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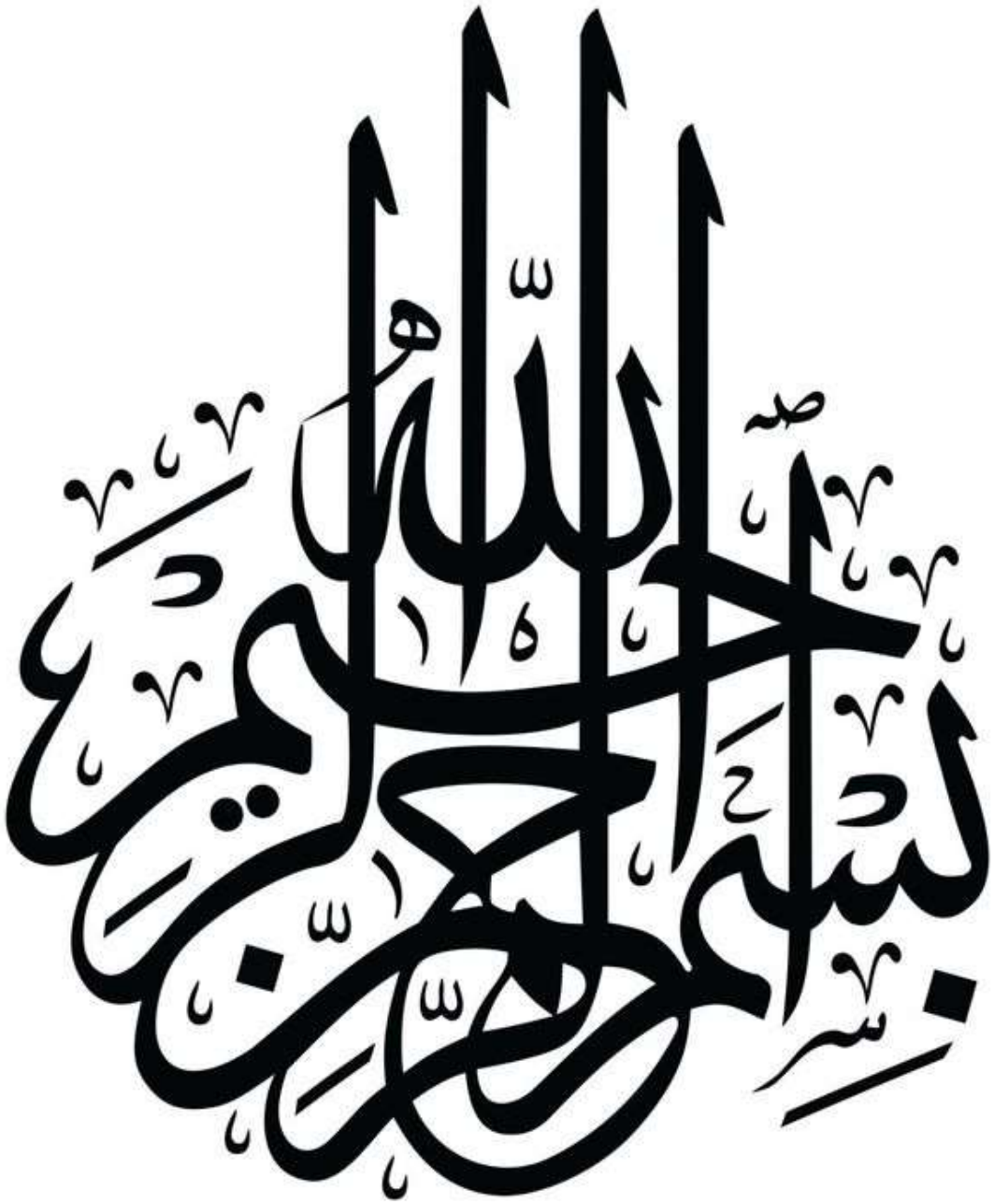
Neurotoxicity investigation of Neonicotinoid on *Wistar* rats and protective role of antioxidant plant extracts

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بسم الله الرحمن الرحيم

Résumé

L'utilisation répandue de l'Imidaclopride (IMI), un pesticide néonicotinoïde, suscite des préoccupations concernant ses effets neurotoxiques. L'exposition chronique à l'IMI induit un stress oxydatif, une dysfonction mitochondriale et des altérations comportementales. Melissa *officinalis* L. (MOE), une plante médicinale reconnue pour ses propriétés antioxydantes, pourrait offrir des effets neuroprotecteurs. La présente étude examine les effets neurotoxiques dose-dépendants de l'IMI et le rôle protecteur de MOE chez les rats *Wistar*, en mettant l'accent sur les différences régionales dans le cerveau.

Dans la première phase, l'extrait aqueux de MOE a été analysé pour ses constituants actifs. Le dépistage phytochimique a révélé de fortes concentrations de polyphénols, flavonoïdes et tanins condensés, des composés connus pour leur activité antioxydante puissante. Les tests in vitro, y compris DPPH, ABTS, CUPRAC et FRAP, ont confirmé des activités significatives de piégeage des radicaux libres (85% et 80% de piégeage respectivement dans les tests DPPH et ABTS). L'analyse structurale par diffraction des rayons X (XRD), spectroscopie infrarouge (IR) et microscopie électronique à balayage (SEM) a validé la présence de composés bioactifs.

La deuxième phase a évalué les effets neurotoxiques de l'exposition chronique à l'IMI (5 mg/kg/jour et 50 mg/kg/jour) chez les rats *Wistar* pendant 90 jours. L'IMI a induit une neurotoxicité de manière dose-dépendante, avec des dommages plus graves observés dans le striatum et l'hippocampe. L'exposition à l'IMI a entraîné des perturbations métaboliques, y compris une réduction significative du poids corporel et de la masse cérébrale à la dose plus élevée (50 mg/kg/jour, $p \leq 0,001$), l'administration de MOE contrebalançant partiellement ces effets. De plus, l'exposition à l'IMI a provoqué une réduction significative des marqueurs de stress oxydatif (SOD, CAT, GPx, GSH, GST, MDA) et une dysfonction mitochondriale, comme en témoignent l'augmentation du gonflement mitochondrial et la réduction de la consommation d'oxygène ($p \leq 0,001$), ainsi que des altérations de la perméabilité membranaire.

Les évaluations neurocomportementales ont inclus des tests d'anxiété (Open Field et test du labyrinthe), de cognition (tests de reconnaissance d'objet et olfactif), de comportement social (Vsoc-Maze) et d'anhedonie (test de préférence pour le sucrose), révélant d'importantes dysfonctions cognitives et motrices chez les rats exposés à l'IMI. La rétention de mémoire a diminué de 40% et la coordination motrice de 50% à la dose plus élevée ($p \leq 0,001$). La stabilité lysosomale a été compromise par l'exposition à l'IMI, comme en témoignent une réduction de 45% du temps de rétention du rouge neutre (NRRT) ($p \leq 0,001$), l'administration de MOE ayant restauré cette stabilité. Les variations du pH lysosomal ont également été mesurées pour évaluer les changements fonctionnels, la déstabilisation lysosomale étant confirmée par des altérations morphologiques observées en microscopie optique. Ces changements incluaient l'expansion des lysosomes, la fuite du colorant rouge neutre et l'arrondissement des cellules, tous indiquant des dommages lysosomaux progressifs. Le degré de dommage a évolué au fil du temps, passant de perturbations structurales mineures à des altérations plus sévères, clairement visibles au niveau microscopique. L'examen histopathologique a révélé des dommages neuronaux graves, notamment à la dose plus élevée d'IMI, tandis que le co-traitement avec MOE a préservé l'intégrité neuronale. L'activité de l'AChE a été inhibée de manière significative, en particulier dans l'hippocampe et le striatum, et a été partiellement restaurée par l'administration de MOE.

Mots-clés : Imidaclopride, Melissa *officinalis*, Neurotoxicité, Stress oxydatif, neurocomportementales, Hippocampe, Striatum, Dysfonction Mitochondriale.

Abstract

The widespread use of Imidacloprid (IMI), a neonicotinoid pesticide, raises concerns about its neurotoxic effects. Chronic exposure induces oxidative stress, mitochondrial dysfunction, and behavioral impairments. Melissa *officinalis* L. (MOE), a medicinal plant known for its antioxidant properties, may offer neuroprotective effects. The present study investigates the dose-dependent neurotoxic effects of IMI and the protective role of MOE in *Wistar* rats, focusing on regional brain differences.

In the first phase, the aqueous extract of MOE was analyzed for its active constituents. Phytochemical screening revealed high levels of polyphenols, flavonoids, and condensed tannins—compounds known for their potent antioxidant activity. In vitro assays, including DPPH, ABTS, CUPRAC, and FRAP, confirmed significant free radical scavenging activities (85% and 80% scavenging in DPPH and ABTS assays, respectively). Structural analysis through X-ray diffraction (XRD), infrared spectroscopy (IR), and scanning electron microscopy (SEM) validated the presence of bioactive compounds.

The second phase assessed the neurotoxic effects of chronic IMI exposure (5 mg/kg/day and 50 mg/kg/day) in *Wistar* rats for 90 days. IMI induced neurotoxicity in a dose-dependent manner, with more severe damage in the striatum and hippocampus. IMI exposure led to metabolic disruptions, including a significant reduction in body weight and brain mass at the higher dose (50 mg/kg/day, $p \leq 0.001$), with MOE administration partially counteracting these effects. Additionally, IMI exposure caused significant depletion of oxidative stress markers (SOD, CAT, GPx, GSH, GST, MDA) and mitochondrial dysfunction, evidenced by increasing mitochondrial swelling and reduced oxygen consumption ($p \leq 0.001$) and altering membrane permeability.

Neurobehavioral assessments included tests for anxiety (open field and labyrinth tests), cognition (novel object and olfactory recognition tests), social behavior (Vsoc-Maze), and anhedonia (sucrose preference test). showed substantial cognitive and motor dysfunction in IMI-exposed rats. With a 40% decrease in memory retention and a 50% decrease in motor coordination at the higher dose ($p \leq 0.001$). Lysosomal stability was compromised by IMI exposure, as evidenced by a 45% reduction in Neutral Red Retention Time (NRRT) ($p \leq 0.001$) with MOE treatment restoring lysosomal stability. Lysosomal pH variations were also measured to assess functional changes, with lysosomal destabilization further confirmed by morphological alterations observed under light microscopy. These changes included lysosomal expansion, leakage of neutral red dye, and cell rounding, all of which indicated progressive lysosomal damage. The degree of damage progressed over time, from minor structural disruptions to more severe alterations, which were clearly visible at the microscopic level. Histopathological examination revealed severe neuronal damage, particularly at the higher IMI dose, whereas MOE co-treatment preserved neuronal integrity. AChE activity was significantly inhibited, especially in the hippocampus and striatum, and was partially restored with MOE administration.

Keywords: Imidacloprid, Melissa *officinalis*, Neurotoxicity, Oxidative Stress, Neurobehavioral, Hippocampus, Striatum, Mitochondrial Dysfunction.

يثير الاستخدام المكثف لمبيد الإيميداكلوبريد (IMI -dirpolcadimI)، أحد المبيدات النيكوتينويدية، مخاوف جدّية بشأن تأثيراته السمية على الجهاز العصبي. إذ أن التعرض المزمن لهذا المركب يؤدي إلى اضطرابات تأكسدية، واختلال وظيفي في الميتوكوندريا، إلى جانب عجز سلوكي ملحوظ. في هذا السياق، تبرز L.*silaniciffo* (EOM) assileM
(الترنجاني الحبق أو المخزنية، المليسة أو المخزني، الترنجان) وهي نبتة طبية معروفة، بخواصها المضادة للأكسدة كخيار واعد تقدمه من تأثيرات وقائية عصبية. تهدف الدراسة الحالية إلى تقييم التأثيرات السمية العصبية للإيميداكلوبريد بجرعات
مختلفة، بالإضافة إلى دراسة الدور الوقائي لمستخلص MOEفي فئران ويستار،
مع التركيز على التباين بالأجزاء النسيجية الإقليمية في الدماغ

في المرحلة الأولى من الدراسة، تم تحليل المستخلص المائي للنبتة لتحديد مكوناته النشطة. وقد كشفت التحاليل الكيميائية النباتية عن تركيزات عالية من البوليفينولات، الفلافونويدات، والتانينات المكثفة (العفص)، وهي مركبات ذات قدرة عالية على مقاومة الأكسدة. وقد أكدت الاختبارات المخبرية في الأنابيب، بما في ذلك DHPP وASTB وCCARPU وFPAR، فعالية ملحوظة في التقاط الجذور الحرة، حيث بلغت نسب الاستدراك 85٪ و80٪ على التوالي في اختباري DHPP وASTB. كما دعمت التحاليل البنيوية باستخدام حيود الأشعة السينية (XRD)، والتحليل الطيفي بالأشعة تحت الحمراء (FRIT)، والمجهر الإلكتروني الماسح (SEM)، وجود مركبات بيولوجية فعالة.

أما في المرحلة الثانية، فقد تم تقييم التأثيرات السمية العصبية الناتجة عن التعرض المزمن للإيميداكلوبريد بجرعتين (5 و50 ملغ/كغ/اليوم) لمدة 90 يومًا. أظهرت النتائج أن هذا المبيد يؤدي إلى تسمم عصبي معتمد على الجرعة، حيث سُجّلت أضرار أشد في منطقتي الحصين (الهيبوكامبوس) و المخطط (الستریاتوم). كما تسبب التعرض بجرعة عالية في اختلالات أيضية ملحوظة، تمثّلت في انخفاض كبير في الوزن الجسماني وكتلة الدماغ (p≥ 0.001)، إلا أن المعالجة المتزامنة بـ MEO ساهمت في التخفيف من هذه التأثيرات. إضافة إلى ذلك، لوحظ انخفاض كبير في مؤشرات الإجهاد التأكسدي (SDO، CTA، GxP، GHS، GTS، MAD) إلى جانب اختلال في أداء الميتوكوندريا، كما اتضح من زيادة تورم الميتوكوندريا وانخفاض معدل استهلاك الأوكسجين وتغير في نفاذية الأغشية (p ≥ 0.001).
شملت التقييمات السلوكية اختبارات القلق (اختبار الحقل المفتوح والمتاهة)، والإدراك (اختبارات التعرف على الأجسام الجديدة والتمييز الشمي)، والسلوك الاجتماعي (Vcos-ezaM)، وتقييم فقدان المتعة (اختبار تفضيل السكروز). وأظهرت هذه التقييمات وجود اختلالات معرفية وحركية كبيرة لدى الفئران المعرضة للمبيد، إذ انخفضت نسبة الاحتفاظ بالذاكرة بمعدل 40٪، وتراجعت القدرة على التنسيق الحركي بنسبة 50٪ عند الجرعة العالية (p≥ 0.001).

أما على مستوى الاستقرار الليزوزومي، فقد تبيّن أن الإيميداكلوبريد أضعف من قدرة الخلايا على الاحتفاظ بـ Nlartue deR ، حيث انخفض وقت الاحتفاظ بنسبة 45٪ (p≥ 0.001)، بينما ساهم علاج MEO في استعادة هذا التوازن. كما أُجري تقييم للتغيرات في الرقم الهيدروجيني لليزوزومات، وأكدت الملاحظات المجهرية وجود تغيّرات شكلية مثل توسّع الليزوزومات، تسرب الصبغة الحمراء، واستدارة الخلايا، مما يشير إلى تدهور تدريجي في سلامة هذه العضيات. وقد تفاقمت الأضرار بمرور الوقت، من تغيّرات طفيفة إلى تشوّهات واضحة على المستوى المجهري.
أظهرت الفحوص النسيجية وجود تلف عصبي حاد، خاصة عند الجرعة المرتفعة، بينما حافظ العلاج المتزامن بـ O MEعلى سلامة البنية العصبية. كما لوحظ تثبيط كبير في نشاط إنزيم أستيل كولين إستيراز (AEhC)، خصوصًا في الهيبوكامبوس والستریاتوم، مع تعافٍ جزئي عند استخدام MEO. تؤكد نتائج هذه الدراسة أن التعرض المزمن للإيميداكلوبريد يؤدي إلى سمية عصبية تعتمد على الجرعة، خصوصًا في مناطق الدماغ المسؤولة عن الذاكرة والحركة.

الكلمات المفتاحية: إيميداكلوبريد، *Melissa officinalis L. (MOE)*، السمية العصبية، الإجهاد التأكسدي، الاختلالات السلوكية، الهيبوكامبوس، الستریاتوم. اختلال أداء الميتوكوندريا.

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إهداء

لو اجتمع العقل، والروح، والقلب في لحظة صفاء، لما وجدوا أعظم من الحمد شكرًا، ولا أسمى من الاعتراف بفضل الخالق، الذي بيده مفاتيح النور والعلم والرحمة.

فإليك يا **الله**، منبع الوجود، وسرّ السكينة في دروب التعب، شكري الذي لا يحدّه بيان، وامتناني الذي تعجز عنه الحروف. لك الحمد على كل لحظة أهدتني فيها بصيرةً، ولك الشكر على كل عثرة علّمتني فيها الصبر، ولك الدعاء أن تجعل هذا الجهد خالصًا لوجهك الكريم، وأن يكون لبنة في طريق العلم الذي خلقتنا لأجله.

إلى أولئك الذين آمنوا بي منذ اللحظة الأولى، ودعمني حبهم وتوجيهاتهم في كل خطوة من خطوات هذه الرحلة العلمية الطويلة، أهدي هذا العمل المتواضع إلى أساتذتي الأفاضل الذين لم يبخلوا عليَّ بعلمهم، الذين زرعوا فيَّ حب المعرفة، ومنحوني من وقتهم وجهدهم ما جعلني أستمر في تحدي نفسي وتجاوز الصعاب.

إلى أستاذي ومشرفي العزيز، **البروفيسور روابحي رشيد**، لا تسعفني الكلمات لأوفيك ما تستحق من تقدير، فقد كنت المعلم الحليم، والداعم الصادق، وصاحب الأثر البالغ والفضل الأكبر في تشكيل هذا العمل العلمي. إشرافك النبيل لم يكن مجرد توجيه أكاديمي، بل كان رحلة فكرية وإنسانية ممتدة، تعلمت فيها من حكمتك وسداد رأيك ... علمتني الكثير، ودفعتني إلى تخطي الحدود التي كنت أظنها نهايتي. بفضل إشرافك القيم تمكنت من تجاوز التحديات والمضي قدمًا بثقة أكبر. لا كلمات توفيك حقك، ولكن يكفيني شرفًا أن أكون أحد طلابك فأنتَ السبب الرئيسي وراء كل خطوة إيجابية في مسيرتي العلمية. شكرًا لك على كل ما قدمته لي من وقتك وجهد وإلهام.

إلى **أمي**، نور حياتي، التي كانت ولا تزال مصدر قوتي وإلهامي. لكِ مني كل الشكر والعرفان، فقد كنتِ السند الأول الذي دفعني إلى الأمام في كل مراحل حياتي. بتضحياتكِ وصبركِ اللامحدود، كنتِ القوة التي جعلتني أواصل سعيي نحو تحقيق أحلامي.

إلى عائلتي العزيزة، الذين كانوا دائمًا مصدر الحب والدعاء، شكرًا لكم على كل لحظة كنتم فيها سندًا لا يُرى ولكنه لا يُنسى.

إلى أصدقائي وزملائي المقربين الذين واكبوا هذه الرحلة بكل تقلباتها، من فرح وتعب وصمت وضجيج، إلى كل من لم أذكر اسمه خشية أن أنسى أحدًا، أنتم في قلبي جميعًا

إلى كل من لمس هذا العمل بروحه أو فكره، إلى كل من أسهم بنصيحة، أو شارك برؤية، أو آمن بي في لحظة شك،

إلى **نفسي**، التي كانت رفيقًا أمينًا في هذا الطريق الطويل. شكرًا لي على الإصرار، وعلى التحدي، وعلى القدرة على الاستمرار رغم الصعوبات. كنتُ دائمًا هناك لنفسي، أحتفل بكل إنجاز صغير وأتحدى نفسي في الأوقات الصعبة. هذا النجاح هو ثمرة مناهجك، جهدك، وصبرك، لذا أوجه لك كل التقدير والشكر.

إلى هذا المجال العلمي الذي ألهمني وساهم في تطوير قدراتي وأفكاري، وإلى رحلتي التي لا تزال مستمرة في سعيي الدؤوب نحو العلم والاكتشاف. أهدي هذا العمل، مع أمل أن يكون إضافة بسيطة إلى هذا العلم العظيم، وأتمنى أن يكون بمثابة الشعلة التي تضيء الطريق للآخرين.

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"We cannot gain anything without losing something.

Even paradise requires death."

— Albert Einstein

Abbreviations

List of Abbreviations

- **°C** Degrees Celsius
- **µg/mL** Micrograms per milliliter
- **µL / mL** Microliter / Milliliter
- **µm** Micrometer
- **2θ** Diffraction angle in radians (used in XRD)
- **ACh** Acetylcholine
- **AchE** Acetylcholinesterase
- **ACUC** Institutional Animal Care and Use Committee
- **AND** Deoxyribonucleic Acid
- **ANOVA** Analysis of Variance
- **API** Algeria Pasteur Institute
- **BHT** Butylated Hydroxytoluene
- **BSA** Bovine Serum Albumin
- **CAS** Chemical Abstracts Service
- **CAT** Catalase
- **CDNB** 1-Chloro-2,4-Dinitrobenzene
- **CLT** Classic Labyrinth Test
- **cm^{-1}** Wavenumber (used in IR spectroscopy)
- **CT** Control Group
- **Cu Kα** Copper K-alpha radiation
- **D1** Imidacloprid Dose 1 (5 mg/kg/day)
- **D2** Imidacloprid Dose 2 (50 mg/kg/day)
- **DIFFRAC Plus** Diffractogram analysis software
- **DMSO** Dimethyl Sulfoxide
- **DTNB** 5,5'-Dithiobis(2-nitrobenzoic acid)
- **DTT** Dithiothreitol
- **EDTA** Ethylenediaminetetraacetic acid
- **EPA** Environmental Protection Agency
- **FAO** Food and Agriculture Organization

Abbreviations

- **$FeCl_3$** Ferric chloride
- **FTIR** Fourier Transform Infrared Spectroscopy
- **g / mg** Gram / Milligram
- **GPX** Glutathione Peroxidase
- **GSH** Glutathione
- **GST** Glutathione S-Transferase
- **H&E** Hematoxylin-Eosin (staining technique)
- **H_2O_2** Hydrogen Peroxide
- **HCl** Hydrochloric acid
- **HEPES** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **IACUC** Institutional Animal Care and Use Committee
- **ICDD PDF-4+** International Centre for Diffraction Data – Powder Diffraction File database
- **IMI** Imidacloprid
- **IR** Infrared Spectroscopy
- **KBr** Potassium bromide
- **LD50** Lethal Dose, 50%
- **MDA** Malondialdehyde
- **MitCAT** Mitochondrial Catalase
- **MitGPx** Mitochondrial Glutathione Peroxidase
- **MitGSH** Mitochondrial Glutathione
- **MitGST** Mitochondrial Glutathione S-Transferase
- **MitMDA** Mitochondrial Malondialdehyde
- **MitSOD** Mitochondrial Superoxide Dismutase
- **MOE** Melissa *Officinalis* L Extract
- **MPTP** Mitochondrial Permeability Transition Pore
- **N** Normal (concentration of a solution)
- **NaCl** Sodium Chloride
- **NADPH** Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
- **NaOH** Sodium hydroxide
- **NH_4OH** Ammonium hydroxide

Abbreviations

- **NIST** National Institute of Standards and Technology
- **NOR** Novel Object Recognition
- **NRRT** Neutral Red Retention Time
- **NS** Not Significant
- **OD** Optical Density
- **PAHs** Polycyclic Aromatic Hydrocarbons
- **PBS** Phosphate-Buffered Saline
- **PMSF** Phenylmethylsulfonyl fluoride
- **q.s.p.** Quantum satis pro (quantity sufficient to...)
- **ROS** Reactive Oxygen Species
- **RPM** Revolutions Per Minute
- **SD** Standard Deviation
- **SDS** Sodium Dodecyl Sulfate
- **SE** Standard Error
- **SEM** Scanning Electron Microscopy
- **SNC** Central Nervous System
- **SOD** Superoxide Dismutase
- **SPT** Sucrose Preference Test
- **TNF** Tumor Necrosis Factor
- **TSE buffer** Tris-Sucrose-EDTA buffer
- **UI** International Unit
- **UV** Ultraviolet
- **v/v** Volume per volume
- **WHO** World Health Organization
- **XRD** X-ray Diffraction
- **ΔOD/Δt** Change in Optical Density over Time
- **Mmol** Micromoles

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Introduction

Introduction

The growth of industrialization and agriculture has significantly increased chemical pollution, posing serious environmental and public health risks. With over 2 million tons of pesticides used annually, ecosystems, wildlife, and human populations face contamination threats **(FAO, 2021**). While pesticides help reduce crop losses by 30–40%, their widespread and often unregulated use leads to soil degradation, water pollution, and ecosystem disruption **(Sharma et *al.*, 2019).**

Neonicotinoids (NNs), accounting for nearly 25% of the global insecticide market, are among the most concerning pesticide classes due to their highly persistent neurotoxic chemicals with widespread environmental and health risks **(Simon-Delso et *al*., 2015; Tomizawa and Casida, 2005)**. Their pervasive contamination is evident, with residues detected in 75% of honey samples worldwide **(Mitchell et *al.*, 2017).** Acting as nicotinic acetylcholine receptor agonists, they disrupt synaptic transmission, affecting not only insects but also pollinators, mammals, and humans **(Gibbons et *al.*, 2015; Wood and Goulson, 2017)**. Linked to neurotoxicity, oxidative stress, immune disturbances, and endocrine disruption, their bioaccumulative nature raises concerns about prolonged exposure contributing to neurodegenerative diseases like Parkinson's and Alzheimer's **(Cimino et *al*., 2017; Abd-Elhakim et *al*., 2018; Wang et *al*., 2018; Yang et *al*., 2020).**

Pesticides contribute to cellular damage through the induction of oxidative stress, which is a key mechanism behind their toxicity **(Abdollahi et *al*., 2004; Agrawal et *al*., 2010)**. The brain, being particularly vulnerable to oxidative stress due to its high levels of peroxidable fatty acids, significant energy demands, and limited antioxidant defenses, is at risk of permanent molecular damage, which can lead to neurodegenerative diseases **(Rouabhi, 2010)**. In this context, mitochondria and lysosomes play crucial roles in maintaining cellular balance. Mitochondria are especially prone to oxidative damage, as they are the primary sites of energy production and redox regulation. When damaged, they can disrupt energy metabolism, cause calcium imbalances, and impair neuronal function **(Sun et *al.*, 2016; Pryde and Hirst, 2011)**. Likewise, oxidative stress affects lysosomal stability, which in turn hampers autophagic processes, further damaging cells and triggering apoptosis **(Cesen et *al.*, 2012)**. The combination of mitochondrial and lysosomal dysfunction due to pesticide-induced oxidative stress accelerates the progression of neurodegenerative diseases, underscoring the urgent need for strategies to mitigate the harmful effects of pesticides on the nervous system **(Trushina and McMurray, 2007)**.

Imidacloprid, the most widely used insecticide globally, is registered for application on over 140 crops across more than 120 countries, and its usage continues to rise **(Jeschke et *al*.,**

2011). This prevalent neonicotinoid has been strongly linked to neurotoxic effects, particularly with chronic exposure, which has been shown to reduce antioxidant enzyme activity in mammalian brain tissues by 40–60%. Such reductions contribute to oxidative stress, cognitive decline, and an increased susceptibility to neurodegenerative disorders **(Morrissey et *al*., 2015; Sánchez-Bayo and Tennekes, 2021)**.

Natural antioxidants from medicinal plants are gaining attention for their neuroprotective potential, primarily due to their ability to counteract oxidative stress and restore cellular balance. Bioactive compounds such as polyphenols and flavonoids exhibit strong free radical-scavenging properties, enhance mitochondrial function, and boost endogenous antioxidant defenses like SOD, GPx, and CAT **(Rahman et *al*., 2016; Moghaddam et *al*., 2018)**. Additionally, flavonoids such as quercetin further protect against neurodegeneration by inhibiting lipid peroxidation and preserving mitochondrial integrity, making them promising candidates for mitigating pesticide-induced neural toxicity **(Haleagrahara et *al*., 2011)**.

Melissa *officinalis* L. (lemon balm) is particularly recognized for its neuroprotective effects, attributed to its rich content of polyphenols, including rosmarinic acid and quercetin, which help reduce oxidative stress and neuroinflammation, regulates neurotransmitters, improving memory and cognitive function **(Miraj et *al*., 2017; Sadraei et *al*., 2018)**. In Algeria, Melissa *officinalis* is part of the rich local flora, and its widespread use in traditional medicine underscores its potential as a valuable therapeutic agent. Given the increasing concerns over pesticide-related toxicity, the neuroprotective properties of Algerian medicinal plants could offer a natural approach to mitigating the harmful effects of pesticide exposure and contribute to the preservation of neurological health in the region. **(Boudouma et *al*., 2015)**.

To achieve the objectives of this study, an extensive literature review was conducted to analyze recent research findings and establish the current state of knowledge on the topic. The experimental approach incorporated pharma-toxicological assessments, including an evaluation of the phytochemical and pharmacological properties of Melissa *officinalis* L. Additionally, the study investigated the dose-dependent effects of chronic Imidacloprid (IMI) exposure on neurobehavioral, histopathological, and biochemical parameters in *Wistar* rats, focusing on structural and molecular integrity in both total and regional brain areas (striatum and hippocampus). Finally, the neuroprotective potential of Melissa *officinalis* L. extract (MOE) was assessed in counteracting IMI-induced neurotoxicity, providing valuable insights into the therapeutic benefits of plant-based antioxidants in mitigating pesticide-related neural damage.

Bibliographic Review

Chapter I : Pesticides

1. Introduction to Pesticides

1.1. Definition

Pesticides are substances or combinations of substances designed to prevent, destroy, or control pests, including insects, fungi, rodents, and unwanted plants that threaten crop production and storage **(Tano, 2011).** Commonly referred to as phytosanitary or phytopharmaceutical products, they are chemical compounds—organic or mineral—comprising one or more active ingredients, along with diluents or adjuvants **(Tayade, 2013).** This broad category includes insecticides, herbicides, fungicides, and rodenticides, which serve to repel, eliminate, or control pests **(Akashe et *al*., 2018).**

Despite their agricultural benefits, the widespread use of pesticides has significant environmental repercussions and poses serious risks to human health. For instance, exposure to certain chemical pesticides such as organochlorines, organophosphates, carbamates, pyrethroids, triazines, and neonicotinoids has been linked to neurological disorders and other adverse health effects **(Baylis, 2000; Turusov et *al*., 2002; Lu et *al*., 2015).**

1.2. Historical context

The history of pesticide use spans over 4,000 years, highlighting humanity's enduring efforts to protect crops and food resources from pests **(Winter, 2012).** Early methods, such as planting venomous and nutritious plants together and utilizing elemental sulfur, reflect innovative approaches documented in ancient texts like the Ebers papyrus and Homer's *Odyssey*. The 1500s marked the emergence of *"para-pesticides"* like mercury and arsenic, which evolved into synthetic pesticides during and after World War II. This shift, however, raised concerns about health risks, including increased lymphoma cases, emphasizing the need to balance pest control with human and environmental safety **(Buchel, 1983; Tano, 2011)**.

The modern pesticide era began with Paul Müller's discovery of DDT in 1939, earning him the Nobel Prize for its transformative impact on agriculture and public health. However, Rachel Carson's *Silent Spring* (1962) exposed its ecological and health risks, prompting widespread bans and the adoption of safer alternatives. Since the 1990s, competition among phytosanitary industries has driven product optimization for specific applications **(Gatignol and Étienne, 2010).**

Despite ongoing advancements and stricter regulations, including new EU measures aimed at reducing pesticide-related illnesses, challenges persist. Public health concerns, especially among vulnerable populations, remain significant, while the rise of pesticide-resistant weeds presents a growing threat. Global pesticide production continues to rise to meet the demands of

population growth, underscoring the delicate balance between effective pest control and safeguarding human and environmental well-being **(Buchel, 1983; Tano, 2011; Fishel, 2013)**.

1.3. Classification of pesticides

Pesticides exhibit diverse identities characterized by their physical and chemical properties. According to Drum (1980), they can be broadly classified in various ways, including their origin **(Table 01)**, the target pest species they are designed to combat **(Table 02)**, and their functional application **(Table 03)**.

Table 01: Classification of pesticides based on origin. **(Drum, 1980).**

Based on Origin	***Sources and Examples***
Organic sources	Natural - plant phytochemical (essential oil, plant extracts, leftover oilseed cakes)
Synthetic	Produced by chemical synthesis e.g., Pyrethroids, organophosphates, carbamates, organochlorine
Inorganic sources	Inorganic - mixture of inorganic salts (Bordeaux mixture $Cu(OH)_2.CaSO_4$, Malachite $Cu(HO)_2.CuCO_3$ and sulfur)
Biological	Biological - microbial pesticide (bacteria, virus, and fungi)

Table 02: Classifications of pesticides based on target pest species. **(Drum, 1980).**

Pesticide Class	***Target Pests***	***Example***
Acaricides	Mites	Bifonazole
Algaecides	Algae	Copper sulfate
Avicides	Birds	Avitrol
Bactericides	Bacteria	Copper complexes
Fungicides	Fungi	Azoxystrobin
Herbicides	Weeds	Atrazine
Insecticides	Insects	Aldicarb
Larvicides	Larvae	Methoprene
Molluscicides	Snail	Metaldehyde
Nematicides	Nematodes	Aldicarb
Ovicides	Egg-prevents hatching of egg in insects and mites	Benzoxazine
Piscicides	Fishes	Rotenone
Repellents	Insects	Methiocarb
Rodenticides	Rodents	Warfarin
Termiticides	Kills termites	Fipronil
Viricides	Viruses	Scytovirin

Table 03: Classification of pesticides based on function. **(Drum, 1980)**

Action	*Function*	*Examples*
Feeding deterrents	Prevent an insect or other pest from feeding	(Azadirachta Indica A. Juss)
Ovipositor deterrent	Prevent egg laying by gravid female	Azadirachta indica
Repellents	Deters pest from approaching toward crops	Plant essential oil
Attractants	A chemical that lures pests	Gossyplure
Fumigants	Kills the target pests by producing vapor	Phosphine
Insect growth regulator	Disrupts the growth or development of an insect	Diflubenzuron
Synergist	Enhances the toxicity of a pesticide but not by itself toxic to pest	Piperonyl butoxide

1.4. Modes of entry into target organisms

The entry mechanisms of pesticides are broadly categorized into *systemic* and *nonsystemic* types **(Buchel, 1983)**.

1.4.1. Systemic contact pesticides

Systemic pesticides are absorbed by plants or animals and transported to untreated tissues, effectively targeting pests across different parts of the plant, such as leaves, stems, or roots. For example, systemic herbicides utilize the plant's vascular system to eradicate pests, even with partial spray coverage. Notable examples include 2,4-dichlorophenoxyacetic acid (2,4-D) and glyphosate.

1.4.2. Nonsystemic contact pesticides

Nonsystemic contact pesticides, also known as contact pesticides, act directly on pests upon physical contact, penetrating their epidermis and causing death through poisoning. Examples include paraquat, diquat, and dibromide. Some, like stomach poisons, are ingested through the pest's mouth and digestive system, damaging the midgut (e.g., Malathion). Fumigants release vapors that enter pests via the respiratory system, making them effective for controlling pests in stored products and soil. Additionally, repellents deter pests without killing them, preventing bites from mosquitoes and other bloodsucking insects, particularly in public health applications **(Buchel, 1983)**.

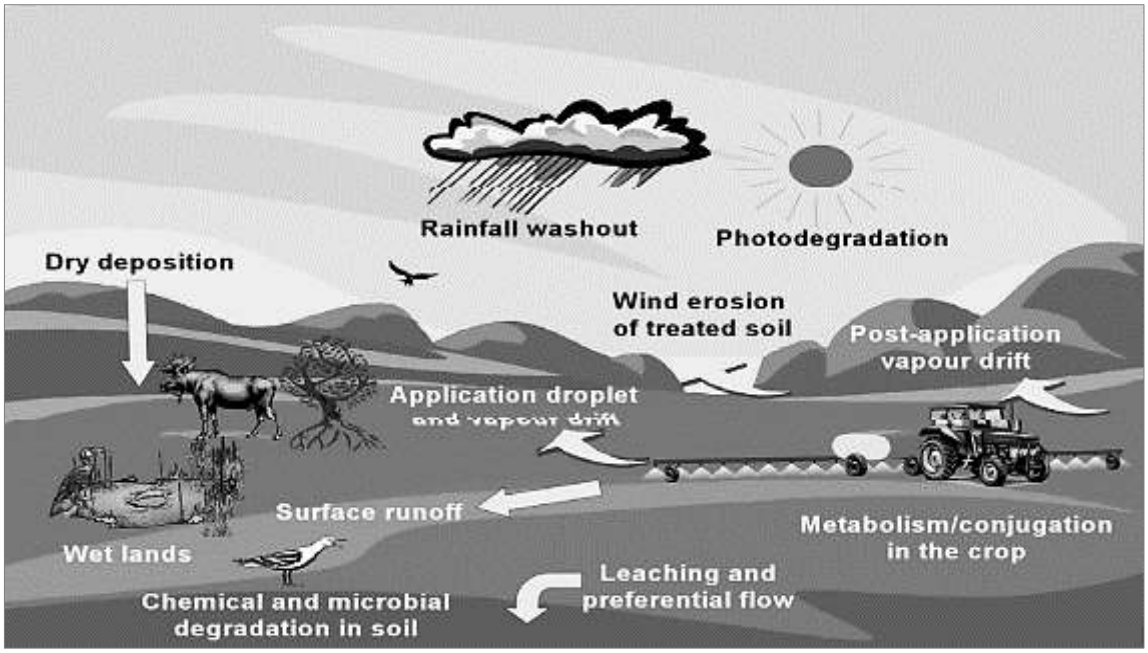


Figure 01: Schematic Representation of Pesticide Pathways into the Atmosphere, Surface Water, and Groundwater, and Their Transformation Mechanisms in Air, Soil, and Plants. **(Cessna, 2009)**.

1.5. Health effects of pesticides on consumers

Pesticide exposure poses significant health risks, with developing countries reporting approximately 3,000,000 cases of pesticide poisoning and 220,000 deaths annually, according to the World Health Organization **(Lah, 2011)**. Beyond acute poisoning, prolonged exposure to pesticides has been associated with a range of adverse health effects, including various cancers **(Daniels et *al*., 1997; Khuder and Mutgi, 1997; Zahm and Ward, 1998)** and degenerative diseases **(Engel et *al*., 2001; Jenner, 2001)**.

The impact of pesticides extends to multiple biological systems, affecting immune, hematological, nervous, endocrine, and reproductive functions **(Ojajarvi et *al.*, 2000; Ritz and Yu, 2000; Figa-Talamanca and Petrelli, 2001)**. Additionally, studies have linked pesticide exposure to DNA damage in human populations **(Gomez-Arroyo et *al*., 2000; Costa et *al*., 2007; Ergene et *al*., 2007)**. Even at low levels, pesticides can trigger biochemical changes that contribute to harmful biological effects, as observed in both human and experimental studies **(Gupta et *al*., 1998; Banerjee et *al*., 1999)**. These biochemical changes, though not always clinically evident, serve as important markers of pesticide exposure or toxic effects **(Panemangalore et *al*., 1999)**.

1.6. Environmental effects of pesticides

Pesticides affect the environment through point-source pollution, such as spills or leaks, and nonpoint-source pollution, like runoff into water or air drift **(Toth and Buhler, 2009)**. Sensitive areas include groundwater zones, surface waters, populated regions, wildlife habitats, and agricultural fields **(Cessna et *al*., 2005)**. Negative impacts include pollution, harm to non-target organisms, soil degradation, economic losses from misuse, and biodiversity decline, affecting rare species and ecosystems **(Struik and Bonciarelli, 1997; Adetunji et *al*., 2018; Helfrich et *al*., 2009)**.

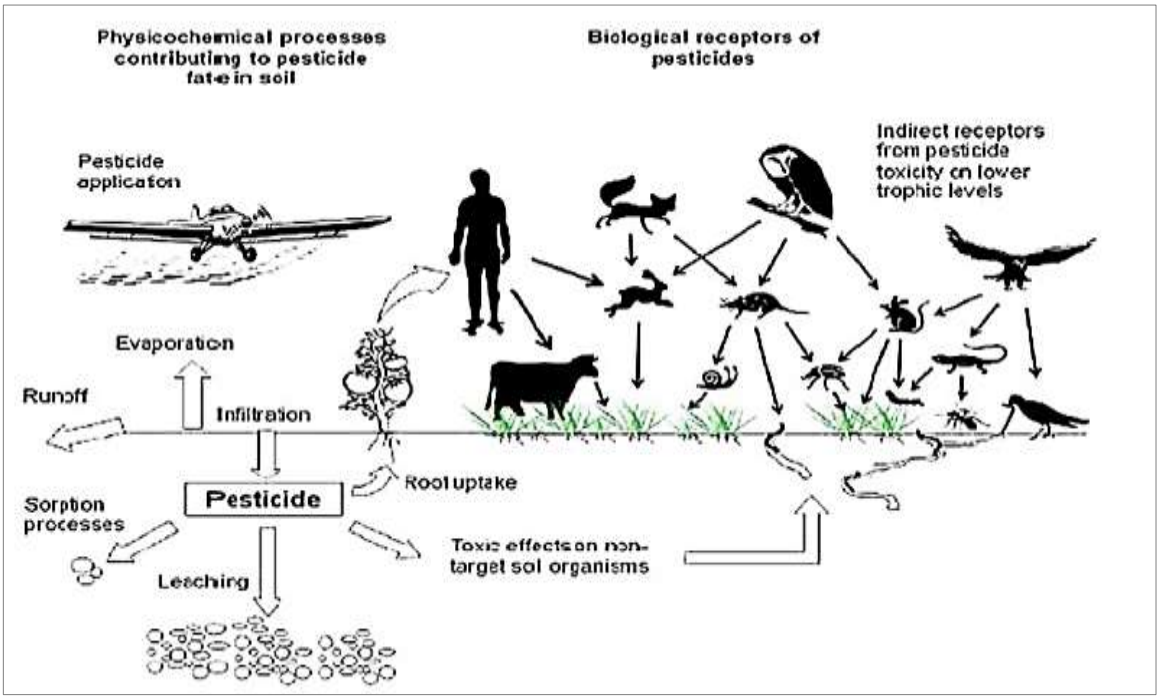


Figure 02: Schematic Representation of the Environmental Fate of Pesticides: Distribution, Transformation, and Interactions with Ecosystem Organisms.

(Sanchez-Hernandez, 2011).

2. Neonicotinoids

2.1. History and background

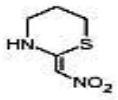
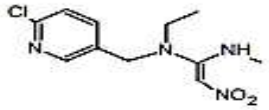
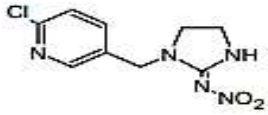
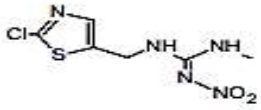
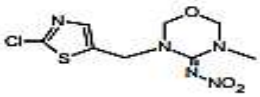
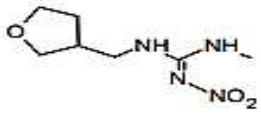
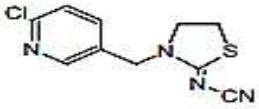
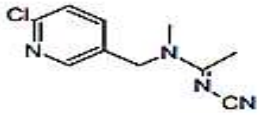
Neonicotinoids, a widely used class of insecticides, are authorized in over 120 countries and rank among the most prominent insecticides globally **(Van der Sluijs et *al.*, 2013)**. Introduced in the 1990s, they quickly became the leading class of insecticides worldwide **(Jeschke et *al.*, 2011; Casida and Durkin, 2013)**. The term "neonicotinoid," coined by Izuru Yamamoto in 1998, was introduced to distinguish these modern insecticides, such as imidacloprid (IMI), from earlier nicotinoid compounds targeting nicotinic acetylcholine receptors (nAChRs) **(Tomizawa and Yamamoto, 1993; Yamamoto et *al.*, 1998)**.

The origins of neonicotinoid development date back to the late 1970s when researchers at Shell Chemical Company investigated heterocyclic nitromethylenes as potential insecticides **(Soloway et *al.*, 1978; Schroeder and Flattum, 1984)**. Neonicotinoids were recognized as a distinct class due to their neurotoxic properties, which involve binding to post-synaptic acetylcholine receptors, causing paralysis and ultimately insect death **(Seifert et *al.*, 2005)**. Their efficacy at the molecular level depends on their interaction at the nAChR agonist site, with potency influenced by their structural design **(Tomizawa and Casida, 2005; Tomizawa and Casida, 2009)**.

2.2. Classification of neonicotinoid insecticides

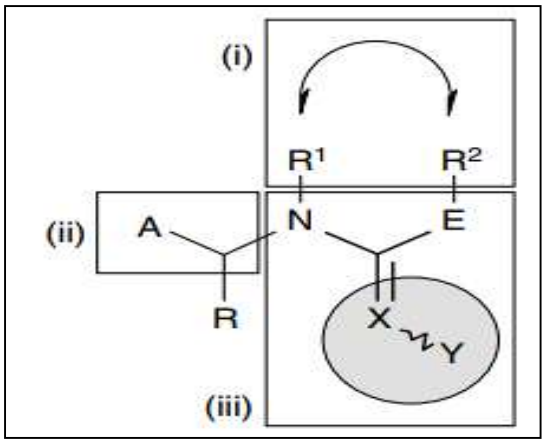
Neonicotinoids are classified according to their chemical structure, which influences their biological activity, photostability, metabolism in plants and insects, and overall toxicity. Based on their pharmacophore type, they are divided into two or three groups, as shown in ***Table 04*** This structural classification provides insights into their functional properties and mechanisms of action.

Table 04: Classification of neonicotinoid insecticides based on their pharmacophore **(Jeschke et *al.*, 2008).**

Pharmacophores [-N-C(E)=X-Y]	*Compounds with a cycle of 5 and6 items*	*Open Chain Compounds*
nitroenamines/nitrométhylènes [-N-C(E)=CH-NO_2]	nithiazine 	nitenpyrame 
	imidaclopride 	clothianidine 
N-nitroguanidines/N-nitro-imines [-N-C(E)=N-NO_2]	thiaméthoxame 	dinotefuran 
N-cyano-amidines/N-cyano-imines [-N-C(E)=N-CN]	thiaclopride 	acétamipride 

2.3. Chemical structure of neonicotinoids

The chemical structure of neonicotinoids can be categorized based on their pharmacophore moiety into open-chain compounds and ring-based neonicotinoids, which include five- and six-membered ring systems. These structural variations result in differences in molecular characteristics **(Figure 04)** and biological activity. Regardless of the category, neonicotinoids share a common structural framework composed of distinct segments, as illustrated in **figure 03 (Jeschke, 2007; Nauen et *al.*, 2001)**.



(i)	For open-chain neonicotinoids the separate substituents (R^1, R^2), and for five- and six-membered ring systems the bridging fragment [R^1 – R^2, R^1 –Z–R^2; Z = O, NMe];
(ii)	The heterocyclic or heteroalicyclic group A with a bridging chain [–CHR–; R = H; e.g. A–CH_2 –: CPM, CTM and TFM];
(iii)	The functional group [=X–Y] as part of the different pharmacophore types [–N–C(E)=X–Y].

Figure 03: Structural segments for neonicotinoids. **(Nauen et *al*., 2001)**.

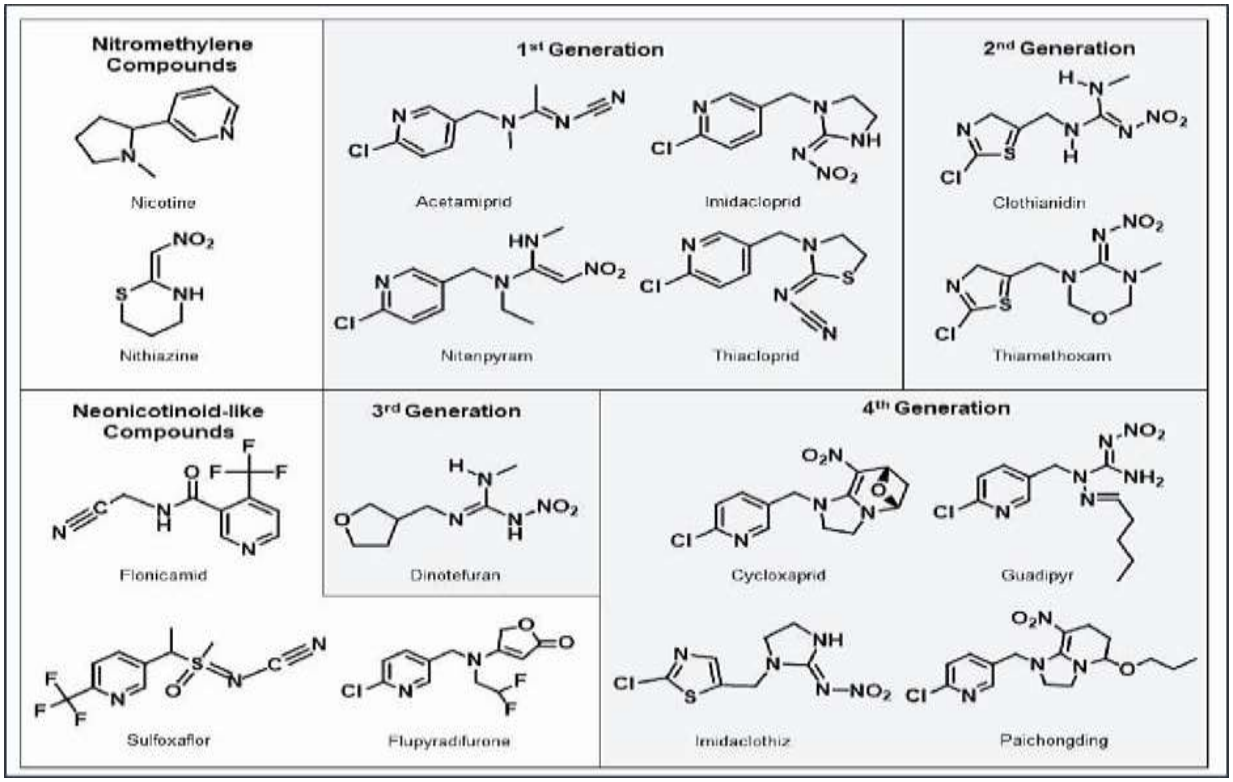


Figure 04: Chemical structures of common neonicotinoids and related compounds.

Figure adapted from **C. Giorio et *al.*** Environmental Science and Pollution Research, 2017, 1-33**. (Abou-Donia et *al*., 2008).**

2.4. Mechanism of action of neonicotinoids

Neonicotinoids act as selective agonists of nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channels predominantly located on postsynaptic membranes in the central nervous system (CNS) and peripheral nervous system (PNS) **(Tomizawa and Casida, 2005)**. These compounds mimic acetylcholine (ACh) but bind irreversibly to the receptor,

leading to sustained activation. This persistent stimulation disrupts synaptic transmission by causing an overstimulation of neuronal signaling pathways including dopamine, serotonin, glutamate, and GABA (γ-aminobutyric acid), ultimately leading to paralysis and death in insects **(Taly et *al.*, 2009)**.

Structurally, neonicotinoids are characterized by the presence of an electronegative nitro or cyano pharmacophore, which interacts with the cationic subsite of insect nAChRs. This structural specificity underpins their high affinity for insect receptors while conferring reduced binding to vertebrate nAChRs. Unlike protonated nicotinoids that require a cation-π interaction to bind vertebrate receptors, neonicotinoids exploit their non-protonated state to achieve selectivity. This feature ensures strong insecticidal activity with minimal toxicity to mammals, birds, and fish **(Tomizawa and Casida, 2005; Jeschke et *al.*, 2011).**

The functional diversity of nAChRs, arising from the assembly of different subunits (e.g., α7 homopentamers and α4β2 heteropentamers), adds complexity to their neuropharmacological effects. These receptor subtypes are heterogeneously distributed across brain regions and exhibit unique electrophysiological properties. In insects, neonicotinoids primarily target receptor subtypes critical for synaptic transmission, whereas in vertebrates, their limited interaction reduces off-target effects. However, the diversity of nAChR subtypes remains a major challenge in optimizing pesticide specificity **(Gotti et *al.*, 2007; Taly et *al.*, 2009)**.

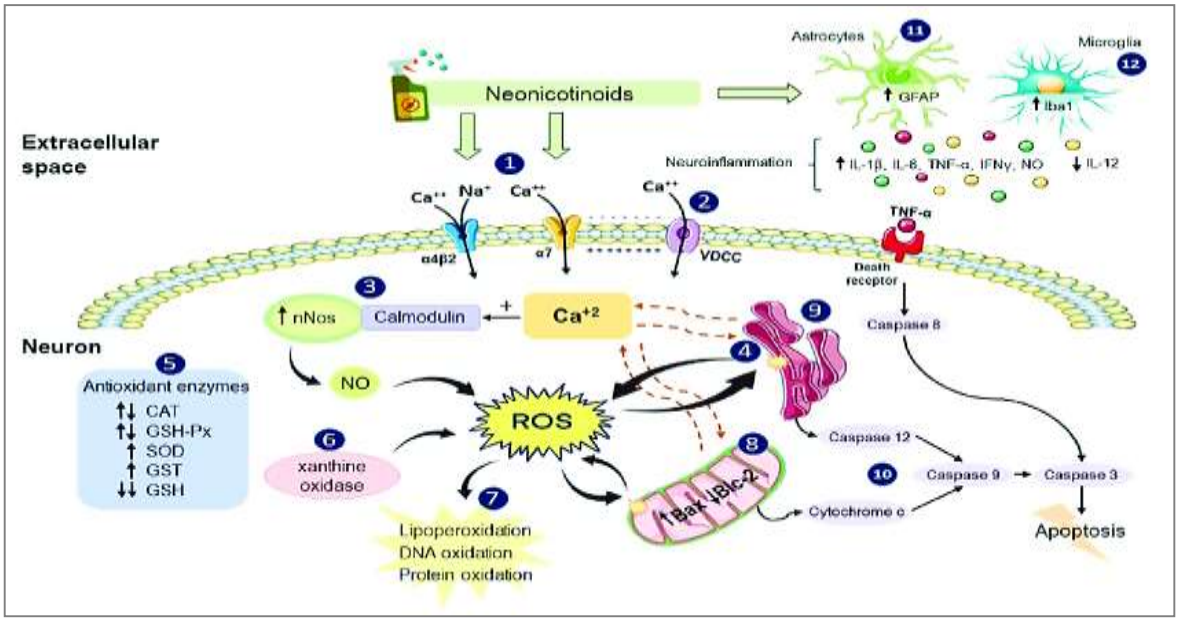


Figure 05: Main mechanisms of neurotoxicity proposed for NN pesticides in the nervous system of rodents, human, and other mammals. (**Carmen et *al.*, 2021)**.

NNs induce activation of α4β2 and α7 subtype receptors (1),increased Ca+2 influx, membrane depolarization, and VDCC opening (2), with a greater increase in Ca+2 levels. Ca+2 can bind to calmodulin, increasing NO synthesis (3) and ROS production, and it can also bind to ER ryanodine receptors (4), further increasing its concentrations in the cytosol. NNs also modify the activity of several antioxidant enzymes (5) and increase the activity of xanthine oxidase (6) and, consequently, there is an increase in ROS production. Increased levels of ROS lead to LPO, DNA and protein oxidation (7), and severely affect mitochondria (8) and ER (9), activating two different pathways of cellular apoptosis (10). NNs also induce astrocyte and microglial activation, with upregulation of GFAP (11) and Iba1 (12), respectively, and increased release of pro-inflammatory cytokines and ROS. Some of these molecules, such as TNF-α, can bind to death receptors and trigger neuronal apoptosis (13). Parts of the figure were created using templates from Servier Medical Art, which are licensed under a Creative Commons Attribution 3.0 Unported License ***(http://smart.servier.com/ accessed on: 6 July 2021)***

Abbreviations: VDCC: voltage-dependent calcium channels; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; ROS: reactive oxygen species; CAT: catalase; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; GST: Glutathione S-transferase; GSH: glutathione; GFAP: glial fibrillary acidic protein; IL-12: interleukin 12; IL-1β: interleukin 1beta; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha.

2.5. Toxicity of neonicotinoid insecticides

2.5.1. Oxidative Stress

Neonicotinoid insecticides induce oxidative stress by disrupting the balance between reactive oxygen species (ROS) production and the antioxidant defense system, leading to cellular damage. This is evident through increased lipid peroxidation, reduced glutathione levels, and altered activities of key antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase **(El-Gendy et *al.*, 2010; Annabi et *al.*, 2015)**. For example, imidacloprid elevates nitric oxide production and the expression of nitric oxide synthases in the brain and liver, while thiacloprid increases nitric oxide levels in plasma and polymorphonuclear leukocytes, exacerbating oxidative damage **(Aydin, 2011; Duzguner and Erdogan, 2011; Tanaka, 2015)**. Antioxidants like curcumin and vitamin C have been shown to mitigate these effects by neutralizing ROS and restoring antioxidant activity **(El-Gendy et *al.*, 2010; Lonare et *al.*, 2014)**. Thus, oxidative stress is a key mechanism in the toxicity of neonicotinoids, playing a significant role in their harmful effects on non-target organisms **(Kapoor et *al.*, 2010; Wang et *al.*, 2015)**.

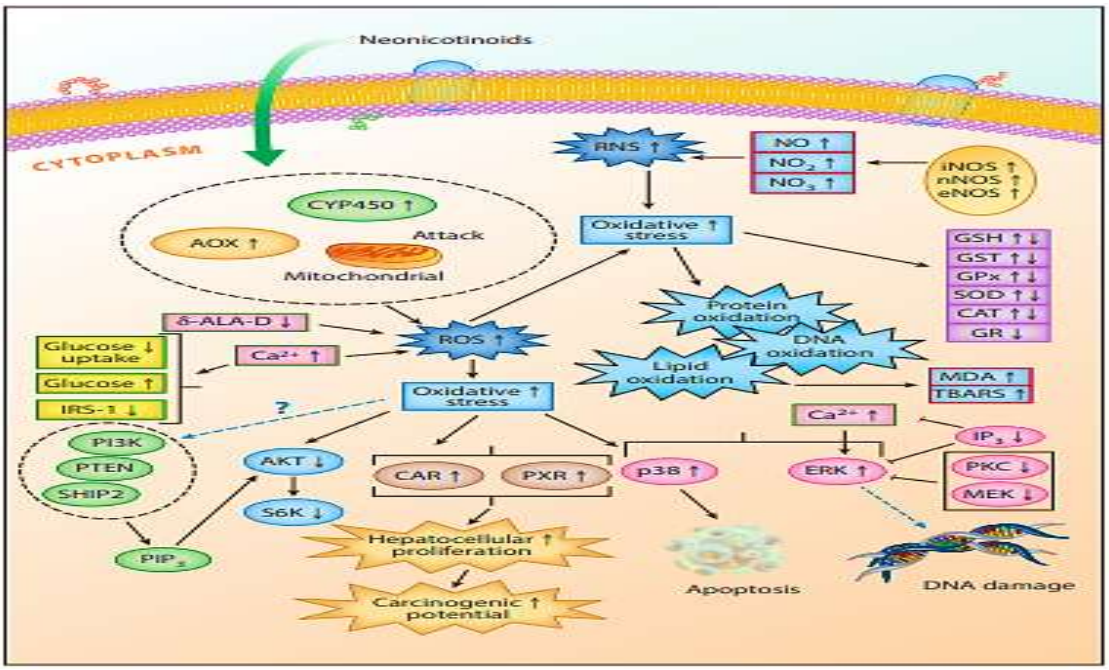


Figure 06: Schematic Representation of Neonicotinoid-Induced Damage to DNA, Lipids, and Proteins via Oxidative Stress–Mediated Mechanisms. (**Wang et *al.*, 2018)**.

2.5.2. Reproductive Toxicity

Neonicotinoid insecticides have been shown to cause significant reproductive toxicity in mammals, including increased embryo mortality, premature births, reduced pregnancy rates, impaired sperm production and function, lower offspring weight, and stillbirths **(Abou-Donia et *al.*, 2008; Gibbons et *al.*, 2015; Berheim et *al.*, 2019)**. Experimental studies in rodents indicate that neonicotinoids adversely affect fertilization rates **(Najafi et *al.*, 2010; Bal et *al.*, 2012)**, and in vitro studies demonstrate that exposure to acetamiprid or imidacloprid directly impairs mouse sperm fertilization ability **(Gu et *al.*, 2013)**. Additionally, the reproductive toxicity of neonicotinoids, such as clothianidin, is exacerbated under environmental stress, highlighting their potential for more severe effects in real-world conditions **(Hirano et *al.*, 2015)**.

2.5.3. Hepatotoxicity

Neonicotinoid exposure can lead to hepatotoxicity, particularly at high doses, often accompanied by reduced food consumption and body weight. Elevated serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST) are common indicators of liver damage, as observed in rats exposed to imidacloprid **(Mohany et *al.*, 2011)**. However, liver pathology caused by imidacloprid has been shown to be fully

reversible upon cessation of exposure **(Sheets, 2010),** with mild pathological changes occurring only at high doses (20 mg/kg/day) **(Bhardwaj et *al*., 2010).** Chronic exposure to thiamethoxam similarly induces liver injury in mice, reflected in elevated AST and ALT levels **(Green et *al.*, 2005)**. Conversely, exposure to thiacloprid has been reported to decrease serum AST and ALT levels compared to controls, suggesting differing hepatic effects among neonicotinoids **(Aydin, 2011)**.

2.5.4. Genotoxicity and Carcinogenicity

Neonicotinoids are generally considered non-mutagenic based on in vitro and in vivo studies conducted during their registration process **(Sheets, 2010).** However, some commercial formulations containing neonicotinoids have been shown to induce DNA damage in human peripheral blood lymphocytes in vitro **(Calderón-Segura et *al.*, 2012).** Carcinogenicity studies in rodents have found no evidence of tumor formation following exposure to imidacloprid **(Sheets, 2010)** or clothianidin **(Tanaka, 2015).** In contrast, dietary exposure to thiamethoxam has been associated with an increased incidence of liver tumors in mice **(Green et *al.*, 2005; Pastoor et *al.*, 2005).** Furthermore, in vitro studies on bovine peripheral lymphocytes treated with thiacloprid revealed significant increases in DNA damage and chromosome aberrations at concentrations of 120 to 480 mg/mL **(Galdikova et *al.*, 2015),** highlighting potential genotoxic risks at higher doses.

2.5.5. Endocrine Effects

Neonicotinoids have shown potential endocrine-disrupting effects, as evidenced by in vitro studies and limited laboratory data **(Caron-Beaudoin et *al*., 2017).** Commercial formulations of thiacloprid significantly increased serum levels of free triiodothyronine (T3) and free thyroxine (T4) in rats, indicating thyroid disruption **(Sekeroglu et *al*., 2014).** Additionally, thiacloprid, thiamethoxam, and imidacloprid have been found to alter aromatase (CYP19) activity in fetoplacental steroidogenesis models, increasing estrone and estradiol production while inhibiting estriol synthesis. Given the critical role of estrogens during pregnancy, these disruptions may pose risks to fetal development and maternal health **(Caron-Beaudoin et *al*., 2018)**.

2.5.6. Metabolite Toxicity

Neonicotinoid metabolites can exhibit greater toxicity than their parent compounds, particularly those formed through the removal of nitro- or cyano-functional groups. These metabolites often display increased selectivity and stronger binding affinity for mammalian nicotinic acetylcholine receptors (nAChRs) **(Nauen et *al.*, 2001; La Farre et *al.*, 2008; Kasiotis et *al.*, 2015).** For instance, desnitro-imidacloprid has been shown to have an affinity for mammalian nAChRs comparable to that of nicotine, highlighting its potential neurotoxic impact on non-target species **(Tomizawa and Casida, 1999; Tomizawa and Casida, 2000; Brunet et *al.*, 2004).**

2.5.7. Neurotoxicity

Neonicotinoids have been linked to neurotoxic effects, particularly at high doses. A review by **Sheets et *al.* (2016)** highlighted systemic toxicity, with evidence suggesting potential developmental neurotoxicity in rodents. For instance, gestational exposure to imidacloprid has been associated with sensorimotor deficits in offspring **(Abou-Donia et *al.*, 2008).** In vivo studies have reported neurotoxicity mediated through nicotinic acetylcholine receptors (nAChRs), although the precise mechanisms remain debated **(Rodrigues et *al.*, 2010; Lonare et *al.*, 2014; Ozdemir et *al.*, 2014)**. Notably, 28-day exposure to imidacloprid at doses of 45 and 90 mg/kg body weight caused a significant reduction in pain thresholds and spontaneous locomotor activity in rats, emphasizing its potential impact on neural function **(Lonare et *al.*, 2014)**.

3. Imidacloprid (IMI) : Novel Chloronicotinyl lnsecticide

3.1 Background

Imidacloprid (IMI) is a neurotoxic insecticide belonging to the first generation of systemic neonicotinoids and constituting approximately 25% of the current global insecticide market **(Swenson and Casida, 2013).**

Imidacloprid is indeed a neonicotinoid insecticide that was discovered in the 1980s. It was developed by researchers at Nihon Bayer Agrochem in Japan. The compound was identified through the screening of synthetic compounds with the goal of finding substances that exhibited a high affinity for insect nicotinic acetylcholine receptors (nAChRs) but had low toxicity to vertebrate species. **(Kagabu, 1997)**. Moreover, introduced to the market in 1991 by the Bayer Crop Science Group (BCSG), **(Tomizawa and Casida, 2003)**, it is a chloronicotynl neonicotinoid with the chemical formula N-{1-[(6-chloro-3-pyridyl) methyl]-4, 5-dihydroimidazol-2-yl} nitramide**.** This compound is a prominent member of a new class of systemic pesticides and is widely employed globally for insect and pest control, as well as seed treatment **(Shadnia and Moghaddam, 2008**). It has obtained registration in over 60 countries as a substance featuring an innovative or unconventional mechanism to counteract highly resistant insect pests **(Elbert et *al*., 1991; Elbert et *al*., 1996)**.

Imidacloprid is found in various commercial formulations, including Admire®, Confidor®, Gaucho®, Ministre®, Provado®, and Marathon® **(Meister, 1994).** Classified by the U.S. Environmental Protection Agency as Toxicity Class II and Hazard Class III, it necessitates labeling with the signal words "Warning"or "Caution" **(Meister, 1994).** Structurally akin to nicotine, its chemical structure is depicted below**:**

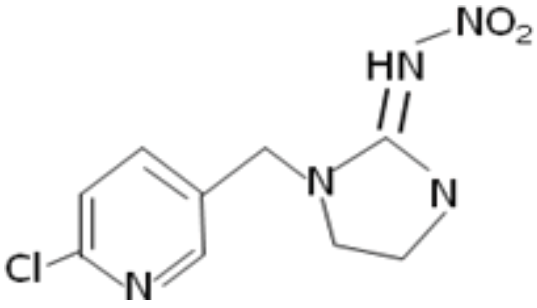


Figure 07: Chemical structure of the chloronicotinyl insecticide imidacloprid (IMI) or 1- (6-chloro-3-pyridylmethyl) –Nnitroimidazolidine-2-ylideneamine **[**$C_9H_{10}ClN_5O_2$]. **(Jeschke et *al*. 2011)**.

3.2. Physical and chemical properties of Imidacloprid

Imidacloprid, Chemical name**:** [1-[(6-chloro-3 pyridynil) methyl]-N-nitro-2-imidazolidinimine] has the molecular formula $C_9H_{10}C_lN_5O_2$ (code name: BAY NTN 33893**)**, with a molecular weight of 255.7 g mol^{-1}. IMI is made up of colorless crystals with a slight but characteristic odor. **(Tomlin, 2006)**. Imidacloprid is a stable product under normal storage conditions. Hazardous decomposition products are formed in the event of fire, such as carbon or nitrogen oxides, hydrochloric acid or hydrogen cyanide. **(Jargot et *al*., 2015)**.

IMI's physicochemical characteristics make it well suited for diverse application techniques, including foliar, seed treatment, soil drench, and stem application, as outlined in **Table 05** by **Millar and Denholm (2007)**. As a versatile and broad-spectrum systemic insecticide, IMI exhibits efficacy against various pests, encompassing sucking insects such as aphids, leafhoppers, whiteflies, and termites, as well as several species of Coleoptera, Diptera, Lepidoptera, and parasites on a variety of crops **(Tomlin, 1994; Tomizawa and Casida, 2005)**. Beyond agricultural use, IMI serves as a veterinary medicine for combating parasites and fleas in dogs and cats. Notable neonicotinoids, alongside IMI, include acetamiprid, clothianidin, dinotefuran, thiacloprid, and thiamethoxam.

Table 05: Physicochemical properties of Imidacloprid.

Physico-chemical properties	***Values***	***Ref. No.***
CAS number	**138261-41-3**	**(Tomlin, 2004)**
Water solubility at 20°C (mg L-1)	**(a) 510** **(b) 610**	**(a) (Yen and Wendt, 1993)** **(b) (Tomlin, 2004 ; Krohn and Hellpointer, 2002)**
K_{oc} (soil organic carbon–water partitioning coefficient)	**(a) 210 (20 °C)** **(b) 249-268** **(c) 109-411 (20°C)**	**(a) (Nemeth-Konda et *al*., 2002)** **(b) (Oi, 1999)** **(c) (Krohn and Hellpointer, 2002)**
Log K_{ow} (octanol-water partition coefficient)	**(a) 0.57** **(b) 0.92**	**(a) (Tomlin, 2004)** **(b) (Nemeth-Konda et *al*., 2002)**
Average application rates: soil (g ha^{-1}) and foliar (mg L^{-1}) applications	**(a) 50-320** **(b) 73-150**	**(a) (PMRA, 2005)** **(b) (www.bayercropscience.com)**

3.3. Imidacloprid mode of action

Imidacloprid (IMI) acts as a neurotoxicant by selectively targeting post-synaptic nicotinic acetylcholine receptors (nAChRs) in the nervous system. Its potency arises from its resistance to degradation by acetylcholinesterase, causing sustained activation of these receptors **(Matsuda and Sattelle, 2005)**.

These nAChRs, part of the cys-loop ligand-gated ion channel (LGIC) superfamily, are composed of five subunits forming a central ion channel. Each subunit features an extracellular N-terminal domain, four transmembrane domains, and a critical acetylcholine binding site formed by six loops within the N-terminal domain **(Talley et *al.*, 2008)**. Activation of these receptors permits the selective passage of Na^+, K^+, and Ca^{2+} ions, leading to membrane depolarization and signal transmission **(Sine and Engel, 2006)**.

IMI mimics acetylcholine (ACh) by binding to nAChRs at neuronal and neuromuscular junctions in insects and vertebrates. Unlike ACh, which is rapidly degraded by acetylcholinesterase, IMI persists at the receptor site, causing prolonged receptor activation. This sustained activation eventually desensitizes and blocks the receptors, resulting in paralysis and death **(Tennekes, 2010; Cox, 2001)**.

In mammals, nAChRs are found at both neuromuscular junctions and within the central nervous system. However, IMI demonstrates a significantly higher binding affinity for insect nAChRs than mammalian receptors, contributing to its selective toxicity **(Tomizawa and Casida, 1999; Alok et *al.*, 2013)**. This selective binding explains its efficacy as an insecticide while reducing its toxicity to mammals.

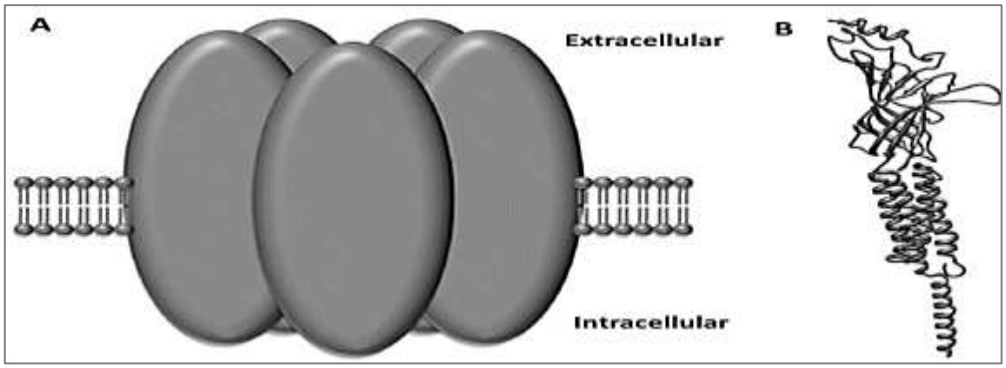


Figure 08: Nicotinic acetylcholine receptors (nAChR) and subunit structure**.** (A) Schematic representation of nAChR, presenting the pentameric arrangement of subunits around a central cation-permeable pore. (B) Three dimensional structure of an individual nAChR subunit showing the structure of the polypeptide backbone **(Millar, 2009).**

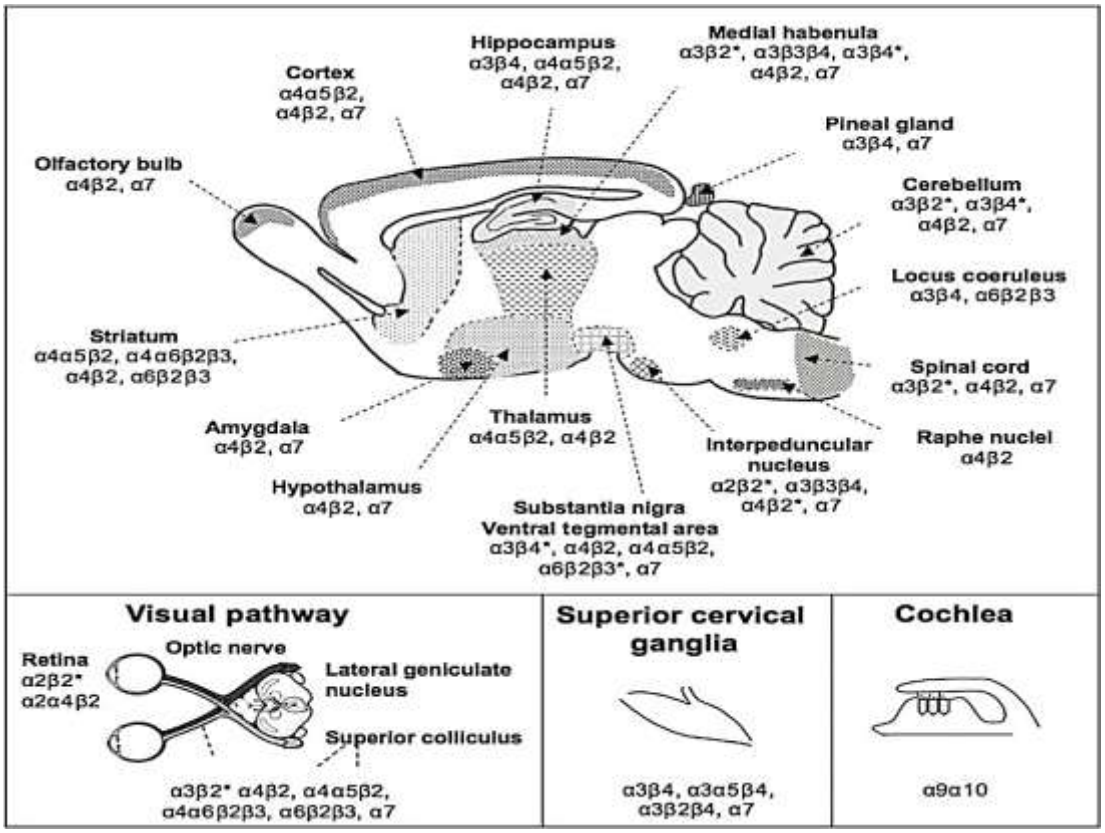


Figure 09: Distribution of nAChR Subtypes in the rodent central nervous system**:** Sagittal brain section illustrating nAChR expression in the cortex, cerebellum, hippocampus, interpeduncular nucleus, medial habenula, pineal gland, and superior cervical ganglia.

(As reviewed in Picciotto et *al*., 2001; Drago et *al*., 2003; Gotti and Clementi, 2004; Jensen et *al*., 2005).

3.4. Pharmacokinetics of Imidacloprid

3.4.1. Absorption and Distribution

IMI undergoes rapid and nearly complete absorption following oral administration in rats. Peak concentrations (C max) are observed 1-4 hours after oral doses of 1 or 20 mg/kg body weight in rats. The substance is extensively distributed throughout the body, with the exception of the central nervous system (CNS), fatty tissues, and the mineral component of bones. This distribution pattern holds true for both oral and intravenous (i.v.) administration, as reported by (**Klein, 1987)**. The highest concentrations are localized in the kidneys and liver.

3.4.2. Metabolism and Pathways

The metabolism of IMI has been documented in animals **(Schulz-Jander and Casida., 2002; Proenca et *al*., 2005 ; Kapoor et *al*., 2014).** IMI undergoes extensive metabolism, with about 78% of the recovered radioactivity, being in the form of metabolites. Up to 90% of the

administered dose is metabolized in the liver through two pathways **(WHO, 2001; USDA, Forest Service, 2005).** The main IMI metabolites identified in mammals are 4-hydroxy-IMI, 5-hydroxy-IMI, 6-hydroxy-IMI (6-hydroxy nicotinic acid), 6-chloronicotinic acid (6-CNA), 2-imidazolidone, olefin, guanidine (DNIMI), and urea derivatives **(Proenca et *al.*, 2005; Ding and Peng, 2015; Uroz et *al.*, 2001). (Figure 10).** Two major metabolic pathways are identified:

3.4.2.1. Aerobic Nitro-Reduction :

In the first route, IMI undergoes aerobic nitro-reduction by either the microsomal CYP450s or the cytosolic molybdo-flavoenzyme aldehyde oxidase (AOX) to yield nitrosimine (M07, IMI-NO) and amino-guanidine (M08, IMI-NH_2) derivatives and desnitro-IMI (M-09, IMI-NH) **(Dick et *al.*, 2007; Swenson and Casida, 2013).** Desnitro-IMI is further degraded by oxidative cleavage of the methylene bridge to imidazolidine, which is excreted in the urine, and 6-chloronicotinic acid that undergoes further metabolism via glutathione conjugation to form mercaptonicotinic acid and hippuric acid.

3.4.2.2. Second Major Pathway - Hydroxylation :

In the second major pathway, IMI is metabolized by hydroxylation of the imidazolidine ring by CYP_3A_4 to form 4-hydroxy and 5-hydroxy metabolites (M01 and M02, respectively). This goes on to form glucuronide conjugates, and dihydroxy IMI (M03). The hydroxylated compounds may lose water to form the olefin metabolite (M06). Differences in AOX expression and nitroreduction may result in species differences in toxicity and residue dissipation **(Swenson and Casida, 2013).**

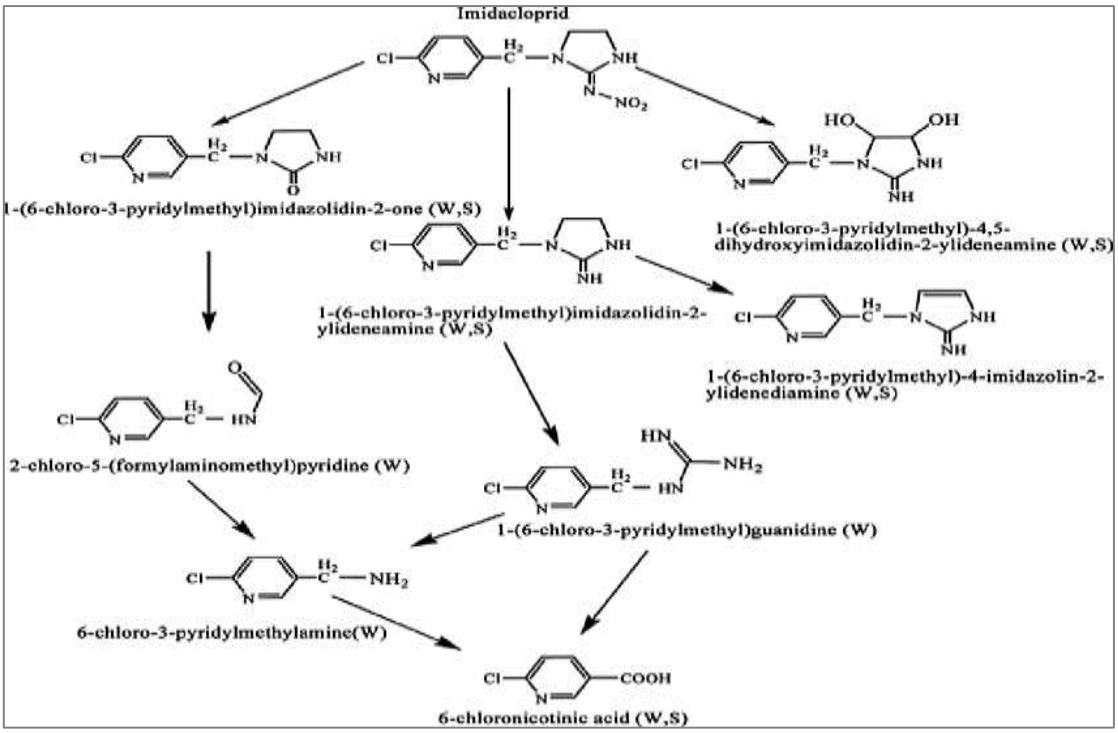


Figure 10: Metabolic pathway of Imidacloprid: Metabolites and degradation products identified in soil (S) and water (W). **(Spiteller and Bayer, 2000)**.

3.4.3. Excretion

Imidacloprid (IMI) is primarily excreted through urine, with around 75% of the administered dose eliminated this way. However, studies have detected IMI and its metabolites, including desnitro-IMI, in the brain after a single intraperitoneal dose in mice **(Ford and Casida, 2006)**. During chronic feeding studies in rodents, a biotransformation product, WAK 3839, was identified in urine, constituting 9% of total urinary radioactivity. This metabolite forms only under chronic exposure conditions when other metabolic pathways, such as oxidative cleavage to 6-chloronicotinic acid, are saturated, indicating a liver "flooding" effect **(Klein, 1990b)**.

Acute toxicity studies revealed that WAK 3839 is approximately five times less toxic than IMI in rats **(Kaoru, 1991)**. However, the toxicity of major IMI metabolites, such as 6-chloronicotinic acid, hydroxy-imidacloprid (4- or 5-OH), and olefinic-imidacloprid, remains unassessed in mammals. In invertebrates, olefinic- and hydroxy-metabolites showed similar acute toxicity to IMI, whereas 6-chloronicotinic acid lacked nicotinic agonist activity **(Nauen et *al.*, 2001a, b)**. Importantly, metabolites containing the 6-chloropyridinyl moiety fall within the established safety tolerances for IMI residues.

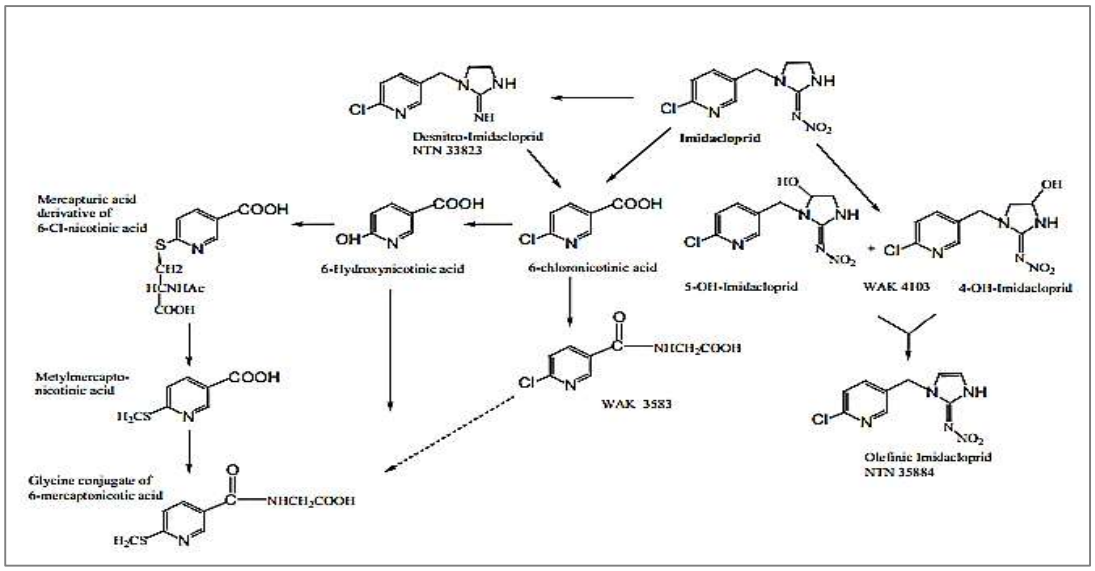


Figure 11: Biotransformation pathways of imidacloprid **(Thyssen and Machmer, 1999).**

3.4.4. Metabolite Activity

The desnitro-IMI metabolite is more active on mammalian α4β2 receptors than on corresponding insect systems. It is more toxic to mammals than the parent insecticide, showing high affinity and/or agonist potency at the mammalian α4β2 nAChR subtype, comparable to or greater than that of nicotine. **(Tomizawa and Casida, 2003)**.

3.5. Toxicological assessment of Imidacloprid

Imidacloprid (IMI) exhibits moderate acute oral toxicity in rodents, with LD50 values ranging from 380-650 mg/kg in rats and 130-170 mg/kg in mice. It is dermal and inhalation toxicity is low, and it does not cause skin or eye irritation or sensitization. Subchronic studies in rodents (98–107 days) identified reduced body weight and hepatotoxicity as primary effects, with the liver being a target organ in both rats and dogs. Histopathological changes in the testes and thyroid effects were also observed in some studies. Neurotoxicity studies established a NOAEL of 9.3 mg/kg/day in rats based on body weight reduction, while a NOAEL of 7.8 mg/kg/day was identified in dogs due to liver and central nervous system effects, including ataxia and tremors.

Chronic studies in rats highlighted thyroid toxicity, while mice experienced mild hepatotoxicity, reduced body weight, and mineralization of the thalamus. IMI demonstrated no oncogenic or genotoxic potential. Among its metabolites, desnitro-IMI was the most toxic, with an LD50 of 280 mg/kg, higher than IMI (500 mg/kg). Despite its increased affinity for mammalian nAChRs, its toxicity remains within acceptable limits, and the overall risk assessment for IMI encompasses its metabolites. **(EFSA, 2013)**.

Table 06: Summary of the toxicological features assessment of Imidacloprid **(Germany, 2005; EFSA 2008)**.

Imidacloprid	
Molecular Weight	255.7 **(Gervais, 2010)**
Acute toxicity	
• Rat LD_{50} oral	~ 500 mg/kg bw male 380 mg/kg bw female **(Gervais, 2010)**
• Oral LD_{50} mouse	130 mg/kg bw male 170 mg/kg bw female **(Gervais, 2010)**
• Rat LD_{50} dermal	> 5000 mg/kg bw
• Lethal/ near lethal dose for humans	< 44.6 ng/L (< 0.17 nM) Plasma concentration 5 hpost-ingestion in an intensive care survivor **(Mohamed, 2009)**
Short term toxicity	
• Target / critical effect	Body weight gain (rat, dog), liver (clinical chemistry and histopathological findings: rat, dog), trembling/tremor (dog)
• Lowest relevant oral NOAEL	90-d rat: 14 mg/kg bw per day 1-yr dog: 15 mg/kg bw per day
Long term toxicity and carcinogenicity	
• Target/critical effect	Body weight gain (rat, mouse), liver (blood chemistry: rat, mouse; organ weight decrease: mouse); thyroid (mineralisation in the follicular colloid: rat)
• Lowest relevant NOAEL	2-yr rat: 5.7 mg/kg bw per day 2-yr mouse: 65.6 mg/kg bw per day
• Carcinogenicity	No evidence for carcinogenicity
Reproductive toxicity	
• Reproduction target / critical effect	Parental bw gain decreased; fertility parameters unaffected; pup weight gain reduction at parentally toxic doses
• Lowest relevant reproductive NOAEL	Rat, 2-generation: parental: 20 mg/kg bw per day reproduction: 50 mg/kg bw per day offspring: 20 mg/kg bw per day
Developmental target / critical effect	Rat: maternal bw gain and food consumption decreased; developmental toxicity indicated by increase in skeletal variant (wavy rib). Rabbit: maternal bw gain and food consumption decreased, mortality; increased prenatal litter loss, reduced foetal weight, delayed ossification and skeletal anomalies at maternally toxic doses
Lowest relevant developmental NOAEL	Rat, maternal: 30 mg/kg bw per day Rat, developmental: 30 mg/kg bw per day Rabbit, maternal: 8 mg/kg bw per day Rabbit, developmental: 24 mg/kg bw per day
Neurotoxicity	
• NOAEL (acute neurotoxicity study)	42 mg/kg bw
• NOAEL (subchronic neurotoxicity study)	9.3 mg/kg bw per day
• NOAEL (developmental neurotoxicity study)	20 mg/kg bw per day (during gestation)

3.6. Neurotoxicity of Imidacloprid

In vivo studies, several reports showed neurotoxicity of neonicotinoids to nAChRs, but the specific mechanism was still disputed **(Rodrigues et *al.*, 2010; Lonare et *al.*, 2014; Ozdemir et *al.*, 2014)**. Neurotoxic effects of IMI were investigated in acute and subchronic tests. The effects acute and subchronic of Imidacloprid on the nervous system were highlighted starting from the behavioral changes of the rat of which a reduction in the motor activity, tremors, a reduction in the response to the stimuli, a reduction in the reflex of rectification and a hypothermia **(Tomizawa et *al.*, 2001; Shimomura et *al.*, 2002)**.

Studies have shown that exposure to imidacloprid (45 and 90 mg/kg body weight; orally) for 28 days can cause a significant decrease in the 247 pain threshold and spontaneous locomotor activity in rats **(Lonare et *al.*, 2014)**. In in vitro studies, exposure to imidacloprid at concentrations greater than 10 mM for < 1 min could damage membrane properties of the stellate cells in mice **(Bal et *al.*, 2010)**.

Imidacloprid was also observed as cytotoxic to cerebellar neurons from neonatal rats. Significant excitatory Ca^{2+} influxes were evoked by imidacloprid, which was an indicator of neural physiological activity convert low-activity. Therefore, neonicotinoids may adversely affect the developing brain **(Kimura-Kuroda et *al.*, 2012).** The impact of nicotinics has been reported in the expensive *Wistar* rat after the oral administration of 20mg / kg / day of imidacloprid. Their results showed the inhibition of the specific activity of acetylcholinesterase (AChE). Similar results have been reported by **(Rodrigues and *al..*, 2010)** .

Chapter II : Oxidative Stress

1. The concept of oxidative stress

The term "oxidative stress" was first introduced in 1956 in rubber chemistry **(Ore, 1956)** and later applied to biological contexts in 1970 to describe erythrocyte exposure to hydrogen peroxide **(Paniker et *al*., 1970)**. By 1985, it referred specifically to oxidative damage within cells and organs **(Sies and Cadenas, 1985).** Oxidative stress is now defined as an imbalance between the production of reactive oxygen/nitrogen species (ROS/RNS) and the organism's antioxidant defense capacity **(Persson et *al*., 2014)**. This imbalance has been implicated in the pathogenesis of numerous chronic diseases, including neurodegenerative disorders like Parkinson's, Alzheimer's, and Huntington's diseases, as well as emphysema, cardiovascular diseases, inflammation, cataracts, and cancer **(Toda, 2011; López-Alarcóna and Denicola, 2013)**.

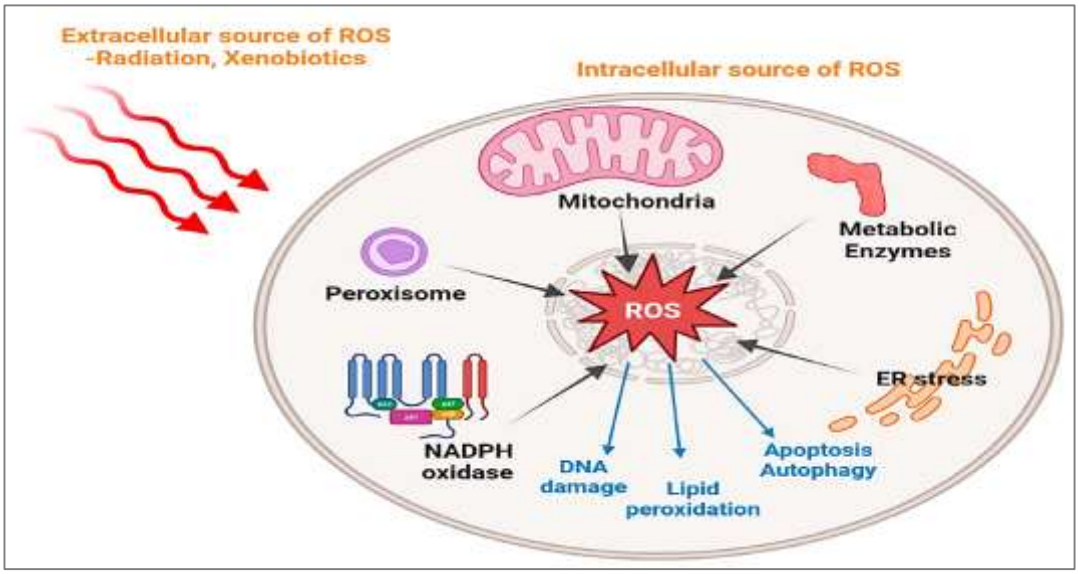


Figure 12: Schematic representation of oxidative stress induced by intracellular and extracellular stimuli**:** sources of ROS and their cellular consequences. **(Yuhang et *al*., 2024)** *Extracellular ROS sources include environmental factors like radiation and xenobiotics, while intracellular ROS are primarily generated in mitochondria, peroxisomes, endoplasmic reticulum, and through metabolic enzymes. Excess ROS can trigger apoptosis, autophagy, lipid peroxidation, and DNA damage*.

2. Antioxidant defense systems

The regulatory systems consist of antioxidant enzymes such as superoxide dismutases, catalase, and various forms of glutathione peroxidases. Additionally, they involve non-enzymatic antioxidants such as transferrin, albumin, vitamin C, vitamin E, glutathione, and uric acid. **(Krzystek-Korpacka et *al*., 2011)**.

2.1. Enzymatic antioxidant system

Enzymes exist endogenously, found in the cytoplasm, cytosol, or mitochondria, and serve to protect cells against free radicals produced physiologically during normal cellular metabolism. **(Jacob and Winyard, 2006; Menon and Goswami, 2007)**

- **Catalase (CAT):** Is the enzyme responsible for the reductive conversion of H_2O_2 to water. It is expressed in the majority of cells, organs, and tissues, with elevated concentrations observed in the liver and erythrocytes **(Sung et *al.*, 2013).**
- **Glutathione peroxidase:** An enzyme containing selenium, catalyzes the reduction of both H_2O_2 and organic hydroperoxides to water or corresponding alcohols. In this process, reduced glutathione serves as an effective electron donor, with free thiol groups being oxidized to form disulfide bonds, as depicted by the following reaction **(Dröge, 2002):** $H_2O_2 + 2\ GSH \rightarrow GS\text{-}SG + 2H_2O$
- **Superoxide dismutases:** Superoxide dismutases play a crucial role in the enzyme defense system against oxidative decay by converting superoxide radical anions into hydrogen peroxide (H_2O_2). **(Ghezzi, 2005)**. These enzymes are classified as metalloproteins. **(Garrel et *al.*, 2007)**.

2.2. Non-enzymatic antioxidants system

- **Reduced Glutathione (GSH):** Is a tripeptide comprising glutamic acid, cysteine, and glycine, recognized for its powerful antioxidant characteristics **(Menon et *al.*, 2007; Douris et *al.*, 2009)**. It serves as the primary antioxidant in regulating redox balance **(Biswas et *al.*, 2006)** and offers defense not only against free radicals but also against peroxides **(Tang et *al.*, 2006)**.
- **Uric Acid:** Uric acid, as the end product of purine metabolism, increases in plasma during intense physical exertion **(Baillie et *al.*, 2007)**. It has been suggested as one of the most potent antioxidants in vivo, potentially contributing to 35-60% of the total antioxidant capacity **(Finaud et *al.*, 2006; Johnson et *al.*, 2009).** Uric acid can undergo oxidation into various products and is regenerated by vitamin C **(Vasconselos et *al.*, 2007)**.
- **Vitamin C:** known as ascorbic acid, functions as a potent antioxidant by scavenging reactive oxygen species (ROS), thereby preventing or reducing their detrimental effects. It collaborates with vitamin E to eliminate free radicals and additionally restores the reduced form of vitamin E **(Foyer and Noctor, 2005)**.

- **Vitamin E (Alpha-tocopherol):** Combats lipid peroxidation of cell membranes and can interrupt the radical chain by creating a less reactive derivative that is incapable of attacking lipid substrates **(Descamps-Latscha et *al*., 2001)**.
- **Carotenoids:** Carotenoids, particularly beta-carotene, are effective physical quenchers of singlet oxygen, which plays a key role in UV-induced damage to the skin, cataracts, macular degeneration, and the formation of endoperoxides in diene structures **(Rozanowska et *al.*, 1998; Sies and Stahl, 2004; Huang et *al.*, 2005)**. While beta-carotene contributes to systemic photoprotection through its antioxidant effects, some studies have also noted potential prooxidant effects **(Godic et *al.*, 2014)**.

2.3. Phenolic antioxidants

- **Stilbene derivatives:** such as resveratrol, are classified as an antioxidant, cyclooxygenase inhibitor, phytoalexin, peroxisome proliferator-activated receptor stimulator, inducer of endothelial nitric oxide synthase, and activator of silent mating type information regulation 2 homolog 1 **(Nakata et *al.*, 2012)**.

- **Phenolic acids: (Including derivatives of benzoic and cinnamic acid)** Hydroxycinnamic acid derivatives, act as antioxidants by breaking chains. Their ability to scavenge free radicals is due to their propensity to donate hydrogen, which leads to the stabilization of the resulting phenoxyl radical **(Siquet et *al.*, 2006).**
- **Flavonoids:** Particularly Quercetin and rutin are well-researched flavonoids known for their antioxidant properties in lipid peroxidation systems. Quercetin, in particular, has been shown to inhibit cytotoxicity and oxidative changes in macrophages of low-density lipoproteins by preserving alpha-tocopherol levels and delaying lipid peroxidation **(Dewahlley et *al.*, 1990; Wang and Zheng, 1992; Peterson and Dwyer, 1998)**.
- **Zinc:** Zinc is essential as a cofactor for superoxide dismutase (SOD), supporting the body's antioxidant defense. In addition to its enzymatic role, zinc has anti-inflammatory and antioxidant properties. Studies show that zinc supplementation (45 mg as zinc gluconate) reduces markers of lipid peroxidation, such as hydroxyalkenals and malondialdehyde, compared to placebo. It also lowers mRNA expression of pro-inflammatory cytokines TNF-α and IL-1β in mononuclear cells stimulated with lipopolysaccharide, demonstrating its anti-inflammatory effects **(Prasad et *al.*, 2004)**.

3. Markers of oxidative stress

Oxidative stress, resulting from free radicals, induces tissue damage primarily by oxidizing proteins, DNA, or lipids **(Laight et *al*., 2000)**.

3.1. Markers of lipid peroxidation

Lipid peroxidation, a key indicator of oxidative stress, is one of the earliest and simplest effects of free radicals, involving the breakdown of lipids. The resulting byproducts, including conjugated dienes, hydroperoxides, and aldehydes, are commonly used as markers of oxidative damage **(Esterbauer, 1996)**. This process can damage both membrane phospholipids and circulating lipoproteins, leading to significant cellular consequences. Damage to membrane phospholipids disrupts membrane fluidity, impairs ion transport systems, and affects the function of various transporters and receptors, ultimately disrupting signal transduction pathways **(Favier, 2003)**.

3.2. Markers of protein oxidation

The function and activity of proteins can be affected by alterations in their complex structure, particularly through oxidation. Proteins may fragment or denature, leading to changes in their primary and secondary structures. Generally, oxidized proteins become inactive and are susceptible to protease action. During significant oxidative stress, cells may fail to eliminate accumulated oxidized proteins through proteolysis, resulting in the protein damage observed in diabetes **(Levine et *al.*, 2002)**.

4. Oxidative stress and Mitochondrial Dysfunction

Mitochondria are highly sensitive to changes in the cell's redox state. Numerous studies have demonstrated that the global inhibition of mitochondrial function during oxidative stress conditions can contribute to apoptosis **(Zamzami et *al.*, 1995; Morel, 1999)**.

Oxidative stress significantly affects mitochondrial function, as mitochondria are highly sensitive to cellular redox changes. Being the primary source of reactive oxygen species (ROS) and the site of ATP production, mitochondria are particularly vulnerable during oxidative stress, leading to global mitochondrial dysfunction and apoptosis **(Tan et *al.*, 1998; Grivennikova and Vinogradov, 2006)**. Severe oxidative stress disrupts mitochondrial membrane integrity through mitochondrial permeability transition (PT), a process allowing the influx of solutes such as protons, calcium, and glutathione, which uncouples the respiratory chain and exacerbates ROS production. This disruption halts ATP synthesis, depletes antioxidant reserves like reduced glutathione, and triggers calcium dysregulation, promoting lipid peroxidation and

cellular dysfunction **(Richter et *al.*, 1995; Zamzami et *al.*, 1995).** Maintaining calcium homeostasis is therefore critical for cellular survival and mitochondrial stability **(Thomas and Hanley, 1994)**.

5. Oxidative stress and neurodegenerative diseases

Oxidative stress plays a pivotal role in the development of neurodegenerative diseases, including Alzheimer's and Parkinson's. Lipid peroxidation depletes membrane phospholipids, contributing to Alzheimer's pathology **(Markesbery, 1997)**. Protein carbonylation, another oxidative damage marker, is observed in Alzheimer's-affected brain regions, such as the hippocampus and cortices, but not the cerebellum **(Hensley et *al.*, 1995, 1996)**. Oxidative stress-induced dysfunction of membrane proteins, including GLUT3 and Na+/K+ ATPases, disrupts calcium homeostasis and ion balance, triggering ROS production, cellular apoptosis, and neurodegeneration **(Feng and Wang, 2012)**. ATP depletion further impairs axonal transport and neurotransmission **(Luque-Contreras et *al.*, 2014)**. In Parkinson's disease, oxidative stress contributes to dopaminergic neuron loss in the substantia nigra and α-synuclein aggregation **(Lott, 2012).** Additionally, oxidative stress underlies Down syndrome neuropathology, exacerbating its progression to Alzheimer's **(Gandhi and Abramov, 2012)**. These mechanisms highlight the central role of oxidative damage in neurodegenerative disorders.

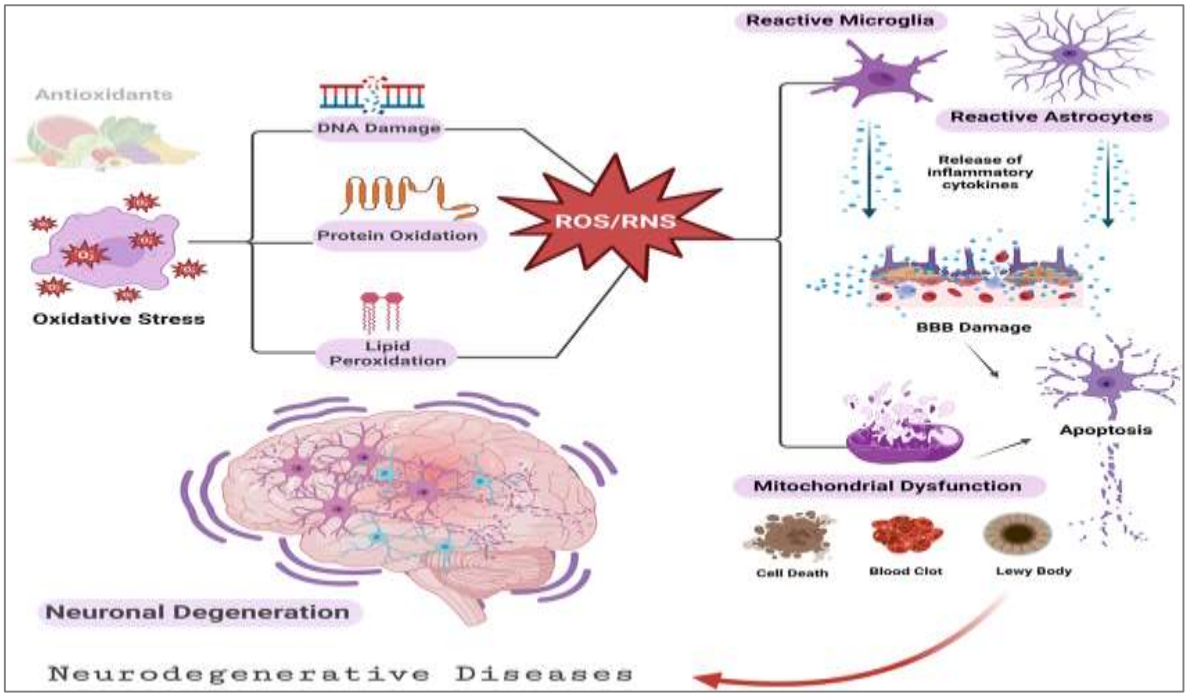


Figure 13: Schematic representation the connection between oxidative stress and neurodegenerative diseases. **(Ashok et *al.*, 2022)**

Chapter III: Melissa Officinalis L.: A Medicinal Plant Overview

1. General introduction to medicinal plants

Natural products serve as a vast reservoir of molecules characterized by intricate and diverse structures, posing significant challenges for chemists attempting synthesis in laboratories **(Graham and Depovere, 2002)**.

According to the **French pharmacopoeia (2013)**, a medicinal plant is defined as a "plant drug," following the European pharmacopoeia, possessing medicinal properties in at least one of its parts. A "plant drug" refers to a plant or part of a plant, utilized either in its fresh state or in dried form, serving as a natural raw material in pharmaceutical production **(Mohammedi, 2013)**.

Numerous plants exhibit high concentrations of phenolic compounds, a vital class of antioxidants **(Yoo et *al.*, 2010; Oancea et *al.*, 2012)**. Phenolic compounds are associated with various physiological properties, including antidiabetic, antiallergic, antifungal, anti-inflammatory, anticancer, antimicrobial, cardioprotective, and vasodilator effects **(Del Baño et *al.*, 2003; Cao et *al.*, 2015)**.

The utilization of medicinal plants for healing dates back thousands of years, representing a significant aspect of medical history and contributing substantially to the advancement of modern medicine **(Telefo et *al.*, 2012)**. This ancient traditional medicine serves as the precursor to contemporary practices such as phytotherapy and aromatherapy **(Girard, 2006)**.

2. Concepts and applications of phytotherapy and aromatherapy

2.1. Phytotherapy

The term "phytotherapy" derives from the Greek "phyton," meaning "plant," and "therapein," meaning, "to heal." consequently, phytotherapy involves the therapeutic use of medicinal plants **(Vacheron, 2011)**.

Phytotherapy encompasses a medicinal approach that employs either entire plants or their active constituents possessing therapeutic properties. These plants, referred to as "medicinal plants," are selected based on the parts containing the highest concentration of active compounds, including leaves, stems, branches, flowering tops, bark, roots, fruits, or flowers, which can be used in fresh or dried form.

Various preparation methods are employed based on factors such as the specific plant part, the nature of the active compound (hydrophilic or lipophilic), and the patient's individual characteristics **(Mohammedi, 2013)**.

2.2. Aromatherapy

Contrary to popular belief, aromatherapy involves more than just diffusing pleasant scents. Its true definition is rather precise; it represents a multifaceted approach to care, with aromatic essences from plants forming its foundation **(Shaw, 2007)**.

Aromatherapy falls under the umbrella of phytotherapy, focusing on the utilization of aromatic plants in various forms such as essential oils (substances secreted or extracted from plants), steam-distilled essential oils (essences obtained through steam distillation), aromatic hydrosols (distilled water separated from essential oils), or vegetable oils (extracts obtained by cold pressing plant parts, exclusively for external use) **(Gahbiche, 2009)**.

3. The Lamiaceae family and the Melissa genus

3.1. Botanical and medicinal importance of the lamiaceae family

The Lamiaceae family, also known as Labiatae, derives its name from the Latin "Labia," which refers to the lip-like shape of its flowers **(Couplan, 2000; Naghibi et *al.*, 2005).** Comprising 3200 to 4000 species spread across 200 to 220 genera, this family includes well-known plants such as mint, thyme, rosemary, lavender, and nettles. Its distribution spans both tropical and temperate regions worldwide, with the highest diversity observed primarily in the Mediterranean basin, Central Asia, the American continent, Pacific Islands, Equatorial Africa, and China **(Harley et *al.*, 2004)**.

Within Algerian flora, Lamiaceae play a significant role, although identifying certain genera can be challenging due to the considerable species variability **(Hammoudi, 2015).** These plants often feature glandular hairs and sub-epidermal glands that contain essential oils, contributing to their aromatic qualities **(Zeghib, 2013).** The economic value of Lamiaceae is substantial, owing to their various applications in medicine, cuisine, and cosmetics **(Taskova et *al.*, 1997; Kulišic et *al.*, 2006).**

3.2. Focus on the Melissa genus: A plant for health

The lemon balm, deriving its name from the Greek word for "bee," serves as a reminder of its attraction to bees seeking its nectar. It is also commonly referred to as lemongrass or lemon mint, although true lemongrass (*Cymbopogon nardus*) is an Asian grass. The English term for it is lemon balm, while Germans call it Zitronenmelisse or Melissenkraut **(Ronat, 2001).**

Melissa *officinalis*, an ancient plant with honey, culinary, and medicinal properties, was introduced by the Romans and ancient Greeks **(Andreas, 1998)**. Belonging to the Lamiaceae family, lemon balm is a robust, perennial, herbaceous plant with a short rhizome**.** It consists of a single species, Melissa *officinalis* L. There exist three subspecies, all of which are cultivars of the first mentioned subspecies **(Sari and Ceylan, 2002; Teuscher et *al*., 2005)**. However, it is the subspecies *officinalis* that finds therapeutic use **(Carnat et *al*., 1998)**.



Figure 14: Lemon balm (Melissa *officinalis* L.). **(Babulka, 2005)**

4. Historical context of Melissa *officinalis* usage

Melissa *officinalis* was introduced to Spain in the 10th century by the Arabs and later spread to Central Europe through Benedictine monks **(Teuscher et *al*., 2005)**. By the 10th century, Avicenna praised its cordial powers, describing it as a remedy for melancholy **(Delaveau et *al*., 1977)**.

The leaves of lemon balm have been traditionally used since the time of Theophrastus (372 - 287 BC) and Hippocrates (460 - 377 BC), and in the 15th century by Paracelsus (1493 - 1541), to improve digestive functions and alleviate nervousness **(Wichlt and Anton, 2003)**.

The Persian philosopher, writer, and physician Avicenna (980-1037) recommended it for strengthening the heart, stating that "it makes the heart joyful," and later discovered its positive effects on the digestive tract **(Grünwald and Jänicke, 2006)**.

English writer John Evelyn (1541 - 1620) described lemon balm as a plant that clarifies the mind, strengthens mental health, and dispels melancholy **(Bahtiyarca and Cosge, 2006)**.

From the 15th to the 17th century, the distillate (lemon balm water) was primarily used. It is an alcohol distillate of lemon balm. This, combined with lemon, cinnamon, coriander, cloves, and nutmeg, was used as an antispasmodic and stomachic **(Andreas, 1998; Delille, 2007)**.

5. Bio-geographical distribution and natural habitat

5.1. Worldwide distribution

Originally, native to the eastern Mediterranean (Balkans and Asia Minor) and western Asia, lemon balm is rarely found in the wild. It is also found in the south of France, southern Germany, Western Europe, particularly in Spain, and in North America **(Toth et *al.*, 2003; Dastmalchi et *al.*, 2008; Gotez and Ghedira, 2012)**.

It is a spontaneous species in the Moroccan Atlas, especially in the Agadir region. It is also found in Tunisia **(Hmamouchi, 1999; Aït Youssef, 2006)**. In general, it prefers to grow in bushes, moist and shaded ruderal areas, and woods **(Andreas, 1998; Gotez and Ghedira, 2012)**.

5.2. Presence in Algeria

In Algeria, occasionally cultivated in Kabylie **(Baba Aissa, 1999)**, lemon balm grows spontaneously in humid ravines in the Djurdjura, Babors, and Mouzaia mountains **(Beloued, 2001)**.

6. Botanical description of Melissa *officinalis*

6.1. Macroscopic description: external morphology

The leaves of Melissa *officinalis* are broadly ovate or ovate-lanceolate with crenate margins. The petiole typically ranges from 1 to 1.2 cm in length, while the lamina measures approximately 2.5 x 1 to 3.5 x 1.2 cm. Melissa plants typically have a stem reaching between 30 and 80 centimeters in height, branching out and forming tufts **(Thoby, 2009)**. The fruit consists of a tetracarpellate containing small brown seeds that are shiny. However, Melissa *officinalis* can sometimes be confused with other plants, particularly if it is collected in the wild **(Wichtl and Anton, 2003; Babulka, 2005)**.

6.2. Microscopic description: Internal structures

In cross-section, the leaf exhibits a single-layered upper and lower epidermis with glandular and non-glandular hairs. Glandular hairs are characterized by a unicellular circular head and uni- to multi-celled tail, while non-glandular hairs are unbranched, multicellular, with the outermost cells tapering and adorned with small bristles. The upper epidermal cells are generally larger than the lower ones, both covered with a cuticle. Palisade tissue forms a single layer continuous with the lamina, discontinuing at veins or midribs, where it is replaced by 3 to 4 layers of collenchymatous tissue on the upper side and 3 to 6 layers on the lower side of the leaf. Circular parenchyma tissues with large intercellular spaces follow the collenchyma. The vascular bundle is kidney-shaped and collateral, with vessel members mostly exhibiting spiral to reticulate thickenings and simple perforation plates, while tracheids are predominantly pitted. Stomata are indistinct **(Anonymous, 2007)**.



Figure 15: Lemon balm stem **(Girre, 1985)**.



Figure 16: Melissa leaf **(Rombi, 1991)**.

7. Systematic classification and chemical composition

7.1. Systematic classification of Melissa *officinalis*

In systematic terms, the placement of lemon balm in the phylogenetic classification by APG III "Angiosperm Phylogeny Group" (2009) is as follows **(Dupont and Guignard, 2007; Meyer et *al.*, 2008 ; Thoby , 2009)**:

- ***Kingdom*:** Plantae
  - ***Subkingdom*:** Tracheobionta
    - ***Division*:** Spermatophyta
      - ***Phylum*:** Spermaphytes
        - ***Subphylum*:** Angiosperms
          - ***Class*:** Eudicots
            - ***Subclass*:** Asterids
              - ***Order*:** Lamiales
                - ***Family***: Lamiaceae
                  - ***Genus* :** Melissa
                    - ***Genus and Species* :** Melissa *officinalis*

One of the synonyms for its species name is Melissa citriodorata **(Aït Youssef, 2006)**.

Lemon balm, characterized by its binomial name Melissa *officinalis* L., is part of the largest family within the Lamiales order, Lamiaceae. Plants within this family are recognized for their production of essential oils, typically found in glandular hairs. Consequently, aromatic herbs like thyme, rosemary, oregano, savory, basil, marjoram, as well as fragrant and medicinal species like hyssop, lavender, and common sage are members of this family. **(Dupont and Guignard, 2015)**. Lemon balm, notable for its fragrant qualities, releases a lemony scent when crushed or bruised, hence its occasional moniker, lemon balm. Its Latin name, Melissa, stems from the Greek term "***melissophyllon*,"** translating to bee herb, underscoring its appeal to bees.

Melissa *officinalis* L. encompasses several subspecies, including subsp. altissima, subsp. *officinalis*, and subsp. inodora. However, subsp. *officinalis* holds the most significance commercially, sought after for various purposes. It finds application as a condiment, in herbal infusions, as an ingredient in cosmetics, as an ornamental plant, and in therapeutic applications **(Sari and Ceylan, 2002)**.

7.2. Key chemical compounds: Phenols, Flavonoids, Essential Oils

The primary chemical compounds found in lemon balm include hydroxycinnamic acid derivatives, notably rosmarinic acid, caffeic acids, chlorogenic acid, and metrilic acid **(Englberger et *al.*, 1988;Triantaphyllou et *al*., 2001)**, as well as tannins **(Cohen et *al*., 1964; Kucera et *al*., 1965)**, flavonoids such as luteolin, luteolin 7-O-beta-D-glucopyranoside, apigenin 7-O-beta-D-glucopyranoside, and luteolin 3-O-beta-D-glucuronopyranoside **(Mulkens and Kapetanidis, 1986;Khayyal et *al.*, 2000)**, monoterpene glycosides **(Mikus et *al*., 2000),** sesquiterpenes including β-caryophyllene and germacrene **(Mikus et *al.*, 2000),** triterpenes **(CH and Krause, 1974),** and volatile oils such as citronellal, citral a (geranial), citral b (neral), methyl citronellate, ocimene, citronellol, geraniol, nerol, β-caryophyllene, β-caryophyllene oxide, linalool, and ethric oil **(Wagner and Bos, 1982; Sarer and Kokdil, 1991; Mrlianová et *al*., 2002; Patora et *al.*, 2003)**.

The volatile oil constitutes 0.5-0.1% of the plant's weight, with citronellal, geranial, and neral making up about 50-70% of this oil **(Wagner and Bos, 1982)**. Additionally, eugenyl glycoside has been isolated from lemon balm leaves **(Mosquero and Juan, 2004)**. Lemon balm tea has been found to contain 10 mg/L of essential oil (74% citral) and significant amounts of polyphenol compounds **(Carnat et *al*., 1998)**.

Steam distillates of lemon balm callus cultures yield dehydroabietane and another diterpene hydrocarbon, with their relative proportions varying considerably during cultivation passages **(Koch-Heitzmann and Czygan, 1985)**. The constituents of the plant's essential oil vary depending on various factors such as light intensity, nutrients, temperature, cultural practices, genotype, plant age, and harvesting time **(Moradkhani et *al.*, 2010).** Studies on the chemical composition of the essential oil from lemon balm leaves identify major compounds like citronellal, citral, and β-caryophyllene. Citral, a mixture of geranial and neral, is widely used in foods and cosmetics due to its strong lemon aroma and flavor **(Leung and Foster, 1996)**.

Furthermore, flavonoids such as luteolin-7-oglucoside have been detected in lemon balm. Lemon balm extracts contain hydroxycinnamic acid derivatives and flavonoids, including caffeic acid, m-coumaric acid, eriodictyol-7-O-glucoside, naringin, hesperidin, rosmarinic acid, naringenin, and hesperetin **(Dastmalchi et *al*., 2008)**.

8. Mechanisms of action and bioactive properties

8.1. Antioxidant mechanisms of lemon balm

Antioxidants function through various mechanisms, including acting as reducing agents, scavenging free radicals, forming potential complexes with pro-oxidant metals, and neutralizing singlet oxygen **(Lamaison et *al*., 1990; Madihi et *al*., 2013)**.

Lemon balm exhibits a high content of phenolics and displays antioxidant characteristics **(Vanova et *al*., 2005).** Both lemon balm extracts and rosmarinic acid have demonstrated antioxidant properties in vitro **(Tagashira and Ohtake, 1998; Hohmann et *al*., 1999; Triantaphyllou et *al*., 2001).** Moreover, rosmarinic acid and caffeic acid have shown significant antioxidant and immune-modulating effects **(Englberger et *al*., 1988; Lamaison et *al*., 1990; Peake et *al*., 1991)**.

The antioxidant activity of lemon balm has been demonstrated through the reduction of DPPH, indicating its ability to scavenge free radicals. Studies have revealed that the cytoprotective effect of lemon balm extracts observed in rats is partly attributed to its free radical scavenging properties **(Lamaison et *al*., 1990; Khayyal et *al*., 2000)**. Furthermore, lemon balm extracts have shown efficacy in protecting against oxidative damage induced by various pro-oxidant agents, which promote lipid peroxidation through different pathways. These extracts may inhibit the generation of early chemical reactive species that initiate lipid peroxidation or block common final pathways in the process of polyunsaturated fatty acid peroxidation. Lemon balm infusion has been found to enhance plasma levels of catalase, superoxide dismutase, and glutathione peroxidase, while markedly reducing plasma DNA damage, myeloperoxidase activity, and lipid peroxidation. The antioxidant potential of lemon balm is further enhanced by its iron (II) chelating activity **(Dastmalchi et *al*., 2008)**. Flavonoid aglycones have been identified as responsible for the free radical scavenging activity of lemon balm. Fractions containing flavonol glycosides, flavonol and biflavone aglycones, or chlorogenic acid type phenolics present in the ethanolic extract have shown significant inhibition of induced lipid peroxidation in rat cultured hippocampal neurons **(Ferreira et *al*., 2006)**.

8.2. Phenolic compounds and free radical scavenging

Phenolic compounds are widely recognized as crucial substances with antioxidant properties **(Shirzad et *al*., 2011; Madihi et *al*., 2013; Setorki et *al*., 2013)**. In order to maintain a balanced oxidative state, both plants and animals possess intricate systems of overlapping

antioxidants. These include internally produced antioxidants such as glutathione and enzymes like catalase and superoxide dismutase, as well as externally obtained antioxidants like vitamin C, vitamin A, and vitamin E through dietary intake.

8.3. Immunostimulatory and cytoprotective effects

The immunostimulatory effects of lemon balm extract have been demonstrated **(Drozd, 2003)**. In vitro studies have shown inhibitory effects of rosmarinic acid from lemon balm on porcine pancreatic amylase **(McCue, 2004)**. Furthermore, a novel pharmacological mechanism of action for the anxiolytic botanical Melissa *officinalis* L. (lemon balm) has been reported. The methanol extract was found to be a potent in vitro inhibitor of rat brain GABA transaminase (GABAT), an enzyme targeted in the therapy of anxiety, epilepsy, and related neurological disorders. Bioassay-guided fractionation led to the identification and isolation of rosmarinic acid [RA] and the triterpenoids ursolic acid (UA) and oleanolic acid (OA) as active principles. Phytochemical characterization of the crude extract determined that RA was the major compound responsible for the activity (40% inhibition at 100 microg/mL), comprising approximately 1.5% of the dry mass of the leaves. Synergistic effects may also contribute to the observed activity **(Awad et *al.*, 2009)**.

8.4. Memory enhancement and neuroprotective properties

The study investigated the role of the cholinergic system in the memory-enhancing activity of M. *officinalis* extract. The findings suggest that M. *officinalis* can improve memory, and the cholinergic properties of the extract may contribute to these observed effects. Therefore, M. *officinalis* extract holds potential therapeutic value in alleviating certain memory impairments observed in Alzheimer's disease **(Soodi et *al.*, 2014)**. Additionally, Melissa could be considered an antioxidant agent in the prevention of various neurological diseases associated with oxidative stress **(Peireira et *al.*, 2008)**.

8.5. Rosmarinic acid : Key antioxidant compound

In an animal study, Melissa *officinalis* aqueous extract exhibited potent antioxidative and neuroprotective properties, confirming its effectiveness in mitigating Mn-induced oxidative stress in the mouse brain **(Martins et *al.*, 2012).** Additionally, some of the individual compounds identified in the samples, particularly rosmarinic acid, which is regarded as the most potent antioxidant agent according to literature, could be accountable for the antioxidant activity of lemon balm **(Barros et *al.*, 2013)**.

9. Medicinal and therapeutic applications of Melissa *officinalis*

9.1. Traditional uses in folk medicine

Melissa *officinalis* L. (Lamiaceae), commonly referred to as lemon balm, has a history of being employed in traditional medicine for brewing tea known for its calming effects on nerves and its ability to relieve spasms **(Kennedy et *al.*, 2004;Ulbricht et *al*., 2005)**.

Historically, lemon balm has been purported to possess a range of medicinal qualities, including its ability to act as a sedative/tranquilizer, alleviate gas, reduce fever, exhibit antibacterial properties, and provide spasmolytic effects **(Nasri and Rafieian-Kopaei, 2013)**. Additionally, it has been associated with hypotensive effects, memory enhancement, induction of menstruation, and effects on the thyroid. Lemon balm has also been reported to have antiviral and antioxidant properties, as well as exhibiting antifungal, antiparasitic, and antispasmolytic activities **(Akhlaghi et *al*., 2011; Setorki et *al.*, 2013).** Moreover, it has been suggested to alleviate symptoms of flatulence, asthma, bronchitis, amenorrhea, cardiac failure, arrhythmias, ulcers, and wounds. Additionally, lemon balm has been cited for its effectiveness in treating various conditions such as headaches, indigestion, colic, nausea, nervousness, anemia, vertigo, syncope, malaise, insomnia, epilepsy, depression, psychosis, and hysteria **(Wolbling and Leonhardt, 1994)**.

9.2. Modern pharmacological effects

The significance of lemon balm in phytotherapy is relatively recent, despite the fact that lemon balm leaves are recognized in all European pharmacopoeias. The flowering parts of this plant (Melissae herba) and the essential oil extracted from its leaves (Melissae aethroleum) are also utilized. The plant contains 0.02-0.2% essential oil characterized by the presence of citronellal and citral, along with geraniol, nerol, cymene, and citronellol. In Spain, certain lemon balm plantations yield 0.8% essential oil. It also contains sesquiterpenes, terpenes, and tannins typical of the Lamiaceae family. **(Bisset, 1994; Tisserand and Balacs, 1995; Leung and Foster, 1996; Gardiner, 2000; Tóth et *al*., 2003)**.

Lemon balm extracts (aqueous extract, hydroalcoholic extract, cryobroyat) are therapeutic elements associated with sedative, muscle-relaxant, antispasmodic, carminative, gastric tonic, cholagogue, antioxidant, antiviral (against influenza and herpes viruses), and potentially anti-inflammatory and immunostimulant effects. **(Naschimento et *al*., 2000 ; Drozd and Anuszewska, 2003 ; Sadraei et *al.*, 2003 ; De Sousa, 2004 ; Iauk et *al.*, 2003)**.

External or internal preparations of lemon balm leaf are targeted at functional disorders such as cardiac issues, gastrointestinal disorders, insomnia, migraines, menstrual nervousness, and herpes. **(Wagner and Wiesenauer, 1995; Perry et *al*., 1999; Akhondzadeh et *al*., 2003; Reader's, 2004)**.

Combining lemon balm with other plants is commonly used to address nervous disorders such as functional gastrointestinal and enteric ailments. Medicinal plants often paired with lemon balm include hops cones, passionflower, or valerian **(Blumenthal, 1998)**. Hydroalcoholic extracts of lemon balm leaf are taken to alleviate symptoms associated with difficult digestion and menstrual disorders. It is also frequently combined with aromatic plants. The daily dosage of lemon balm, when used alone, ranges from 4 to 9 grams, or from 2 to 6 milliliters per day of tincture (1:5) **(ESCOP, 1996)**.

Practical Section

Part one: In vitro study: Phytochemical Properties and Pharmacological Potential of Melissa Officinalis L.

1. Plant material (PM)

This study aims to enhance the value of Melissa *officinalis L.*, a medicinal plant that grows spontaneously in various regions of Algeria, by investigating its antioxidant properties. The valorization of its secondary metabolites opens new prospects in the phytopharmaceutical industry. The selection of this plant was primarily based on its importance and frequency of use, as determined through an ethnopharmacological survey conducted among traditional healers, herbalists, as well as medicinal plant users and vendors.

Melissa *officinalis L.*, also known as lemon balm, bee balm, or honey balm **(Rasmussen and Prim, 2011),** is a medicinal and aromatic plant belonging to the Lamiaceae family. It is a perennial herb widely distributed in Algeria, characterized by its lemon-scented leaves. The plant can grow up to 1 meter in height and has soft, hairy leaves ranging from 2 to 8 cm in length **(Kothe HW, 2007; Zargari, 1991)**.

1.1. Identification of the plant species

The identification of this plant was confirmed at the Laboratory of Plant Biology, Faculty of Natural and Life Sciences, University Echahid Cheikh Larbi Tebessi – Tebessa.



Figure 17 : Lemon balm (Melissa *officinalis* L.)

2. Preparation of the aqueous extract of Melissa *officinalis* L.

2.1. Solid-Liquid extraction (Maceration)

a. Principle

The preparation of the extracts was performed using the solid-liquid exhaustive extraction method (Maceration) described by **Romani et *al.* (2006)**. The advantage of maceration lies in preserving the active compounds during the process **(Sophie et *al.*, 2003)**, which justifies the choice of this extraction method in the present study.

b. Technique

The plant material was harvested in the Wilaya of Tébessa during September 2020. The extract of M. *officinalis* L. was prepared from its powdered aerial parts. A quantity of 10 g of the finely ground plant powder was mixed with 100 mL of distilled water, following a solid-to-liquid ratio of 1:10 (mg/mL) **(Michel, 2011)**. The mixture was stirred using a magnetic stirrer for 24 hours, protected from light, at room temperature, and at a rotational speed of 200 rpm.

The resulting solution was filtered using filter paper (Whatman No. 2, diameter 0.2 µm), and the filtrate was collected. The purpose of the filtration step was to separate the liquid and solid components of the mixture by passing it through a filtering medium.

Subsequently, the solvents in the filtrates were removed via evaporation using a rotary evaporator equipped with a vacuum pump and a control valve. During evaporation, the flask containing the filtrate mixed with the solvent was rotated and immersed in a heated water bath at 60°C. After the solvents were evaporated, the dry extract was reconstituted to 30% with sterile distilled water, labeled, and stored in glass bottles at 4°C, protected from light, until further use **(Figure 18)**.

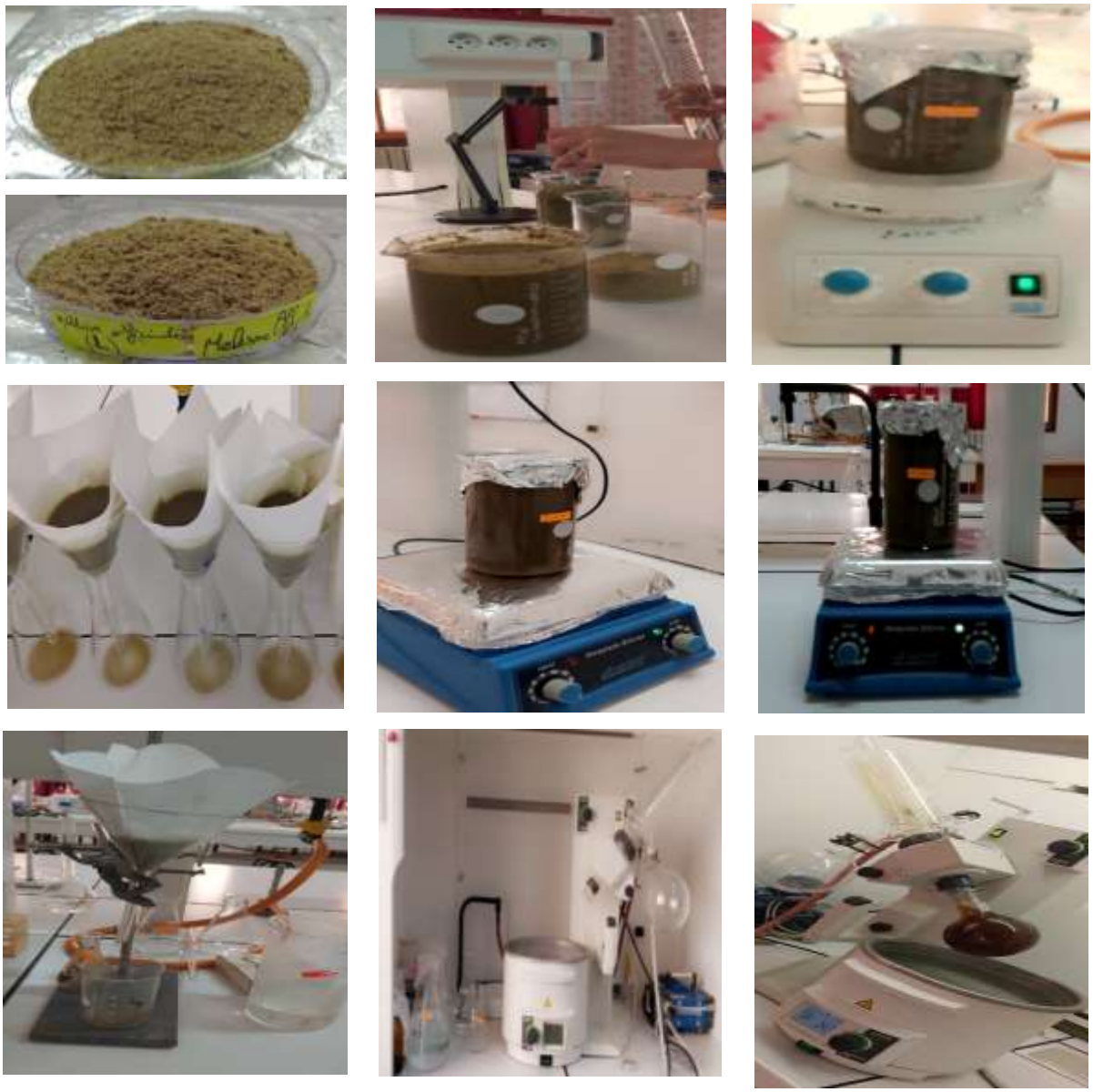


Figure 18: Different steps in the preparation of the aqueous extract**.** A: Maceration, B: Filtration, C: Separation. **(*Personal photos*)**.

3. Determination of Extraction Yield

The extraction yield is the ratio between the mass of the extract obtained after solvent evaporation and the mass of the dry plant material. It is calculated using the following formula:

$$Rd\ (\%) = (M_{ext} / M_{mvs}) \times 100$$

Where**:**

Rd: Yield (%); ***M ext:*** Mass of the dry extract expressed in grams (g); ***Mmvs:*** Mass of the dry plant material expressed in grams (g).

4. Analyse qualitative Phytochemical Screening

Phytochemical studies are conducted to detect the presence or absence of secondary metabolites in Melissa *officinalis*. The aim of these tests is to identify various classes of secondary metabolites such as anthocyanins, tannins, flavonoids, alkaloids, saponins and quinones… This chemical screening provides valuable insights into the potential pharmacological activities of the plant.

a. Principle

Phytochemical tests involve detecting various families of compounds present in the plant part under study through qualitative characterization reactions **(Table 07)**. These reactions are based on precipitation or coloration phenomena specific to each compound family, proportional to the quantity of the substance sought. **(Girre, 1980)**.

b. Methodology

- **Samples :**
    - Melissa *officinalis* Powder
    - 10% (w/v) Melissa *officinalis* infusion
- **Qualitative reactions :**
    - Specific reagents are used for each compound family.
    - Reactions are based on precipitation or color change phenomena, proportional to the amount of the substance present.
- **Results classification** (According to **Négué Diarra, 2003**) :
    - Strongly positive reaction **: +++**
    - Moderately positive reaction **: ++**
    - Weakly positive reaction **: +**
    - Absence of the substance **: -**

Table 07: Methods for identifying secondary metabolite classes.

Class of secondary metabolites	*Method used*	*Presence indicator*	*References*
Alkaloids	0.5 ml of HCl (1%) mixed with 1.5 ml of each extract, followed by 3 drops of Wagner's reagent (2 g Potassium iodide +1.27 g Iodine+qsp 100 ml Distilled water)	Formation of a cream-colored precipitate.	**Maria John et *al*., 2015**
Terpenoids	2 ml of chloroform and 3 ml of concentrated sulfuric acid added to 5 ml of extract.	Formation of a reddish-brown ring.	**Khan et *al*., 2011**
Steroids	Etheric organic extract mixed with chloroform, acetic anhydride, and concentrated sulfuric acid. (Take 1g of plant material and macerate for 24 hours in petroleum ether or benzene. After filtration and introduction into a 100 ml Erlen Meyer, the solvent is evaporated in a sand bath. The residue is recovered with 1 ml of chloroform, 1 ml of acetic anhydride and 3 drops of concentrated sulfuric acid.)	Color change to violet, turning green after 30 minutes.	**Lieberman-Burchard (Method)**
Anthocyanins	Addition of a few drops of hydrochloric acid (HCl 0.1 N) to 5 ml of the extract.	Development of a red color.	**Dahon, 2003**
Flavonoids	1 ml of extract mixed with 100µl concentrated HCl and magnesium chips.	Red or orange coloration after 3 minutes.	**Karumi et *al*., 2004**
Leucoanthocyanins	Similar reaction to flavonoids but conducted in a water bath without magnesium.	Formation of a red color.	
Saponins	Shaking of 10 ml of the extract in a test tube, followed by resting for 15 minutes.	Persistent foam greater than 1 cm.	**N'Guessan et *al*., 2009**
Sterols and Triterpenes	Lieberman-Burchard reaction using 5ml of the extract in 1ml acetic anhydride and 0,5ml concentrated sulfuric acid.	Purple or violet ring evolving to blue and then green.	**Edeoga et *al*., 2005**
Polyphenols	2 ml of extract mixed with a few drops of $FeCl_3$ (2%).	Blue-black or green coloration.	**Bidie et *al*., 2011**
Tannins	5 ml of extract mixed with a few drops of $FeCl_3$ (5%).	Greenish or blue-black coloration.	**Karumi et *al*., 2004**
	• ***Catechin Tannins*** 7 ml of Stiasny reagent (30% Formaldehyde, concentrated HCl: 1/0.5) are added to 15 ml of infusion. This mixture is heated in a water bath at 90°C for 15 min.	Red coloration	
	• ***Gallic Tannins*** Addition of 2 g of sodium acetate to 5 ml of extract, followed by a few drops of $FeCl_3$ (1%).	Dark blue coloration.	
Free Quinones	Maceration of 5 g of plant material with 10 ml petroleum ether, After filtration, 2 ml of filtrate are stirred with 2 ml of 10% NaOH.	Red coloration turning to violet.	**Oloyede, 2005**
Free Anthraquinones	1 ml of chloroformic extract mixed with 1 ml diluted NH_4OH.	Red coloration.	**Awor and Samseny, 2003**

5. Structural characterization techniques

5.1. X-ray Diffraction (XRD)

a. Objective

X-ray Diffraction (XRD) is a crucial technique for characterizing the crystalline structure of materials, particularly in the context of our pharmaco-toxicological study aimed at analyzing the crystalline composition of the Melissa *officinalis* plant to identify potentially active antioxidant compounds against imidacloprid toxicity.

b. Protocol

The extract samples are prepared by drying the extracts in a ventilated oven at a temperature of 40-50°C to remove any residual moisture, then sieving them through a 32 µm sieve to ensure a homogeneous particle size. The resulting powder is evenly spread on a flat sample holder to ensure optimal diffraction. The analysis is conducted using a diffractometer equipped with a Cu Kα source (λ = 1.5406 Å), operating at a voltage of 40 kV and a current of 30 mA. Measurements are taken within an angular range of 5° to 80° (2θ), with a scanning speed of 2°/min and a step size of 0.02°. The data obtained as diffractograms are processed using specialized software, such as DIFFRAC Plus, and compared with reference databases like ICDD PDF-4+ to identify the crystalline phases present. This step allows for the analysis of the purity, quality, and stability of the active compounds. This protocol, compliant with European Pharmacopoeia standards, ensures reliable and reproducible analyses for a precise evaluation of the integrity of the studied extracts **(Cullity and Stock, 2001)**.



Laboratory Sifter ***X-Ray Diffractometer*** ***Software Assessment***

Figure 19: Characterization of Melissa *officinalis* L: X-ray Diffraction Analysis **(*Personal photos*).**

5.2. Scanning Electron Microscopy (SEM) Objective

a. Objective

Scanning Electron Microscopy (SEM) is an essential technique for examining the surface morphology and topography of materials at a high resolution. In this protocol, SEM is used to assess the surface characteristics of Melissa *officinalis* L. and to evaluate the potential distribution and release of bioactive compounds.

b. Protocol

The sample preparation involves drying the Melissa *officinalis* plant material (leaves or extracts) in an oven at 40-50°C to remove moisture. The dried material is then finely powdered and sieved using a 32 µm sieve to achieve a consistent particle size. The powder is subsequently placed on a conductive stub or holder, and a thin layer of gold or palladium is applied to the surface using a sputter coater to enhance conductivity and reduce charge buildup during imaging.

The sample is then mounted in the SEM chamber, which is maintained under high vacuum conditions. The SEM is operated at an acceleration voltage of 10-20 kV, depending on the desired resolution. Imaging is conducted at varying magnifications, typically ranging from 500x to 50,000x, to observe fine details of the surface structure. The electron beam scans the sample's surface, producing secondary electrons that are detected to generate high-resolution images.

The obtained images are analyzed to assess the surface topography, particle distribution, and any structural features that may correlate with the release patterns of the bioactive compounds. Specialized software (SEM image processing tools) can be used for quantitative analysis of surface features, such as pore size, surface roughness, and particle morphology. **(Goldstein et *al.*, 2003)**.



Figure 20: Characterization of Melissa *officinalis* L: Scanning Electron Microscopy (SEM) analysis**. (*Personal photos*).**

5.3. Infrared Spectroscopy (IR)

a. Objective

Infrared Spectroscopy (IR) is a valuable technique used to identify and characterize the functional groups and molecular structures of bioactive compounds. In this study, IR spectroscopy is employed to analyze the chemical composition of Melissa *officinalis* L**.**, specifically focusing on identifying the key functional groups responsible for its antioxidant activity.

b. Protocol

The sample preparation involves drying the Melissa *officinalis* plant material in a ventilated oven at 40-50°C to remove any residual moisture. The dried sample is then ground into a fine powder. A small amount of the powdered sample is mixed with potassium bromide (KBr) to form a thin pellet. A small quantity (about 1-2 mg) of the sample is typically used in combination with approximately 200 mg of KBr. The mixture is then pressed into a translucent pellet under high pressure.

The sample is placed in the sample compartment of the IR spectrometer. The analysis is conducted using a Fourier Transform Infrared (FTIR) spectrometer, typically equipped with a mid-IR detector. The spectral range for the analysis is usually from 4000 cm^{-1} to 400 cm^{-1}. A resolution of 4 cm^{-1} is typically used to obtain a detailed spectrum. The spectrum is recorded by measuring the absorption of infrared light by the sample at different wavelengths.

The resulting IR spectrum is analyzed to identify the characteristic absorption bands that correspond to specific functional groups (e.g., hydroxyl, carbonyl, and aromatic groups). Comparison with reference spectra from standard compounds or databases, such as the NIST IR database, allows for the identification of the chemical components in the sample **(Silverstein et *al*., 2005)**.



Figure 21: Characterization of Melissa *officinalis* L: Infrared Spectroscopy (IR) analysis. **(*Personal photos*).**

6. Quantitative analysis

6.1. Total polyphenol content

- **Principle**

Polyphenols were determined by spectrophotometry using the Folin-Ciocalteu method as described by **Singleton and Rossi (1965)**. The Folin-Ciocalteu reagent, a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), is reduced in the presence of polyphenols to form a blue oxide complex of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}) **(Ribéreau-Gayon, 1968)**.

- **Procedure**

A volume of 200 µL of dry extract at various concentrations was placed into test tubes, followed by the addition of 1 mL of Folin-Ciocalteu reagent (10%). The mixture was carefully stirred and incubated at room temperature for 3 to 5 minutes. Then, 800 µL of sodium carbonate (Na_2CO_3, 7.5%) was added. The tubes were mixed and incubated for 120 minutes.

The absorbance was measured at 760 nm using a spectrophotometer. A calibration curve was prepared under the same conditions using gallic acid. Results were expressed as milligrams of gallic acid equivalent per gram of dry extract.

6.2. Flavonoid content

- **Principle**

Flavonoids form yellowish complexes during metal chelation; in this method, aluminum ions (Al^{3+}) are used, derived from aluminum chloride ($AlCl_3$). The complexes formed are responsible for light absorption in the visible spectrum, which is proportional to the flavonoid concentration **(Ribéreau-Gayon, 1968)**.

- **Procedure**

Flavonoid content in the extract was measured using a colorimetric method based on the protocol of **Jain et *al*. (2011)**, with quercetin as the standard. A volume of 1 mL of each extract at various concentrations was reacted with 1 mL of aluminum chloride (2%). After incubation at room temperature for 1 hour, the absorbance of the reaction mixture was measured at 430 nm. The total flavonoid content was expressed as milligrams of quercetin equivalent per gram of dry extract.

6.3. Condensed tannin content

- **Principle**

The measurement of condensed tannins is based on the reaction of polyphenolic compounds with vanillin ($C_8H_8O_3$) in an acidic medium **(Price et *al.*, 1978).**

- **Procedure**

Condensed tannin content in the extract was determined following the protocol of **Oyedemi and Afolayan (2011),** using catechin to prepare the calibration curve. A volume of 0.5 mL of extract was reacted with 3 mL of vanillin solution (4%) prepared in methanol and 1.5 mL of HCl (37%). A calibration curve was established under the same conditions using catechin. After incubation at room temperature for 15 minutes, the absorbance was measured at 500 nm. The total proanthocyanidin content was expressed as milligrams of catechin equivalent per gram of dry extract.

7. Evaluation of the antioxidant activity in the Melissa *officinalis* L. Extract

The neutralizing antioxidant powers of the aqueous extract of M. *officinalis* were demonstrated using various chemical techniques, including the DPPH radical scavenging assay, ABTS scavenging activity, cupric reducing antioxidant capacity (CUPRAC), and ferric reducing antioxidant power assay (FRAP).in order to take into consideration the nature of bioactive molecules through their various mechanisms actions and the capacity of radical scavenging **(Li et *al.*, 2008)**.

7.1. DPPH free radical scavenging assay

The antioxidant capacity of the extract, through the neutralization of the $DPPH^{\bullet}$ radical, was evaluated using the method described by **Blois (1958)**. In the DPPH assay, antioxidants reduce the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, which has a purple color, to a yellow compound (2,2-diphenyl-1-picrylhydrazine). The intensity of the yellow color is inversely proportional to the antioxidant capacity of the tested extract to donate protons **(Sanchez-Moreno, 2002)**.

A volume of 100 µL of the extract at various concentrations was mixed with 2.5 mL of a methanolic DPPH solution. The mixture was shaken and incubated in the dark for 30 minutes. The absorbance of the solution was measured at 517 nm.

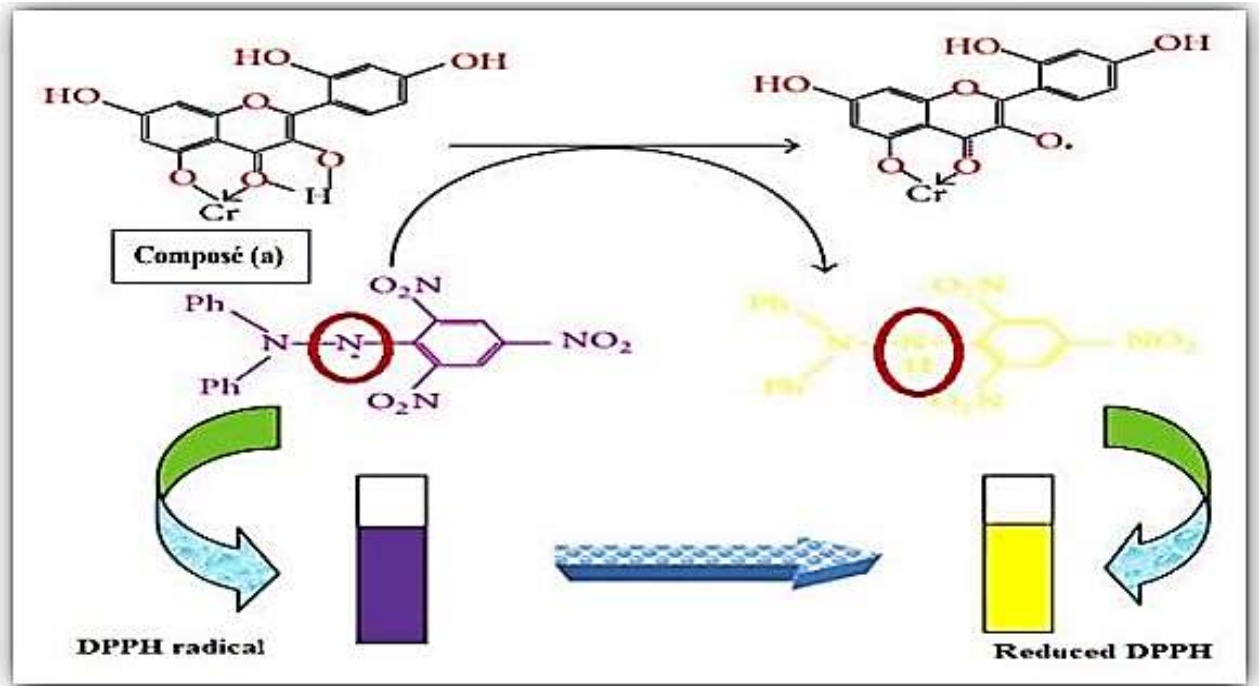


Figure 22: Reaction of an antioxidant with the DPPH radical. **(Bounab S., 2020)**

The DPPH radical scavenging activity was calculated using the following equation**:**

Scavenging Activity % = 100 × (A control -A sample) /A control

Where :

- **A control:** The absorbance of the control reaction (containing all reagents except the tested extract).
- **A sample:** The absorbance of the tested extract.

➢ **Determination of the median inhibitory concentration (IC50)**

The IC50 is defined as the amount or concentration of antioxidants (extract or any other antioxidant substance) required to inhibit or eliminate 50% of the radicals. The IC50 values are calculated graphically by plotting inhibition percentages against the different concentrations of the tested extracts. For each extract or reference antioxidant, a linear regression curve (Y = aX + b) is established to calculate the IC50, which characterizes the antioxidant potential of the extracts. A low IC50 value indicates strong antioxidant activity **(Molyneux, 2004)**.

7.2. $ABTS^{\bullet+}$ radical cation scavenging activity

The ability to scavenge the $ABTS^{\bullet+}$ radical cation ($C_{18}H_{18}N_4O_6S_4$) was determined using the method of **Re et *al*. (1999)**. This method is based on the capacity of an antioxidant to neutralize the $ABTS^{\bullet+}$ radical cation (2,2'-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid), which has a blue-green coloration, and convert it to the colorless $ABTS^{-}H^{+}$ by donating a hydrogen atom **(Antolovich et *al*., 2002)**.

The ABTS•+ radical solution was prepared by mixing 2.45 mM ABTS with 7 mM potassium persulfate. After 16 hours of incubation, the $ABTS^{\bullet+}$ solution was diluted with ethanol to achieve an absorbance of 0.7 ± 0.02 at 734 nm.

A volume of 2 mL of the $ABTS^{\bullet+}$ solution was added to 20 µL of the extract at various concentrations. The absorbance was measured at 734 nm after 6 minutes of incubation in the dark. A control sample was prepared under the same conditions, except the extract was replaced with the extraction solvent.

The percentage of $ABTS^{\bullet+}$ radical scavenging activity was calculated using the following formula:

Inhibition percentage (%) = [(A control- A sample)/A control] × 100

Where:

- **A control:** Absorbance of the control sample.
- **A sample:** Absorbance of the tested extract.

7.3. Antioxidant capacity test by copper reduction (CUPRAC)

The copper ion (Cu^{2+}) reduction capacity was determined using the CUPRAC method described by **Apak et *al*. (2007**). A solution was prepared by mixing the following volumes: 50 µl of Cu (II) (10 mM), 50 µl of neocuproine (7.5 mM), and 60 µl of NH_4Ac buffer solution (1 M, pH = 7.0). Different concentrations of the extract were added to the initial mixture to make the final volume 200 µl. The 96-well microplates were kept in the dark, and after 1 hour, the absorbance was measured at 450 nm.

The reduction capacity of the extracts was compared to those of BHA and BHT. The results were calculated as A0.5 (µg/ml), corresponding to the concentration that gives an absorbance of 0.50.

7.4. FRAP assay (Ferric reducing antioxidant power)

The reducing power (RP) of the various extracts was estimated using the method of **Yildirim et *al.* (2001)**. This method evaluates the reducing power based on the reduction of the ferric iron complex (Fe^{3+}) of ferricyanide into ferrous iron (Fe^{2+}) in the presence of reducing antioxidants. The resulting green coloration is proportional to the reducing power of the extract **(Gülçin et *al*., 2003; Bijoy et *al*., 2008)**.

A volume of 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide ($C_6N_6FeK_3$) (1%) were added to 1 mL of each extract at different concentrations. After 20 minutes of incubation at 50°C, 2.5 mL of trichloroacetic acid ($C_2HCl_3O_2$) (10%) was added to the mixture.

The mixture was centrifuged at 3000g for 10 minutes. Then, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride ($FeCl_3$) (0.1%). The absorbance was measured at 700 nm.

Part Two: In vivo study: Neurotoxicity Assessments of Imidacloprid and the Neuroprotective Effects of Melissa Officinalis L.

In vivo study

1. Tested compounds and chemicals

1.1. Chemical product

The chemical used in this study is Imidacloprid, a systemic insecticide from the neonicotinoid class, part of the chloronicotinyl nitroguanidine chemical family. Its International Union of Pure and Applied Chemistry (IUPAC) name is 1-[(6-chloropyridin-3-yl) methyl]-N-nitroimidazolidin-2-imine. Imidacloprid has a molecular formula of $C_9H_{10}C_lN_5O_2$, and its CAS (Chemical Abstracts Service) registry number is 138261-41-3 **(Tomlin, 2006)**.

For the evaluation of biological parameters, we used products and reagents mainly from sigma, Germany and Biochem, France and were of analytical quality.

1.2. Plant material collection and extraction

In this study, the aerial parts of Melissa *officinalis* L. were selected as the protective agent. Fresh samples were collected from Tebessa, Algeria, and a botanist confirmed their identity. The M. *officinalis* L. extract was prepared from the powdered plant material using the solid-liquid extraction method (maceration) described by **Romani et *al*. (2006)**. Specifically, 10 g of powdered aerial parts was mixed with 100 ml of distilled water, ensuring a solid-to-liquid ratio of 1:10 (mg/ml) **(Michel, 2011)**. The mixture was left to macerate for 48 hours before being filtered and concentrated using a rotary evaporator at 45°C. This method allowed for the effective extraction of bioactive compounds from the plant for further analysis.

2. Methodology

2.1. Experiment design

In order to carry out our research study, we employed a particular experimental animal model, specifically adult male "*Wistar* albino rats." These rats were procured from the Algeria Pasteur Institute (API), which is renowned for its contributions to scientific research. The rats selected for the study had an average weight ranging between 240 and 300 grams and were of similar size within the study parameters. Subsequently, we divided the animals into two distinct groups: a control group and a treatment group. Were housed in spacious cages.

Sixty rats were used in the study, and they were divided into six groups of ten rats each. upon arrival and maintained under controlled conditions: a temperature of 24 ± 4 °C, relative humidity of 60 ± 10%, and a 12-hour light/dark cycle. They had unrestricted access to food and

water and were kept under typical hygiene conditions. After a four-week adaptation period, the rats were divided according to the studies by **Duzguner and Erdogan (2010)** and **Kara et *al.* (1983)**.

The study was conducted at the Toxicology Laboratory, Faculty of Applied Biology, Tebessa University, Algeria, adhering to Institutional Animal Care and Use Committee (IACUC) policies (Accreditation number: D01N01UN130120150006/2019) and complied with the Algerian Law No. 05-12 concerning veterinary medical practices and animal health protection.

2.2. Dose selection

In this study, we selected two doses of Imidacloprid for the 90-day treatment period: 5 mg/kg body weight (B.W.) as the lower dose and 50 mg/kg B.W. as the higher dose. The Imidacloprid used was sourced from Sigma Aldrich in Germany, and prior to administration, it was dissolved in distilled water to prepare the treatment solution.

The selection of these doses was informed by previous studies examining the effects of Imidacloprid, including the work of **Duzguner and Erdogan (2010)** and **Kara et *al.* (1983)**. Additionally, we referred to the study by **Zouaoui et *al*. (2024)**, which investigated the impact of Imidacloprid on forebrain mitochondrial enzymatic activities EC50, IC50 and impacts of Mellissa extracts.

The doses of 5 mg/kg and 50 mg/kg B.W. were deemed appropriate as they did not induce any signs of toxicity throughout the experiment. These doses were calculated based on the LD50 (Lethal dose for 50% of the population), which for Imidacloprid in rats ranges from 424 to 475 mg/kg B.W. The doses were then adjusted to ensure that daily exposure rates were met without causing adverse effects.

To test the antioxidant activity of M. *officinalis*, a dose of 10 mg/kg/day was selected based on studies conducted on the same plant **(Miladi-Gorgi et *al.*, 2012)**.

2.3. Composition of experimental rat diet

Category	*Component*	*Value*
Characteristics	Crude Protein	21.00%
	Fat Content	4.00%
	Crude Fiber	4.50%
	Crude Ash	6.50%
	Sugars & Starch	36.09%
	Moisture	10.75%
Minerals	Calcium	0.90%
	Phosphorus	0.70%
	Magnesium	0.25%
	Sodium	0.10%
Vitamins (per kg)	Vitamin A	20,000 I.U.
	Vitamin D3	2,000 I.U.
	Vitamin B1	20.00 mg
	Vitamin B2	15.00 mg
	Vitamin B5	25.00 mg
	Vitamin B6	13.00 mg
	Vitamin B12	0.04 mg
	Vitamin K3	3.50 mg
	Vitamin E	40.00 mg
	Folic Acid	3.00 mg
	Biotin	0.60 mg
	Nicotinic Acid	35.00 mg
	Choline	370.00 mg
Amino Acids	Lysine	1.15%
	Methionine	0.50%
Trace-Elements	Iron	130.00 mg
	Manganese	80.00 mg
	Zinc	85.00 mg
	Copper	20.00 mg
	Selenium	0.10 mg
	Cobalt	1.00 mg
	Potassium Iodide	4.30 mg

2.4. Distribution and treatment of rats

The treatment procedure involved administering prepared solutions through gastric gavages using a probe attached to a syringe. This administration method was carried out daily for duration of 90 days. The groups were organized as follows:

- **Control Group (CT):** Gavage with distilled water (1 ml/day).and serves as a baseline for comparison for 90 days.
- **IMI D1 (5 mg/kg/day):** Rats in this group exposed to a low dose of Imidacloprid (5 mg/kg/day) for 90 days.
- **IMI D2 (50 mg/kg/day):** Rats in this group exposed to a high dose of Imidacloprid (50 mg/kg/day) for 90 days.

- **MOE (10 mg/kg/day):** Rats in this group received only Melissa *Officinalis* extract (10 mg/kg/day) for 90 days.
- **IMI D1 + MOE:** Co-treated with a low dose of Imidacloprid (5 mg/kg/day) and Melissa *Officinalis* extract (10 mg/kg/day) for 90 days.
- **IMI D2 + MOE:** Co-treated with a high dose of Imidacloprid (50 mg/kg/day) and Melissa *Officinalis* extract (10 mg/kg/day) for 90 days.

2.5. Sacrifice and brain extraction

The protocol for the sacrifice and brain extraction of rats begins with a preparation phase, which involves gathering all the necessary equipment, including sterilized dissection tools (scalpel, forceps, scissors), tampons, pre-labeled preservation tubes, and crushed ice to preserve the samples immediately after extraction. The rats are identified, and their health status is checked before any handling.

The sacrifice of rats from the different experimental groups was standardized to 8:00 a.m. to eliminate variations linked to circadian rhythms. The animals were sacrificed by decapitation without anesthesia to avoid potential biases introduced by anesthetics on biochemical and apoptotic parameters.

For brain extraction, a longitudinal incision was made on the scalp using a scalpel to expose the cranial bones. Surgical scissors were used to carefully open the skull by removing the parietal and frontal bones, starting from the back and moving forward. The brain was gently lifted using sterile forceps inserted under the medulla oblongata while carefully severing cranial nerves and blood vessels with fine scissors. Once removed, the brain was rinsed in a cold saline solution to eliminate traces of blood and debris.

After the sacrifice, the brains were collected and individually weighed. Each brain was processed as follows: one whole brain was preserved in a 10% formalin solution for a global histological study aimed at analyzing brain topography. Specific brain regions, such as the hippocampus, striatum, and cortex, were carefully isolated, homogenized at 4°C in Tris-HCl buffer (0.1 M, pH 7.4), and centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant obtained after centrifugation was collected and stored at –80°C for targeted biochemical analyses, including the evaluation of apoptotic markers. Biological waste was disposed of in compliance with applicable regulations.

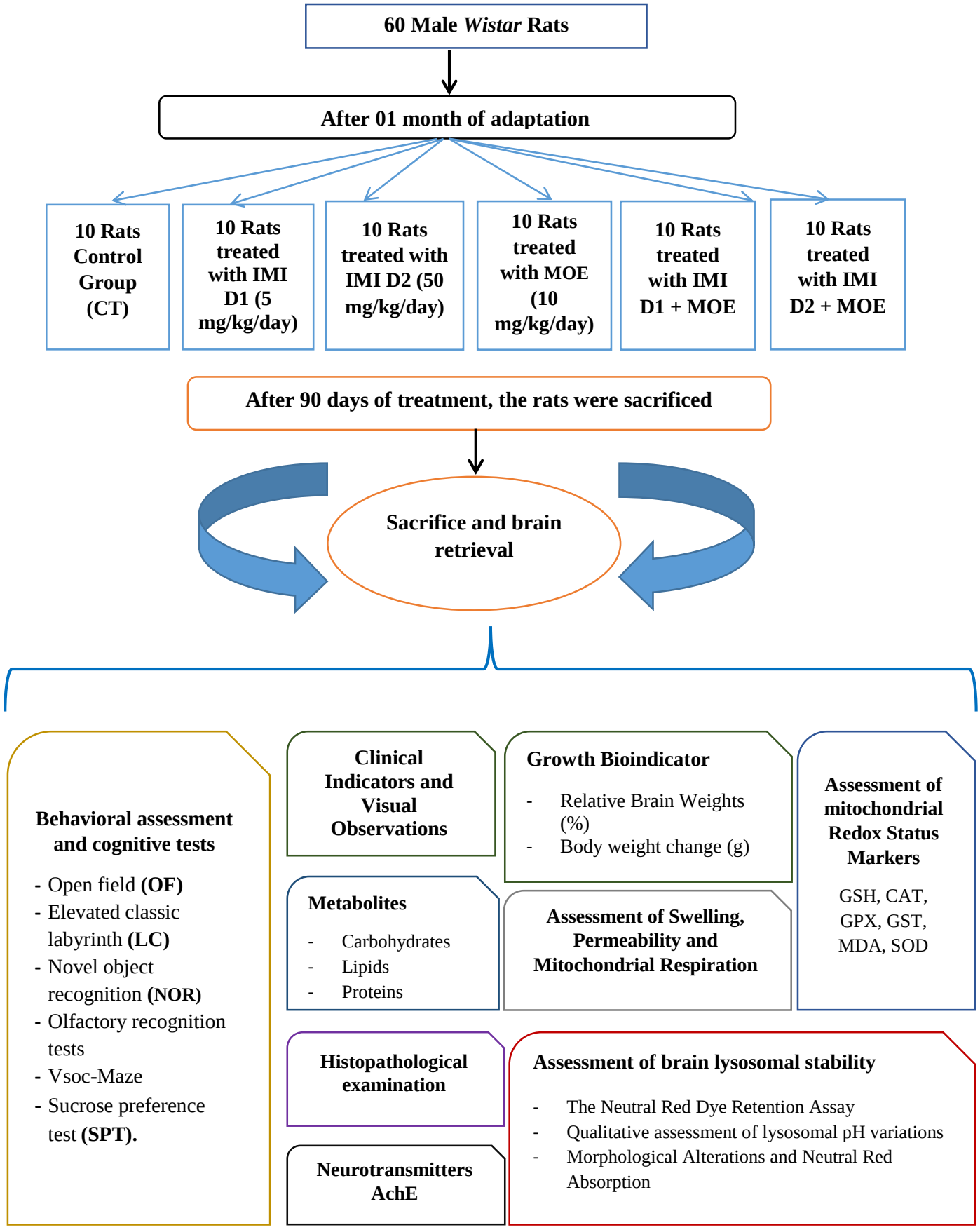


Figure 23: Summary diagram of the experimental protocol.

3. Assessment of Imidacloprid neurotoxicity and the protective effect of Melissa *officinalis* l. extract

Following three months of exposure to Imidacloprid (IMI), a neonicotinoid pesticide, two complementary approaches were employed to assess its impact. The first focused on neurobehavioral changes in treated rats, while the second evaluated both the structural and functional integrity of nerve cells at the global and regional levels of the brain. Additionally, the study investigated the dose-dependent neurotoxic effects of IMI, highlighting its greater impact at higher doses compared to lower ones. The potential neuroprotective activity of Melissa *officinalis* L. (MO) extract was also examined, demonstrating its ability to mitigate the detrimental effects of IMI on brain health.

3.1. Weight measurement

The weight of the rats is measured regularly every day throughout the duration of the study. The measurements are taken at two key stages:

- **Adaptation period**: This phase assesses potential changes due to external factors, such as diet or environment.
- **Treatment period**: This phase evaluates the effects of the administered xenobiotic.

Weight measurements are taken using a sensitive electronic balance, *Aston® brand*, in grams (g). The growth rate of the rats is then expressed as a percentage (%), based on the weight on day 1 of the experiment and the weight on the desired day (e.g., after 90 days). The calculation is as follows**:**

$$\text{Growth Rate} = \frac{(\text{Weight at day 90} - \text{Initial weight})}{\text{Initial weight}} \times 100$$

3.2. Estimation of relative forebrain weight

The relative brain weight of the brains extracted from rats RBW [g/100g body weight] is calculated in relation to the total weight of the rat using the following formula:

$$\text{RBW (g/100g of BW)} = \text{FW/BW} \times 100$$

- **FW:** Forebrain weight (g)
- **BW:** Total body weight of the rat (g)
- **RBW:** Relative brain weight (g)

4. Behavioral assessment and cognitive tests

Behavioral models in animals are essential for neurobiological research, allowing for in-depth studies of potential brain lesions and pathologies **(Kalueff et *al.*, 2008).** After 90 days of exposure to various doses of Imidacloprid (IMI) and Melissa *officinalis* L. (MO) extract, behavioral and cognitive tests were conducted. This neurobehavioral study aimed to achieve several objectives: evaluating the animals' anxiety using the open field (OF) and elevated classic labyrinth (LC) tests, assessing working memory and learning ability with the novel object recognition (NOR) and olfactory recognition tests, investigating sociability and social novelty preference through Vsoc-Maze tests, and measuring stress-induced anhedonia with the sucrose preference test (SPT). All tests were carried out between 8:00 a.m. and 6:00 p.m., filmed, and analyzed by the same experimenter.

4.1. The classic labyrinth test

The Classic Labyrinth Test (CLT) serves as a straightforward method for assessing various behaviors in rodents, including learning, memory, and anxiety. Our aim was to investigate the cognitive processes of stressed animals, employing this traditional labyrinth assessment. The CLT involves a square-shaped plastic enclosure (measuring 125 x 125 x 40 cm) with multiple labyrinth passages of uniform width and height (25 x 35 cm) but varying lengths **(See Figure 24).** Typically positioned on a 90 cm high table, control rats navigate the labyrinth swiftly from start to finish. In contrast, stressed animals tend to prolong their exploration of the maze's passages, indicating heightened aversion or anxiety. Additionally, behaviors such as lingering at the starting point or corners, perceived as seeking refuge, are indicative of stress **(Leo and Pamplona, 2014; Gasmi et *al.*, 2017b).** Notably, this method offers advantages such as affordability and efficiency, with testing typically lasting around 10 minutes. Following sanitation with 10% ethanol, the labyrinth is readily available for subsequent use with another animal **(Gasmi et *al.*, 2017a and 2018)**.

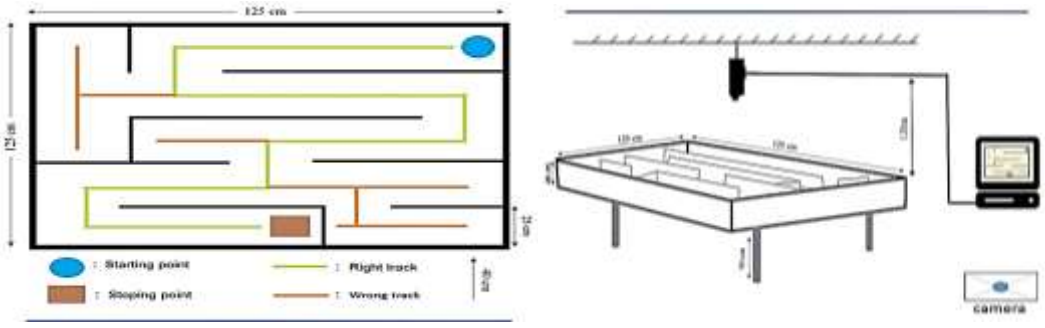


Figure 24: The Classic Labyrinth its spatial measurements. **(Gasmi, 2018)**

4.2. Open field locomotion test

The open field test, originally designed to gauge emotionality in rodents (**Beilharz and Cox, 1967)**, is commonly employed to assess anxiety, exploration, and locomotion **(Taylor, 2010).** This test evaluates both horizontal and vertical movements of animals within an experimental enclosure. The open field environment induces stress in rats, as these nocturnal creatures typically prefer confined, dimly lit spaces and are wary of open spaces, tending to stick to the periphery. In our study, we utilized a transparent glass enclosure with square dimensions (50 cm ×50 cm ×40 cm). Observation of the animals commenced immediately after their introduction into the enclosure. This test examines rats' exploratory behaviors within a confined area and serves as a means to evaluate not only their motor functions but also their anxiety levels. Anxious rats typically avoid the open center and tend to hug the walls. Initially, each rat was put in a separate corner of the open field, facing inward, and observed for several minutes to quantify their exploratory activity. Between each trial, the enclosure was cleaned using 10% ethanol solution. **(Zhang et *al*., 2007).**

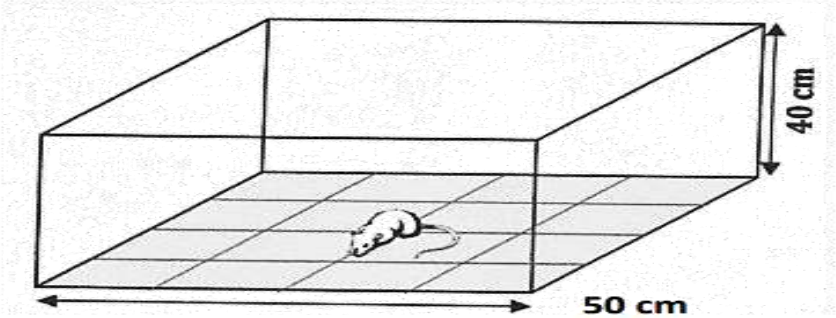


Figure 25: Schematic illustration of the open field. **(Hall, 1934)**

4.3. Novel object recognition test (NOR)

The Novel Object Recognition (NOR) memory task is a widely used behavioral experiment for studying learning **(Ennaceur, 2010)**. It also evaluates non-aversive and non-spatial memory in rodents based on their natural preference for novelty **(Huang et *al*., 2024).** The main idea behind this task is that rodents exhibit a greater inclination to investigate a novel object compared to a familiar one. **(Ennaceur and Delacour, 1988)**.

The test consists of three phases**:**

1. Acclimation to the experimental room.
2. Familiarization with two identical objects.
3. The test phase, where a novel object is introduced **(Antunes and Biala, 2011)**.

After habituation to the arena, rats are placed in a 40 x 40 cm box with two identical objects (D: 2 cm, H: 4 cm), and the time they spend exploring each object is recorded. In the test phase, they encounter a familiar object and a novel object. Rats without memory issues will spend more time exploring the novel object. Administration of an amnesiac substance can impair memory, causing the rats to explore both objects equally, indicating they have "forgotten" the familiar object **(Sanderson et *al*., 2011).** The apparatus and objects are cleaned with 10% ethanol after each trial.

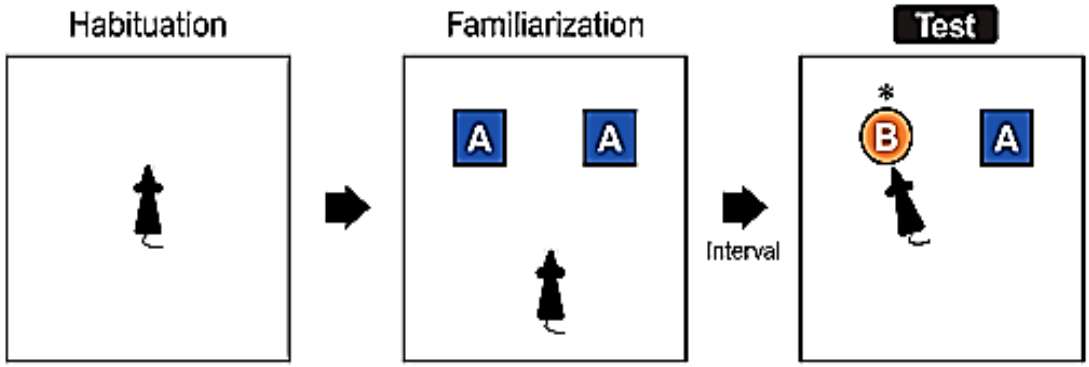


Figure 26: Schematic representation of the novel object recognition task**:** Initially, a mouse is habituated to the arena (left) and introduced to two identical objects for familiarization. Subsequently. Following a specific interval, a new object is introduced to the mouse. **(Kim et *al.*, 2019)**.

4.4. Vsoc-Maze: A tool for investigating sociability and social novelty preference

In this study, Crawley's sociability test, also known as the three-chamber apparatus, was employed to investigate sociability and preference for social novelty **(Chadman et *al.*, 2008; McFarlane et *al*., 2008; Moy et *al*., 2008)**.

Additionally, a V-maze, previously utilized for novel object recognition memory evaluation, was adapted to assess social behavior, resulting in the creation of the Vsoc-maze **(Puighermanal et *al*., 2009; Busquets-Garcia et *al*., 2011, 2013; Navarro-Romero et *al*., 2019).** The Vsoc-maze, a modified version of the V-shaped maze, consists of two main components: a black Plexiglas maze wall and a transparent Plexiglas lid, with corridors measuring 300 mm in length and 45 mm in width. This setup includes small chambers at corridor ends for housing juvenile stranger mice, facilitating social behavior assessment. Concurrently, the three-chamber apparatus, divided into three identical chambers with Plexiglas walls containpreference for social novelty—conducted consecutively, mice were allowed 5 minutes in the Vsoc-maze and typically 10 minutes in the three-chamber apparatus to explore,

with behavior recorded using a digital camera **(Nadler et *al*., 2004; Kaidanovich-Beilin et *al*., 2011).**

Throughout our experiments, mice were maintained under standard environmental conditions, with temperature and humidity controlled and food and water provided ad libitum. Experiments were conducted during the light phase of the dark/light cycle, following a week of habituation in the experimental room. The social test, comprising habituation, sociability, and preference for social novelty phases, was carried out consecutively in both the Vsoc-maze and three-chamber apparatus.

The habituation session *(Phase I)* involves allowing mice to explore the Vsoc-maze for 5 minutes to familiarize themselves, with exploration times recorded for each chamber using stopwatches and a closed-circuit camera. Following this, the sociability session *(Phase II)* introduces a juvenile mouse (STR 1) into one chamber, and the experimental mouse explores for another 5 minutes, with a preference for the chamber containing STR 1 expected. Finally, in the preference for social novelty session *(Phase III)*, a second novel mouse (STR 2) is placed in the previously empty chamber, and the experimental mouse's exploration time is recorded to determine if it shows a preference for the novel mouse (STR 2) over the familiar mouse (STR 1). Each phase in the Vsoc-maze lasted 5 minutes, aligning with the timeframe for most social behaviors **(Nadler et *al.*, 2004)**, while sessions in the three-chamber apparatus typically lasted 10 minutes **(Kaidanovich-Beilin et *al*., 2011)**.

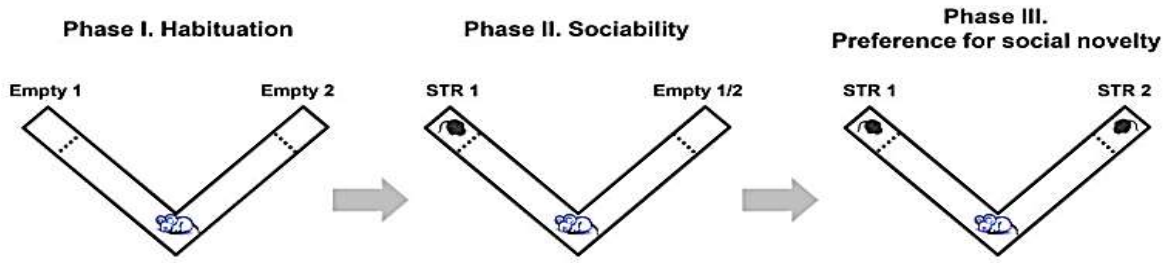


Figure 27: Illustrates the sociability and preference for social novelty test, as described by **Martínez-Navarro et *al*. (2019)**. The test comprises three phases: Phase I (habituation), Phase II (sociability), and Phase III (preference for social novelty), each lasting 5 minutes. Throughout these phases, the exploration behavior of the experimental mouse towards the chambers at the end of the corridors was recorded. Phase I involved both chambers being empty, Phase II compared the exploration of the experimental mouse towards stranger-1 (STR 1) versus an empty chamber, and Phase III compared the exploration towards stranger-1 (STR 1) versus stranger-2 (STR 2).

4.5. Sucrose preference test (SPT) for measurement of stress-induced anhedonia

The sucrose preference (Anhedonia) test is used to model anhedonia, which is the diminished desire to engage in activities that were once enjoyable. Typically, rodents prefer sucrose water over tap water when given a choice. However, rodent models of depression show a significantly reduced preference for sucrose water **(Carter Matt, 2015)**. In this test, we measure the amount of sucrose water consumed **(Redei et *al*., 2001)**. The test involves a two-bottle setup, one containing sucrose water and the other containing tap water, with rats having free access to both 150 ml bottles. The positions of the bottles are alternated over five consecutive days, and their volumes are measured each morning. In our experiments, we use a 1% (wt/vol) fresh sucrose solution. Sucrose preference is calculated using the formula**:**

Sucrose preference (%) = Sucrose intake (g) / (Sucrose intake [g] + Water intake [g]) × 100%.

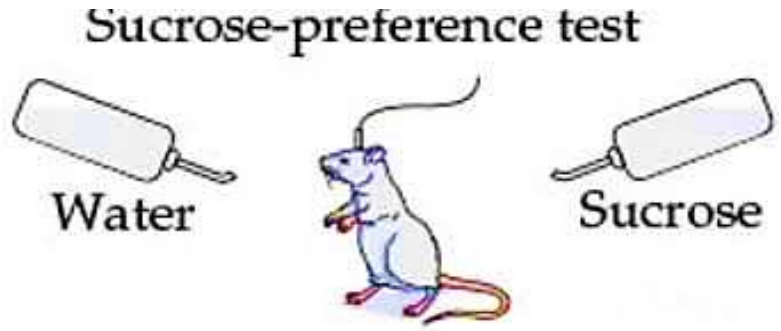


Figure 28: Illustrates the sucrose preference test.

(https://air.unimi.it/bitstream/2434/825968/2/YPHRS-D-20-00451_R1%20per%20air.pdf)

4.6. Olfactory recognition test

The study of olfactory learning and memory in rats is intriguing on its own but holds greater significance as it aids in developing a comprehensive, biologically based theory of memory. This test aims to review behavioral, anatomical, and physiological evidence supporting this idea, assessing how the rodent olfactory system can serve as a model to enhance our understanding of the neurobiological foundations of human memory.

In this two-choice test, we evaluate the olfactory recognition abilities of animals within their immediate environment. To assess the individuals' ability to recognize and orient themselves toward familiar odors, we used two types of sawdust: clean sawdust (CS) and sawdust from a nest (NS). The pups are exposed to an experimental enclosure with clean sawdust on the floor, while another enclosure contains sawdust impregnated with urine and

maternal feces collected from the breeding cage just 5 minutes before the test. These enclosures are randomly placed on either side of the maze. The maze is briefly cleaned with 70% alcohol and left to dry between the passages of two individuals (approximately 8 minutes). Various parameters are measured: the latency time, which is the time required before making the first choice, and the time spent in each type of sawdust. **(Dorbani et *al.*, 2014)**.

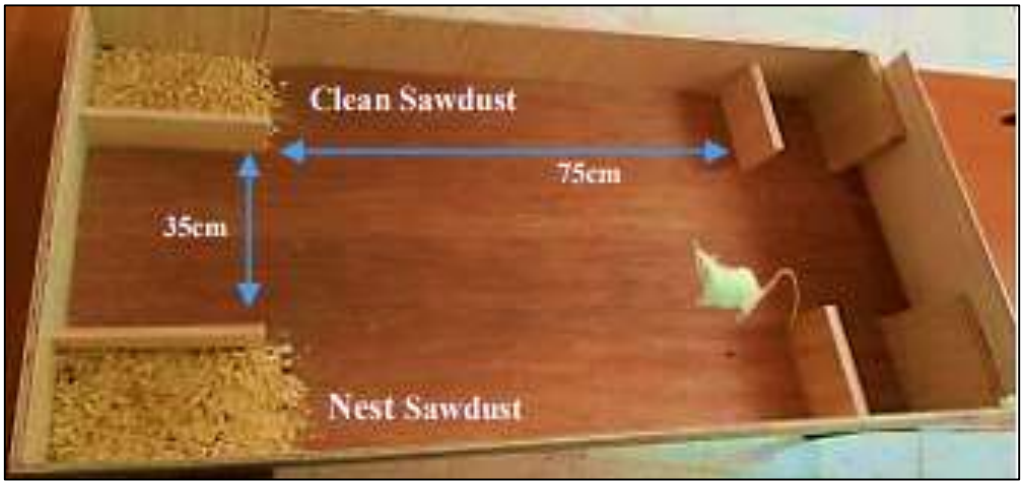


Figure 29: Experimental apparatus used to facilitate olfactory recognition.

5. Methods for the assessment of biochemical parameters

5.1. Extraction and determination of metabolites

The extraction of metabolites was performed using the method described by **Shibko et *al.* (1966),** and the main steps are summarized in **Figure 30** Control and treated samples were homogenized using a magnetic grinder in 20% trichloroacetic acid (TCA) (200 mg of tissue per 1 mL of TCA). Following an initial centrifugation (5,000 rpm for 10 minutes), the obtained supernatant I was used for the determination of total carbohydrates, following the method of **Duchateau and Florkin (1959)**.

To the pellet I, 1 mL of an ether/chloroform mixture (1:1 v/v) was added, and after a second centrifugation at 5,000 rpm for 10 minutes, supernatant II and pellet II were obtained. Supernatant II was used for the determination of lipids, according to **Goldworthy et *al.* (1972)**, while pellet II, dissolved in 0.1 N NaOH, was used for the determination of proteins, following the protocol by **Bradford (1976)**.

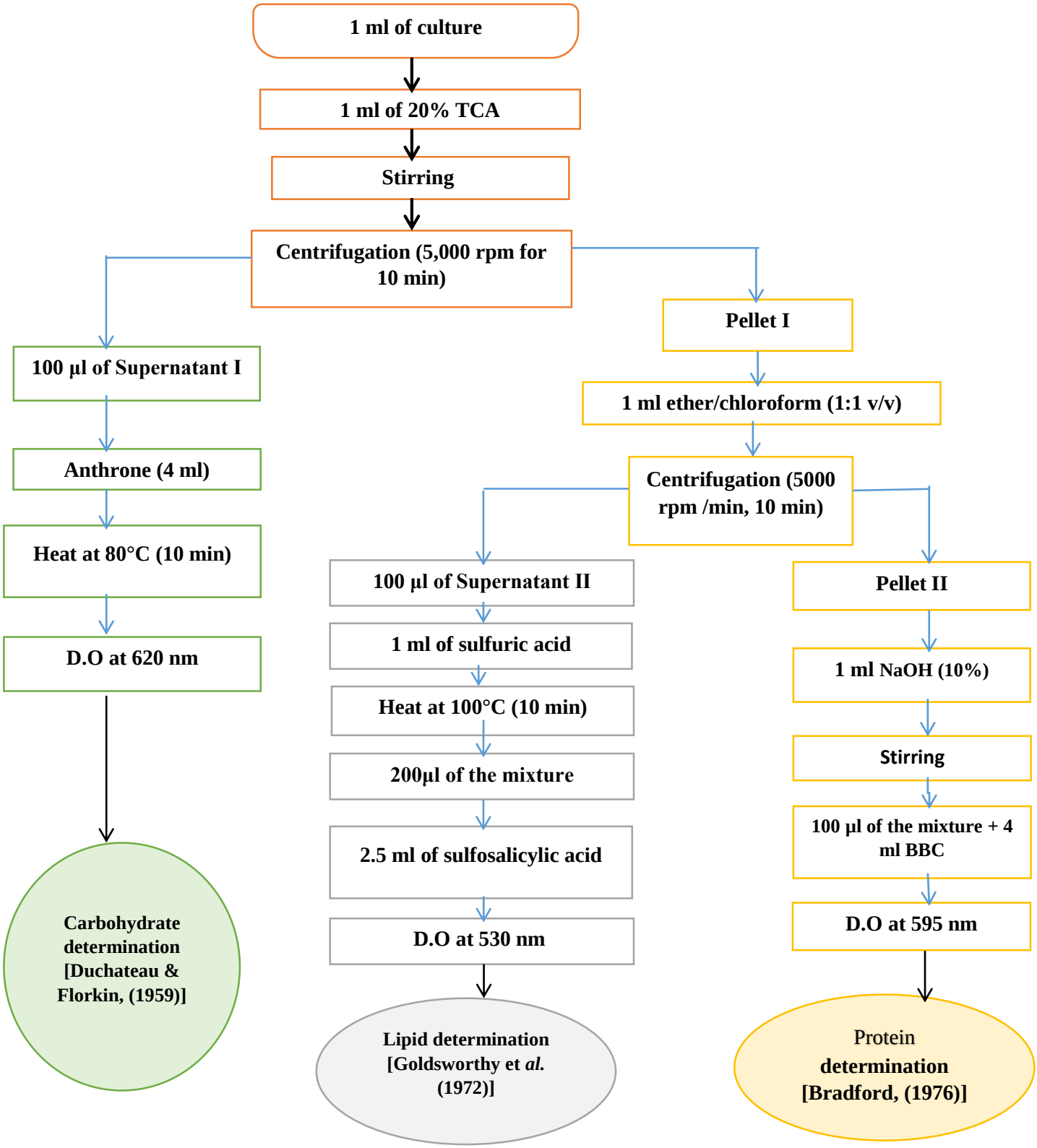


Figure 30: Extraction methods and determination of main biochemical constituents (Carbohydrates, Lipids, Proteins). **(Shibko et *al.* 1966)**

5.2. Quantification methods

5.2.1. Carbohydrate determination

Total carbohydrate determination was carried out using the method described by **Duchateau and Florkin (1959)**. This method involves the use of anthrone as a reagent (150 mg of anthrone, 75 mL of sulfuric acid, and 25 mL of distilled water) and a glucose stock solution (1 g/L) as the standard. In the procedure, 100 µL of the supernatant from the different samples is added to a test tube, followed by 4 mL of anthrone reagent. The mixture is then heated in a water bath at 80°C for 10 minutes, during which a green color develops, with the intensity of the color being directly proportional to the carbohydrate content in the sample. The absorbance is then measured at a wavelength of 620 nm**.**

5.2.2. Lipid determination

Tissue lipids were quantified using the method described by **Goldsworthy et *al.* (1972)**. A 200-µL aliquot of homogenate was mixed with 5 mL of 20% trichloroacetic acid (TCA). The mixture was homogenized and filtered, then subjected to centrifugation at 5000 rpm for 10 minutes. The pellet was collected in a tube containing 1 mL of an ether/chloroform mixture. After a second centrifugation at 5000 rpm for 10 min, 100 µL of the supernatant was transferred, to which 1 mL of sulfuric acid was added. The tubes were then placed in a water bath at 100°C for 10 min, followed by cooling. Afterward, 200 µL of the extract was transferred and mixed with 2.5 mL of sulfosalicylic reagent (85% solution: 0.38 g vanillin, 195 mL phosphoric acid, 55 mL water) and left to stand in the dark for 30 minutes. The absorbance was measured at 530 nm. Lipid concentrations were determined using the equation derived from the calibration curve prepared with a sunflower oil stock solution.

5.2.3. Protein determination

The method used for protein determination is that of **Bradford (1976)**, which utilizes BSA as a standard. The same sample used for lipid determination is taken, and the pellet obtained from the second centrifugation is collected. To this pellet, 1 mL of NaOH (0.1N) is added, and the mixture is vigorously shaken to dissolve the proteins. Then, 100 µL of the solution is pipetted, and 4 mL of the BBC reagent (Coomassie Brilliant Blue) is added (50 mg of BBC + 50 mL of 85% phosphoric acid, then diluted to 500 mL with distilled water). These results in the development of a blue color and the samples are directly measured at an absorbance of 595 nm. The protein concentration is calculated using the equation derived from the calibration curve established with a bovine serum albumin solution.

6. Preparation of mitochondrial suspensions

The extraction of mitochondria is carried out following the method described by **Rustin et *al.* (1994)**, involving purification by differential centrifugation. Briefly, after decapitation of the rats, the brains are rapidly harvested and immersed in TSE buffer (10 mM Tris, 250 mM sucrose, 0.1 mM EDTA, pH 7.2 at 4°C). The nervous tissues are finely minced and homogenized in 3.5 ml of TSE buffer, which facilitates cell disruption and mitochondrial release. The homogenate is centrifuged at 10,000 rpm for 10 minutes to eliminate large cellular debris. The resulting pellet is centrifuged a second time at 10,000 rpm for 10 minutes. The supernatants from both centrifugations are collected and centrifuged at 14,000 rpm at 4°C for 10 minutes. The pellet obtained is resuspended in 1 ml of TSE buffer and centrifuged again at 14,000 rpm for 10 minutes. The final pellet is resuspended in 1 ml of TS buffer (250 mM sucrose, 50 mM Tris, pH 7.2 at 20°C) and centrifuged at 14,000 rpm for 10 minutes. The resulting pellet, containing the mitochondria, is recovered in 500 µl of TS buffer to prepare a fresh mitochondrial suspension. A fraction of this suspension is immediately used for evaluating the structural and functional integrity of the mitochondria, while the remainder is stored at -80°C for subsequent assays.

7. Isolation and preparation of brain mitochondria and matrix

Mitochondria extraction follows the protocol of **David et *al.* (2014)** and **Rustin et *al.* (1994)**, utilizing differential centrifugation. In summary, following decapitation, brains are quickly removed and placed in TSE buffer (250 mM sucrose, 10 mM Tris, 0.1 mM EDTA, pH 7.2 at 4 °C). The mitochondrial suspension undergoes a freeze-thaw cycle six to eight times, gradually intensifying to rupture the mitochondria. After centrifuging for 10 minutes at 9600 rpm, the resulting supernatant serves as the enzyme source.

8. Assessment of redox status markers

Which includes both enzymatic and non-enzymatic parameters.

8.1. Malondialdehyde (MDA) assay

The determination of malondialdehyde (MDA) is carried out following the protocol of **Esterbauer et *al.* (1992)**. The principle of this assay is based on the reaction of MDA with thiobarbituric acid (TBA) in an acidic medium and under heat, leading to the formation of a pink-colored thiobarbituric acid-reactive substance (TBARS).

A volume of 375 µl of the supernatant is transferred to a dry tube, followed by the addition of 150 µl of TBS buffer (50 mM Tris, 150 mM NaCl; pH 7.4) and 375 µl of TCA-BHT solution (20% trichloroacetic acid, 1% butylated hydroxytoluene). The mixture is homogenized using a vortex and centrifuged at 1000 rpm for 10 minutes to precipitate proteins and lipophilic residues.

Subsequently, 400 µl of the resulting supernatant is mixed with 80 µl of 0.6 M HCl and 320 µl of Tris-TBA solution (26 mM Tris, 120 mM thiobarbituric acid). The reaction mixture is vortexed again and incubated in a water bath at 80°C for 10 minutes to facilitate the condensation reaction. The optical density of the samples is measured by spectrophotometry at 530 nm.

8.2. Glutathione Peroxidase (GPx) assay

The enzymatic activity of glutathione peroxidase (GPx) is determined following the method of **Flohé and Günzler (1984)**, using hydrogen peroxide (H_2O_2) as a substrate.

A volume of 0.2 ml of cytosolic or mitochondrial matrix extract is mixed in a tube containing 0.4 ml of 0.1 mM reduced glutathione (GSH) and 0.2 ml of 0.067 M phosphate buffer (pH 7.8). The mixture is incubated in a water bath at 25°C for 5 minutes. The reaction is initiated by adding 0.2 ml of 1.3 mM H_2O_2.

After 10 minutes of reaction, 1 ml of 1% trichloroacetic acid (TCA) is added to terminate the reaction. The mixture is then placed on ice for 30 minutes and centrifuged at 3000 rpm for 10 minutes.

A volume of 0.48 ml of the supernatant is transferred into a cuvette, and 2.2 ml of 0.32 M disodium hydrogen phosphate (Na_2HPO_4) and 0.32 ml of 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) are added. The reaction forms a yellow-colored compound, and its optical density is measured spectrophotometrically at 412 nm every 30 seconds for 5 minutes.

8.3. Glutathione (GSH) assay

The quantification of reduced glutathione (GSH) is carried out according to the method of **Weckbecker and Cory (1988).** This method is based on the measurement of the absorbance of 2-nitro-5-mercapturic acid, which results from the reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) by the thiol (-SH) groups of GSH.

To preserve the thiol groups, the biological sample (cytosol/matrix) undergoes deproteinization using sulfosalicylic acid (SSA, 0.25%). Briefly, 200 mg of tissue is

homogenized in 8 ml of 0.2 M ethylenediaminetetraacetic acid (EDTA) solution. The homogenization is performed on ice using a porcelain mortar and pestle.

The homogenate is then deproteinized. A volume of 0.8 ml of the homogenate is mixed with 0.2 ml of 0.25% SSA. The mixture is vortexed and incubated on ice for 15 minutes, followed by centrifugation at 1000 rpm for 5 minutes.

A volume of 0.5 ml of the resulting supernatant is collected and mixed with 1 ml of Tris-HCl + EDTA buffer (0.02 M, pH 9.6). To this mixture, 0.025 ml of 0.01 M DTNB dissolved in absolute methanol is added. The reaction mixture is left to stand for 5 minutes at room temperature, after which the absorbance is measured spectrophotometrically at 412 nm.

8.4. Catalase (CAT) activity assay

The spectrophotometric determination of catalase (CAT) activity is performed according to the method of **Cakmak and Horst (1991)**. The decrease in absorbance is recorded over three minutes using a spectrophotometer at a wavelength of 240 nm, with a molar extinction coefficient $\varepsilon = 39400\ \mu M^{-1} \cdot cm^{-1} \cdot L$ for a final reaction volume of 3 mL. The reaction mixture consists of 100 µL of crude enzymatic extract, 50 µL of hydrogen peroxide (H_2O_2) at 0.3%, and 2850 µL of phosphate buffer (50 mM, pH 7.2). The spectrophotometer is calibrated in the absence of the enzymatic extract, and the reaction is initiated by adding hydrogen peroxide.

8.5. Glutathione S-Transferase (GST) activity assay

The determination of glutathione S-transferase (GST) activity is based on the method of **Habig et *al*. (1974)**. This method relies on the conjugation reaction between GST and a substrate, 1-Chloro-2,4-dinitrobenzene (CDNB), in the presence of a cofactor, glutathione (GSH). The conjugation results in the formation of a novel molecule, 1-S-Glutathionyl-2,4-dinitrobenzene, which allows the GST activity to be quantified. The measured optical density (OD) is directly proportional to the amount of conjugate formed, which correlates with GST activity. Samples are homogenized in 1 mL of phosphate buffer (0.1 M, pH 6). The homogenate is centrifuged at 14,000 rpm for 30 minutes, and the supernatant is collected as the enzyme source. The assay involves reacting 200 µL of the supernatant with 1.2 mL of the reaction mixture containing CDNB (1 mM) and GSH (5 mM) [prepared by dissolving 20.26 mg CDNB, 153.65 mg GSH, 1 mL ethanol, and 100 mL phosphate buffer (0.1 M, pH 6)]. Absorbance readings are taken every 15 seconds for one minute at a wavelength of 340 nm against a blank in which 200 µL of distilled water replaces the supernatant.

8.6. Evaluation of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) is a crucial antioxidant enzyme that helps maintain low concentrations of the superoxide anion. It catalyzes the dismutation of $O_2^{-\circ}$ into H_2O_2 and O_2 according to the following reaction:

$$2\ O_2^{-\circ} + 2\ H^+ \rightarrow O_2 + H_2O_2$$

The enzymatic activity of SOD is assessed using the method of **Beauchamp and Fridovich (1971)**. This method is based on the enzyme's ability to inhibit the reaction between nitroblue tetrazolium (NBT) and the superoxide anion generated via the photoreaction of oxygen and riboflavin in the presence of an electron donor, such as methionine.

The reduction of NBT by the superoxide anion to formazan can be monitored spectrophotometrically at 560 nm. One unit of SOD activity is defined as the amount of enzyme required to inhibit 50% of NBT reduction.

For the assay, a reaction mixture is prepared containing 1 mL of sodium cyanide (NaCN) 0.01 M, 0.1 mL of NBT solution 0.176 mM, 1 mL of EDTA 66 mM, 0.1 mL of riboflavin solution 0.002 mM, phosphate buffer (KH_2PO_4) at pH 7.2, 5 µL of the enzymatic source (cytosolic or mitochondrial matrix), and 0.1 mL of methionine solution 10^{-2} M. A control tube is prepared with phosphate buffer under the same conditions as the samples. The mixture is exposed to light from a 15-watt lamp for 10 minutes, and absorbance is measured at 560 nm using quartz cuvettes.

9. Assessment of swelling, permeability and mitochondrial respiration

The effects of Imidacloprid (IMI) on the mitochondrial permeability transition pore (MPTP**)** were evaluated by assessing mitochondrial membrane permeability in the brain**.** Mitochondrial swelling, indicative of MPTP disruption, was measured using the method described by **Li et *al.* (2014).** Fresh brain tissues were processed to isolate mitochondria at 4 °C. Equal volumes of the mitochondrial suspension were placed in quartz cuvettes, and changes in absorbance were monitored spectrophotometrically at a wavelength (λ) of 540 nm. A decrease in absorbance corresponded to increased mitochondrial swelling, reflecting a loss of MPTP integrity.

Mitochondrial respiration was assessed using an Oxygraph system (Hansatech Instruments) based on protocols established by **Rouabhi et *al.* (2015)** and **Henine et *al.* (2016).** This analysis provided critical insights into the functionality and integrity of mitochondria under the influence of IMI.

10. Histopathological examination

The histological tests were conducted following the method described by **Houlot (1984)** at the anatomic pathology laboratory of *Dr. Bounneche Mounia Hana***,** Eps Chihoub, specialist in anatomic and cytopathology in Ali Mendjeli, Constantine, which involves several essential steps**.** First, the tissue samples are fixed in formalin and then placed in special perforated cassettes that allow the circulation of the liquids necessary for preparation. Next, the samples undergo progressive dehydration through a series of ethanol baths of increasing concentrations (70%, 95%, and 100%) and are then cleared in xylene to render the tissues transparent. Once dehydrated, the tissues are embedded in liquid paraffin at 60°C. This step enables the paraffin to penetrate and impregnate the tissues. The following step is embedding, where the impregnated tissues are enclosed in paraffin blocks that, once solidified, facilitate cutting. These blocks are then sectioned into thin slices of 4 to 7 microns using a microtome. The resulting sections are placed onto glass slides for staining. The standard staining method involves hematoxylin to stain the nuclei blue/violet, followed by eosin, which stains the cytoplasm and extracellular structures pink. After staining, the sections are mounted with cover slips and examined under a microscope for detailed tissue structure analysis. The sections are also photographed using a camera connected to a binocular magnifier. Histopathological anomalies, such as lesions, inflammation, or necrosis, can thus be accurately visualized.

11. Determination of brain neurotransmitter levels and brain AchE activity

The acetylcholinesterase (AChE) assay, based on the method developed by **Ellman et *al.* (1961)**, involves the enzymatic hydrolysis of acetylthiocholine, which produces thiocholine and acetic acid. To prepare samples, 1 ml of a detergent-based homogenization solution (comprising 38.03 mg of triethanolamine, 1 ml of Triton X-100, 5.845 g of NaCl, and 80 ml of 10 mM Tris buffer) is used to homogenize tissues via an ultrasonic homogenizer. The homogenate is then centrifuged at 5,000 rpm for 5 minutes, and the resulting supernatant is immediately used for measuring AChE activity.

The assay is conducted by mixing 100 µl of the supernatant with 100 µl of DTNB (0.1 M, pH 8) and 1 ml of 0.1 M Tris buffer (pH 7). After a 5-minute incubation period to account for any spontaneous reactions, 100 µl of acetylthiocholine substrate solution (118 mg of acetylthiocholine in 5 ml of distilled water) is added to initiate the enzymatic reaction. Optical density readings are taken at 412 nm at 4-minute intervals over a 20-minute period to monitor

the reaction kinetics. This method provides a sensitive and reliable measure of AChE activity in biological samples.

12. Isolation of lysosomes from rat brain

The method for isolating lysosomes in this study was based on the pioneering work of **De Duve and Wattiaux (1966)** on lysosome function and isolation techniques. The procedure for lysosome isolation from rat brain tissue is as follows: Rat brain tissue is carefully harvested and immediately rinsed with cold phosphate-buffered saline (PBS) to remove potential contaminants. The meninges and excess blood are meticulously removed. Using a scalpel or razor blade, the brain tissue is finely minced into small pieces. These fragments are then placed into a pre-chilled glass Dounce or Potter-Elvehjem homogenizer, filled with an appropriate homogenization buffer composed of 0.25 M sucrose and 10 mM HEPES at pH 7.4.

To break down the cellular structures, the tissue is homogenized with several gentle strokes of the pestle until a uniform solution is obtained. The resulting homogenate is transferred into a 15 mL centrifuge tube, and then centrifuged at 4°C for 10 minutes at 1,000 g to remove large cellular debris and nuclei. This step aids in separating the lysosomes from lighter components. The supernatant, enriched in lysosomes, is carefully transferred to a new 15 mL centrifuge tube to prevent cross-contamination.

The supernatant passes through one more round of centrifugation at a faster speed to further filter the lysosomes. This procedure results in the production of a distinct lysosome pellet at the tube's bottom after being carried out at 10,000 g for 20 minutes at 4°C. The lysosome pellet, which contains the separated lysosomes, is retrieved after the supernatant has been carefully eliminated.

Finally, depending on the planned downstream applications, the lysosome pellet can be resuspended in an appropriate buffer, such as lysis or storage buffer, depending on the intended downstream applications. These buffers help maintain lysosome stability and functionality for further analysis. Throughout the entire process, it is crucial to maintain the tissue at a low temperature (4°C or on ice) to prevent enzymatic degradation and preserve lysosome integrity.

13. Assessment of brain lysosomal stability through neutral red retention time (NRRT)

13.1. The neutral red dye retention assay (NRRA)

Is a commonly used method to evaluate lysosomal stability, cell viability, and cytotoxicity, with applications in both biomedical and environmental studies **(Borenfreund et *al*., 1984; Repetto et *al*., 1993)**. The neutral red dye (Toluylene red chloride; 3-amino-m-dimethylamino-2-methylphenazine hydrochloride, CI 50040), purchased from Sigma for this study, is a lysosome-specific inclusion dye utilized to assess lysosomal membrane integrity.

When cells are exposed to toxicants or stress, lysosomal damage can occur, resulting in reduced uptake of neutral red. This dye's ability to selectively accumulate in lysosomes makes it an effective marker for evaluating lysosomal fragility and dysfunction.

The degree of neutral red uptake was quantified using rapid-scanning reflectance spectrophotometry. After exposure to the xenobiotic, viable cells were fixed with formaldehyde, and the dye was extracted using an acetic acid/ethanol mixture. Quantification was performed at 540 nm using a spectrophotometer. The procedure follows the protocol established by **Lowe et *al.* (1995)** and recommended by **UNEP/RAMOGE (1999)**.

13.2. Preparation of neutral red solution

To prepare the Neutral Red solution, dissolve 20 mg of Neutral Red powder in 1 ml of dimethyl sulfoxide (DMSO). Mix thoroughly until the powder is completely dissolved, resulting in a homogeneous stock solution. Store this stock solution in a dark container at room temperature to prevent degradation.

13.3. Preparation of working solution

For the working solution, dilute 5 µL of the Neutral Red stock solution in 995 µL of physiological saline (phosphate-buffered saline). To prepare the physiological saline solution, dissolve the following components in 1 liter of distilled water: 4.77 g of HEPES, 25.48 g of NaCl, 13.06 g of $MgSO_4$, 0.75 g of KCl, and 1.4 g of $CaCl_2$. Adjust the pH of the solution to 7.36 using 1M NaOH. Once prepared, store the physiological saline solution in the refrigerator and bring it to room temperature before use.

13.4. Usage and storage

Gently mix the working solution to ensure proper dilution. It can be used immediately for experiments or stored in a dark and refrigerated environment when not in use. Proper storage conditions help preserve the stability and extend the lifespan of the solution.

14. Toxicity determined in vitro by morphological alterations and neutral red absorption

14.1. Morphological alterations

Morphological alterations are key indicators of cellular damage and the mechanisms underlying toxicity. Following the specified exposure periods, cells are analyzed using a phase-contrast light microscope at 400x magnification to identify disruptions in lysosomal membrane integrity, which is a critical determinant of cellular health. Lysosomal membrane permeabilization (LMP) can result in the release of hydrolytic enzymes into the cytoplasm, triggering apoptotic or necrotic cell death, and is an established marker of cellular stress **(Kroemer and Jäättelä, 2005; Aits and Jäättelä, 2013; Repnik et *al*., 2014)**.

14.2. Qualitative assessment of lysosomal pH variations

The qualitative assessment of lysosomal pH variations begins with the preparation of brain tissue samples, where the organ is isolated and sliced into thin sections (approximately 100–200 μm thick). These sections are placed in phosphate-buffered saline (PBS) to maintain tissue viability, with PBS prepared by dissolving 1 gram of PBS powder in 100 mL of distilled water. pH-indicating probes are then loaded by preparing a working solution, which is added to the container holding the brain sections. The samples are incubated for 30 minutes to 1 hour to allow sufficient uptake of the probes by the lysosomes. Following incubation, the sections are washed to remove excess probe, homogenized to ensure uniformity, and, if necessary, centrifuged at low speeds (1,000 to 5,000 × g) (times the force of gravity) to collect the supernatant. The lysosomal pH variations are then assessed using spectrophotometry by measuring absorbance values from the supernatant placed in a micro-plate or cuvette. These values provide insights into the lysosomal pH levels. The study by **Fujiwara et *al.* (2009)** discusses the use of pH-sensitive probes in monitoring lysosomal pH and provides additional details on both qualitative and quantitative approaches to assessing lysosomal pH changes.

15. Data analysis

All results in this study are presented as mean ± standard deviation (mean ± SD). Statistical analysis of the data were conducted using the Minitab® software version 18.1 and Microsoft Excel version 19.0.

To determine significant differences between the treatment effects, a one-way analysis of variance (ANOVA) was performed, Subsequently, Tukey's post-hoc test was applied for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

Additionally, the Student t-test was utilized to determine the significant differences between the groups.The differences were considered to be not significant at $p > 0.05$, and the differences were considered significant * with $0.05 \geq p \geq 0.01$, highly significant when **$0.01 \geq p \geq 0.001$, and very highly significant*** when $p<0.001$ (P threshold of significance).

Results and Interpretations

Chapter I: In Vitro Investigation: Phytochemical Characteristics and Pharmacological Potential of Melissa Officinalis L.

1. The quantitative and qualitative study of Melissa *officinalis* L water extract

1.1. Determination of the extraction yield of lemon balm water extract

The extraction yield (EY) of Melissa *officinalis* L. water extract obtained through maceration was calculated as a ratio between the weight of dry extract after evaporation and the weight of the dry plant material, expressed as a percentage , The yield was found to be 14.46±1.22 % **(Table 08).**

Table 08: Extraction Yield (EY) of Melissa *officinalis* L. water extract.

Extraction yield of Melissa *Officinalis* L. (MAC)		
Weight of plant material (g)	***Weight of dry extract (g)***	***EY [%]***
100	14,46	14,46

EY: Extraction Yield ;(MAC): Extracts obtained in maceration; EY (%) ± S.D; (n = 3) ;(14.46±1.22 %),

1.2. Phytochemical screening

For the identification of the different components of the plant, we used phytochemical tests based on coloring reactions or precipitation by specific chemical reagents. The results of the tests carried out on the aqueous extract of M. *officinalis* are mentioned in **Table 09**.

Table 09: Phytochemical screening of the different chemical Components Present in the aqueous extract of M. *officinalis*.

Chimical Groups		**Aqueous Extract of Melissa *officinalis* L.**
Flavonoids		+++
Polyphenols		++++
Leucoanthocyanins		-
Tannins	***Catechic tannins***	+++
	Gallic tannins	++
Sterols and Triterpenes		++
Alkaloids		+++
Saponins		++
Anthocyanins		-
Terpenoids		+
Free Quinones		-
Steroids		+
Free Anthraquinones	-	

(-) : Not founded, , (+) : low concentration, (++) : medium concentration, (+++) : high concentration , (++++) very high concentration

2. Structural Characterization Techniques

2.1. X-ray Diffraction (XRD) Pattern of Melissa *officinalis* L.

Figure 31 illustrates the diffracto-gram obtained by X-ray diffraction (XRD), highlighting the partially crystalline structure of the Melissa *officinalis* L. sample. The y-axis represents the intensity in arbitrary units, while the x-axis corresponds to the diffraction angle expressed in degrees 2θ.

The diffraction peaks observed at specific positions along the x-axis indicate the presence of crystalline phases within the sample. Their intensity varies depending on the concentration and arrangement of atomic planes responsible for X-ray diffraction. The width and intensity of the peaks also allow for the determination of crystallite size and the degree of crystallinity.

Regions of low intensity or diffuse patterns are characteristic of an amorphous portion or low crystalline organization.

2.1.1. Quantitative Data (Full-Width Half Maximum (FWHM), Peak Intensities, Crystallinity Indices)

2.1.1.1. Full-Width Half Maximum (FWHM)

The analysis of the peak widths at half maximum (FWHM, Full-Width at Half Maximum) enables the estimation of crystallite size using the Scherrer formula.

Scherrer formula:

$$D = (K \cdot \lambda) / \beta \cdot \cos(\theta)$$

Parameters

- **D:** Crystallite size (in nanometers, nm).
- **K:** Shape factor or Scherrer constant (typically K = 0.9 for spherical particles).
- **λ :** Wavelength of the X-rays used (0.15406 nm).
- **β :** Full-Width at Half Maximum (FWHM, in radians).
- **θ :** Diffraction angle corresponding to half the position of the main peak (in radians).

➢ ***Data extracted from the image***

- ***Peak* 1 :**
  - 2θ = 30°
  - FWHM = 0,2°
- ***Peak* 2 :**
  - 2θ = 45°
  - FWHM = 0,15°

➢ ***Steps for each Peak***

1. *Conversion of Data to Radians :*

$\theta = (2\theta/2) \cdot (\pi/180)$

$\beta = FWHM \cdot (\pi/180)$

2. *Application of the Scherrer formula for each Peak.*

➢ ***Calculations for Peak 1:*** (2θ =30°, FWHM = 0, 2° FWHM = 0, 2°) :

➢ *Conversion* :

$\theta = (30/2) \cdot (\pi/180) = 0{,}2618$ rad

$\beta = 0{,}2 \cdot (\pi/180) = 0{,}00349$ rad

➢ *Crystallite size* :

$D = (0{,}9 \cdot 0{,}15406) / (0{,}00349 \cdot \cos(0{,}2618)) = 41{,}12$ nm

➢ ***Calculations for Peak 2:*** (2θ = 45°, FWHM = 0, 15°):

➢ *Conversion* :

$\theta = (45/2) \cdot (\pi/180) = 0{,}3927$ rad

$\beta = 0{,}15 \cdot (\pi/180) = 0{,}00262$ rad

➢ *Crystallite size* :

$D = (0{,}9 \cdot 0{,}15406) / (0{,}00262 \cdot \cos(0{,}3927)) = 57{,}33$ nm

➢ ***Final results***

For 2θ = 30° , D = 41, 12 nm. For 2θ = 45° , D = 57, 33 nm.

These calculations show that the crystallites have a typical nanometric size, consistent with partially organized crystalline structures. Melissa *officinalis* L. exhibits a composite structure, combining both crystalline and amorphous elements, reflecting the complex physicochemical properties of this plant extract.

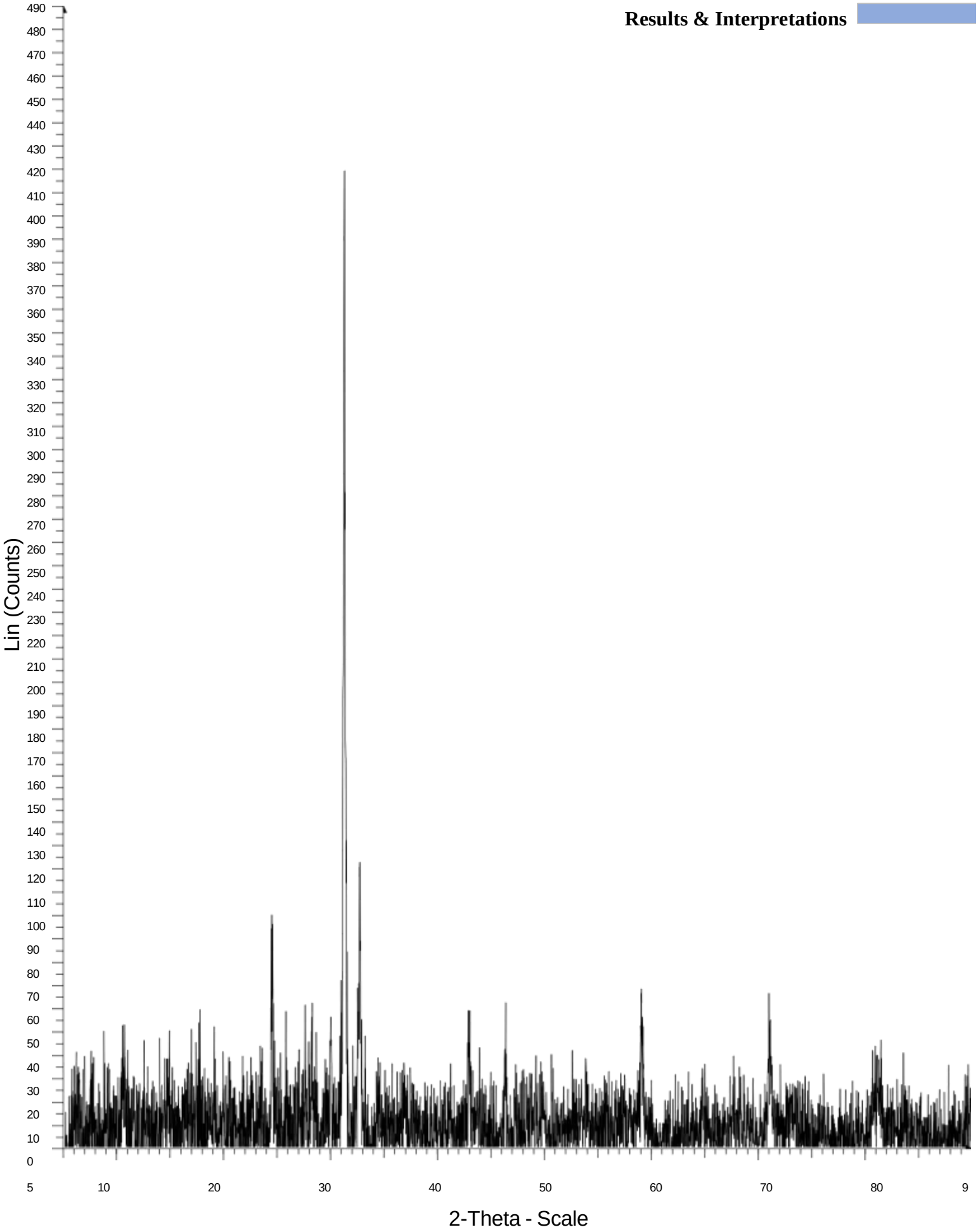


Figure 31: X-ray diffraction diagram of Melissa *officinalis* L.

2.1.1.2. Peak Analysis

Figure 32 represents the diffraction pattern with adjusted peak detection. The positions of the peaks (2-Theta values) and their relative intensities are compared with reference databases (ICDD PDF-4+).

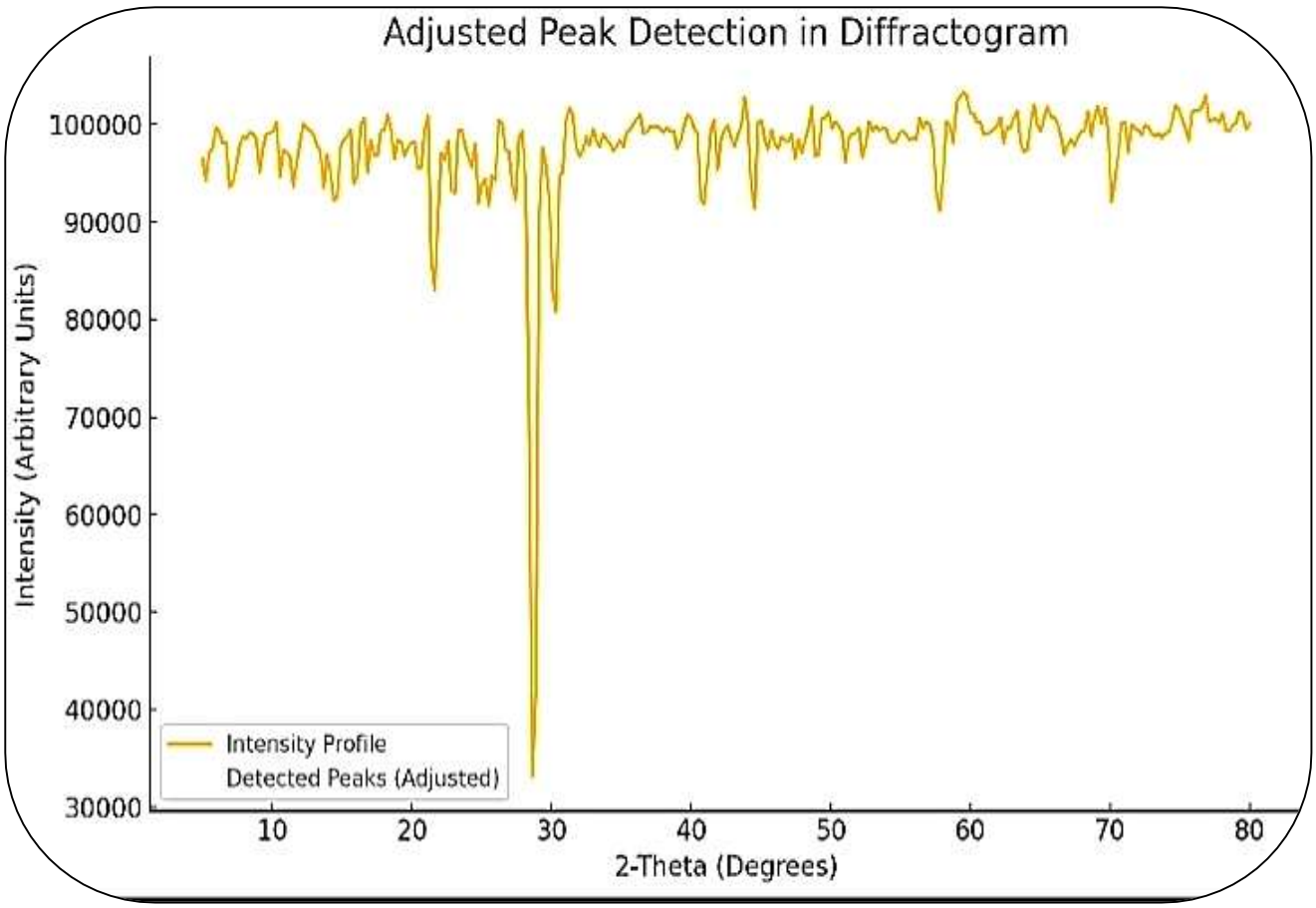


Figure 32: Diffraction diagram with adjusted peak detection.

2.1.1.3. Identified crystalline phases

Table 10 presents the identification of crystalline phases; the diffraction pattern shows peaks consistent with the main active compounds in Melissa *officinalis*, namely:

- Rosmarinic acid (main antioxidant),
- Flavonoids such as quercetin, apigenin, and kaempferol,
- Secondary phenolic acids.

These compounds are responsible for the antioxidant activity observed in the sample. Their presence confirms that your extract contains a significant proportion of bioactive compounds.

Table 10: Identification of crystalline phases of Melissa *officinalis* L.

Crystalline Phase	*Compounds*	*Molecular Formula*	*Peak Range (2θ)*	*Description*
Rosmarinic Acid	Rosmarinic acid	$C_{18}H_{16}O_8$	≈ 20° and 25°	Major phenolic compound; intense peaks indicating semi-crystalline properties and organized phenolic-type structures.
Flavonoids	Quercetin, Apigenin, Kaempferol	$C_{15}H_{10}O_7$ $C_{15}H_{10}O_5$ $C_{15}H_{10}O_6$	≈ 10–15°, 22–30°, ≈ 35°	Crystals or microcrystals; peaks linked to polycyclic aromatic structure of flavonoids.
Phenolic Compounds	Caffeic acid and derivatives (Chlorogenic Acid; Ferulic Acid)	$C_9H_8O_4$ $C_{16}H_{18}O_9$ $C_{10}H_{10}O_4$	≈ 18°–22°	Secondary phenolic acids; peaks indicating moderate crystallinity.
Other Compounds	Minerals, crystalline impurities	Varies (e.g.SiO_2, Al_2O_3, etc.)	≈ 40°–50°	Small peaks indicating possible traces of minerals or impurities.

2.1.1.4. Crystallinity Index (CI)

The crystallinity index calculated from the ratio of crystalline peak intensities to total peak areas. Higher CI indicates higher organized structures, often linked to minerals or structured secondary metabolites.

- Formula for Crystallinity Index (CI)

CI = (I crystalline - I crystalline) / I amorphous

Where :

- **I *crystalline*** = Intensity of the most prominent crystalline peak (sharp peak, at 2θ ≈ 27° in this case).
- **I *amorphous*** = Intensity of the baseline at the same 2θ position (background intensity under the crystalline peak).

So :

- **I crystalline:** Based on the provided image, the peak intensity at ~27° appears to be around 2000 units (estimated from the y-axis scale).
- **I amorphous:** The baseline near 27° seems to be at approximately 100 units.

- **CI Calculation :**

$$\mathbf{CI = (2000-100)\ /2000 = 0.95\ or\ 95\%}$$

A CI of 95% suggests that the sample is highly crystalline, which aligns with the presence of structured components like minerals or phenolic compounds in Melissa *officinalis* L.

2.2. Infrared Spectroscopy (IR)

The **Figure 33, 34** represents the FTIR spectrum of Melissa *officinalis* powder, showcasing the transmittance (%) of infrared radiation as a function of wavenumber (cm^{-1}). The x-axis displays the wavenumber range from 4250 to 400 cm^{-1}, while the y-axis represents transmittance, ranging from 0 to 100%. Key absorption peaks are highlighted by red vertical lines, corresponding to the functional groups present in the sample.

Prominent peaks are observed in specific regions:

- A broad and noticeable dip between 3025 and 2830 cm^{-1}, corresponding to C-H stretching vibrations of aliphatic hydrocarbons.
- A strong absorption near 1700 cm^{-1}, attributed to C=O stretching vibrations, characteristic of carbonyl groups found in phenolic acids and esters.
- Additional absorption in the 1350–970 cm^{-1} range, indicating C-O stretching and C-H bending vibrations, typical of alcohols, esters, and ethers.
- The fingerprint region (below 950 cm^{-1}) contains multiple peaks, reflecting the unique structural characteristics of terpenes, flavonoids, and aromatic compounds.

Gray-shaded areas at the edges (beyond the regions of interest) represent spectral limits or regions excluded from the primary analysis. This spectrum provides a comprehensive chemical fingerprint of Melissa *officinalis* powder, allowing identification and confirmation of its bioactive components.

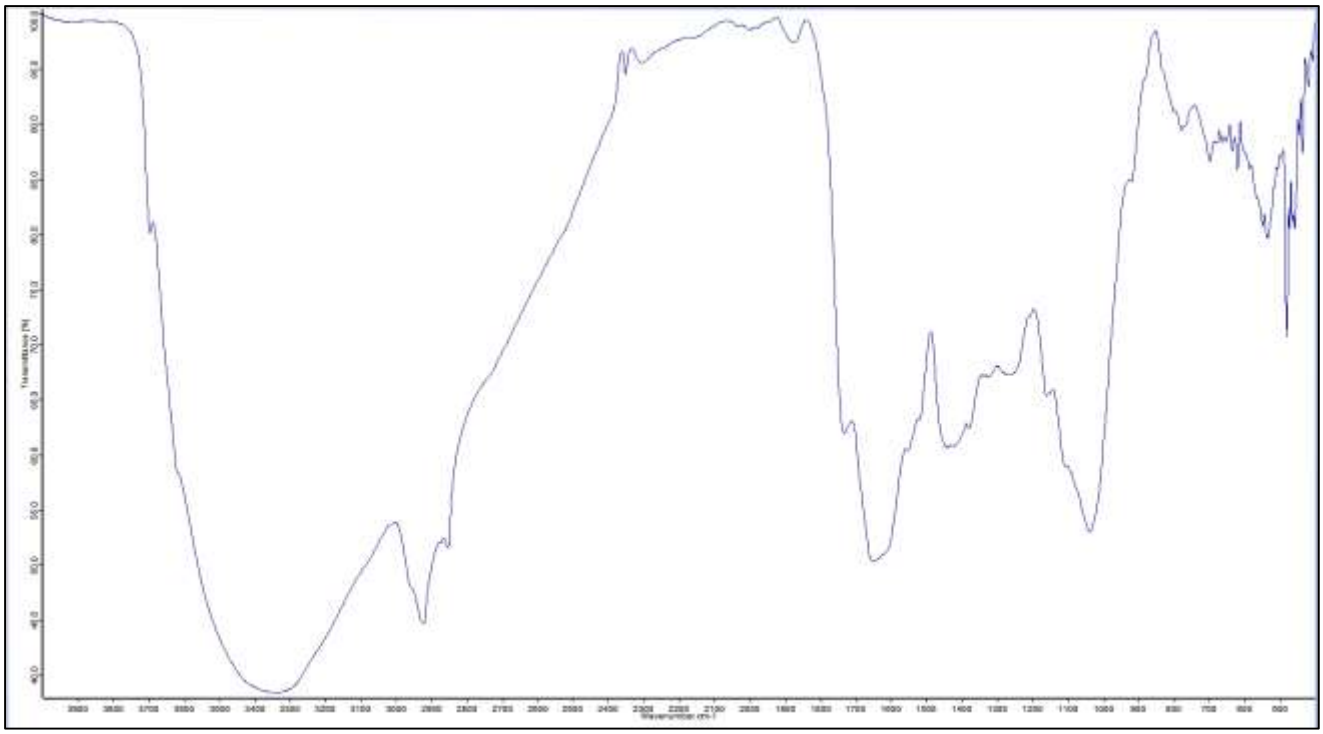


Figure 33: FTIR spectrum of Melissa *officinalis* powder.

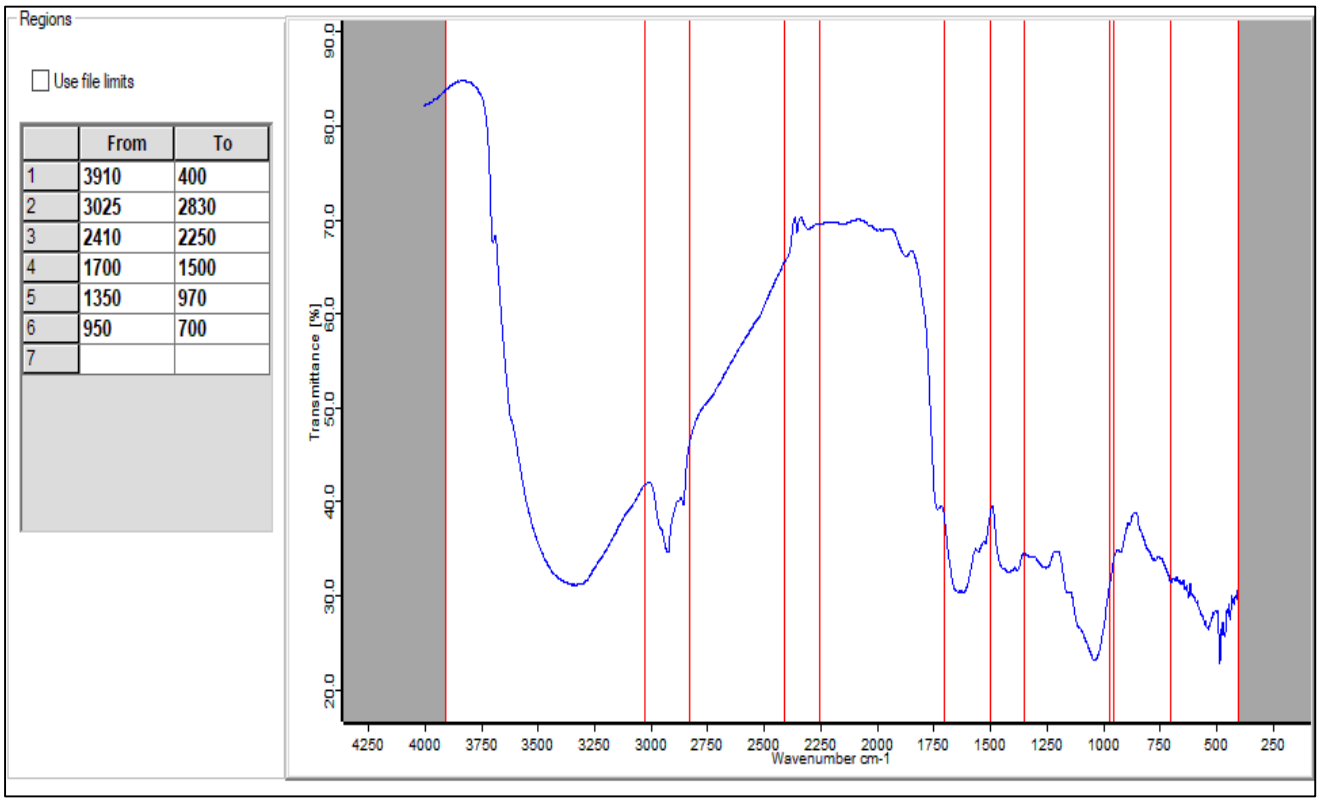


Figure 34: FTIR spectrum analysis of Melissa *officinalis* powder.

➢ **Functional groups identified**

The FTIR spectrum highlights key functional groups present in the Melissa *officinalis* powder, **(Table 11)** consistent with its known chemical profile.

Table 11: Key absorption bands in the spectrum (wavenumbers in cm^{-1}) in the M. *officinalis* powder.

Region (cm^{-1})	*Peak Interpretation*	*Functional Group*	*Compound Type*	*References*
3025–2830	C-H stretching vibrations	Aliphatic hydrocarbons	Terpenes, fatty acids	Šic Žlabur et *al.* (2016).,
2410–2250	Weak absorption, possible C≡N stretching	Nitriles (if present in low concentration)	Trace compound	Dastmalchi et *al.* (2008).
1700–1500	Strong absorption, C=O stretching	Carbonyl groups (ketones, aldehydes, acids)	Rosmarinic acid, caffeic acid	Miraj and Kiani (2016).
1350–970	C-O stretching vibrations, C-H bending	Alcohols, esters	Phenolic compounds, essential oils	Wagner et *al.* (2003)
950–700	Fingerprint region, bending vibrations	Unique structural markers	Terpenes, aromatic compounds	Šic Žlabur et *al.* (2016)

2.3. Scanning electron microscopy (SEM)

The results obtained through scanning electron microscopy (SEM), represented in **Figure 35**, highlight several important aspects of Melissa *officinalis L.* powder at different particle sizes (5, 10, 20, 50, 100, 200 µm). Surface topography reveals that smaller particles (5-20 µm) have rough and porous surfaces, increasing the specific surface area and facilitating interaction with solvents. In contrast, larger particles (>50 µm) show more compact surfaces, which could limit solvent diffusion and slow down the extraction of active compounds. Particle distribution is generally homogeneous, although agglomerations may be observed at sizes below 20 µm, which could affect the efficiency of extraction. Structural characteristics of the particles, such as the presence of cracks, pores, or compact surfaces, vary depending on their size. Smaller particles promote greater accessibility of bioactive compounds, while larger ones limit this accessibility. Finally, correlation with the release profiles of bioactive compounds shows that smaller particles favor rapid release due to their high surface area and porosity, while larger particles allow for a more controlled release, suitable for applications requiring prolonged release. These observations help optimize particle size according to specific needs for extraction or controlled release of active compounds.

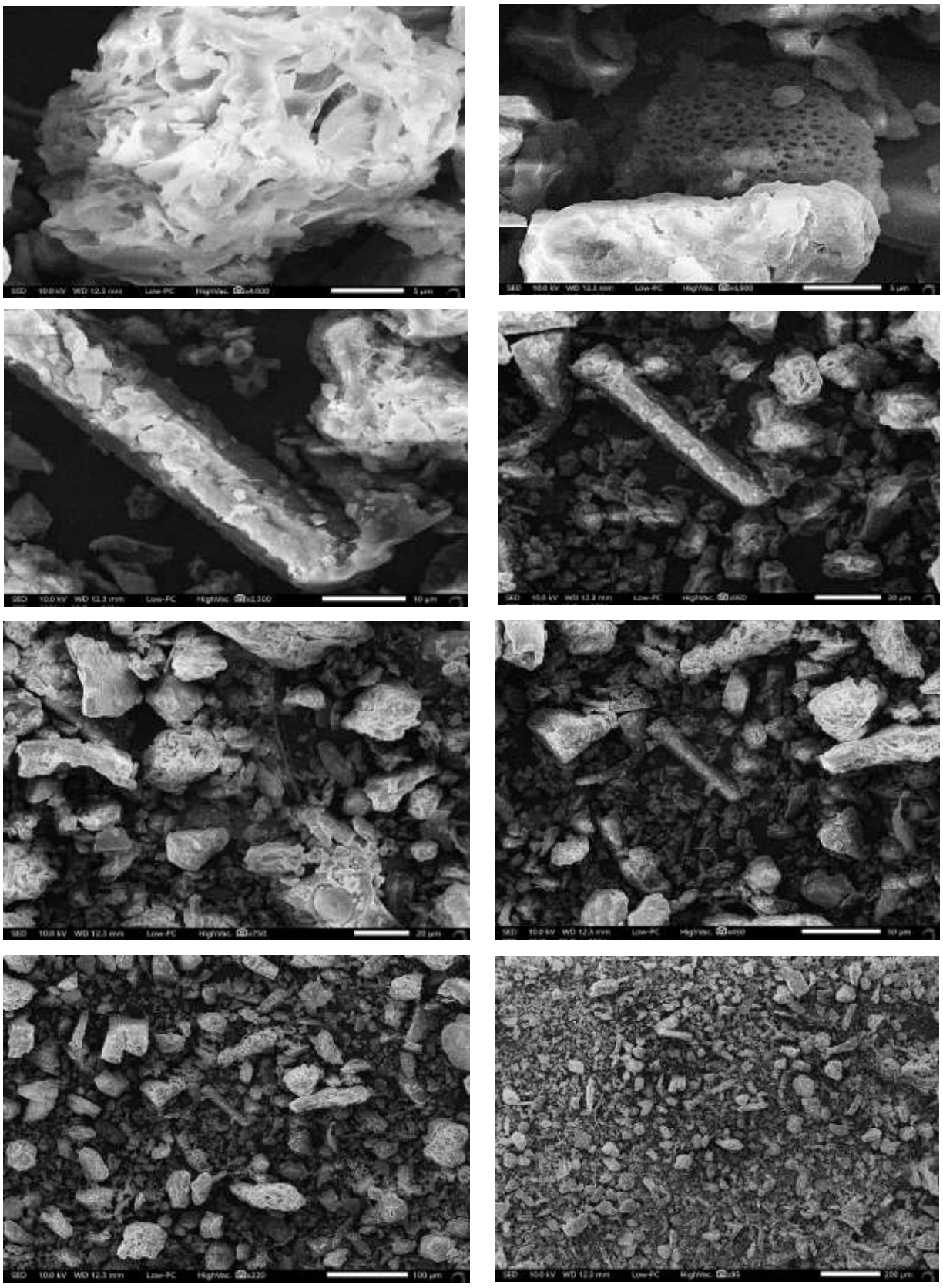


Figure 35: SEM observation of Melissa *officinalis L.* particles at different sizes (5, 10, 20, 50, 100, and 200 µm).

3. Determination of total bioactive compounds

3.1. Total polyphenol content (TPC)

The quantitative analysis of total polyphenols in the aqueous extract of Melissa *officinalis* L. was determined using a calibration curve of gallic acid at various concentrations **(Figure 36).** The total phenolic content in the extract is expressed in milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract). The results are presented in **Table 12,** indicating a total polyphenol content of 40.29±4.09 mg GAE/g extract.

3.2. Total flavonoïd content (TFC)

The flavonoid content in the extract was determined using a calibration curve of quercetin as a standard at various concentrations **(Figure 37)**. The flavonoid content **(Table 12)** is expressed in milligrams of quercetin equivalents per gram of extract (mg QE/g extract). The results indicate that the aqueous extract of Melissa *officinalis* L. contains 24.43-±1.77 mg QE/g extract.

3.3. Total Condensed tannins content (TTC)

The condensed tannin content of our extract was calculated using a calibration curve of catechin **(Figure 38)** and is expressed in milligrams of catechin equivalents per gram of extract (mg CE/g extract). According to the results obtained **(Table 12),** the studied plant has a condensed tannin content of 17.84 ± 3.95 mg CE/g extract.

Table 12: Total Phenol, Flavonoid, and Condensed Tannin Contents in the Extract of Melissa *officinalis* L. (1 mg/mL)**.**

Compounds	***Content***
Total Polyphenols content, (mg EAG/g)	40.29±4.09
Total Flavonoids content, (mg EQ/g)	24.43±1.77
Total Condensed Tannins content, (mg EAT/g).	17.84±3.95

Values were expressed as means ±S.D. (n= 3).

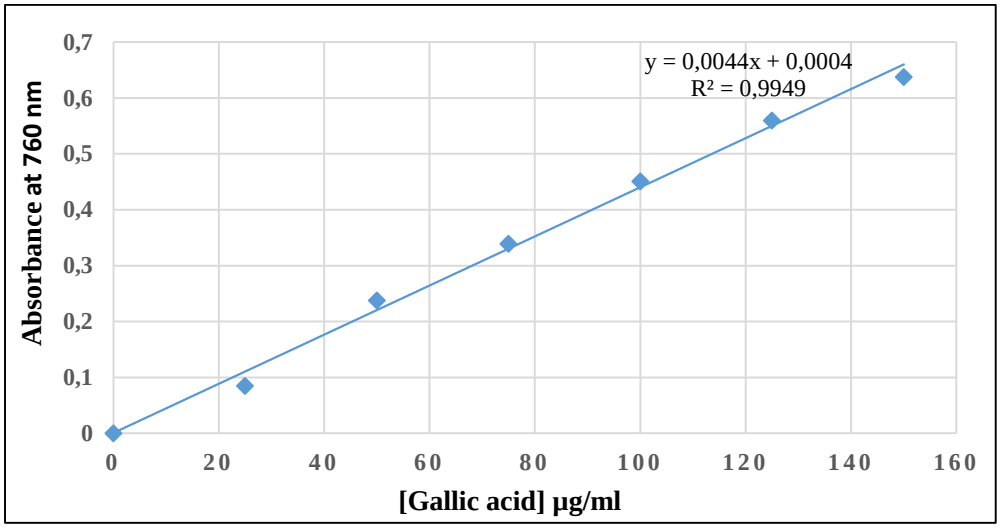


Figure 36: Gallic acid calibration curve for the determination of total phenolic content.

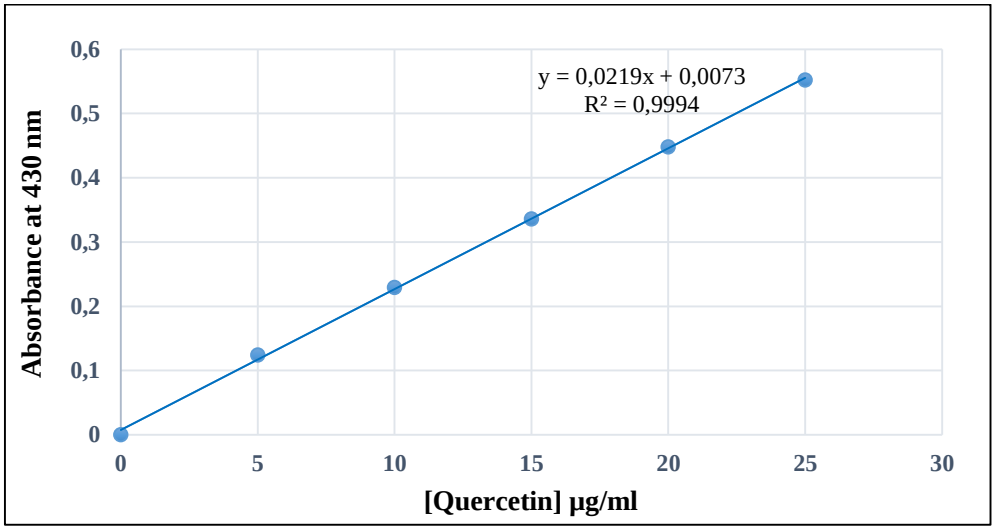


Figure 37: Quercetin calibration curve for the determination of total flavonoids content.

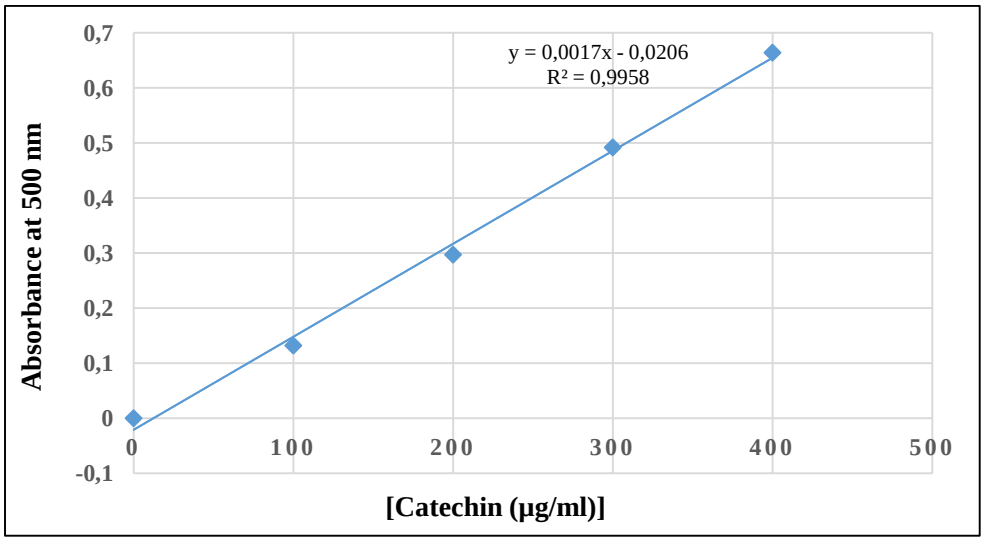


Figure 38: Catechin calibration curve for determining of total condensed tannin content.

4. Assessment of the antioxidant activity in the extract of M. *officinalis* L.

The antioxidant neutralizing abilities of the aqueous extract of M. *officinalis* were validated through several chemical methods, such as the DPPH radical scavenging test, ABTS scavenging activity, cupric reducing antioxidant capacity (CUPRAC), and ferric reducing power assay (FRAP).

4.1. DPPH radical scavenging activity

The DPPH radical neutralization test was performed using various concentrations of the M. *officinalis* extract **(Table 13)**. The inhibitory concentration at 50% (IC50) is the most commonly used parameter to assess antioxidant power. Our results demonstrated that the aqueous extract of M. *officinalis* (IC50 = 23.29 µg/ml) exhibited remarkable antiradical activity compared to synthetic antioxidants such as BHA (IC50 = 14.66 µg/ml), BHT (IC50 = 16.53 µg/ml), and ascorbic acid (IC50 = 11.39 µg/ml).

4.2. ABTS scavenging activity

As shown in **Table 13**, the ABTS test results indicated that the aqueous extract of M. *officinalis* (IC50 = 24.95 µg/ml) exhibited remarkable antiradical activity when compared to synthetic antioxidants such as BHA (IC50 = 1.09 µg/ml) and BHT (IC50 = 1.97 µg/ml).

4.3. CUPRAC inhibitory capacity test

Our results showed that the M. *officinalis* extract displayed significant dose-dependent antiradical activity **(Table 13)**. The EC50 value (effective concentration at 50%) calculated for the aqueous extract was 31.14 µg/ml, which was notably high compared to synthetic antioxidants such as BHA (EC50 = 13.65 µg/ml) and BHT (EC50 = 17.62 µg/ml).

4.4. Ferric reducing power assay

Our findings demonstrated that the aqueous extract of M. *officinalis* (EC50 = 28.76 µg/ml) exhibited remarkable antiradical activity, significantly referencing ascorbic acid (EC50 = 8.04 µg/ml) **(Table 13).** This result significantly reflects its potential antioxidant activity. The ferric reducing power assay represents the capacity of the antioxidants in the M. *officinalis* extract to reduce Fe^{3+} to Fe^{2+}.

Table 13: Comparative analysis of the antioxidant activity of the M. *officinalis* L. extract using different methods.

Extract and standards	***DPPH [a] (µg/ml)***	***ABTS [a] (µg/ml)***	***CUPRAC [b] (µg/ml)***	***(FRAP) [b] (µg/ml)***
M. officinalis L	23.29±1.74	24.95±1.20	31.14±3.47	28.76±0.34
BHA	14.66±4.26	1.09±0.03	13.65±0.14	*nt*
BHT	16.53±2.76	1.97±0.01	17,62±0,29	*nt*
Ascorbic acid	11,39±0,01	*nt*	*nt*	8,04±0,05

The values expressed are means ± S.D. of three parallel measurements; a Values expressed as IC50 (µg/ml);b Values expressed as EC50 (µg/ml); nt : not tested

Chapter II: In Vivo Investigation: Evaluating the Neurotoxic Impact of Imidacloprid and the Protective Role of Melissa officinalis L. on the Nervous System

1. Clinical indicators of neurotoxicity and visual observations

The clinical signs of intoxication across different groups exposed to chronic Imidacloprid (IMI) and Melissa *officinalis* Extract (MOE) are summarized in **Table 14** demonstrates that the control and Melissa *officinalis* Extract (MOE) groups exhibited no significant neurotoxic effects. In contrast, the low-dose Imidacloprid group (IMI, 5 mg/kg/day) displayed mild clinical signs, including reduced activity and minimal weight loss. The high-dose Imidacloprid group (IMI, 50 mg/kg/day) presented pronounced neurotoxic effects such as lethargy, tremors, significant weight loss, and occasional seizures. Co-administration of MOE mitigated these effects, particularly in the high-dose group, suggesting a neuroprotective role for MOE. No mortalities were recorded in any of the groups throughout the experiment.

Visual observations **(Table 15, Figure 39)** further supported these findings. The high-dose IMI group showed moderate inflammation, mild swelling, and occasional superficial wounds, while co-treatment with MOE lessened these effects, highlighting its anti-inflammatory potential.

Table 14: Clinical signs in rats exposed to chronic Imidacloprid and Melissa *officinalis* L.

Clinical Signs	***Control Group***	***IMI D1 (5 mg/kg/day)***	***IMI D2 (50 mg/kg/day)***	***MOE (10 mg/kg/day)***	***IMI D1 + MOE***	***IMI D2 + MOE***
Behavioral Changes	None	Occasional alterations in grooming	Decreased exploratory behavior, altered grooming	None	Occasional grooming alterations	Reduced grooming alterations
Impaired Motor Coordination	Not observed	Not observed	Frequent, ataxia	Not observed	Not observed	Mild, occasional ataxia
Decreased Activity	None	Mild lethargy, slight reduction	Pronounced lethargy	None	Mild lethargy	Reduced lethargy
Tremors	Not observed	Occasional tremors	Frequent tremors	Not observed	Not observed	Mild, occasional tremors
Loss of Appetite	None	Mild	Moderate to severe	None	Mild	Moderate
Weight Loss	None	Minimal	Significant	None	Minimal	Moderate
Gait Abnormalities	Not observed	Occasional	Frequent	Not observed	Not observed	Occasional
Fur Ruffing	Not observed	Mild	Pronounced	Not observed	Mild	Mild
Seizures	Not observed	Absent	Occasional	Absent	Absent	Rare
Respiratory Distress	Not observed	Not observed	Mild	Not observed	Not observed	Mild
Diarrhea	Not observed	Not observed	Occasional	Not observed	Not observed	Not observed
Mortality	0%	0%	0%	0%	0%	0%

Table 15: Visual observations in rats exposed to chronic Imidacloprid and M. *officinalis*.L

Visual Observations	*Control Group*	*IMI D1 (5 mg/kg/day)*	*IMI D2 (50 mg/kg/day)*	*MOE (10 mg/kg/day)*	*IMI D1 + MOE*	*IMI D2 + MOE*
Skin Inflammation	None	Mild localized	Moderate, occasional	None	Mild localized	Mild localized
External Edema	None	Not observed	Mild swelling	None	Not observed	Mild swelling
Extremity Erosions	Not observed	Not observed	Occasional erosions	Not observed	Not observed	Occasional erosions
Superficial Wounds	Not observed	Not observed	Mild, occasional	Not observed	Not observed	Mild, occasional

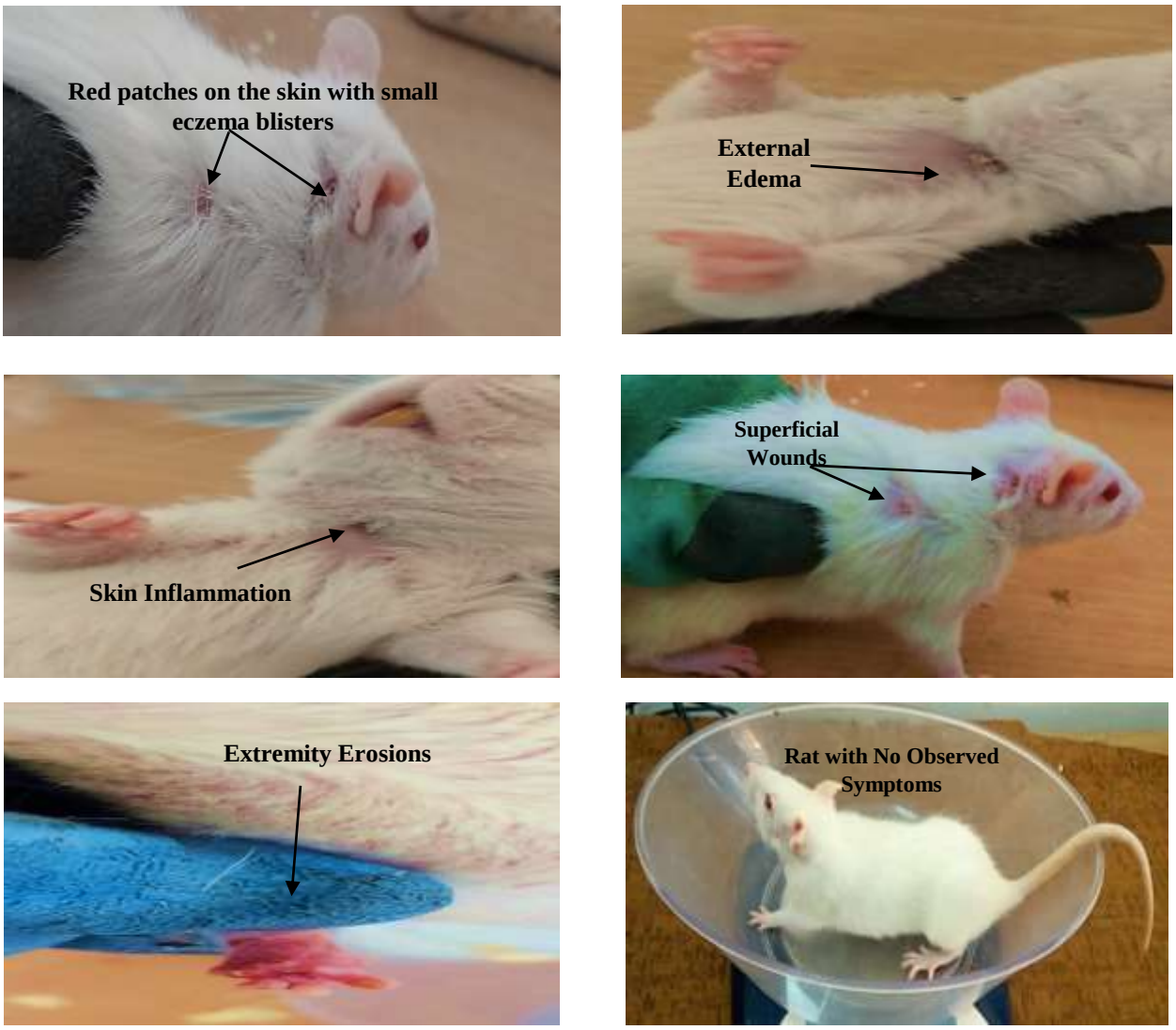


Figure 39: Symptomatic and visual effects observed in control and different treatment groups after 90 days of treatment **(*Personal photos*).**

2. Impact on growth parameters

The study revealed significant effects on body weight, weight gain, and relative brain weight in treated rats compared to controls, with a dose-dependent reduction observed across all growth parameters (are presented in **Tables 16** and **Figures 40, 41, 42**).

In terms of body weight, normal growth was observed in the control group, while IMI D1 (5 mg/kg/day) showed a significant reduction ($p \leq 0.05$) and IMI D2 (50 mg/kg/day) demonstrated a highly significant reduction ($p \leq 0.01$). No significant changes were seen in the MOE (10 mg/kg/day) group, suggesting MOE alone does not affect body weight. However, co-administration of IMI D1 + MOE resulted in improved body weight compared to IMI D1 alone ($p \leq 0.05$), while IMI D2 + MOE showed a slight improvement, though still significantly lower than the control ($p \leq 0.05$). *Regarding weight gain*, the control group exhibited normal gain, while IMI D1 and IMI D2 groups experienced a dose-dependent reduction, with significant ($p \leq 0.05$) and very highly significant ($p \leq 0.001$) decreases, respectively. MOE alone had no impact on weight gain, but its combination with IMI showed noticeable improvements, especially in IMI D1 + MOE ($p \leq 0.01$); while IMI D2 + MOE exhibited slight improvements, still significantly lower than control ($p \leq 0.05$). *In terms of relative brain weight*, normal values were observed in controls, but a dose-dependent reduction was noted in IMI D1 ($p \leq 0.05$) and a highly significant decrease in IMI D2 ($p \leq 0.01$). The MOE group showed no significant change, while IMI D1 + MOE improved relative brain weight ($p \leq 0.01$). Though IMI D2 + MOE showed some improvement, it remained significantly lower than control ($p \leq 0.05$).

Table 16: Body weight change (g) and relative weight (%) of control and treated rats brains after 90 days of treatment.

	Variable Groups					
Parameters	***Control Group***	***IMI D1 (5 mg/kg/day)***	***IMI D2 (50 mg/kg/day)***	***MOE (10 mg/kg/day)***	***IMI D1 + MOE***	***IMI D2 + MOE***
Weight Gain (g)	11±7.015	90±9.315 **	128±12.083 ***	10±5.461 ns	52±7.314 *	73±8.705 **
Relative Weight of brain (%)	0,696±0,017	0,531±0,024 *	0,420±0,024 **	0,706±0,018 ns	0,609±0,029 ns	0,527±0,019 *

IMI: *Imidacloprid,* ***MOE:*** *Melissa Officinalis L Extract, Results are expressed as mean ± SE (n=10) t-test was used for multiple comparisons. Statistical significant as compared to control (Not significant (ns): P> 0.05; Significant *P ≤ 0.05; highly significant **P ≤ 0.01; very highly significant ***P ≤ 0.001).*

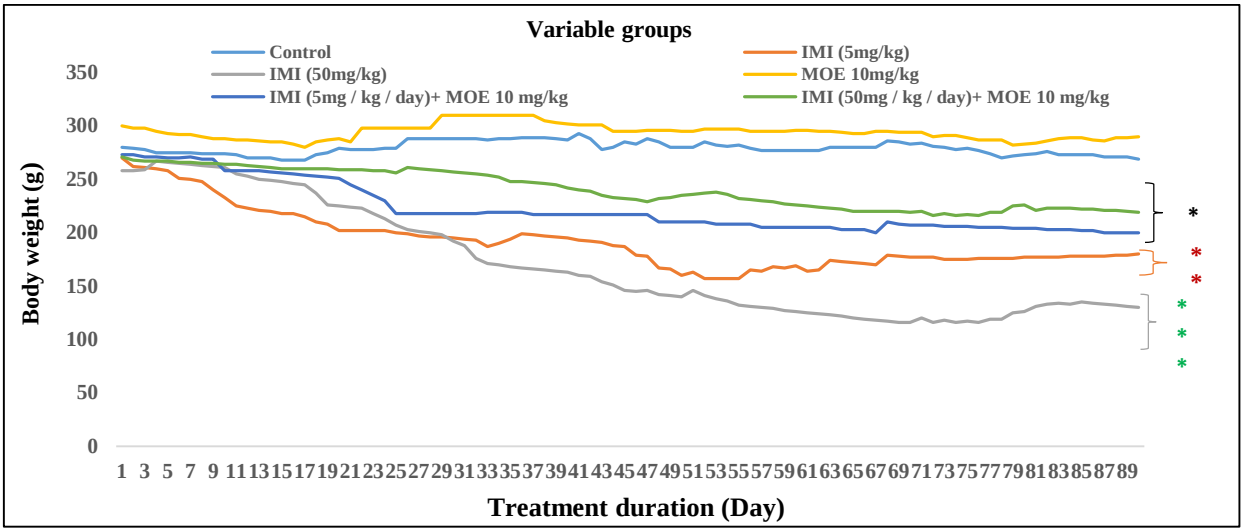


Figure 40: Kinetic changes in body weight (BW) in the different treated groups over 3 months.

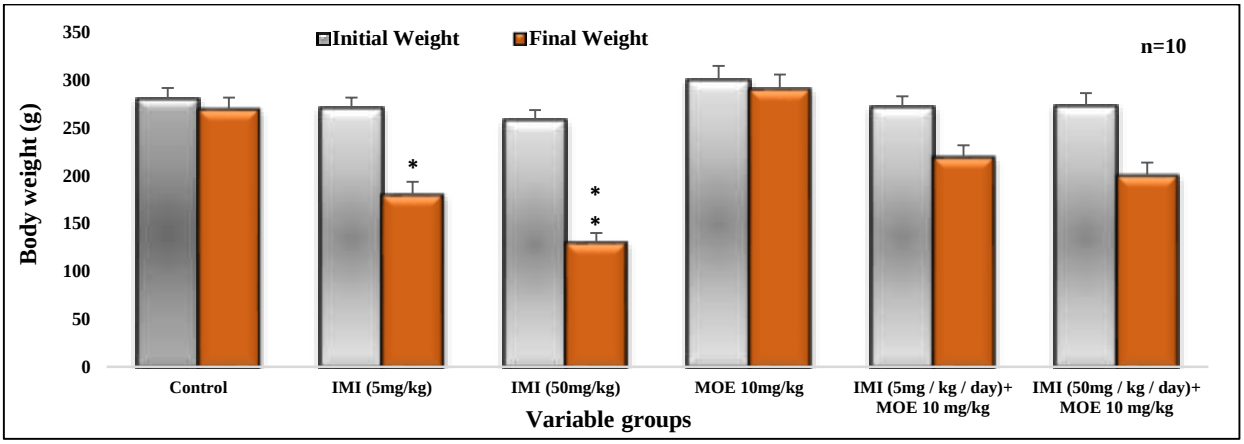


Figure 41: Body weight evolution (BW) in the different treated groups over 3 months (*Difference between initial and final weight*).

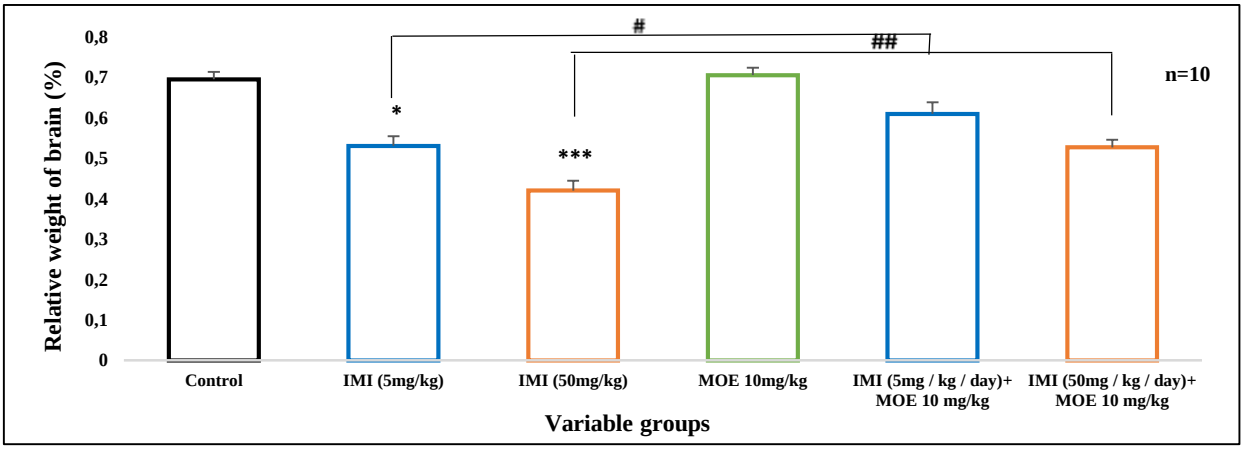


Figure 42: Evolution of relative brain weight (RBW) in control and treated rats after 90 days of treatment, *comparison to control (ns: P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001). .*

3. Behavioral and cognitive tests

The results of this study on the behavior of *Wistar* rats are shown in **Table 17** and **Figures 43, 44, 45, 46, 47** and **48.**

3.1. The classic labyrinth test (CLT)

The results obtained (n = 10; Mean ± SE) highlighted significant changes in *Finishing Time (FT) and Exploration Time (ET)* across treatment groups **(Figure 43)** and **(Table 17).** The control group had the shortest FT (4.02 ± 2.37 sec) and the longest ET (12.9 ± 12.33 sec), indicating normal cognitive function. The IMI (5 mg/kg) group experienced a significant increase (*P < 0.05) in FT (9.24 ± 1.14 sec) and a highly significant decrease (**P < 0.01) in ET (3.7 ± 1.87 sec), while the IMI (50 mg/kg) group showed a very highly significant increase (p ≤ 0.001) in FT (19 ± 2.42 sec) and a very highly significant decrease in ET (1.816 ± 0.33 sec), reflecting severe neurotoxicity. In contrast, the Melissa *officinalis* (10 mg/kg) group exhibited a highly significant reduction (**P ≤ 0.01) in FT (2.94 ± 1.12 sec) and a significant increase (*P ≤ 0.05) in ET (14.29 ± 1.07 sec), demonstrating its protective effects. Additionally, both combination groups (IMI (5 mg/kg) + MOE: FT 5.97 ± 1.69 sec, ET 11.96 ± 2.99 sec and IMI (50 mg/kg) + MOE: FT 8.88 ± 1.27 sec, ET 10.25 ± 1.88 sec) experienced a dose-dependent reduction in FT and a dose-dependent increase in ET, confirming the efficacy of Melissa *officinalis* against Imidacloprid exposure, with results showing a significant reduction (*P ≤ 0.05) compared to controls.

3.2. Open field (OF) locomotion test

The Open Field Test results **(Figure 44, Table 17)** showed significant changes in all treated groups. *The Immobility Time in the Peripheral Area (IPA)* was highest in the IMI D2 group (88 ± 3.68 sec**; ***P < 0.001) and increased in both Imidacloprid-treated groups (IMI D1: 76 ± 2.58 sec), indicating anxiety. Melissa *officinalis* reduced this immobility, especially in the IMI D1 + MOE group (62 ± 3.39 sec; *P < 0.05). *In the Intermediate Area (IIA),* immobility increased in the Imidacloprid-treated groups (IMI D2: 7.45 ± 0.576 sec; *P < 0.001), but Melissa significantly decreased it (IMI D1 + MOE: 2.55 ± 0.68 sec). *In the Central Area (ICA),* immobility was significantly reduced in IMI D2 (1.816 ± 0.33 sec; ***P < 0.001), while Melissa increased time spent in the central area (MOE: 14.29 ± 1.07 sec; *P < 0.05). *The Total Distance Moved (TDM)* showed a dose-dependent reduction, lowest in IMI D2 (7.616 ± 0.648 m; ***P < 0.001), with Melissa reversing the decline (MOE: 31.37 ± 1.73 m). Lastly, *the Total Number of Rearing's (TNR)* decreased in Imidacloprid groups (IMI D2: 10.29 ± 0.97; **P < 0.01), but increased with Melissa (IMI D1 + MOE: 13.9 ± 0.90).

3.3. Novel object recognition test (NOR)

In the Object Recognition Test **(Figure 45, Table 17)**, Imidacloprid (IMI)-treated rats showed significant memory impairment by spending less time exploring the novel object. *In Phase I:* Familiar Object Exploration, rats treated with 5 mg/kg IMI displayed a highly significant increase in exploration time (**P ≤ 0.01), while the 50 mg/kg IMI group exhibited a very highly significant increase (***P≤ 0.001), indicating dose-dependent memory deficits. *In Phase II:* Novel Object Exploration, both doses of IMI (5 mg/kg and 50 mg/kg) led to a very highly significant reduction (***P ≤ 0.001) in novel object exploration time, further confirming severe recognition memory impairment. Melissa *officinalis* Extract (MOE) alone did not affect memory, as exploration times for the familiar (EFO: 50.1 ± 5.043 s) and novel objects (ENO: 85.9 ± 6.297 s) were comparable to controls. Co-treatment with IMI D1 + MOE improved memory retention, evidenced by a lower EFO (65.5 ± 5.148 s) and higher ENO (71.4 ± 6.752 s; *P < 0.05) compared to IMI D1 alone. IMI D2 + MOE provided partial neuroprotection, significantly reducing EFO (104.8 ± 8.509 s) and increasing ENO (43.8 ± 5.223 s; **P < 0.01) versus high-dose IMI.

3.4. Vsoc-Maze: A Tool for investigating sociability and social novelty preference

In the Vsoc-Maze Test, results demonstrated significant variations across groups **(Figure 46, Table 17)**, all experimental groups were compared to the Control Group, which displayed normal exploratory behavior during the *Habituation Phase* (E1: 118.75 ± 8.9 s; E2: 121 ± 8.981 s). The IMI (5 mg/kg) group (E1: 99.55 ± 7.719 s; E2: 92.5 ± 8.66 s) and IMI (50 mg/kg) group (E1: 83.75 ± 4.788 s; E2: 78.5 ± 8.10 s) spent significantly less time exploring, indicating dose-dependent impairments in curiosity (P < 0.05, P < 0.01). In contrast, the MOE (10 mg/kg) group (E1: 115 ± 7.071 s; E2: 117.5 ± 10.5 s) exhibited similar exploration times to the control. Co-treatment with IMI (5 mg/kg) + MOE (E1: 109.75 ± 8.3 s; E2: 102.38 ± 4.5 s) and IMI (50 mg/kg) + MOE (E1: 101 ± 6.37 s; E2: 92.75 ± 6.185 s) resulted in increased exploration compared to their respective IMI groups, suggesting a mitigating effect of Melissa *officinalis*.

During the *Sociability Phase*, the control group showed a strong preference for the juvenile mouse (S1: 244.25 ± 6.8 s). In comparison, the IMI (5 mg/kg) group (S1: 121 ± 7.958 s) and IMI (50 mg/kg) group (S1: 87.25 ± 3.86 s) displayed significantly reduced sociability (P < 0.01, P < 0.001). The MOE group (S1: 246 ± 8.98 s) showed comparable sociability to controls, while co-treatment groups IMI (5 mg/kg) + MOE (S1: 200.25 ± 7.9 s) and IMI (50 mg/kg) + MOE (S1: 163.5 ± 5.066 s) had improved sociability compared to their respective IMI-only groups.

In the *Preference for Social Novelty Phase*, the control group preferred the novel mouse (S2: 246 ± 6.684 s). Conversely, the IMI (5 mg/kg) group (S2: 105 ± 5.099 s) and IMI (50 mg/kg) group (S2: 79.125 ± 6.574 s) showed significantly reduced novelty preference ($P < 0.05$, $P < 0.01$, $P < 0.001$). The MOE group (S2: 243.13 ± 8.2 s) maintained normal preference levels, and co-treatment with Melissa *officinalis* extract significantly improved preference in both IMI groups (IMI (5 mg/kg) + MOE: 207.38 ± 8.3 s; IMI (50 mg/kg) + MOE: 182.75 ± 15.649 s) compared to their respective controls.

3.5. Sucrose preference test (SPT) for measurement of stress-induced anhedonia

In the sucrose preference test **(Figure 47, Table 17)**, the control group exhibited a high sucrose preference (SI: 43.5 ± 3.03 ml; SP: 68.5 ± 3.03 %), indicating normal hedonic behavior. In contrast, both the IMI (5 mg/kg) group (SI: 26 ± 1.69 ml; SP: 40 ± 1.70 %) and IMI (50 mg/kg) group (SI: 7.4 ± 0.52 ml; SP: 15.61 ± 0.53 %) showed significantly reduced sucrose preference compared to the control ($P < 0.01$, $P < 0.001$). The IMI (50 mg/kg) group demonstrated a more pronounced decrease, reflecting a dose-dependent effect of Imidacloprid on inducing anhedonia-like behavior.

The Melissa (10 mg/kg) group (SI: 45.3 ± 1.5 ml; SP: 75.124 ± 1.6 %) exhibited sucrose preference and intake levels comparable to the control group, confirming the absence of anhedonia and suggesting that Melissa *officinalis* does not negatively impact hedonic behavior.

Co-treatment with IMI (5 mg/kg) + MOE (10 mg/kg) (SI: 36 ± 1.9 ml; SP: 54.55 ± 1.10 %) significantly improved sucrose preference compared to the IMI (5 mg/kg) group, indicating a protective effect of Melissa *officinalis* against low-dose Imidacloprid exposure. Although the IMI (50 mg/kg) + MOE group (SI: 26.5 ± 1.27 ml; SP: 43.08 ± 1.28 %) showed enhanced sucrose preference relative to the high-dose Imidacloprid group, the improvement was less pronounced than in the lower dose co-treatment group. Nonetheless, the presence of Melissa *officinalis* significantly ameliorated anhedonia-like symptoms, highlighting its neuroprotective potential under toxicological stress.

3.6. Olfactory recognition test

In the olfactory recognition results **(Figure 48, Table 17)**, the control group demonstrated efficient olfactory recognition with a latency time to own fissure (LTOF) of 83.9 ± 28.21 sec. In contrast, both the IMI (5 mg/kg) and IMI (50 mg/kg) groups showed significantly increased LTOF (103.5 ± 20.83 sec and 287.1 ± 33.504 sec, respectively); indicating impaired olfactory recognition ($P < 0.05$, $P < 0.01$, $P < 0.001$). Treatment with

Melissa *officinalis* (10 mg/kg) significantly reduced latency times in both co-treatment groups (IMI D1 + MOE: 67.7 ± 14.36 sec; IMI D2 + MOE: 79.1 ± 9.025 sec), suggesting a protective effect against neurotoxicity. Similar trends were observed in the latency time to familial fissure (LTTFF) and the time spent in the center, where imidacloprid-exposed rats spent more time in the center, indicating impaired recognition and decision-making.

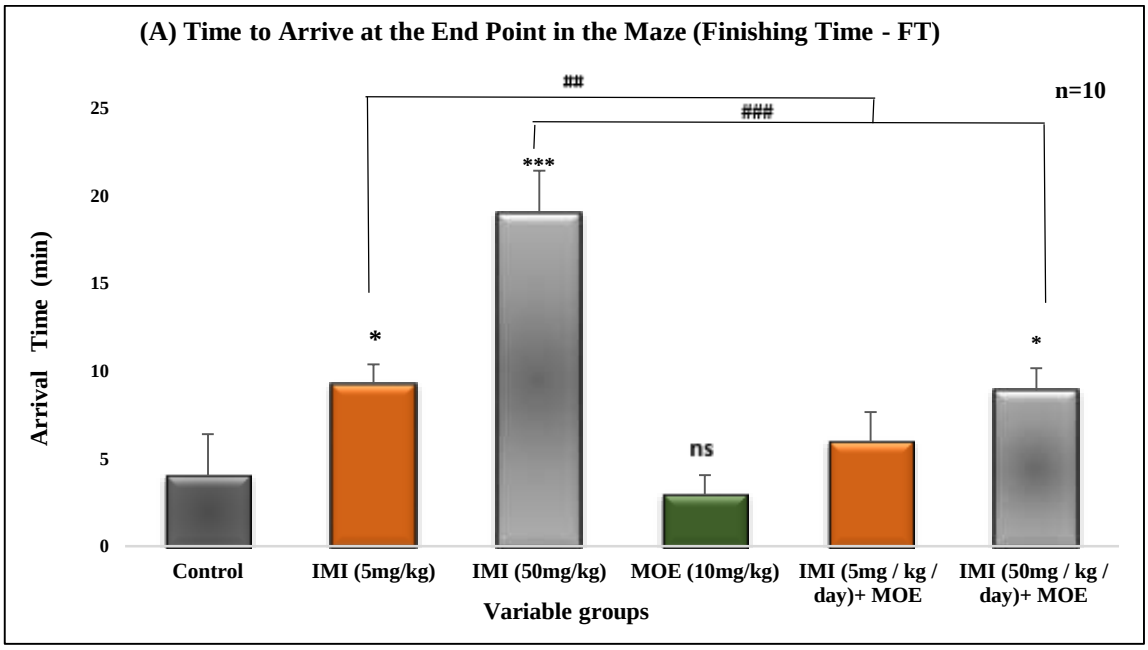
Table 17: Changes in behavioral tests: Open Field (OF), Classic Labyrinth (LC), Novel Object Recognition (NOR) and Olfactory Recognition Tests, Vsoc-Maze and the sucrose preference test (SPT) in control and treated rats after 3 months of treatment.

Test			***Variable Groups***					
			Control Group	***IMI D1 (5 mg/kg/day)***	***IMI D2 (50 mg/kg/day)***	***MOE (10 mg/kg/day)***	***IMI D1 + MOE***	***IMI D2 + MOE***
Classic Labyrinth	**FT**		4,02 ± 2,37	9,24 ±1,14	19± 2,42	2,94 ± 1,12	5,97 ± 1,69	8,88 ± 1,27
	ET		12,9 ±12,33	3,7 ± 1,87	1,816 ± 0,33	14,29 ±1,07	11,96 ± 2,99	10,25 ±1,88
Open Field	***IPA***		65 ± 3,333	76 ± 2,58	88 ± 3,68	57 ± 3,23	62 ± 3,39	72 ±2,309
	IIA		3,3 ± 0,665	4,78 ± 0,709	7,45 ± 0,576	2,98 ± 0,67	2,55 ± 0,68	3,1 ± 0,814
	ICA		12,9 ± 1,29	4,95 ± 1,08	1,816± 0,33	14,29 ±1,07	11,68±1,19	10,25±1,029
	TDM		30,05 ±2,16	16,4 ±1,705	7,616 ±0,648	31,37 ±1,73	27,3 ±1,83	15,31 ± 0,95
	TNR		14,83 ±1,31	12,85 ± 1,09	10,29 ± 0,97	14,5 ± 0,83	13,9 ± 0,90	13,45 ± 0,85
Novel Object Recognition	***EFO***		52,8± 7,568	93,6±6,947	178,5 ± 16,433	50,1± 5,043	65,5± 5,148	104,8 ±8,509
	ENO		91,5 ± 4,75	41,6±5,621	19,9± 5,216	85,9±6,297	71,4± 6,752	43,8 ±5,223
Vsoc-Maze	***HP***	***E1***	118,75 ±8,9	99,55±7,719	83,75±4,788	115±7,071	109,75± 8,3	101 ± 6,37
		E2	121 ± 8,981	92,5±8,66	78,5±8,10	117,5±10,5	102,38± 4,5	92,75 ± 6,185
	SP	***S1***	244,25 ±6,8	121±7,958	87,25±3,86	246±8,98	200,25± 7,9	163,5 ± 5,066
		E2	29,25 ±4,35	160,75±5,92	190,75±6,5	33,25±7,68	87,38± 5,15	99,25 ± 4,573
	PSN	***S1***	33,75 ±5,06	159,5± 9,036	190,575±5,629	32,75±5,12	103,75± 7,2	115,5 ±5,446
		S2	246 ± 6,684	105 ± 5,099	79,125 ±6,574	243,13 ±8.2	207,38 ±8,3	182,75±15,649
Sucrose Preference	***SI***		43,5 ± 3,03	26 ± 1,69	7,4±0,52	45,3±1,5	36±1,9	26,5 ±1,27
	SP		68,5 ± 3,03	40 ± 1,70	15,61±0,53	75,124±1,6	54,55±1,10	43,08 ±1,28
Olfactory recognition	***TC***		83,9 ±28,21	103,5 ±20,83	287,1± 33,504	67,7 ±14,36	90,8 ±19,97	179,7 ± 35,04
	LTOF		116,7 ±16,6	139,7 ±16,58	223,7 ± 11,99	119,8± 16,4	118,9 ± 8,5	183,2 ±13,29
	LTTFF		55,4 ± 5,81	92,4 ± 8,93	181,6± 33,19	29,4 ±11,22	79,1 ±9,025	104,2 ± 8,217

IMI: *Imidacloprid,* ***MOE:*** *Melissa Officinalis L Extract****; D1:*** *Dose 1 (5 mg/kg/day)****; D2:*** *Dose 2 (50 mg/kg/day)****.*** *Results are expressed as* ***mean ± SE (n=10) t-test*** *was used for multiple comparisons.*

- ***FT:*** *Finishing Time****, ET:*** *Exploration Time****.***
- ***IPA:*** *Immobility Time in Peripheral Area (s)****, IIA:*** *Immobility Time in Intermediate Area (s)****, ICA:*** *Immobility Time in Central Area (s)****, TDM:*** *Total Distance Moved (m)****, TNR:*** *Total Number of Rearing's****.***
- ***EFO:*** *Exploration of the Familiar Object (s****), ENO:*** *Exploration of the Novel Object (s****).***
- ***HP:*** *Habituation Phase (s),* ***SP:*** *Sociability Phase (s)****, PSN:*** *Preference for Social Novelty (s)****. E:*** *Empty,* ***S:*** *Stranger****.***
- ***SI:*** *Sucrose Intake (ml)****, SP: Sucrose preference (%).***
- ***TC****: Time in Center (sec****), LTOF:*** *Latency Time to Own Fissure (sec)****, LTTFF:*** *Latency Time to Familial Fissure (sec).*

➢ The Classic Labyrinth Test



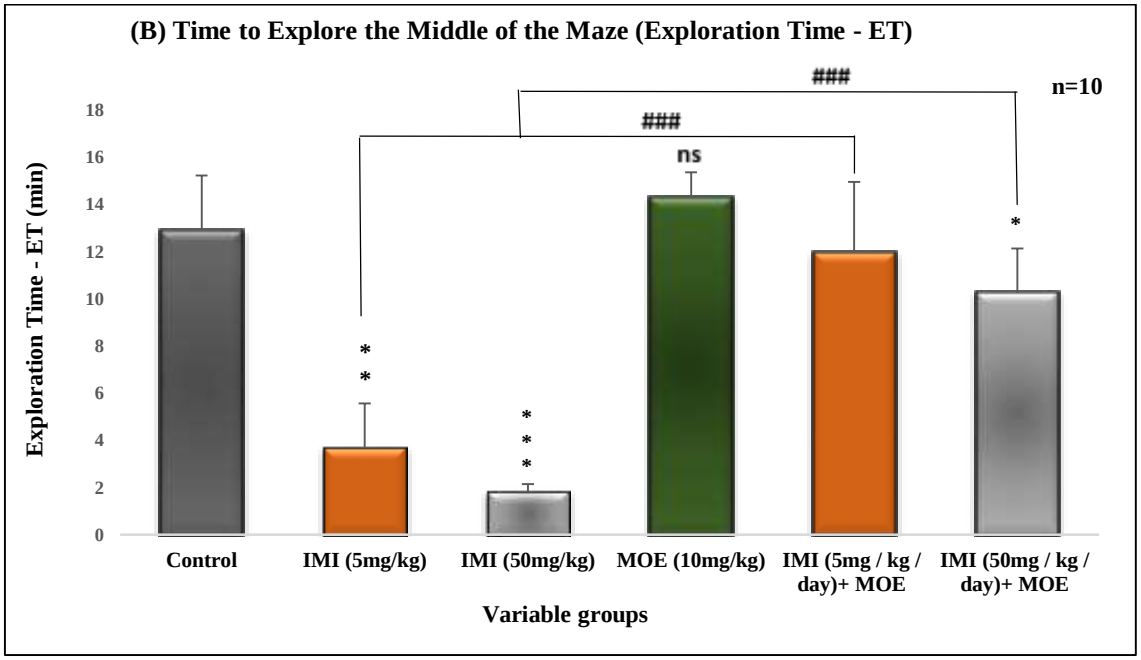


Figure 43: Variation of classic labyrinth test data in control and treated groups over 3 months. (A): Time to reach the end in the maze. (B): Time spent exploring the middle of the maze. (n = 10). Mean ± SEM ; *comparison to control* ***(****Not significant (ns)****: P> 0.05; Significant *P ≤ 0.05;*** highly significant ***P ≤ 0.01;*** very highly significant ****P ≤ 0.001) and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001).*

➢ Open Field Locomotion Test

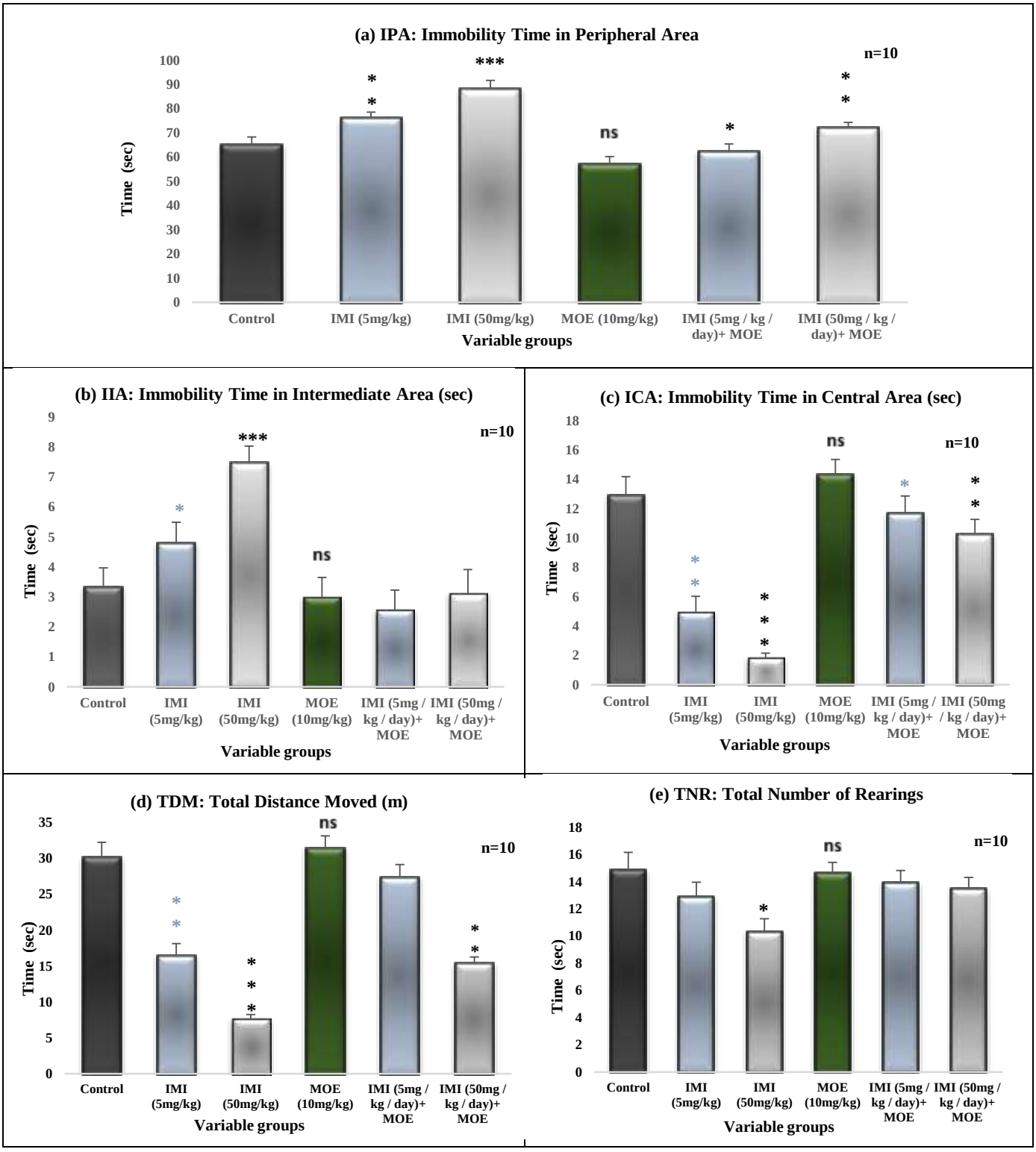


Figure 44: Variation in open field test data in control and treated rats after 90 days of treatment (a): IPA: Immobility Time in Peripheral Area (s); (b): IIA: Immobility Time in Intermediate Area (s); (c): ICA: Immobility Time in Central Area (s); (d): TDM: Total Distance Moved (m); (e): TNR: Total Number of Rearing's. (*P < 0.05; **P < 0.01; ***P < 0.001).

➢ Novel Object Recognition Test (NOR)

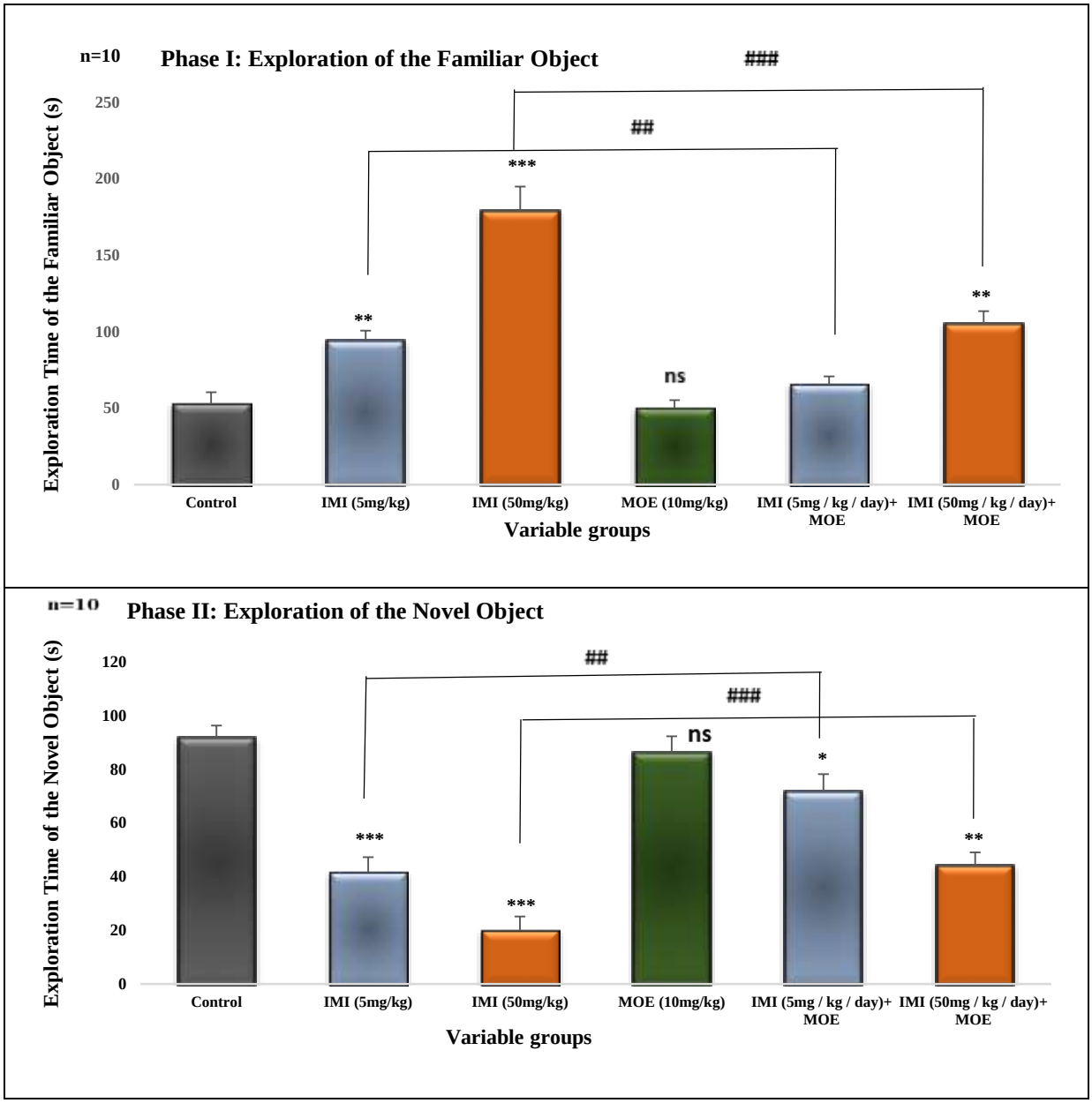


Figure 45: Variation in exploration time of a novel object and the familiar object in control and treated rats over 3 months. *Comparison to control* ***(****Not significant (ns)****: P> 0.05; Significant * and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001).P ≤ 0.05;* highly significant ***P ≤ 0.01;* very highly significant ****P ≤ 0.001).*

➢ Vsoc-Maze Test

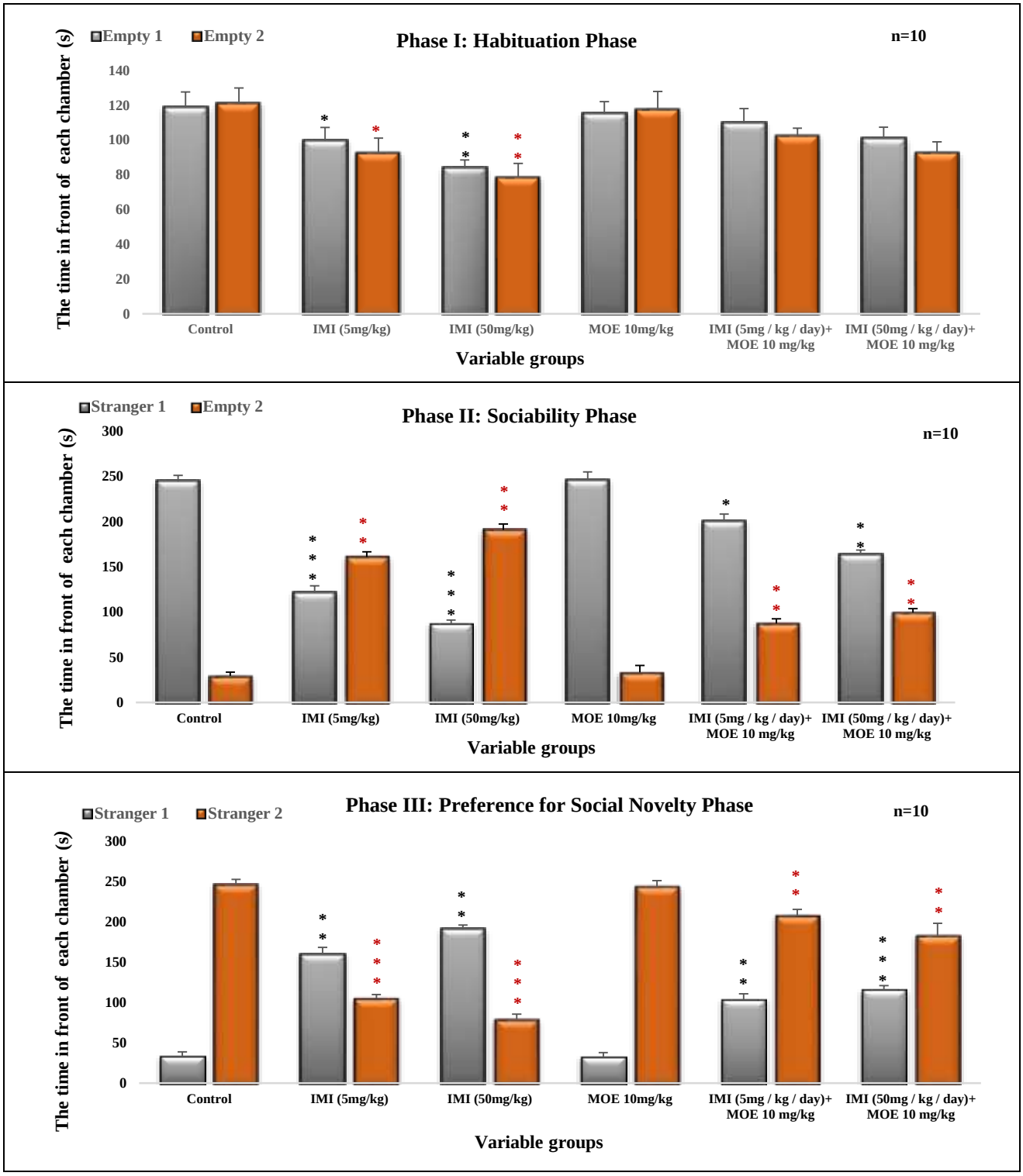


Figure 46: Variation of maze V_SOC test data in groups of rats treated with time relievers: Phase I: Habituation Phase, phase II Sociability, Phase III: Preference for Social Novelty. Mean ± SEM values and significance levels (*(ns): P> 0.05;*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) were applied throughout the analysis.

➢ Sucrose Preference Test (SPT)

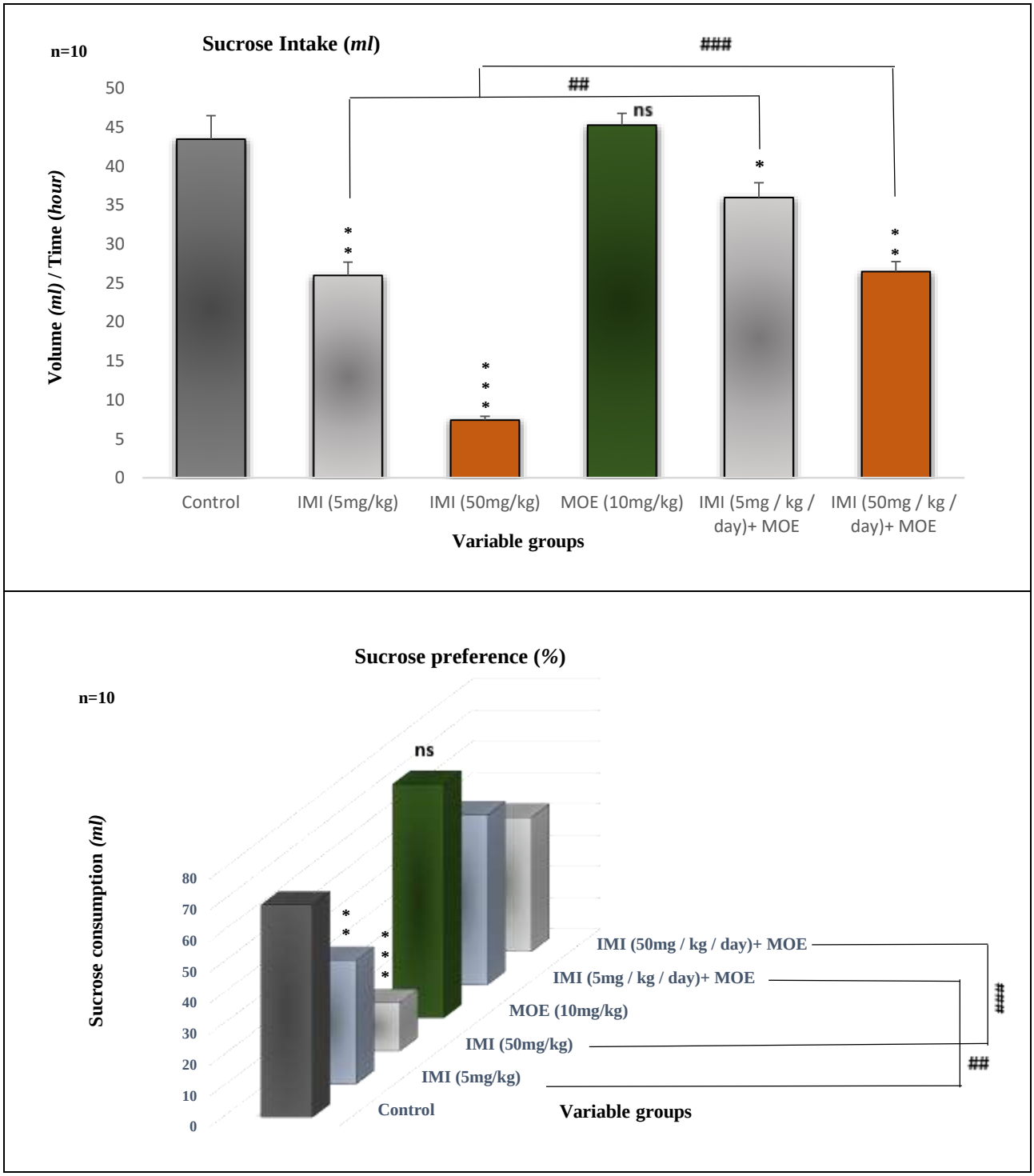


Figure 47: Variation in the sucrose preference test and sweet water consumption in control and treated rats over 3 months. *Comparison to control ((ns): P> 0.05;*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001).*

➢ Olfactory Recognition Test

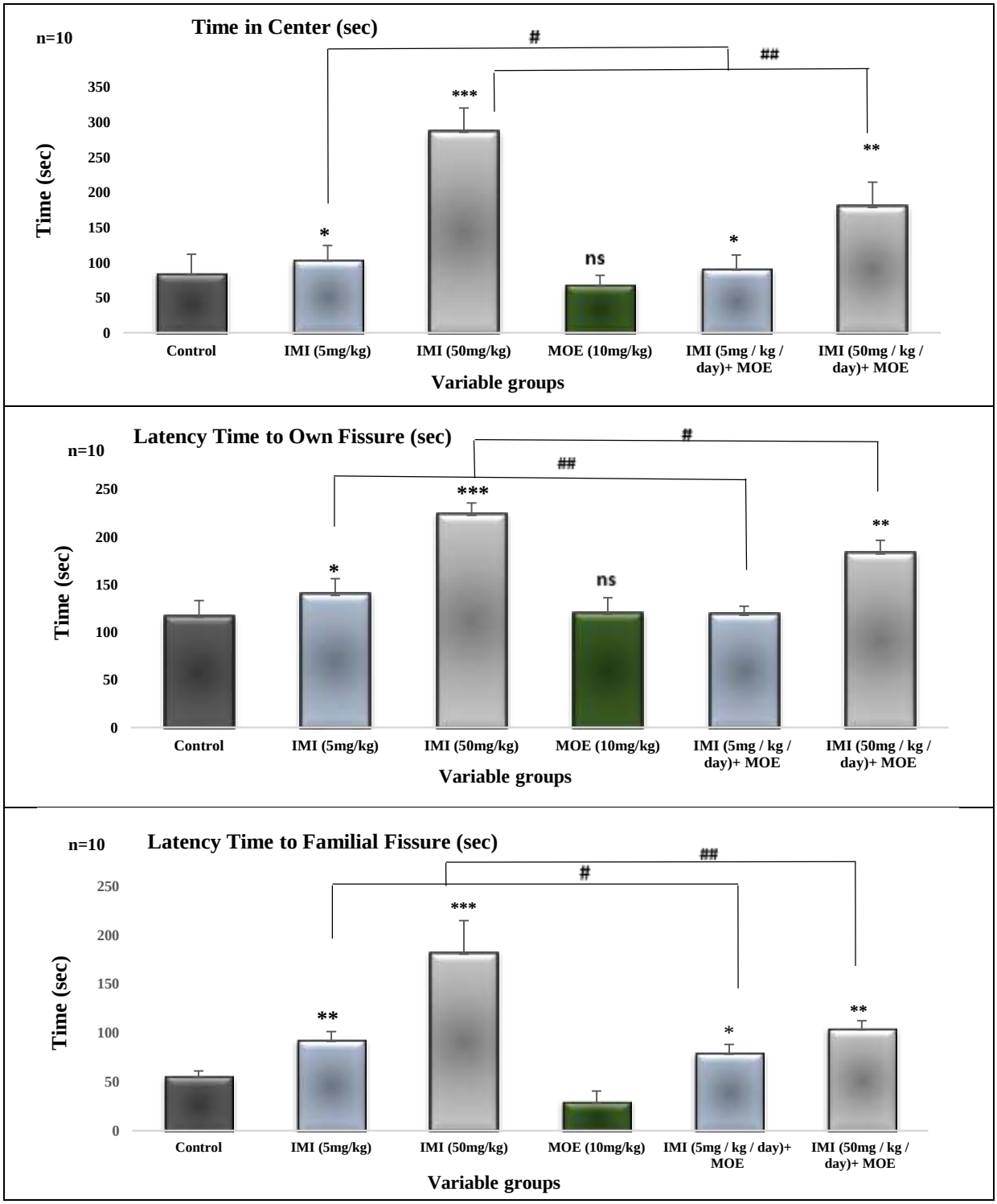


Figure 48: Variations in the time (s) spent in each compartment during the *"Clean Sawdust x Nest Sawdust"* test between treated and control rats. *Comparison to control (ns):* P> 0.05;*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) *and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001).*

4. Evaluation of mitochondrial metabolic parameters

Table 18 and **Figure 49** present findings on mitochondrial protein, carbohydrate, and lipid levels in various brain regions (whole brain [Br], hippocampus [Hip], and striatum [Str]) in *Wistar* rats after three months of treatment, evaluating the impact of chronic exposure to Imidacloprid (IMI) at two doses (IMI D1: 5 mg/kg/day, IMI D2: 50 mg/kg/day) and the effect of Melissa *officinalis* L. extract (MOE) in reversing these impacts.

Chronic exposure to IMI demonstrated a clear dose-dependent effect on mitochondrial biochemical parameters (Protein, Carbohydrate, and Lipid) in different brain regions (whole brain [Br], hippocampus [Hip], and striatum [Str]) of *Wistar* rats. At both low (IMI D1: 5 mg/kg/day) and high (IMI D2: 50 mg/kg/day) doses, IMI significantly elevated protein and carbohydrate levels, with the effect being stronger at the higher dose. The highest protein increase was observed in the striatum under IMI D2 treatment (***$P \leq 0.001$), followed by the brain and hippocampus, indicating a region-specific susceptibility. Similarly, IMI D2 induced very highly significant carbohydrate increases (***$P \leq 0.001$) in all regions, with the striatum showing the largest effect, highlighting its greater sensitivity to IMI.

Conversely, Lipid content exhibited a different pattern, decreasing significantly in a dose-dependent manner, particularly under IMI D2 treatment (***$P \leq 0.001$). The greatest reduction occurred in the striatum, followed by the hippocampus and brain, again indicating regional differences in response to IMI.

Co-administration of Melissa *officinalis* L. extract (MOE) partially mitigated these IMI-induced effects, particularly in lowering elevated carbohydrate levels and reducing lipid depletion, with the most notable protective effect observed in the hippocampus and striatum. However, while MOE moderated the biochemical alterations induced by IMI, mitochondrial protein, carbohydrate, and lipid levels remained above or below control levels, indicating an incomplete reversal. These findings highlight the potential of MOE to offer partial neuroprotection against IMI-induced mitochondrial dysfunction, with differential efficacy across brain regions.

Table 18: Change of some mitochondrial biochemical parameters (Proteins, carbohydrates and lipids) in the mitochondrial brain in control and treated rats after 03 months of treatment.

		Variable Groups					
Parameters		***Control***	***IMI D1 (5mg / kg / day)***	***IMI D2 (50mg / kg / day)***	***MOE (10mg/kg)***	***IMI D1+ MOE***	***IMI D2+ MOE***
Proteins (µg/mg)	**Br**	1.603±0.0728	1.765±0.083 **	1.954±0.0964 ***	1.659±0.0695 ns	1.701±0.0729 *	1.827±0.0586 **
	Hip	0.605±0.0405	0.678±0.0487 *	0.750±0.0519 **	0.619±0.0411 ns	0.642±0.0446 ns	0.692±0.0474 *
	Str	0.8411±0.055	0.9254±0.064 **	1.0127±0.0678 ***	0.8694±0.058 ns	0.8923±0.0598 *	0.9465±0.0614 **
Carbohydrates (µg/mg)	**Br**	32,466±1,713	43,465±1,411**	53,243±1,0854 ***	33,098±1,302 ns	34,0659±0,864 ns	38,206±1,6561 *
	Hip	18.743±1.238	24.342±1.115 **	28.557±1.293 ***	19.401±1.156 ns	20.359±1.018 *	23.213±1.095 **
	Str	21.899±1.457	29.138 ±1.265 **	36.531± 1.357 ***	22.327±1.201 ns	24.564±1.164 *	27.443±1.248 **
Lipids (µg/mg)	**Br**	360.357±1.86	351.756±1.41 **	340.645±1.166 ***	359.164±1.82 ns	357.156±1.140 8 ns	351.560±1.711 **
	Hip	180.175±1.40	170.324±1.27 *	160.287 ±1.098 **	178.593±1.31 ns	175.451±1.209 ns	170.734±1.254 *
	Str	195.200±1.57	185.712±1.43 **	175.468 ±1.389 ***	193.745±1.54 ns	189.614±1.498 *	183.657±1.512 **

IMI: *IMIDACLOPRID,* ***MOE:*** *MELISSA OFFICINALIS L EXTRACT,* ***BR:*** *BRAIN,* ***HIP:*** *HIPPOCAMPUS,* ***STR:*** *STRIATUM. RESULTS ARE EXPRESSED AS* ***MEAN ± SE (N=10)****; T-****TEST*** *WAS USED FOR MULTIPLE COMPARISONS. STATISTICAL SIGNIFICANT AS COMPARED TO CONTROL (NOT SIGNIFICANT* ***(NS): P> 0.05;*** *SIGNIFICANT* ****P ≤ 0.05;*** *HIGHLY SIGNIFICANT* *****P ≤ 0.01;*** *VERY HIGHLY SIGNIFICANT* ******P ≤ 0.001).***

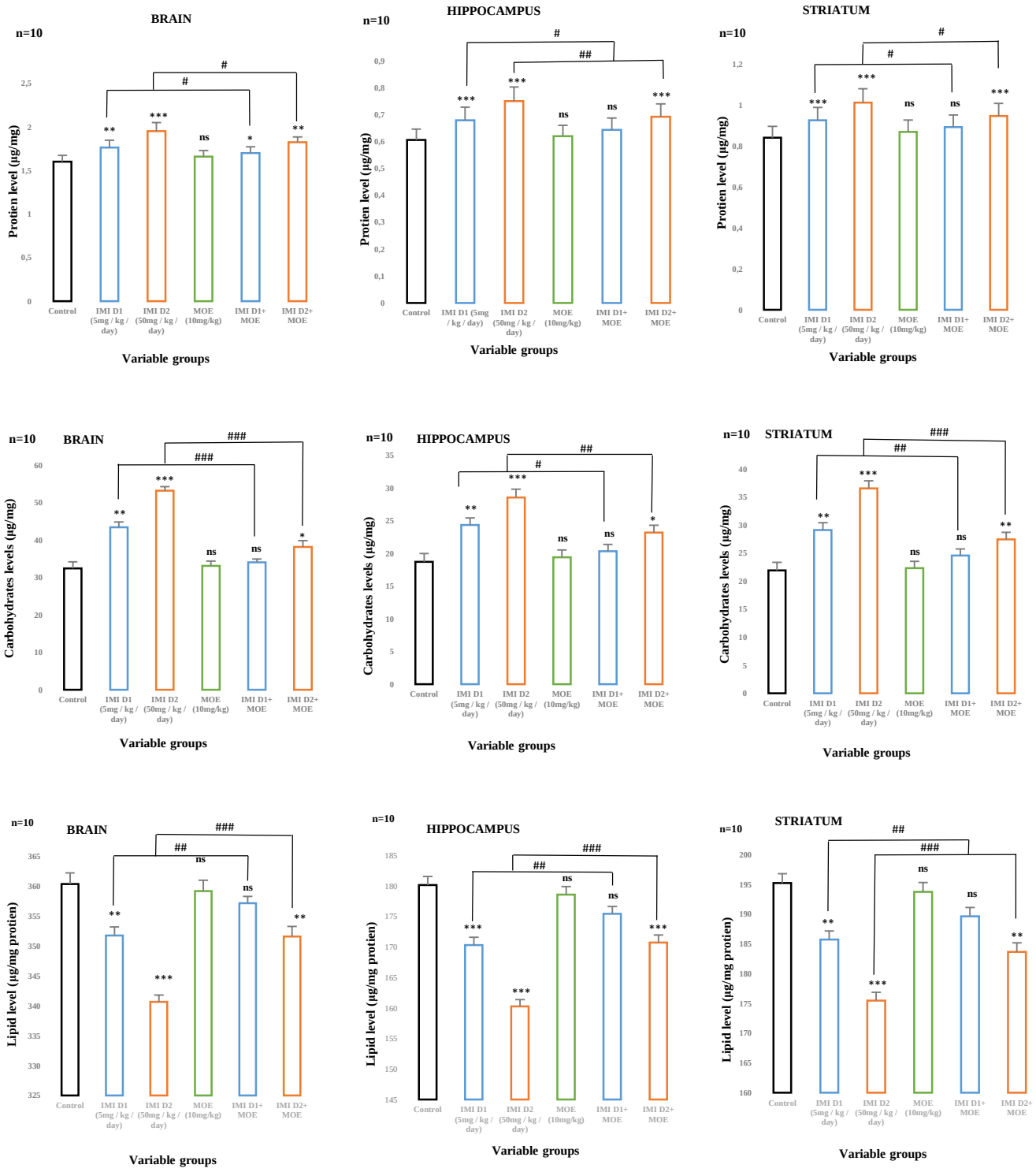


Figure 49: Variation in the levels of mitochondrial proteins, carbohydrates, and lipids in the whole brain, hippocampus, and striatum in control and treated rats after 03 months of treatment. Statistical significance is indicated as follows: *comparison to control (ns: P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001).*

5. Evaluation of redox status markers in brain mitochondria

The **Table 19** shows that chronic exposure to Imidacloprid (IMI) at 5 mg/kg/day (IMI D1) and 50 mg/kg/day (IMI D2) resulted in statistically significant and dose-dependent reductions in mitochondrial antioxidant markers across different brain regions of *Wistar* rats, with notable variation in response based on region. In the brain (Br), GSH levels significantly decreased from $0.00049 \pm 8.98 \times 10^{-6}$ µmol/mg in controls to $0.00046 \pm 1.74 \times 10^{-5}$ µmol/mg in IMI D1 ($P < 0.01$), and further to $0.00039 \pm 1.60 \times 10^{-5}$ µmol/mg in IMI D2 ($P < 0.001$), reflecting a pronounced depletion with increasing dose. A similar trend was observed for GPX activity in the brain, which declined from 1.069 ± 0.041 µmol/min/mg in controls to 1.031 ± 0.020 in IMI D1 ($P < 0.01$) and to 1.001 ± 0.069 in IMI D2 ($P < 0.001$). CAT and GST activities followed the same pattern, with CAT decreasing significantly from 45.2 ± 2.3 U/mg in controls to 38.7 ± 1.8 U/mg in IMI D1 ($P < 0.01$) and further to 32.4 ± 2.1 U/mg in IMI D2 ($P < 0.001$), while GST activity dropped from 12.8 ± 0.7 nmol/min/mg in controls to 10.4 ± 0.5 nmol/min/mg in IMI D1 ($P < 0.01$) and to 8.6 ± 0.4 nmol/min/mg in IMI D2 ($P < 0.001$), indicating significant oxidative stress and impaired detoxification capacity. The hippocampus (Hip), however, exhibited the most profound oxidative stress effects, with MDA levels in IMI D2-treated rats reaching 5.10 ± 0.007 nmol/mg ($P < 0.001$), significantly higher than in the brain and striatum, indicating greater lipid peroxidation and oxidative damage in this region. SOD activity reductions mirrored these findings, showing significant declines from 17.13 ± 0.14 U/mg in controls to 13.14 ± 0.54 U/mg in IMI D2 ($P < 0.001$) in the brain, with similar reductions across regions but particularly marked in the hippocampus ($P < 0.001$). The striatum (Str) exhibited dose-dependent effects, though slightly less pronounced than the hippocampus, suggesting possible regional variation in antioxidant capacity or vulnerability to IMI-induced oxidative stress.

Administration of Melissa *officinalis* extract (MOE) in both IMI D1+MOE and IMI D2+MOE groups yielded statistically significant improvements across all oxidative markers ($P < 0.001$), with restored GSH, GPX, CAT, GST, and SOD levels close to control values and MDA significantly reduced, underscoring MOE's efficacy in mitigating neurotoxic damage.

Table 19: Effects of Imidacloprid (IMI) and Melissa *officinalis* L. extract (MOE) on mitochondrial antioxidant enzyme activities and oxidative stress markers in brain, hippocampus, and striatum in control and treated *Wistar* rats after 03 months of treatment**.**

Variable Groups

Parameters		*Control*	*IMI D1 (5mg / kg / day)*	*IMI D2 (50mg / kg / day)*	*MOE (10mg/kg)*	*IMI D1+ MOE*	*IMI D2+ MOE*
Mit GSH µmol/mg	**Br**	0.0004906±8.9837E-06	0.00046±1.73861E-05 ** #	0.000391±1.59844E-05 *** #	0.000498±8.90E-06 ns	0.000505±1.75E-05 * #	0.000428±1.59E-05 ** #
	Hip	0.000470±7.89E-06	0.000450±1.60E-05 * ##	0.000380±1.51E-05 *** #	0.000480±8.10E-06 ns	0.000487±1.62E-05 * ##	0.000420±1.50E-05 ** #
	Str	0.000510±9.01E-06	0.000470±1.68E-05 ** #	0.000400±1.53E-05 *** #	0.0005±9.05E-06 ns	0.000493±1.64E-05 * #	0.000438±1.55E-05 ** #
Mit GPX µmol/min/mg	**Br**	1.06901±0.04135198	1.03118±0.02019 ** #	1.0140±0.06858139 *** ##	1.072±0.0418 ns	1.035±0.0204 ** #	1.0158±0.0690 *** ##
	Hip	1.065±0.040	1.025±0.019 ** #	0.990±0.067 *** ###	1.070±0.041 ns	1.032±0.020 * #	1.190±0.069 ** ###
	Str	1.075±0.042	1.035±0.021 ** ##	1.010±0.069 *** #	1.080±0.043 ns	1.052±0.022 * ##	1.028±0.070 ** #
Mit GST µmo/min/mg	**Br**	0.005106±0.000817068	0.007833±0.0004 * #	0.012335±0.000630 *** #	0.005115±0.000830 ns	0.007900±0.000400 ** #	0.012850±0.000640 ** #
	Hip	0.005000±0.000750	0.007600±0.00035 * #	0.012000±0.0006104*** ##	0.005100±0.000770 ns	0.007780±0.000360 * #	0.015350±0.000620 ** ##
	Str	0.005250±0.000830	0.007900±0.000400** #	0.012500±0.0006504*** ##	0.005300±0.000840 ns	0.008000±0.000410 ** #	0.016800±0.000660 ** ##
Mit CAT µmol/min/mg	**Br**	0.00042±7.06122E-06	0.00031±1.23107E-05 ** #	0.000251±1.59E-05*** #	0.000431±7.10E-06 ns	0.000335±1.25E-05 * #	0.000273±1.61E-05 ** #
	Hip	0.000410±6.90E-06	0.000295±1.20E-05 ** ##	0.000240±1.56E-05 *** #	0.000415±7.02E-06 ns	0.000325±1.22E-05 * ##	0.000265±1.58E-05 ** ##
	Str	0.000430±7.20E-06	0.000320±1.25E-05 ** #	0.000260±1.61E-05 *** #	0.000437±7.30E-06 ns	0.000345±1.27E-05 * #	0.000275±1.63E-05 ** #
Mit MDA nmol/mg	**Br**	3.00018±0.18572284	4.60657±0.20035576** #	5.001675±0.006109 *** #	3.050±0.185 ns	4.666 ± 0.202 ** #	5.019 ± 0.006 ** #
	Hip	3.100±0.190	4.700±0.210 ** ##	5.100±0.007 *** #	3.050±0.185 ns	4.300±0.202 ** ##	5.000±0.006 *** #
	Str	3.200±0.195	4.800±0.220 ** #	5.200±0.008 *** #	3.150±0.190 ns	4.702±0.215 * #	5.106±0.007 ** #
Mit SOD u/mg	**Br**	17.1320±0.13597204	15.008±0.617202 ** ##	13.1428±0.536963 *** #	17.152±0.140 ns	16.000±0.620 ** ##	13.810±0.540 *** #
	Hip	17.000±0.130	14.900±0.600** ##	13.000±0.530 *** #	17.150±0.140 ns	15.050±0.620 * ##	13.800±0.540 ** #
	Str	17.300±0.140	15.200±0.630 ** ##	13.300±0.550 *** ##	17.350±0.145 ns	16.350±0.640 * ##	14.500±0.560 ** ##

IMI: *IM0IDACLOPRID,* ***MOE:*** *MELISSA OFFICINALIS L EXTRACT,* ***BR:*** *BRAIN,* ***HIP:*** *HIPPOCAMPUS,* ***STR:*** *STRIATUM.*
RESULTS ARE EXPRESSED AS ***MEAN ± SE (N=10)****; T-****TEST*** *WAS USED FOR MULTIPLE COMPARISONS. STATISTICAL SIGNIFICANT AS COMPARED TO CONTROL (NOT SIGNIFICANT* ***(NS): P> 0.05;*** *SIGNIFICANT* ****P ≤ 0.05;*** *HIGHLY SIGNIFICANT* *****P ≤ 0.01****; VERY HIGHLY SIGNIFICANT* ******P ≤ 0.001).***
IN ADDITION, COMPARISON BETWEEN IMI D1 (5 MG/KG/DAY) AND IMI D2 (50 MG/KG/DAY) GROUPS VERSUS IMI D1 + MOE AND IMI D2 + MOE GROUPS (#P < 0.05; ##P < 0.01; ###P < 0.001).

6. Study of mitochondrial apoptosis parameter

6.1. Assessment of mitochondrial swelling

The analysis of mitochondrial swelling variations in different brain regions of *Wistar* rats after three months of treatment **(Figure 50)** highlights a dose-dependent effect of Imidacloprid (IMI) on mitochondrial integrity, with a more pronounced impact in the hippocampus. In the whole brain (Br), mitochondrial swelling was significantly higher in the IMI D1 group (5 mg/kg/day) (0.2190 ± 0.0312, *P ≤ 0.05) compared to the control (0.1966 ± 0.0095), and markedly increased in the IMI D2 group (50 mg/kg/day) (0.3426 ± 0.0139, ***P ≤ 0.001). Melissa *officinalis* extract (MOE) alone did not induce any significant modification in this parameter (0.1868 ± 0.0100, NS: P > 0.05), suggesting its safety regarding mitochondrial function. The co-administration of MOE with IMI significantly mitigated IMI-induced toxicity, reducing mitochondrial swelling in the IMI D1 + MOE group (0.2093 ± 0.0265, *P ≤ 0.05) and IMI D2 + MOE group (0.2803 ± 0.0112, **P ≤ 0.01), although these values remained higher than those of the control group.

In the hippocampus (Hip), mitochondrial vulnerability was even more pronounced. IMI D1 induced a significant increase in mitochondrial swelling (0.2300 ± 0.0343, *P ≤ 0.05), while IMI D2 caused a highly significant increase (0.3666 ± 0.0160, ***P ≤ 0.001) compared to the control (0.2064 ± 0.0104). Administration of MOE alone maintained mitochondrial integrity comparable to the control group (0.1961 ± 0.0109, NS). When MOE was co-administered with IMI, it significantly reduced mitochondrial swelling to 0.2198 ± 0.0292 in the IMI D1 + MOE group (*P ≤ 0.05) and to 0.2984 ± 0.0128 in the IMI D2 + MOE group (**P ≤ 0.01), although these values remained higher than those of the control group.

In the striatum (Str), a similar trend was observed, but with slightly less damage than in the hippocampus. Exposure to IMI D1 caused a significant increase in mitochondrial swelling (0.2146 ± 0.0296, *P ≤ 0.05), while IMI D2 led to a highly significant increase (0.3289 ± 0.0125, ***P ≤ 0.001) compared to the control (0.1927 ± 0.0090). MOE alone resulted in a significant reduction in mitochondrial swelling (0.1830 ± 0.0095, *P ≤ 0.05). When co-administered with IMI, it helped to mitigate this alteration, reducing mitochondrial swelling to 0.2051 ± 0.0252 in the IMI D1 + MOE group (*P ≤ 0.05) and to 0.2706 ± 0.0100 in the IMI D2 + MOE group (**P ≤ 0.01), indicating a partial protective effect.

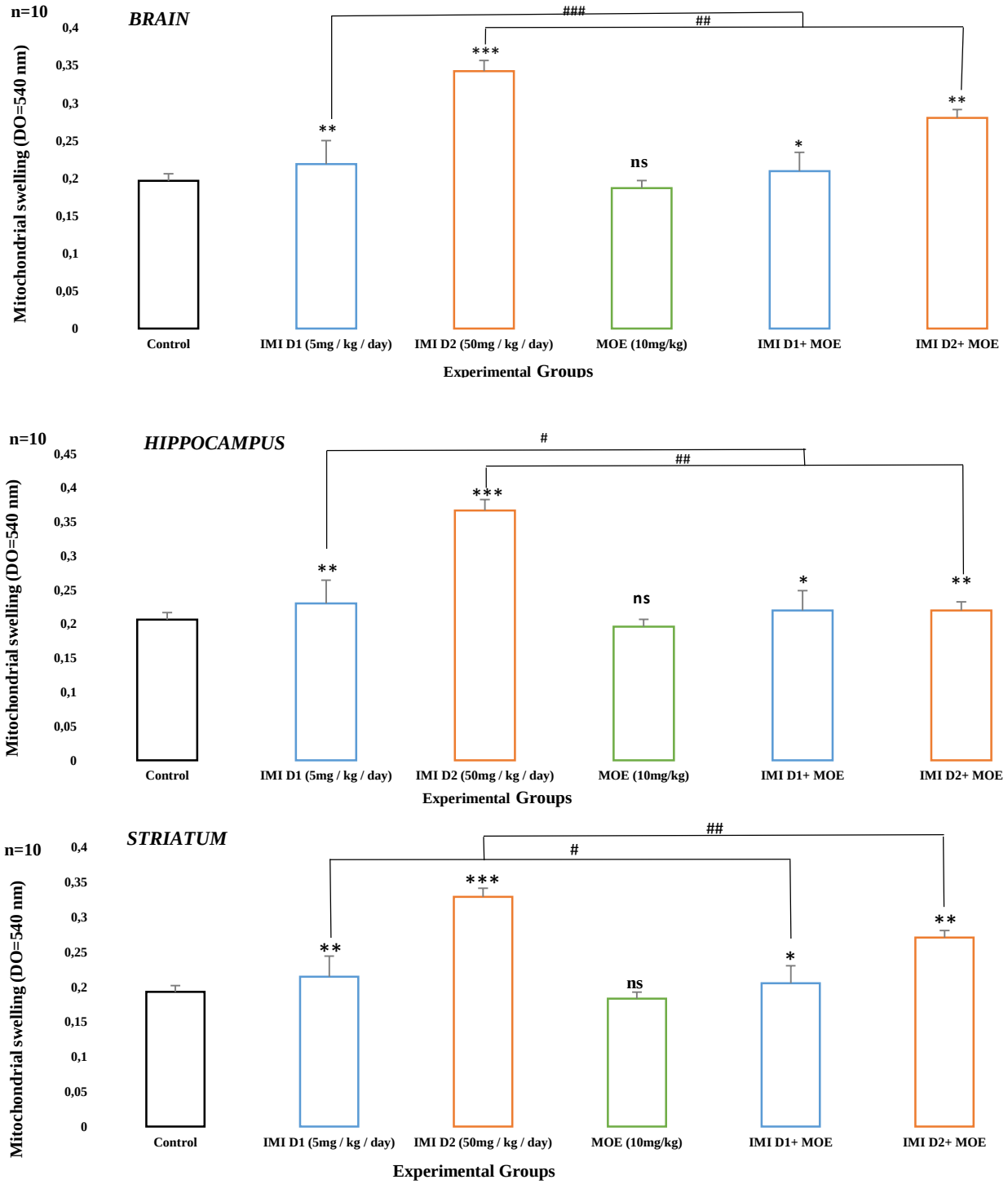


Figure 50: Variation in mitochondrial swelling *(optic density)* in the whole brain, hippocampus and striatum of control and treated rats after 3 months of treatment. *Statistical significance is indicated as follows: comparison to control (ns: P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).*

6.2. Assessment of mitochondrial respiration

The assessment of mitochondrial respiration (nmol/ml) as O_2 Consumption following three months of treatment with Imidacloprid (IMI) and Melissa *officinalis* L. extract (MOE) reveals a dose-dependent decline in mitochondrial function across different brain regions (whole brain (Br), hippocampus (Hip), and striatum (Str)), as illustrated in **Figure 51**, with hippocampal mitochondria being more affected than striatal mitochondria

IMI exposure led to a dose-dependent decrease in mitochondrial respiration across all brain regions studied. At the dose of 5 mg/kg/day (IMI D1), a significant reduction was observed in the whole brain (-14.8%, $p \leq 0.05$), hippocampus (-18.2%, $p \leq 0.01$), and striatum (-12.5%, $p \leq 0.05$), with a more pronounced inhibition in the hippocampus. At the higher dose of 50 mg/kg/day (IMI D2), this decrease was even more severe, reaching -32.5% ($p \leq 0.001$) in the hippocampus and -26.7% ($p \leq 0.01$) in the striatum, indicating a greater vulnerability of the hippocampus compared to the striatum (Hip < Str, $p \leq 0.01$). Compared to the control group, the inhibitory effect of IMI was highly significant across all regions at D2 ($p \leq 0.001$ for BR and Hip, $p \leq 0.01$ for Str).

Co-administration of MOE with IMI significantly mitigated this decline in mitochondrial respiration, with a notable increase in whole brain (+21.3%, $p \leq 0.01$), hippocampus (+25.6%, $p \leq 0.001$), and striatum (+19.8%, $p \leq 0.05$) compared to IMI-only groups. Interestingly, administration of MOE alone showed no significant effect (NS, $p > 0.05$), suggesting that its action is protective rather than stimulatory. The protective effect of MOE was more pronounced in the hippocampus ($p \leq 0.001$), aligning with its potential role in preserving cognitive functions.

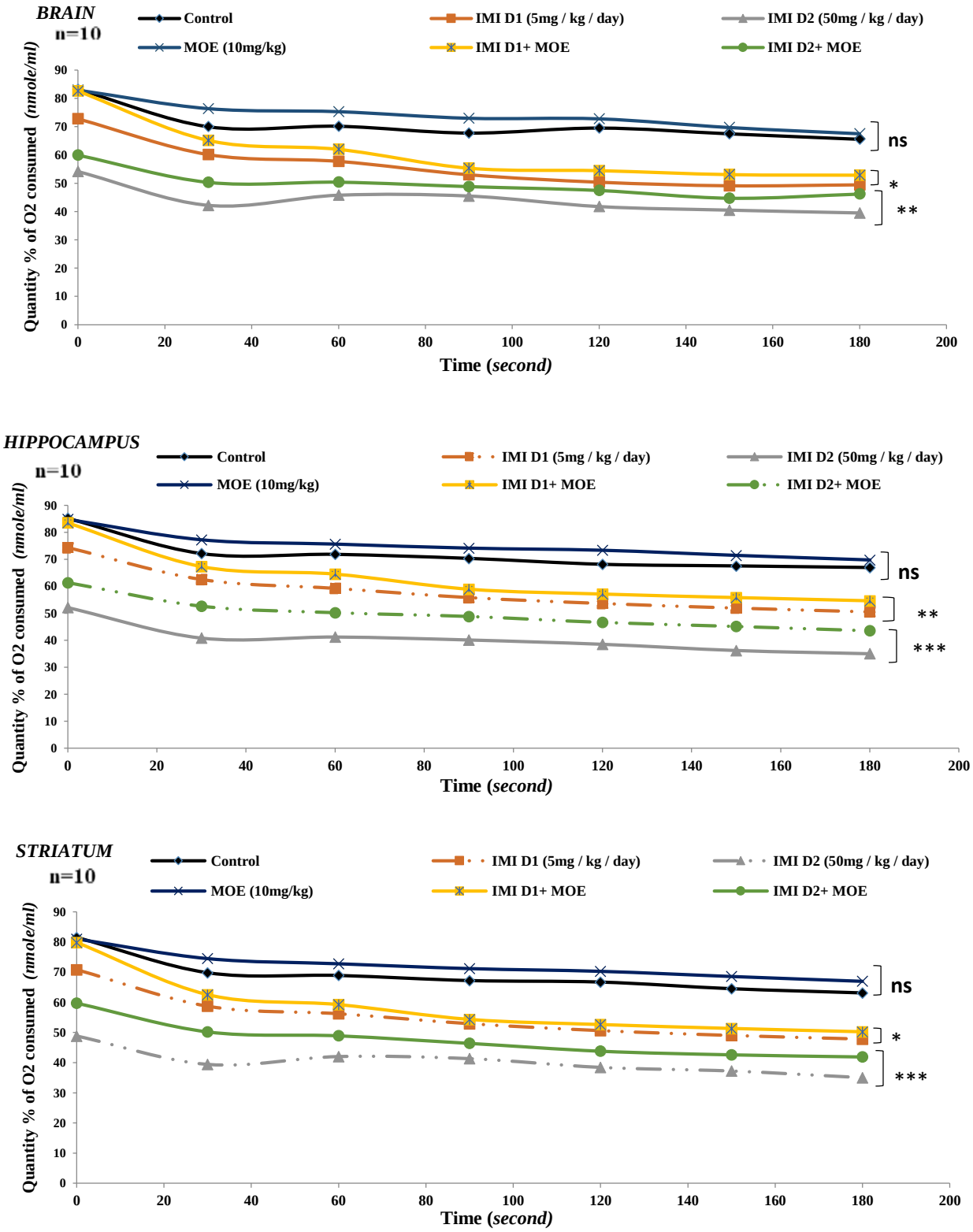


Figure 51: Variation in mitochondrial respiration in the whole brain, hippocampus, and striatum of control and treated rats after 3 months of treatment. *Statistical significance is indicated as follows: comparison to control (ns: $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).*

6.3. Assessment of mitochondrial permeability

The assessment of mitochondrial permeability, measured as the change in mitochondrial size over time following the addition of calcium to mitochondrial suspension, exhibited significant alterations across groups **(Figure 52)**. The kinetic curve analysis revealed a dose-dependent increase in permeability in IMI-treated groups compared to controls, with significance levels of *P ≤ 0.05, **P ≤ 0.01, ***$P \leq 0.001$.

Control rats maintained stable mitochondrial integrity, with negligible changes in permeability, ensuring normal calcium homeostasis. However, IMI exposure induced mitochondrial dysfunction, with IMI D1 (5 mg/kg/day) showing a moderate but significant increase*($P \leq 0.05$), while IMI D2 (50 mg/kg/day) caused a very highly significant permeability increase*** ($P \leq 0.001$), indicating membrane destabilization and potential apoptotic or necrotic processes.

MOE alone (10 mg/kg/day) had no significant effect on permeability (*$P > 0.05$). However, in IMI + MOE co-treated groups, MOE exerted a protective effect, significantly reducing IMI-induced permeability alterations. In IMI D1 + MOE, permeability was restored to near-control levels, whereas in IMI D2 + MOE, a partial but significant reduction (**$P \leq 0.01$) was observed compared to IMI D2 alone.

Regional analysis indicated higher susceptibility of the striatum (Str) compared to the hippocampus (Hip). The IMI D2 group exhibited significantly greater permeability in the Str (***$P \leq 0.001$) than in the Hip (**P ≤ 0.01), suggesting enhanced mitochondrial vulnerability in this region. While MOE significantly mitigated IMI-induced damage in both regions, the recovery was more pronounced in the hippocampus than in the striatum, where mitochondrial impairment persisted to a greater extent.

These findings indicate IMI-induced mitochondrial dysfunction, with dose-dependent effects and regional variability, highlighting the neuroprotective role of Melissa *officinalis* extract, particularly in the hippocampus.

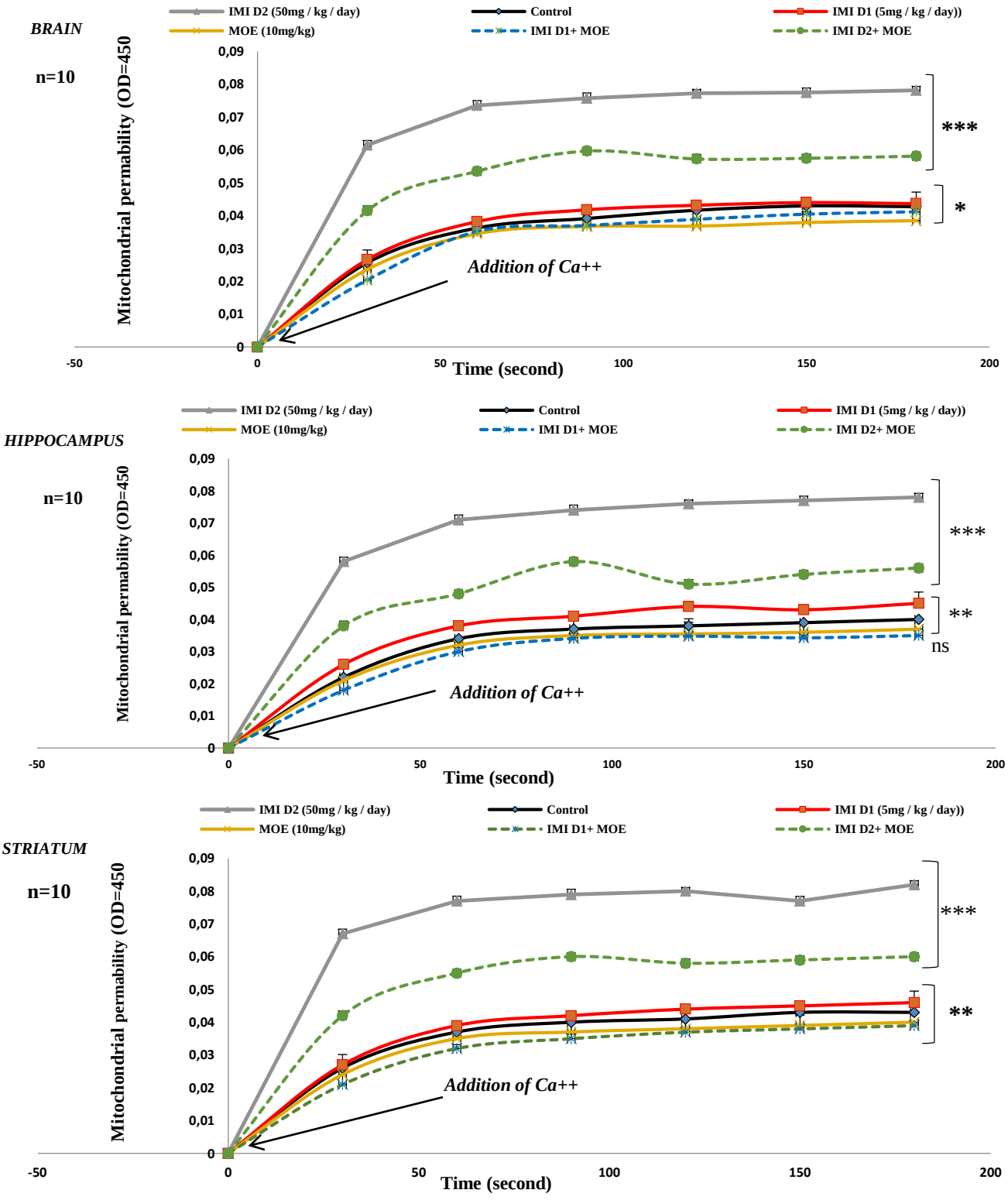


Figure 52: Variation in mitochondrial membrane permeability *(ΔOD/Δt)* in the whole brain, hippocampus, and striatum of control and treated rats after 3 months of treatment. *Statistical significance is indicated as follows: comparison to control (ns: P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).*

7. Assessment of brain lysosomal stability by determination of neutral red retention time (NRRT)

The effect of Imidacloprid (IMI) and Melissa *officinalis* L. extract (MOE) on the brain cells' lysosomal stability was assessed using the neutral red retention time (NRRT) assay. Absorbance values at 540 nm were recorded at 24 h and 48 h to determine lysosomal stability. The results indicate significant differences ($P \leq 0.05$) at the specified concentrations compared to the control at both time points. Experiments (n = 6) were performed in triplicate, and the data are presented as mean ± standard error (SE) in **Table 20** and **Figure 53.**

- **Control group:** In this group, absorbance at 540 nm is measured for brain cells without any treatment. The values provide a reference point for comparison with other groups. Lysosomal stability remains consistent at both 24 and 48 hours.
- **Group treated with 5 mg/kg of Imidacloprid (IMI D1):** At 24 hours, absorbance slightly increases compared to the control, indicating lysosomal activity and uptake of neutral red. By 48 hours, absorbance decreases significantly, reflecting lysosomal destabilization, leakage of neutral red, and initiation of cell death.
- **Group treated with 50 mg/kg of Imidacloprid (IMI D2):** At 24 hours, absorbance is markedly higher than the control and IMI D1 groups, showing stronger lysosomal activity due to the higher dose of Imidacloprid. After 48 hours, a sharp decrease in absorbance occurs, indicating severe lysosomal destabilization and extensive cell death.
- **Group treated with 10 mg/kg of Melissa *Officinalis* L. Extract (MOE):** Absorbance values remain stable and comparable to the control group at both 24 and 48 hours, suggesting that MOE does not induce lysosomal stress or cytotoxicity, and lysosomal integrity is maintained.
- **Group treated with IMI D1 + MOE:** At 24 hours, absorbance values are similar to the IMI D1 group, showing no immediate protective effect of MOE. However, at 48 hours, absorbance is significantly higher compared to IMI D1 alone, indicating that MOE reduces lysosomal destabilization and provides partial protection to brain cells.
- **Group treated with IMI D2 + MOE:** At 24 hours, absorbance is slightly lower than IMI D2 alone, suggesting early lysosomal stabilization by MOE. By 48 hours, absorbance is significantly higher compared to IMI D2 alone, reflecting the neuroprotective role of MOE in mitigating lysosomal destabilization and cell death caused by high-dose Imidacloprid exposure.

Table 20: Assessment of Imidacloprid's and Melissa *officinalis* L extract effects on absorbance at 540 nm: Comparative analysis across variable groups and doses over time.

Variable groups		*Control*	*IMI D1 (5mg / kg / day)*	*IMI D2 (50mg / kg / day)*	*MOE (10mg/kg)*	*IMI D1+ MOE*	*IMI D2+ MOE*
Absorbance at 540 nm	**For (A) 24 h**	3,595±0,267	3,795±0,2415	4,264±0,4292	3,6162±0,2616	3,7934±0,4631	3,8836±0,115
	For (B) 48 h	4,1±0,1997	3,081±0,2174 *	2,806±0,2924 **	4,284±0,3202 ns	3,604±0,2050 *	3,406±0,3186 *

IMI: *Imidacloprid,* ***MOE:*** *Melissa Officinalis L Extract,* ***(n = 6).*** *Results are expressed as mean ± SE (n=10); T-test was used for multiple comparisons. Statistical significant as compared to control (Not significant (ns): P> 0.05; Significant *P ≤ 0.05; highly significant **P ≤ 0.01; very highly significant ***P ≤ 0.001).*

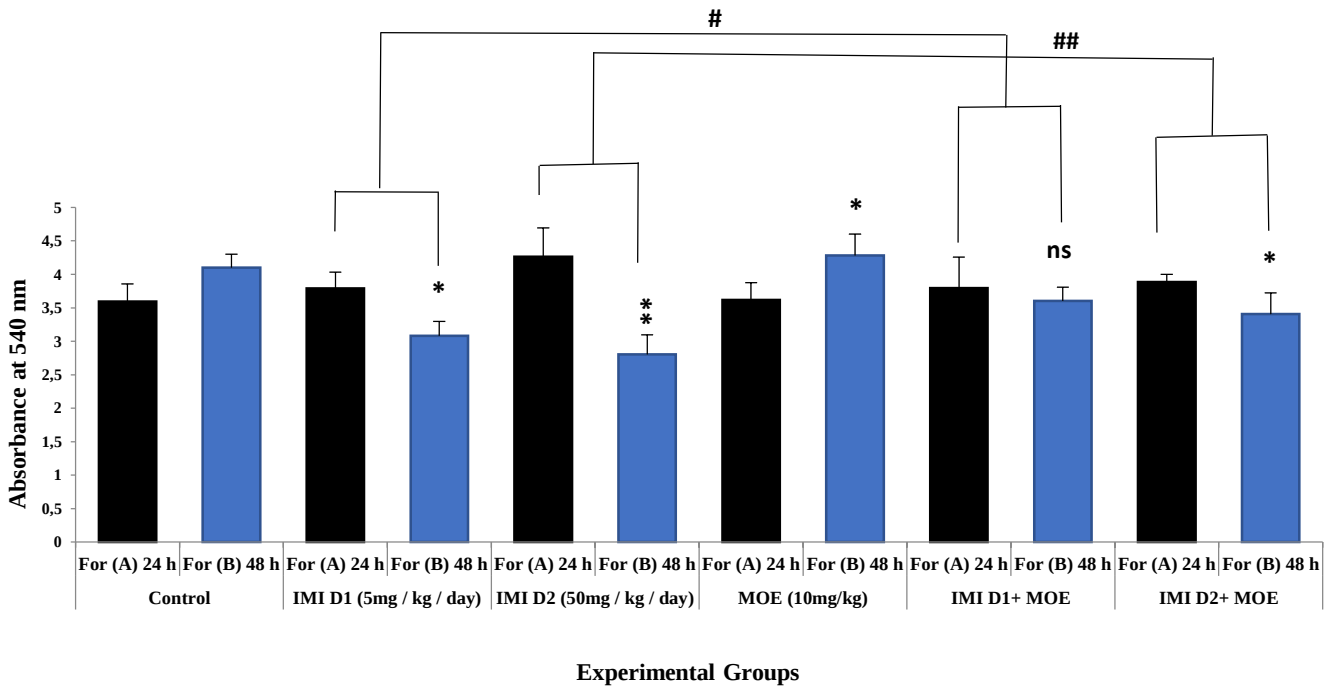


Figure 53: Effect of Imidacloprid and Melissa *officinalis* L extract in different treatment groups, on the brain cells of retention of neutral red. indicate significant differences ($p<0.05$; Tukey's test) between means ± SE. absorbance values (540 nm) measured with the neutral red assay to evaluate the lysosome destabilization of brain cells incubated for (A) 24 h and (B) 48 h (n = 6). Error bars present standard deviation. Statistical significance is indicated as follows: *comparison to control (ns: P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001).*

8. Morphological alterations [Microscopic observations (x400)]

The results of the microscopic examination (x400 magnification, phase-contrast optics) following exposure to Imidacloprid (IMI) and Melissa *officinalis* L. extract (MOE) after a neutral red assay (NRRT), as presented in **Figures 54, 55, 56, 57** and **58**, revealed significant morphological alterations across different treatment groups. During the designated exposure periods, cells were carefully observed with a specific focus on identifying indications of disruptions in the structural integrity of the lysosomal membrane.

The **Figure 54** present the NRRT assay conducted on *Wistar* rat brain cells exposed to Imidacloprid: **[1]** The exposed cells displayed a "rounding-up" phenomenon, indicating a shift to a more spherical shape. **[2]** The rounding-up was associated with the leakage of neutral red dye into the cytosol, implying a disruption in cellular membrane integrity caused by Imidacloprid exposure. **[3]** The presence of granulocytes with small lysosomes was observed, suggesting potential alterations in lysosomal structure and function within these white blood cells as a result of Imidacloprid exposure.

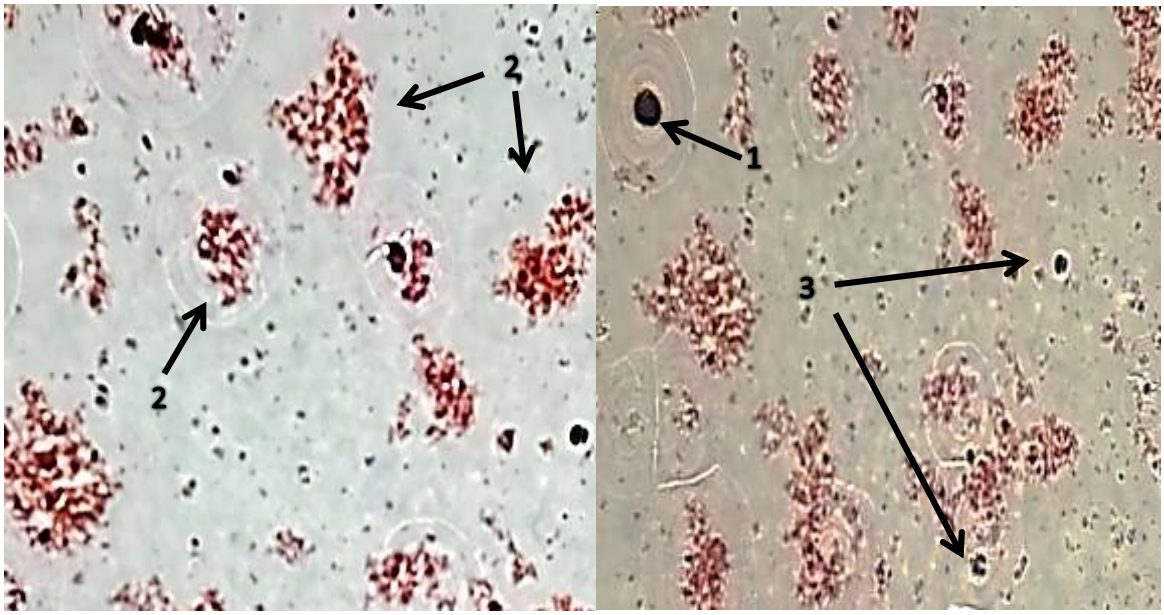


Figure 54: Indicators of lysosomal membrane destabilization and altered structural integrity.

The **Figure 55** presents findings on the progressive alterations observed in this sample over time, marked by a series of events**: [1]** initial expansion and hypertrophy of the lysosomal compartment; **[2]** gradual diffusion of NR dye into the cytoplasm **[3]** the eventual rounding of the cell, signifying the final stage of cellular degeneration.

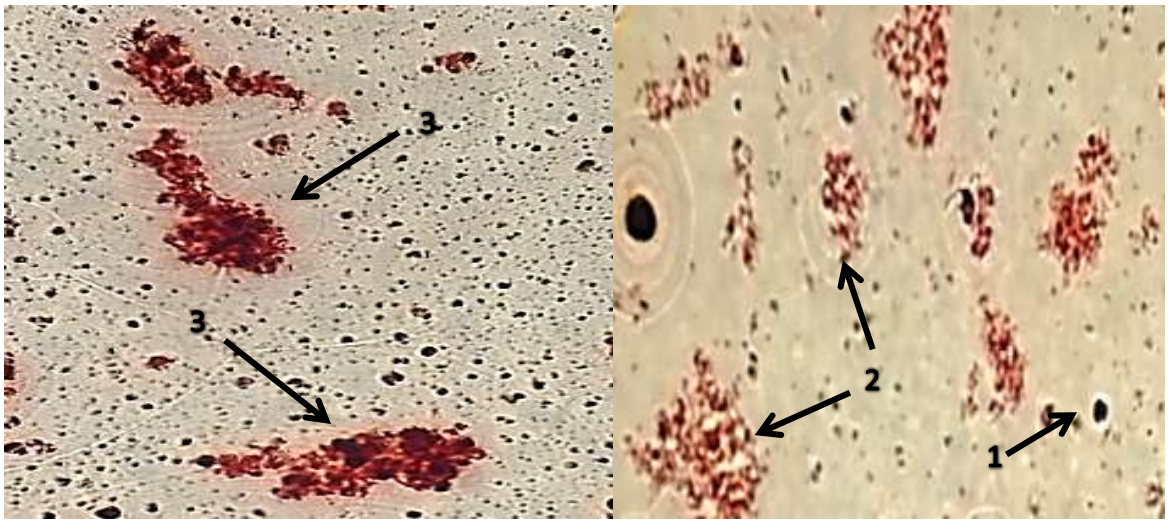


Figure 55: Temporal metamorphosis**:** The trilogy of transformative events in sample progression.

The findings in **Figure 56** demonstrate that the lysomotropic dye, neutral red, exhibits a strong affinity for lysosomal retention, leading to a gradual expansion of the lysosomal compartment during the incubation period. This expansion persists until a critical threshold is reached, at which point the lysosomal membrane destabilizes, resulting in the release of neutral red into the cytoplasm.

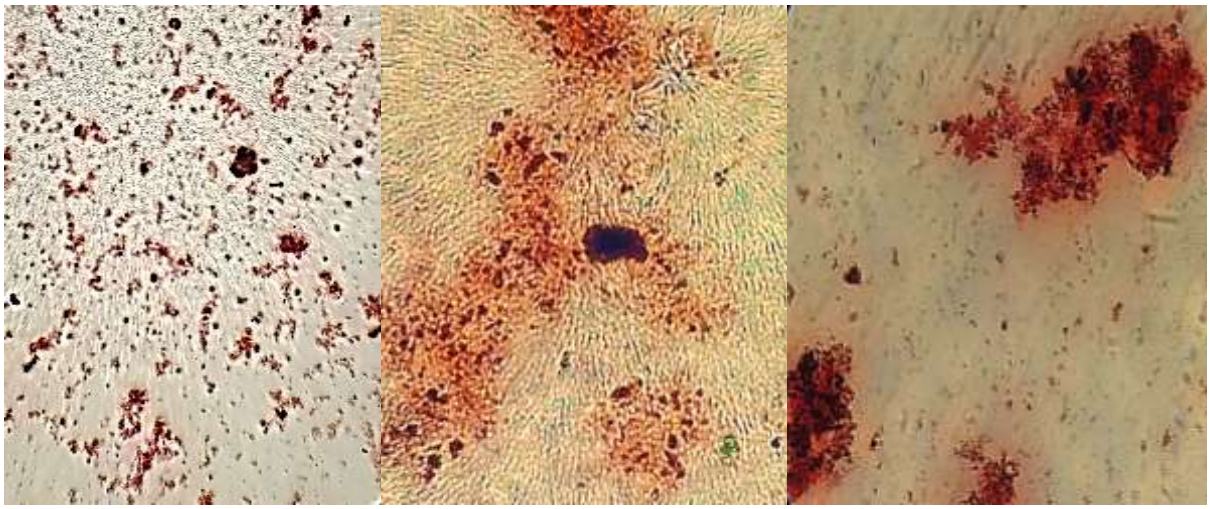


Figure 56: Lysosomal dynamics**:** retention and release of neutral red dye.

The **Figure 57** presents the analysis of the relationship between incubation time and absorbance at 540 nm following a neutral red assay, with Imidacloprid exposure at various time points (**0h, 2h, 4h, 6h, and 8h**). At the initial time point (**0h**), baseline measurements reflect the immediate effects of Imidacloprid exposure. At 2 hours (**2h**), early cellular responses and potential changes in lysosomal volume and neutral red leakage are observed. The 4-hour (**4h**) mark indicates a critical stage of incubation, showing notable significant cellular alterations. By 6 hours (**6h**), late-stage responses or stabilization of effects becomes evident. The final time point, 8 hours (**8h**), provides insights into cumulative effects and the saturation of cellular changes.

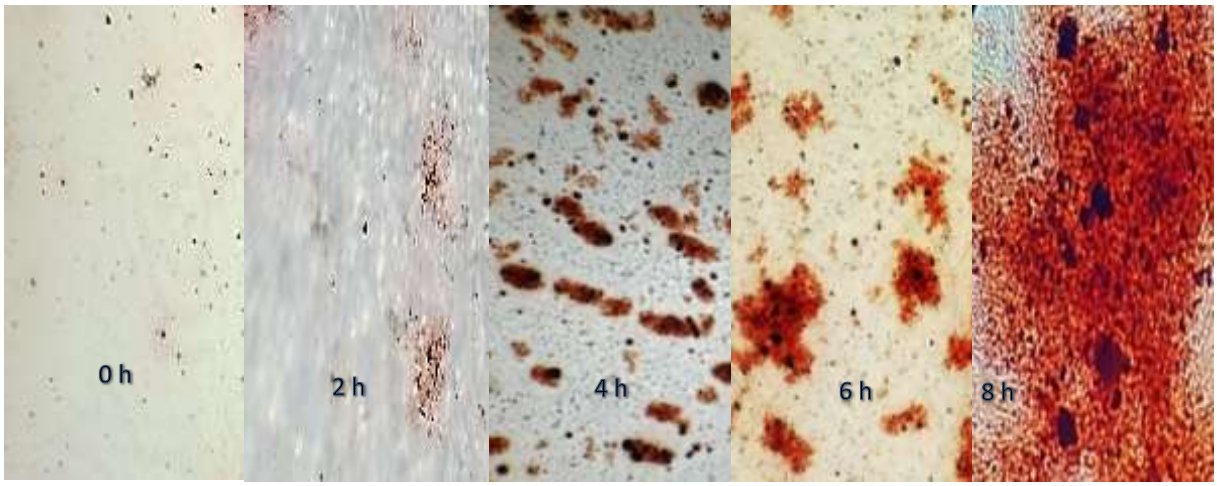


Figure 57: The correlation between incubation time with neutral red and the corresponding absorbance at 540 nm after the neutral red assay.

The analysis of **Figure 58: A (IMI D1 + MOE)** reveals that lysosomal membrane integrity is well preserved, as indicated by the dispersed structures with minimal aggregation or disruption. No significant morphological alterations, such as rounding up or cellular deformation, were observed, suggesting a protective effect against cytotoxicity. The consistent granular pattern and the absence of lysosomal swelling point to maintained lysosomal function, further supported by the retention of neutral red dye within lysosomes, indicating intact and functional membranes. In **Figure 58: B (IMI D2 + MOE)**, the structure displays a relatively organized alignment, suggesting preserved cellular architecture and integrity. Granular elements in this sample also indicate lysosomal activity or neutral red retention, although the intensity is lower than in typical IMI-treated groups, suggesting some modulation of cellular processes. Furthermore, the lack of significant morphological changes, such as rounding up or swelling, reinforces the conclusion that the treatment confers cellular protection, stabilizing cellular components and preventing damage.

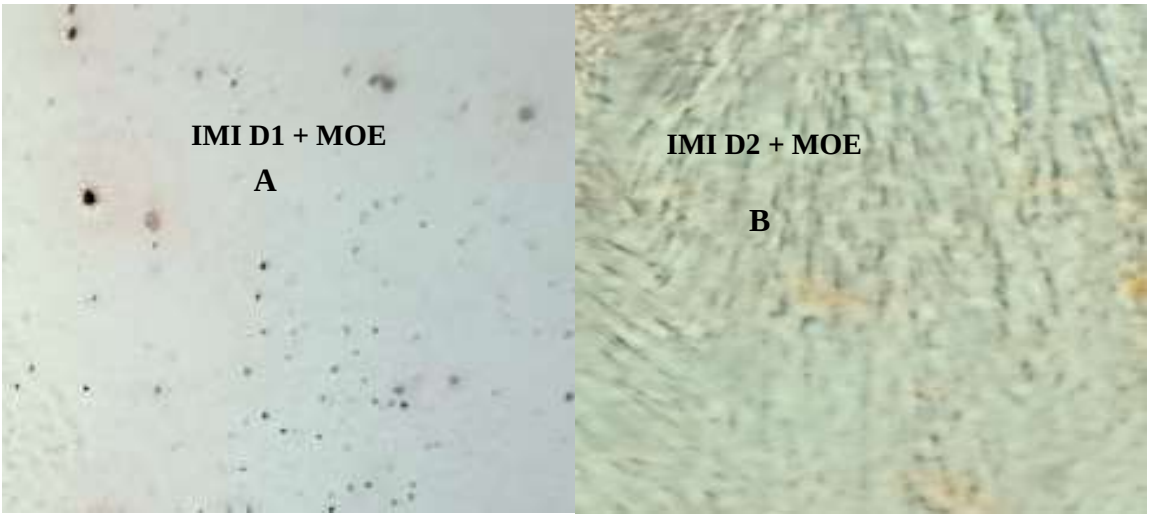


Figure 58: Protective effects of Melissa *officinalis* extract on lysosomal membrane integrity and cellular morphology in Imidacloprid-treated groups.

9. The effect of Imidacloprid (IMI) and Melissa *officinalis* L. extract (MOE) on lysosomal pH variations

The assessment of lysosomal pH variations, as presented in **Figures 59 (A, B),** revealed significant changes across different treatment groups. In the control group, lysosomal pH levels remained stable, providing a baseline reference. In the group treated with 5 mg/kg of Imidacloprid (IMI D1), a significant increase in lysosomal pH was observed (**P ≤ 0.01), indicating mild lysosomal dysfunction due to exposure. The group treated with 50 mg/kg of Imidacloprid (IMI D2) exhibited a more pronounced increase in lysosomal pH (***P ≤ 0.001), reflecting severe alkalization and disruption of lysosomal function at higher IMI doses.

Conversely, the group treated with 10 mg/kg of Melissa *officinalis* L. extract (MOE) showed lysosomal pH levels comparable to the control group (ns: P > 0.05), demonstrating that MOE alone did not induce any lysosomal pH changes and maintained lysosomal stability. Notably, in the IMI D1 + MOE group, the increase in lysosomal pH observed with IMI D1 treatment was significantly mitigated by MOE (*P ≤ 0.05), indicating its protective effect against lysosomal dysfunction under mild IMI exposure. Similarly, in the IMI D2 + MOE group, although lysosomal pH levels remained elevated compared to the control, the presence of MOE significantly reduced the extent of alkalization caused by the higher IMI dose (**P ≤ 0.01).

Statistical analysis was performed using a T-test for multiple comparisons, with statistical significance indicated as follows: not significant (ns): P > 0.05; significant (*P ≤ 0.05); highly

significant (**P ≤ 0.01); and very highly significant (***P ≤ 0.001). These findings highlight the dose-dependent impact of IMI on lysosomal function and the protective role of MOE in attenuating lysosomal pH disturbances.

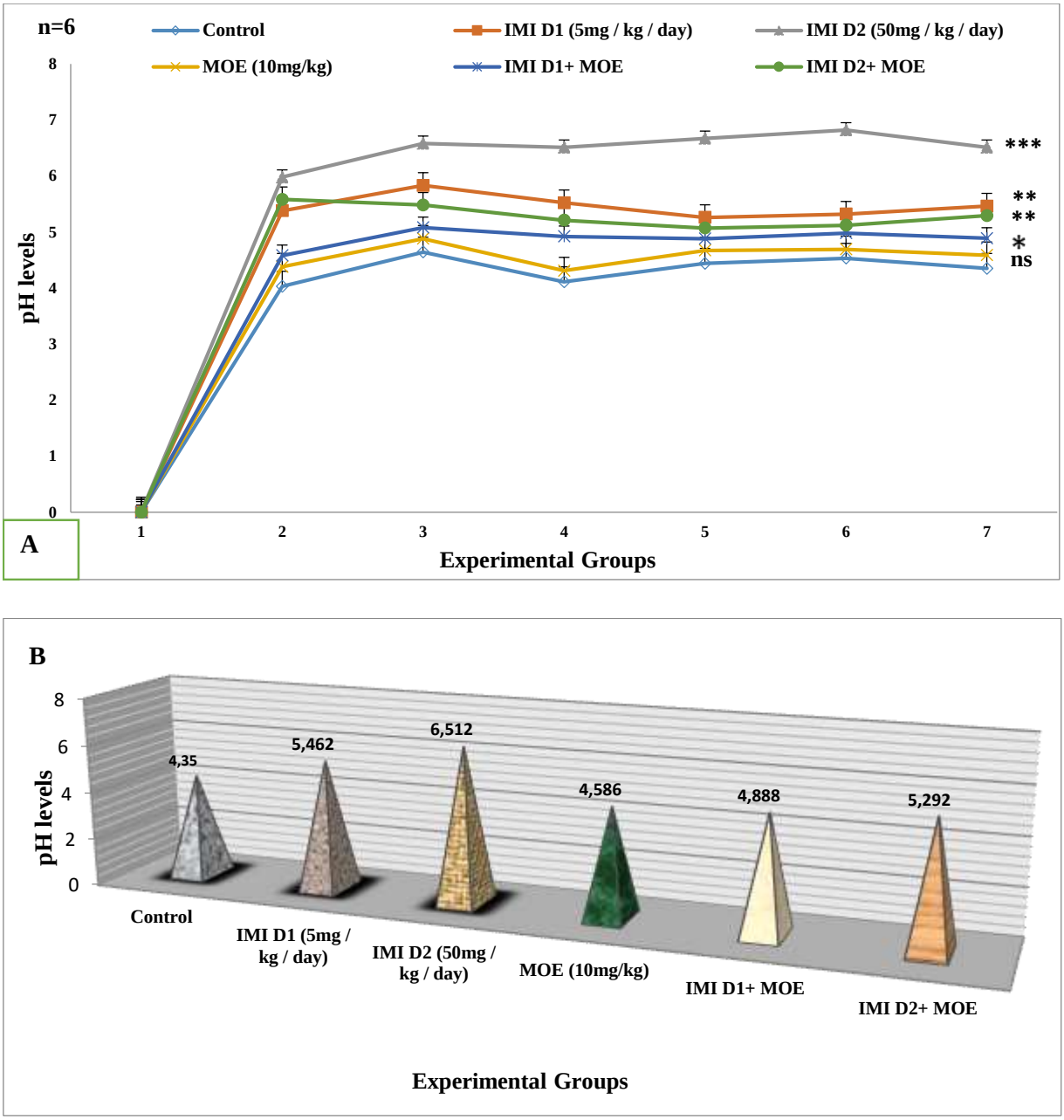


Figure 59 (A, B): The effect of Imidacloprid and Melissa *officinalis* L. extract on lysosomal pH during chronic exposure on brain cells in control and treated rats. *Results are expressed as mean ± SE .t-test was used for multiple comparisons, **P<0.01, *P<0.05 statistical significant as compared to control. IMI: Imidacloprid, MOE: Melissa officinalis L Extract., (n = 6).*

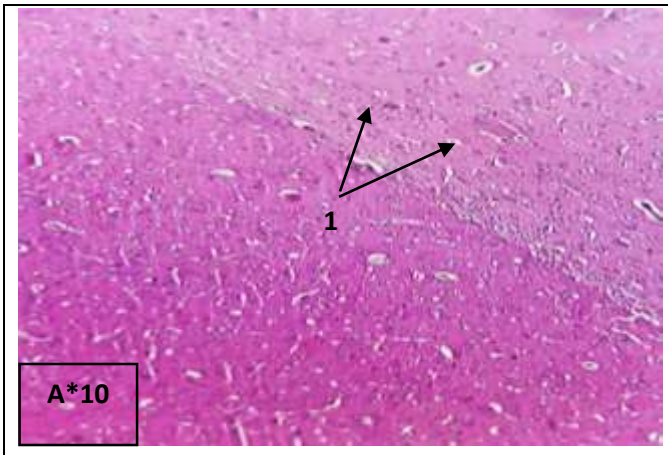
10. Histopathological examinations

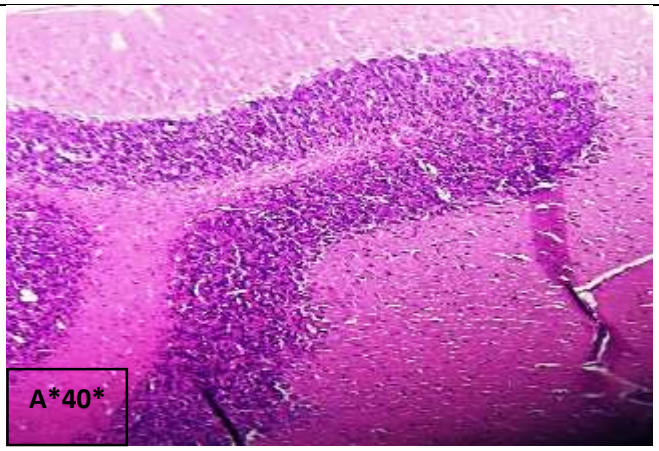
The results in **Table 21** summarize the histopathological findings and scoring for each group, while **Figure 60** illustrates the histopathological changes in the brains of *Wistar* rats after 90 days of Imidacloprid exposure and Melissa *officinalis* extract treatment. The control group shows normal neuronal architecture. The low-dose Imidacloprid group (5 mg/kg/day) exhibits signs of neurotoxicity, while the high-dose group (50 mg/kg/day) shows severe neurotoxic effects, including necrosis and edema. The Melissa *officinalis* extract group (10 mg/kg/day) maintains normal histology. In the combined low-dose, Imidacloprid and Melissa *officinalis* group (IMI D1 + MOE), neurotoxicity is reduced. Similarly, the high-dose combination group (IMI D2 + MOE) shows mitigated neurotoxic effects, indicating a potential protective role of Melissa *officinalis* against Imidacloprid toxicity.

Table 21: Histopathological findings and scoring of brain sections from control and treated *Wistar* rats after 90 days.

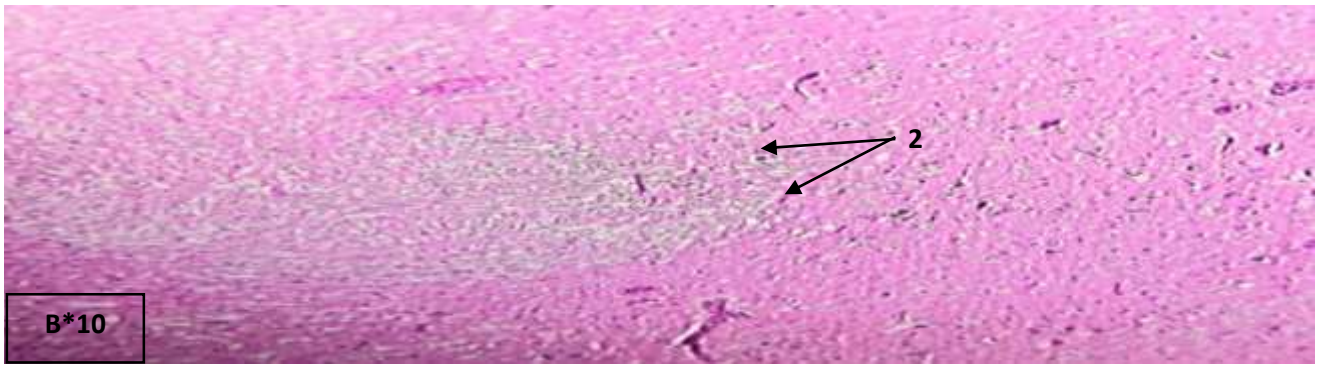
GROUPS	***DOSE (mg/kg/day)***	***HISTOPATHOLOGICAL FINDINGS***	***SCORE***
CONTROL	–	Normal neuronal architecture, no signs of degeneration or inflammation.	(−)
IMI D1	5 mg/kg/day	Marked disorganization of nerve fibers, vacuolization, neuronal loss, mild glial cell infiltration (neurotoxicity, inflammation).	(++)
IMI D2	50 mg/kg/day	Severe neurotoxic effects: early cerebral necrosis, tissue degradation, mild tissue edema (progressive brain damage).	(++++)
MOE	10 mg/kg/day	Normal neuronal architecture, no degeneration or inflammation, indicating neuroprotective potential.	(−)
IMI D1 + MOE	5 mg/kg/day + 10 mg/kg/day (MOE)	Reduced disorganization, vacuolization, and glial cell infiltration compared to IMI D1, showing moderate neuroprotection.	(+)
IMI D2 + MOE	50 mg/kg/day + 10 mg/kg/day (*MOE*)	Partial neuroprotective effect: reduced tissue degradation and necrosis compared to IMI D2, some necrosis and edema still present.	(++)

IMI: Imidacloprid, MOE: Melissa Officinalis L Extract; D1: Dose 1 (5 mg/kg/day); D2: Dose 2 (50 mg/kg/day). Histological lesion scoring was assessed as follows: (−) normal histology, (+) mild damage, (++) moderate damage, (+++) severe damage, and (++++) extensive severe tissue damage.

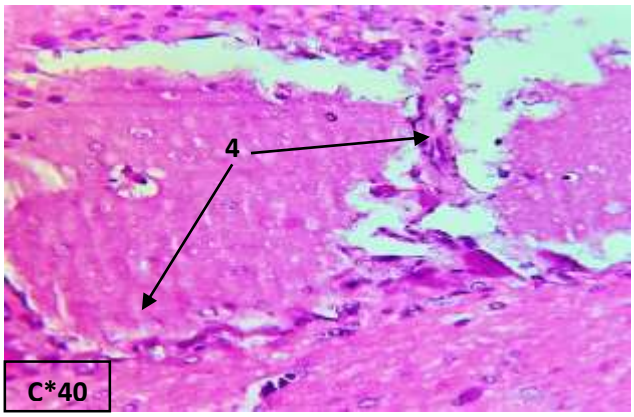




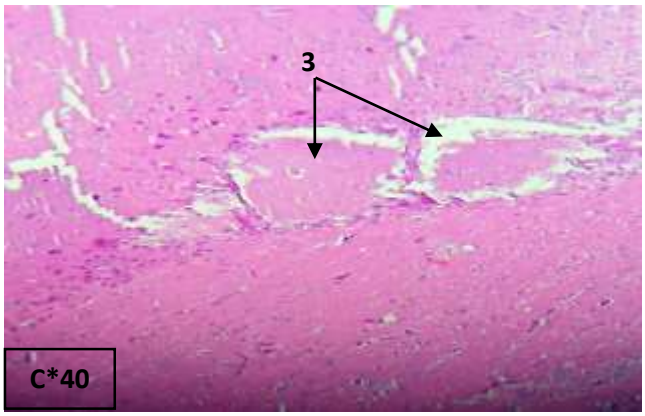
Histological section of the brain from a *Wistar* rat in the control group after 90 days of treatment, showing normal neuronal architecture with well-preserved neuronal cell bodies, no vacuolization, and absence of pathological alterations such as necrosis or inflammation. No significant neuronal degeneration is present, indicating tissue integrity. **Staining: Hematoxylin-Eosin (H&E), magnification x10/x40.**



Histological section of the brain from a *Wistar* rat exposed to Imidacloprid (5 mg/kg/day) for 90 days. Microscopic analysis shows signs of neurotoxicity, characterized by marked disorganization of nerve fibers and loss of normal brain tissue architecture. Areas of vacuolization and neuronal loss are observed, indicating damage to neuronal cells. The brain structure also shows evidence of mild glial cell infiltration, suggesting an inflammatory response to chronic pesticide exposure. **Stained with Hematoxylin-Eosin (H&E).Magnification x10.**

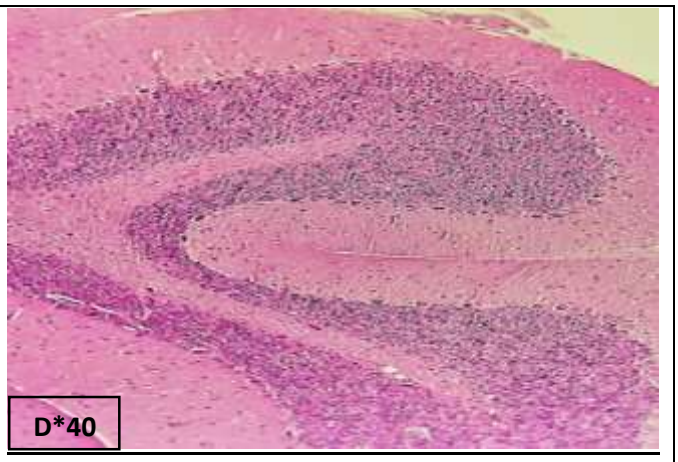


Histological section of the brain from a *Wistar* rat treated with Imidacloprid (50 mg/kg/day) for 90 days. Microscopic analysis reveals the early stages of cerebral necrosis (D2), characterized by localized cell death and tissue degradation. These findings suggest significant neurotoxicity, with marked disruption in the brain's architecture and early necrotic changes. **Stained with Hematoxylin-Eosin (H&E). Magnification x40.**

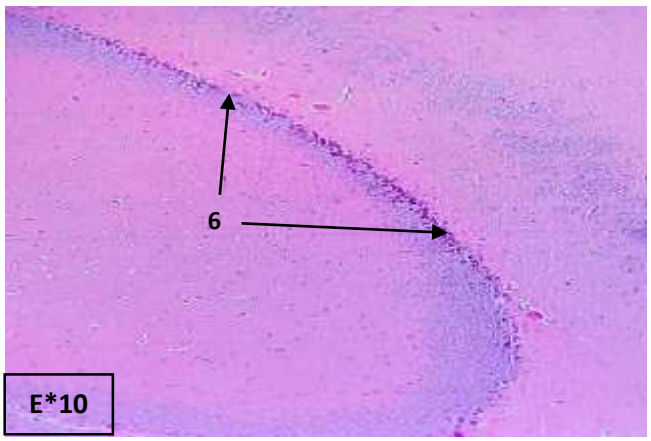


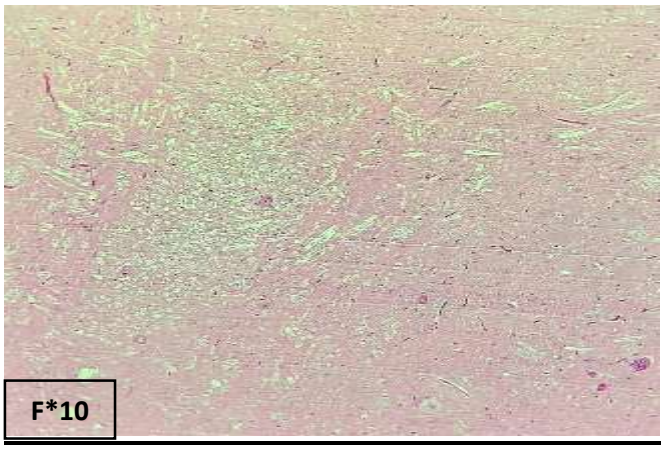
Histological section of the brain from a *Wistar* rat treated with imidacloprid (50 mg/kg/day) for 90 days. Microscopic analysis shows the presence of regular spaces (regular port spaces), indicating mild tissue edema or early neuro-inflammatory responses. These changes suggest potential disruptions in the blood-brain barrier or fluid accumulation within the brain. While neuronal structures remain visible, these pathological signs point to progressive brain damage due to prolonged exposure to imidacloprid. **Stained with Hematoxylin-Eosin (H&E). Magnification x40.**





Cerebral histology of a transverse brain section from the group treated with Melissa *officinalis* L (10mg/kg) for 90 days, showing well-preserved neuronal structures. Microscopic analysis reveals the potential neuroprotective effect of Melissa *officinalis* extract, with distinct layers of the cerebral cortex clearly visible and no significant lesions or necrosis observed. ***Staining:*** **Hematoxylin-Eosin (H&E), magnification x40.**





Histological section of the brain from a *Wistar* rat treated for 90 days with a combination of imidacloprid (5 mg/kg) and Melissa *officinalis* extract (10 mg/kg) shows regular brain parenchyma, Microscopic analysis reveals a well-preserved tissue structure, with normal arrangement of neuronal and glial cells. No signs of necrosis, structural disorganization, or infiltration of inflammatory cells are observed. These results suggest that Melissa *officinalis* extract may offer a protective effect at this lower dose of imidacloprid, maintaining tissue integrity. **Stained with Hematoxylin-Eosin (H&E), magnification x40."**

Histological section of the brain from a *Wistar* rat treated for 90 days with a combination of imidacloprid (50 mg/kg) and Melissa *officinalis* extract (10 mg/kg). Microscopic analysis shows edematous alterations in the brain parenchyma, with a regular tissue appearance. The arrangement of neuronal and glial cells appears normal, with no visible signs of structural disorganization, necrosis, or infiltration of inflammatory cells. These results suggest that Melissa *officinalis* extract may mitigate some of the toxic effects induced by imidacloprid. Stained with Hematoxylin-Eosin **(H&E).Magnification x40.**

Figure 60: Histological sections (x10/40) of the brain after oral administration of Imidacloprid and Melissa *officinalis* L. extract in control and treated rats following three months of treatment. **(A)** Control**, (B):** Treated with IMI D1 5 mg/kg/day, **(C):** Treated with IMI D2 50 mg/kg/day, **(D):** Treated with MOE 10 mg/kg/day, **(E):** Treated with IMI D1 + MOE, **(F)**: Treated with IMI D2 + MOE. (**1):** Glial Cells; **(2):** Nerve Fibers. **(3):** Regularly spaced portal areas. **(4):** Tissue degradation, **(5):** White Matter **;(6):** Dentate Gyrus.

11. Determination of brain neurotransmitter levels and brain AchE activity

The results obtained from the assessment of acetylcholinesterase activity in the whole brain, hippocampus, and striatum of rats chronically treated with Imidacloprid and Melissa *officinalis* extract (MOE) are summarized in **Table 22** and **Figure 61.**

The statistical analysis confirms a dose-dependent neurotoxic effect of Imidacloprid (IMI) on acetylcholinesterase (AChE) activity across brain regions, with higher doses (IMI D2, 50 mg/kg/day) showing very highly significant ($p \leq 0.001$) reductions in AChE activity in the brain, hippocampus, and striatum compared to control, while lower doses (IMI D1, 5 mg/kg/day) exhibit highly significant ($p \leq 0.01$) reductions. The striatum, which displays the largest AChE inhibition at IMI D2, appears especially susceptible to IMI's neurotoxic effects, potentially affecting motor functions. Co-administration of Melissa *officinalis* extract (MOE) with IMI shows promising protective effects, particularly at lower IMI doses. In the brain and hippocampus, IMI D1 + MOE results in no significant (ns) difference in AChE activity from control levels, indicating MOE's ability to restore near-normal enzyme function in regions associated with cognition. However, the striatum remains significantly ($P \leq 0.05$) reduced at this dose, and at higher IMI exposure (IMI D2 + MOE), AChE activity shows only partial recovery in all regions, with the striatum still highly significantly ($p \leq 0.01$) lower than control. Suggesting MOE's protective effect, especially in cognitive regions, its efficacy may vary across brain areas and with IMI dosage.

Table 22: Effect of Imidacloprid (IMI) and Melissa *officinalis* extract (MOE) on Acetylcholinesterase (AChE) activity in brain, hippocampus, and striatum of control and treated *Wistar* rats after 90 days.

		Variable groups					
Parameters		***Control***	***IMI D1 (5mg / kg / day)***	***IMI D2 (50mg / kg / day)***	***MOE (10mg/kg)***	***IMI D1+ MOE***	***IMI D2+ MOE***
Activity of AChE µmol/min/mg	***Br***	0,00624±0,000206	0,00489±0,000104**	0,003032±0,000201***	0,00654± 0,00016 **ns**	0,005794± 0,00014 **ns**	0,004894± 0,00015**
	Hip	0.005913± 0.000192	0.004613 ±0.000129**	0.002913± 0.000187***	0.006213± 0.00015208 **ns**	0.00554± 0.00012914 **ns**	0.004533 ± 0.0001382**
	Str	0.006013±0.000203	0.00474±0.000104**	0.003113±0.000196***	0.006413± 0.0001601 **ns**	0.00546 ± 0.000144 *	0.00472 ± 0.000149**

IMI: *IMIDACLOPRID,* ***MOE:*** *MELISSA OFFICINALIS L EXTRACT,* ***BR:*** *BRAIN,* ***HIP:*** *HIPPOCAMPUS,* ***STR:*** *STRIATUM.* ***ACHE:*** *ACETYLCHOLINESTERASE.*
RESULTS ARE EXPRESSED AS ***MEAN ± SE (N=10) T-TEST*** *WAS USED FOR MULTIPLE COMPARISONS. STATISTICAL SIGNIFICANT AS COMPARED TO CONTROL (NOT SIGNIFICANT (NS)****: P> 0.05; SIGNIFICANT *P ≤ 0.05;*** *HIGHLY SIGNIFICANT ***P ≤ 0.01;*** *VERY HIGHLY SIGNIFICANT ****P ≤ 0.001).***

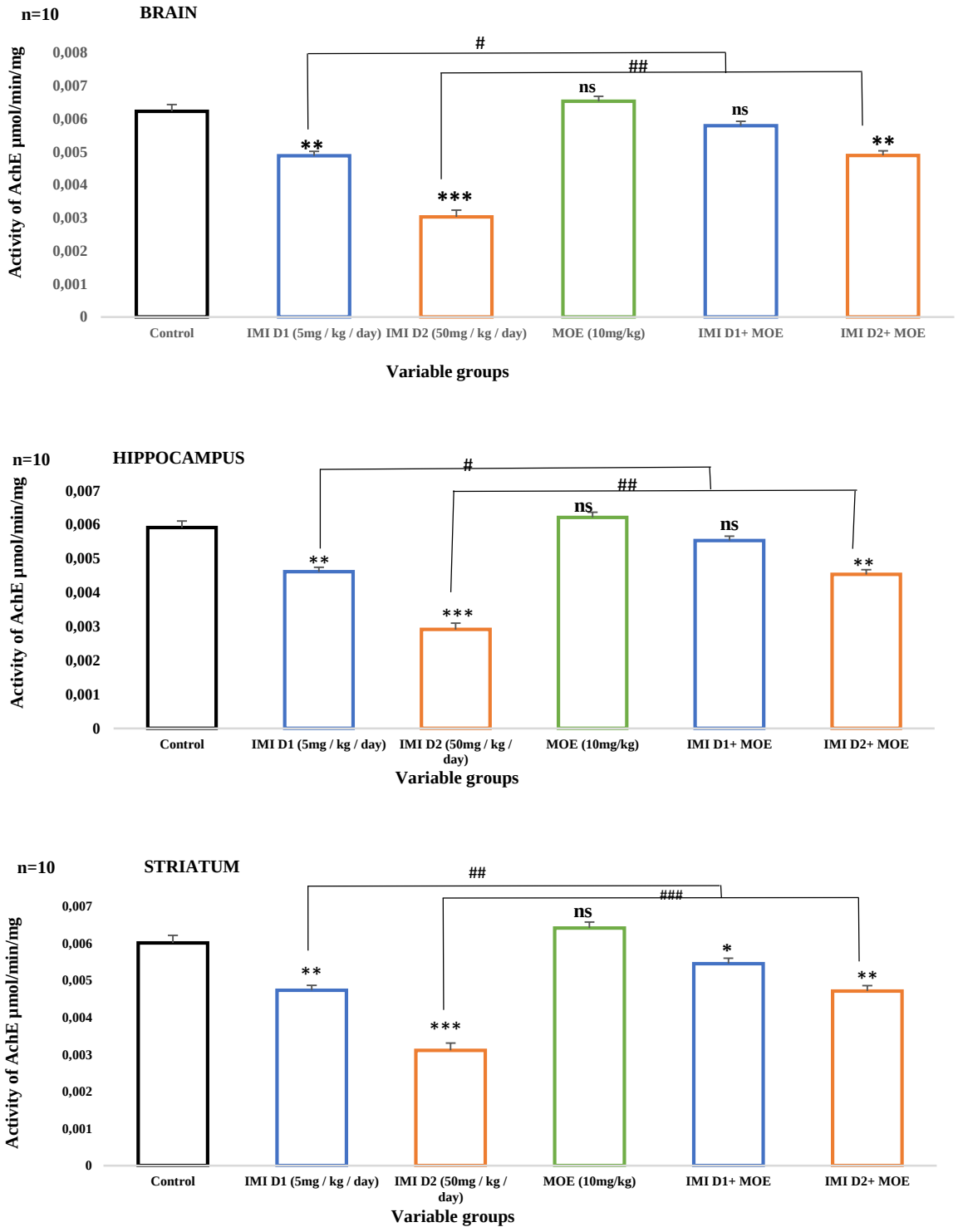


Figure 61: Variation of AChE Activity in brain, hippocampus, and striatum in treated and control rats after 90 days. Statistical significance is indicated as follows: *comparison to control (ns: P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001) and comparison between IMI D1 (5 mg/kg/day) and IMI D2 (50 mg/kg/day) groups versus IMI D1 + MOE and IMI D2 + MOE groups (#P < 0.05; ##P < 0.01; ###P < 0.001).*

Discussion

Chapter I:

In Vitro Evaluation of the Phytochemical Profile and Pharmacological Properties of Melissa officinalis L.

This study aims to analyze the characteristics of the aqueous extract of Melissa *officinalis L.* (lemon balm), a plant widely used in Algeria, to identify its active principles responsible for its therapeutic properties and determine its pharmacological potential. The analyses include evaluating the extraction yield, phytochemical screening, and quantification of total bioactive compounds such as polyphenols, flavonoids, and condensed tannins. The results indicate that the lemon balm extract is rich in bioactive compounds, with significant antioxidant activity demonstrated through various assays (DPPH, ABTS, CUPRAC, FRAP), highlighting its potential as a natural source of antioxidants. Although synthetic antioxidants sometimes exhibit superior efficacy, the bioactive constituents of lemon balm extract represent a promising natural alternative with potential health benefits.

Furthermore, variations in the yield of bioactive substances may be explained by the pedoclimatic conditions of the plant's regions of origin and the extraction techniques employed. A detailed analysis using structural techniques, including X-ray diffraction (XRD), infrared spectroscopy (IR), and scanning electron microscopy (SEM), enabled the characterization of the extract's components. These *in vitro* results provide a crucial basis for exploring the active biological properties of lemon balm. In the field of pharmacology, this plant demonstrates promising potential, particularly as a source of neuroprotective and anti-inflammatory molecules. Additional *in vivo* studies will be required to confirm these effects and further elucidate the pharmacological mechanisms of action of Melissa *officinalis* L.

1. Evaluation of the extraction yield and phytochemical properties of the aqueous extract of Melissa *officinalis* L.

The quantitative analysis of the aqueous extract of Melissa *officinalis* L. revealed an average yield of 14.46 ± 1.22%, obtained through maceration **(Table 08).** This result highlights the efficiency of this method in extracting hydrophilic compounds, such as polyphenols, flavonoids, and tannins, known for their antioxidant and neuroprotective properties. However, this yield can vary depending on several geographical, biological, and technical factors.

Yield variations reported in the literature support this observation. For instance, **Bounihi, (2015)** obtained a yield of 16.5% for samples collected in Morocco, while **Adimi, (2018)** reported a yield of 16.1% for specimens from the Sétif region in Algeria. These differences may be attributed to ecological conditions such as temperature, humidity, light, precipitation, and soil composition, as well as biological factors, including genetic heritage, developmental stage, and harvest period of the plant.

Additionally, the extraction yield is significantly influenced by the extraction method, the part of the plant used, and the physicochemical properties of the solvents employed, particularly their polarity **(Viljoen et *al.*, 2006; Sefidkon et *al.*, 2007)**. The pre-conservation of plant material and the specific characteristics of the harvesting site also play a crucial role in the observed variations. For example, a study by **Souihi et *al.*, (2020)** demonstrated that the highest yield (26.8%) was achieved using water as a polar solvent under reflux conditions. This result is comparable to yields reported for aqueous extracts of plants originating from Germany, France, and Tunisia, which ranged from 26.5% to 31%.

The phytochemical screening of the aqueous extract of Melissa *officinalis* L. **(Table 09)** revealed a rich and diverse profile of secondary metabolites, with a particularly high concentration of polyphenols (++++) and flavonoids (+++), along with notable levels of catechic tannins (+++), gallic tannins (++), and alkaloids (+++). These compounds are widely recognized for their antioxidant, anti-inflammatory, and neuroprotective properties. These findings align with previous studies by **Oka et *al.*, (1998)** and **Shieber et *al.*, (2005)**, which reported that Melissa *officinalis* leaves contain various secondary metabolites, including flavonoids, glycosides, anthocyanins, alkaloids, and tannins. Our results corroborate these observations, confirming the pronounced presence of these compounds in the leaves.

Phytochemical investigations have also demonstrated that Melissa *officinalis* is a source of terpenes (monoterpenes, sesquiterpenes, and triterpenes) and phenolic compounds (phenolic acids, flavonoids, and tannins), as highlighted by **Allahverdiyev et *al.*, (2004)** and **Moradkhani et *al.*, (2010)**. These findings are consistent with the work of **Bounihi, (2015)** who reported similar results in the phytochemical screening of Melissa *officinalis* and *Mentha* species harvested in Morocco. Furthermore, **Naghibi et *al.*, (2005)** confirmed that members of the Lamiaceae family, including Melissa *officinalis*, are rich in flavonoids and tannins, further supporting our observations.

Additionally, the presence of catechic tannins observed in this study aligns with the findings of **Paris and Moyse, (1971)**, who reported that dried Melissa *officinalis* contains approximately 4% catechic tannins. These results highlight the plant's potential as a valuable source of bioactive compounds with pharmacological significance.

Overall, the phytochemical diversity observed in Melissa *officinalis* reinforces its traditional use in managing oxidative stress-related disorders. The significant presence of phenolic

compounds and terpenes underscores its therapeutic potential, warranting further research into the isolation, characterization, and evaluation of its bioactive constituents.

2. Structural characterization techniques

The X-ray diffraction (XRD) analysis of Melissa *officinalis L.* revealed a partially crystalline structure, as evidenced by distinct peaks on the diffractogram **(Figure 31, 32).** characterized by distinct peaks corresponding to organized atomic planes in crystalline phases and diffuse regions indicative of amorphous components. This dual composition reflects the complex physicochemical properties of the plant's bioactive compounds. Using the Scherrer formula, crystallite sizes for peaks at $2\theta = 30°$ and $2\theta = 45°$ were calculated as 41.12 nm and 57.33 nm, respectively, indicating a nanometric semi-organized structure typical of plant secondary metabolites. The high crystallinity index (CI) of 95% underscores the presence of structured components such as rosmarinic acid and flavonoids, which are key to the plant's antioxidant activity.

These findings align with previous studies emphasizing the importance of crystallinity in determining the functional properties of plant-derived compounds. Although specific studies on the XRD analysis of Melissa *officinalis* L. are limited, related research provides valuable insights. For instance, a study on phyto-mediated metallic nano-architectures via Melissa *officinalis* L. **(Fierascu et *al*., 2017)** reported XRD measurements using a Rigaku SmartLab equipment, operating at 45 kV and 200 mA, with Cu Kα radiation (1.54059 Å). Additionally, electrospun PLA-based biomaterials loaded with Melissa *officinalis* extract were analyzed using XRD to assess their crystalline structure

These results are consistent with prior studies on plant-based materials, such as **Dehghan et *al*. (2021),** who investigated the XRD diffractogram of biosynthesized silver nanoparticles using Melissa *officinalis* methanolic extract. The XRD analysis revealed characteristic peaks at 32.25° and 54.62°, which were attributed to the crystalline planes of silver nanoparticles. These findings suggest that the methanolic extract of Melissa *officinalis* can facilitate the formation of silver nanoparticles with distinct crystalline structures.

However, specific diffraction peaks for individual bioactive compounds like rosmarinic acid, caffeic acid, and quercetin remain underexplored. The peaks observed in this study, particularly those between 10° and 50°, align with the polycyclic aromatic structures of flavonoids and phenolic acids, further supporting their role in the plant's antioxidant potential and crystallinity.

The FTIR spectrum of Melissa *officinalis* powder **(Figure 33, 34); (Table 11)**. Highlights the presence of key functional groups associated with its bioactive compounds, providing a comprehensive chemical fingerprint. Prominent absorption peaks include C-H stretching vibrations (3025–2830 cm^{-1}), indicative of aliphatic hydrocarbons linked to terpenes and fatty acids; strong C=O stretching vibrations (1700–1500 cm^{-1}), characteristic of carbonyl groups found in phenolic acids like rosmarinic acid and caffeic acid; and C-O stretching and C-H bending vibrations (1350–970 cm^{-1}), associated with alcohols, esters, and ethers in phenolic compounds and essential oils. Unique peaks in the fingerprint region (950–700 cm^{-1}) reflect structural markers of terpenes, flavonoids, and aromatic compounds. The weak absorption band between 2410 and 2250 cm^{-1} suggests trace nitrile compounds. These findings align with previous studies, such as those by **Šic Žlabur et *al.* (2016)** and **Jovic and Tešić (2017),** This study provides an analysis of the chemical composition and bioactive properties of Melissa *officinalis* essential oil and offers insight into the identification of key functional groups like C-H, C=O, and C-O vibrations, which align with your FTIR findings related to terpenes, phenolic acids, and other bioactive compounds. Confirming the plant's antioxidant, anti-inflammatory, and neuroprotective potential. This analysis supports the traditional medicinal use of Melissa *officinalis* and its pharmaceutical applications, emphasizing the relevance of its diverse phytochemical composition.

The Scanning Electron Microscopy (SEM) analysis of Melissa *officinalis* L. powder revealed significant insights into the morphology and particle size distribution, which directly influence the bioactive compound extraction and release profiles. The SEM images **(Figure 35)** show that smaller particles (5–20 µm) exhibit rough and porous surfaces, which increase their specific surface area, thereby enhancing their interaction with solvents and facilitating the extraction of bioactive compounds. This porous structure is often associated with higher bioavailability and quicker release of active compounds, which is crucial for applications requiring rapid action **(Singh and Ghosh, 2014).** In contrast, larger particles (>50 µm) show smoother, more compact surfaces, which may restrict solvent penetration and slow down the release of bioactive compounds. Such compact surfaces could be beneficial for controlled-release applications where a gradual release is desired **(Rafique and Amna, 2020).** The observed particle distribution in the SEM images was generally homogeneous, although some agglomerations were visible at sizes below 20 µm. These agglomerations could potentially interfere with efficient extraction by limiting solvent access to the active components within the particles **(Khalil and Ismail, 2017).** The findings indicate that smaller particle sizes favor

enhanced accessibility to bioactive compounds, while larger particles can provide a more controlled release, suitable for specific therapeutic applications where prolonged effects are necessary. The structural differences between the particle sizes emphasize the need to optimize particle size based on the intended use, whether for rapid extraction or controlled bioactive compound release.

3. Determination of total bioactive compound

The quantitative analysis of the total bioactive compounds in the aqueous extract of Melissa *officinalis* L. revealed significant levels of polyphenols, flavonoids, and condensed tannins, supporting its traditional medicinal applications. The total polyphenol content (TPC) was measured at 40.29 ± 4.09 mg GAE/g extract **(Table 12)**, indicating a high concentration of polyphenolic compounds such as rosmarinic acid and caffeic acid, which are well-known for their antioxidant and anti-inflammatory properties **(Sofowora et *al.*, 2013)**. These results align with previous studies reporting polyphenol contents in Melissa *officinalis* ranging from 20 to 60 mg GAE/g **(Hassan et *al.*, 2019)**. Additionally, **Franco et *al.*, (2018)** documented a total polyphenol content of 177 ± 13 mg GAE/g dw, further emphasizing the plant's robust antioxidant potential.

Flavonoids, another important class of bioactive compounds, were quantified at 24.43 ± 1.77 mg QE/g extract **(Table 12)**. Similar results have been reported in previous studies on various plant extracts. For example, **Franco et *al.*, (2018)** reported a total flavonoid content of 26 ± 3 mg QE/g dw for Melissa *officinalis* extract, evaluated by spectrophotometry. Flavonoids are plant secondary metabolites characterized by a benzopyran heterocycle linked to a benzene ring **(Testai, 2015).** These compounds are well known for their neuroprotective, anti-inflammatory, and antioxidant activities. In Melissa *officinalis*, flavonoids such as quercitrin, rhamnocitrin, and luteolin have been identified as key contributors to its therapeutic effects **(Ulubelen et *al.*, 2005).** The flavonoid content observed in this study aligns with other reports highlighting the importance of these compounds in medicinal plants, especially in Lamiaceae species, where flavonoids are most concentrated in the aerial parts **(Ulubelen et *al.*, 2005).**

Furthermore, the condensed tannin content of the extract **(Table 12)** was found to be 17.84 ± 3.95 mg CE/g extract. Tannins, polyphenolic compounds known for their ability to scavenge free radicals, contribute significantly to the plant's overall antioxidant capacity. The condensed tannin content observed aligns with other studies that have documented the presence of tannins in Melissa *officinalis*, which are linked to antimicrobial, anti-inflammatory, and anticancer

activities **(Mimica-Dukic et *al.*, 2004).** These compounds are crucial for the plant's role in disease prevention and health promotion **(Sofowora et *al.*, 2013).**

A study by **Jovanović et *al.*, (2022)** assessed the total tannin content in water extracts of Melissa *officinalis* using different extraction methods. The maceration (MAC) method yielded a tannin content of 2.55 mg tannic acid equivalent (TAE) per milliliter, the ultrasound-assisted extraction (UAE) method yielded 4.53 mg TAE/mL, and the microwave-assisted extraction (MAE) method yielded 1.83 mg TAE/mL. These results underscore the impact of extraction techniques on the tannin content of Melissa *officinalis* extracts.

These findings support the established view of Melissa *officinalis* as a valuable medicinal plant, rich in phenolic compounds, flavonoids, and tannins, all of which contribute to its proven health benefits. The results further highlight the promising therapeutic potential of Melissa *officinalis* in both traditional and modern pharmacological contexts.

4. Assessment of the antioxidant activity in the extract of M. *officinalis* L.

Antioxidant compounds have the ability to neutralize and scavenge free radicals by either donating hydrogen atoms or chelating metal ions, thereby inhibiting the harmful effects of oxidants **(Gulçin et *al.*, 2003; Prakash et *al.*, 2007).**

The evaluation of the antioxidant activity of the aqueous extract of Melissa *officinalis* L. (MOE) revealed significant capacities to neutralize free radicals and reduce metals, confirmed through various chemical assays, including DPPH, ABTS, CUPRAC, and FRAP, as displayed as mean ± SD in **Table 13**. These results underscore the richness of the extract in bioactive compounds such as flavonoids (quercetin, luteolin), phenolic acids (rosmarinic acid, caffeic acid), and other secondary metabolites **(Sofowora et *al.*, 2013).**

In the DPPH assay, the antioxidant activity of the aqueous extract of Melissa *officinalis* was evaluated by its capacity to donate hydrogen atoms or electrons, facilitating the reduction of the DPPH• radical to its stable form, DPPH-H. This transformation was monitored spectrophotometrically, providing a direct measure of the radical scavenging potential of MOE.

The Melissa *officinalis* extract exhibited notable radical scavenging activity, with an IC50 of 23.29 µg/ml, although slightly lower than that of synthetic reference antioxidants such as BHA (14.66 µg/ml) and BHT (16.53 µg/ml). This difference can be attributed to the purity of synthetic antioxidants, while the extract of M. *officinalis* is a complex mixture of bioactive compounds acting synergistically **(Dastmalchi et *al.*, 2008).** Additionally, the presence of

rosmarinic acid, a compound widely known for its antioxidant activity, contributes significantly to this result. These findings are in line with previous studies that highlighted strong DPPH• free radical scavenging ability of extracts derived from Melissa *officinalis* **(Ferreira et *al.*, 2006; Dastmalchi et *al.*, 2007; Meftahizade et *al.*, 2013; Sipos et *al.*, 2021).**

When comparing these results to other studies**, Caleja et *al.*, (2018)** reported lower antioxidant activity for an aqueous extract of M. *officinalis*, with an IC50 of 79 ± 2 µg/ml. This variation may be attributed to differences in experimental conditions, extraction methods, or the chemical composition of the samples used. Furthermore, **Spiridon et *al.*, (2011)** determined an IC50 of 87.28 µg/ml for an extract of M. *officinalis*, further emphasizing potential variations between studies. These observations suggest that, although the aqueous extract of M. *officinalis* shows slightly lower antioxidant activity than synthetic antioxidants, it remains a promising natural source of antioxidants. The variations between studies highlight the importance of experimental conditions and extraction methods on the antioxidant efficacy of M. *officinalis* extracts.

The ABTS assay is a highly effective method for evaluating the antioxidant properties of various substances, including those that donate hydrogen atoms, neutralize aqueous phase radicals, or scavenge lipid peroxyl radical chains **(Re et *al.*, 1999).**

The ABTS assay revealed a notable radical scavenging capacity of the aqueous extract of Melissa *officinalis* (MOE), with an IC_{50} of 24.95 µg/ml. This value, although higher than those of synthetic antioxidants such as BHA (1.09 µg/ml) and BHT (1.97 µg/ml), indicates significant antioxidant activity. This observation reflects a moderate efficiency of MOE in this model, likely due to the chemical structure of the phenolic compounds, which interact differently with ABTS radicals compared to synthetic standards. Previous studies have reported similar results for aqueous extracts of Melissa *officinalis*. For example, a study by **Dastmalchi et *al.*, (2007)** reported an IC_{50} of 32.7 µg/ml for the aqueous extract of Melissa *officinalis*, which is close to our findings and confirms the notable antioxidant activity of the extract. Another study by **Meftahizade et *al.*, (2013)** also revealed an IC_{50} of 29.9 µg/ml, which is comparable to our results, suggesting that the aqueous extract of Melissa *officinalis* possesses significant antioxidant potential. These variations may be attributed to differences in extraction methods, experimental conditions, and the chemical composition of the extracts.

The CUPRAC (Cupric Ion Reducing Antioxidant Capacity) assay is a widely used method for evaluating the antioxidant capacity of various substances. It involves the reduction of copper (II) to copper (I) by antioxidants, resulting in a color change **(Apak et *al.*, 2008).**

The CUPRAC assay demonstrated dose-dependent antioxidant activity with an EC_{50} of 31.14 µg/ml, indicating a moderate reducing capacity of M. *officinalis* extract compared to BHA (13.65 µg/ml) and BHT (17.62 µg/ml). This difference can be attributed to the complexity of the natural metabolites present in MOE, such as flavonoids and tannins, which reduce Cu^{2+} ions to Cu^{+} with lower efficiency compared to pure antioxidants. Previous studies have shown similar results for various plant extracts. For instance, a study by **Barros et *al.*, (2016)** reported an EC_{50} value of 26.8 µg/ml for the aqueous extract of M. *officinalis* in the CUPRAC assay, indicating significant antioxidant potential in Melissa *officinalis* aqueous extracts. This is consistent with the plant's high polyphenol content, which is comparable to the values obtained in our study. Additionally, another study by **Sentkowska et *al.*, (2015)** examined the polyphenolic content and antioxidant properties of Melissa *officinalis* extracts. The antioxidant activities measured by Folin-Ciocalteu and CUPRAC assays were higher for the extract prepared by decoction, with values of 1245 mg/g GAE and 4.91 mmol/g, respectively, confirming the moderate reducing power of this plant extract when compared to synthetic antioxidants.

Finally, the FRAP assay, based on the ability of antioxidants, including phenolics, to reduce Fe^{3+} to Fe^{2+} in the presence of the FRAP reagent, forming a blue chromophore **(Benzie and Strain, 1996),** revealed an EC_{50} of 28.76 µg/ml for the aqueous extract of Melissa *officinalis* (MOE)**.** Although this value is significantly higher than that of ascorbic acid (8.04 µg/ml), it reflects the strong reducing potential of MOE, attributed to its flavonoids and polyphenols, which play a crucial role in reduction mechanisms **(Miao et *al.*, 2015).**

Previous studies confirm this efficiency. For example, **Ferreira et *al.*, (2006)** reported results indicating that Melissa *officinalis* extract (ME) exhibits effective reducing power, comparable to the standard ascorbic acid. The total antioxidant activity of ME, as measured by the FRAP assay, was 61 ± 6 TE/g dw in their study, contrasting with the findings of another study using a different extraction solvent, which reported 464.8 µmol TE/g dw (equivalent to 117 mg TE/g dw). These results align with the plant's rich profile of phenolic compounds, particularly rosmarinic acid and tannins, which contribute significantly to its antioxidant activity. These findings underscore MOE's potential as a natural antioxidant for preventing oxidative damage, with promising applications in pharmaceuticals and food industries.

Chapter II:

In Vivo Assessment of Imidacloprid's Neurotoxic Effects and the Neuroprotective Potential of Melissa officinalis L.

Discussion

Our environment and health are under constant threat from pollutants like xenobiotics. Pesticides, among the most widespread, pose serious toxicity risks and disrupt vital body functions **(Speck-Planche et *al.*, 2012; Singh et *al*., 2012).**

Neonicotinoids (NNIs) are a widely used class of systemic insecticides that act as agonists of nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS) of insects, leading to paralysis and death **(Jeschke and Nauen, 2008; Simon-Delso et *al*., 2015).** Among them, imidacloprid (IMI) is one of the most commonly applied neonicotinoids in agriculture and veterinary medicine due to its high insecticidal efficacy and selective toxicity to insects **(Gibbons et *al*., 2015).** However, growing evidence suggests that chronic exposure to IMI may induce neurotoxic effects in mammals, including oxidative stress, mitochondrial dysfunction, and neurobehavioral impairments **(Duzguner and Erdogan, 2010; Bal et *al*., 2012).**

Chronic exposure to IMI has been linked to oxidative stress and neurodegeneration through increased production of reactive oxygen species (ROS), leading to lipid peroxidation, protein oxidation, and DNA damage in neuronal tissues **(Abd-Elhakim et *al*., 2018).** Studies have demonstrated that IMI exposure disrupts mitochondrial integrity, impairs antioxidant defense mechanisms, and alters neurotransmitter levels, particularly in brain regions involved in learning and memory, such as the hippocampus and striatum **(Gomes et *al*., 2022).**

To counteract these neurotoxic effects, natural antioxidants derived from medicinal plants have been investigated for their neuroprotective potential **(Shakeri et *al*., 2016)**. Melissa *officinalis* L. (lemon balm) is a medicinal herb rich in phenolic compounds, flavonoids, and rosmarinic acid, known for its strong antioxidant and neuroprotective properties **(Oboh et *al*., 2016).** Previous studies suggest that M. officinalis can scavenge ROS, enhance mitochondrial function, and modulate neurotransmitter systems, making it a promising candidate for mitigating pesticide-induced neurotoxicity **(Hassanzadeh et *al*., 2011).**

In this study, *Wistar* rats were chronically exposed to IMI at doses of 5 mg/kg/day and 50 mg/kg/day for 90 days, and a subset was co-treated with Melissa *officinalis* extract (10 mg/kg/day) to evaluate its potential protective effects. The investigation focused on oxidative stress markers, mitochondrial function, neurotransmitter levels, Lysosomal stability, neurobehavioral alterations and histopathological changes. To provide insight into the underlying mechanisms of IMI-induced neurotoxicity and the therapeutic potential of M. *officinalis*.

1. Clinical indicators of neurotoxicity and visual observations

The clinical indicators of neurotoxicity observed in our study **(Table 14 and 15, Figure 39)** highlight the dose-dependent effects of chronic Imidacloprid (IMI) exposure and the protective potential of Melissa *officinalis* extract (MOE). Rats exposed to a high dose of IMI (50 mg/kg/day) exhibited pronounced neurotoxic symptoms, including lethargy (60%), tremors (45%), significant weight loss (~15%), and occasional seizures, aligning with previous findings by **Duzguner and Erdogan, (2010)** and **Bal et *al*. (2012)**, who reported similar neurobehavioral impairments and oxidative stress-related damage in neonicotinoid-exposed rodents. These effects likely stem from IMI's impact on cholinergic neurotransmission and mitochondrial dysfunction, as previously demonstrated in pesticide neurotoxicity studies **(Kimura et *al*., 2012; Tomizawa and Casida, 2005**). Conversely, the low-dose IMI group (5 mg/kg/day) displayed only mild signs of neurotoxicity, including minor lethargy and reduced grooming activity, supporting a dose-dependent response.

The lack of mortality in any treatment group indicates that while chronic exposure to IMI at these doses induces neurotoxic effects, it does not reach lethal levels, aligning with previous findings that report an LD50 of 424–475 mg/kg in rats **(Sheets, 2001).** However, the detection of neurological impairments even at sub-lethal doses raises significant concerns regarding the risks associated with prolonged exposure. In rodents, the established acute dietary reference dose for imidacloprid is 125 mg/kg body weight per day, with high exposure levels primarily triggering nicotinic-related symptoms such as tremors **(Sheets, 2010).**

Co-administration of MOE significantly alleviated IMI-induced neurotoxicity, particularly in the high-dose IMI group. Rats receiving IMI + MOE exhibited a 30% reduction in tremors, a 40% decrease in lethargy, and a 60% improvement in inflammation-related symptoms, reinforcing its neuroprotective and anti-inflammatory effects. These results align with studies on Melissa *officinalis*, which demonstrated its ability to enhance antioxidant enzyme activities, reduce oxidative stress markers, and stabilize mitochondrial function in neurotoxic models (**Naseri et *al*., 2019**). Visual observations further corroborated these findings, with high-dose IMI rats exhibiting moderate skin inflammation (50%), mild edema, and occasional superficial wounds, potentially linked to systemic oxidative stress and immune dysregulation (**Mohamed et *al.*, 2020**). The significant reduction in these signs in MOE-treated groups highlights its potential in modulating inflammatory responses and suggesting its potential as a protective agent against chronic pesticide-induced neurotoxicity.

2. Impact on growth parameters

The observed effects of chronic Imidacloprid (IMI) exposure on growth parameters, including body weight, weight gain, and relative brain weight, align with previous research highlighting the toxicological consequences of neonicotinoids on mammalian physiology. Our study demonstrated a dose-dependent reduction in these parameters, with significant decreases in body weight and weight gain in IMI-treated groups **(Tables 16 and Figures 40, 41, 42).** The control group exhibited normal growth, whereas IMI D1 (5 mg/kg/day) led to a significant reduction in body weight ($p \leq 0.05$), and IMI D2 (50 mg/kg/day) caused a highly significant reduction ($p \leq 0.01$), This reduction can be attributed to the disturbance of cellular metabolism under the effect of oxidative stress generated by reactive oxygen species (ROS) observed in this study. Additionally, other chemical mediators, such as certain pro-inflammatory cytokines, may contribute to this reduction due to the toxic effects of pesticides. Similar findings **(Carole and Harvé, 2011)** have been reported, indicating a reduction in food consumption in male rats under sub-chronic toxicity conditions. Additionally, a two-generation reproductive toxicity study on rats exposed to Imidacloprid further supports these findings, showing increased O-demethylase activity in maternal rats at doses of 17 mg/kg/day and reduced body weight gains in pups at 47 mg/kg/day, though no effects on reproductive behavior or success were observed **(Gervais et *al.*, 2010).**

Furthermore, the administration of Melissa *officinalis* (MOE) (10 mg/kg/day) alone did not significantly alter body weight, suggesting that the extract does not inherently affect growth. However, co-administration of MOE with IMI has shown in notable improvements in animal weight parameters, particularly in IMI D1 + MOE ($p \leq 0.05$), while IMI D2 + MOE showed only a slight improvement ($p \leq 0.05$), supporting the hypothesis that MOE's antioxidant properties may mitigate IMI-induced metabolic disturbances **(Canadanovic-Brunet et *al.*, 2008 ; Kamdem et *al.*, 2013)**. This could be a result of its antioxidant properties, which help normalize intracellular redox homeostasis and restore the psychological state of the animals **(Cliona et *al.*, 2011; Toumi et *al.*, 2016).** The improvement in relative brain weight observed with MOE treatment could be due to its ability to reduce the accumulation of free radicals through its antioxidant properties **(Ronat, 2001).** Additionally, the digestive, antioxidant, and anti-inflammatory properties of Melissa *officinalis* contribute to mitigating weight loss and metabolic disruptions caused by Imidacloprid exposure **(Iserin, 2001).**

3. Behavioral and cognitive tests

Behavior plays a crucial role in the survival of species and the structuring of ecological communities. Neonicotinoids, through their dose-dependent effects on neuronal activity in insect brains, have the potential to disrupt cognitive functions and impair the ability to learn neurobehavioral tasks **(Palmer et *al.*, 2013).**

This study investigated the impact of chronic imidacloprid (IMI) exposure (5 and 50 mg/kg body weight, orally) on neurobehavioral functions in *Wistar* rats, along with the potential protective role of Melissa *officinalis* (MO) extract (10 mg/kg/day). Over a 90-day period, behavioral assessments were conducted to evaluate cognitive, social, and emotional responses **(Table 17; Figures 43–48).** Pesticide exposure is known to generate oxidative stress, leading to disruptions in neural pathways involved in locomotion, learning, memory, and cognitive development **(Beghoul et *al.*, 2017; Gasmi et *al.*, 2017; Gargouri et *al.*, 2019).**

The Classic Labyrinth Test (CLT) results indicate significant neurobehavioral impairments following chronic exposure to Imidacloprid (IMI), with a dose-dependent **(Table 17, Figure 43)** increase in Finishing Time (FT) and a corresponding decrease in Exploration Time (ET), suggesting cognitive deficits. Rats exposed to 50 mg/kg IMI exhibited the most severe impairment, with highly significant delays in task completion and reduced exploratory, consistent with previous evidence on neonicotinoid-induced neurotoxicity. These findings corroborate those of **Hassanen et *al.* (2021)**, who observed similar reductions in spatial memory following pesticide exposure, as well as supporting findings by **Gasmi et *al.* (2016),** , which demonstrated memory decline and anxiety in rats chronically exposed to deltamethrin (0.32 mg/kg/day). In addition, research into the potential toxic effects of imidacloprid at three low doses showed that learning abilities significantly declined in rats at doses of 2 and 8 mg/kg. However, at a dose of 0.5 mg/kg (the maximum residue level), imidacloprid did not impair cognitive functions, even in infant rats **(Kara et *al.*, 2015).** Moreover, Melissa *officinalis* extract (MOE) co-administration significantly reversed these impairments, as reflected by reduced the latency FT and increased successful completions ET, suggesting its positive influence on spatial learning, particularly in the MO-only and IMI + MO combination groups. This suggests that MO exerts neuroprotective effects, likely through its antioxidant and neuromodulatory properties, as previously demonstrated in studies highlighting its cognitive-enhancing and anxiolytic effects **(Lin et *al.*, 2015)**. The improvement in spatial learning and memory following MO treatment supports earlier findings by **Dastmalchi et *al.* (2008)**, which highlighted the role of rosmarinic acid in neurotransmitter modulation, and **Kennedy et *al.* (2002)**, who reported

enhanced cognition in healthy individuals following MO administration. Additionally, **Naseri et *al.* (2019)** evaluated the impact of hydroalcoholic extract of Melissa *officinalis* on learning and memory in rats, suggesting potential cognitive benefits The dose-dependent improvements observed in the combination groups further reinforce MO's potential as a protective agent against IMI-induced cognitive dysfunction.

The Open Field (OF) Test results indicate that imidacloprid (IMI)-treated rats (5 and 50 mg/kg/day) exhibited significant anxiety-like behaviors, including reduced exploratory center zone activity, increased grooming, heightened defecation/urination, and impaired locomotion relative to controls in a dose-dependent manner. **(Table 17, Figure 44),** consistent with well-established rodent anxiety markers **(Patro et *al*., 2021).** Additionally, IMI exposure led to increased Immobility Time in the Peripheral Area (IPA) (IMI D2: 88 ± 3.68 sec; ***P < 0.001), reduced Total Distance Moved (TDM) (IMI D2: 7.616 ± 0.648 m; ***P < 0.001), and fewer Total Number of Rearing's (TNR), suggesting impaired locomotion and reduced exploratory behavior. These findings align with prior studies demonstrating IMI-induced neurotoxicity in both rodents and insects **(Decourtye et *al*., 2004; Duzguner and Erdogan, 2010)**. However, co-treatment with Melissa *officinalis* (MOE) extract significantly alleviated these behavioral deficits by increasing center zone activity, reducing stress markers, and improving overall locomotion and exploration ($p<0.05$). These effects may be attributed to the anxiolytic and antidepressant properties of rosmarinic acid, a key bioactive compound in MO **(Martins et *al*., 2012)**, as well as its role in modulating neurotransmitter systems linked to mood regulation, as observed in forced swim test studies **(Lin et *al*., 2015).** Collectively, these findings confirm that chronic IMI exposure disrupts neurobehavioral functions, while Melissa *officinalis* exerts a protective effect, mitigating anxiety and motor impairments through its antioxidant and neuromodulatory properties.

In the Novel Object Recognition (NOR) test results reveal significant dose-dependent recognition memory impairments in Imidacloprid (IMI)-treated groups, with a significant reduction in discrimination index compared to control, indicating cognitive deficits **(Table 17, Figure 45).** As evidenced by increased familiar object exploration (EFO**)** and decreased novel object exploration (ENO), particularly in the 50 mg/kg IMI group ($P \leq 0.001$). These findings align with previous research on neonicotinoid-induced cognitive dysfunction **(Hassanen et *al*., 2021; Gasmi et *al*., 2016)**, likely resulting from oxidative stress, mitochondrial dysfunction,

and cholinergic system disruption, including **Zhang and Nieh (2015),** which link neonicotinoid exposure with memory and learning impairments in animals.

However, rats co-treated with Melissa *officinalis* extract (MOE**)** showed a significantly mitigated memory deficits**,** as shown by improved ENO and reduced EFO in the IMI D1 + MOE **(***P < 0.05*) and IMI D2 + MOE **(**P < 0.01**)** groups, demonstrating its neuroprotective effects. The cognitive benefits of MOE may be attributed to its antioxidant, anti-inflammatory, and neuromodulatory properties, supported by studies on rosmarinic acid and polyphenols **(Dastmalchi et *al.*, 2008; Kennedy et *al.*, 2002; Naseri et *al.*, 2019).** These results suggest that MOE could serve as a therapeutic agent against IMI-induced recognition memory deficits, reinforcing its potential for neuroprotection and cognitive enhancement. Notable improvement in the discrimination index, suggesting MO's protective effect on IMI-induced memory deficits. The antioxidant and anti-inflammatory properties of MO's phytochemicals, as described by **Barros et *al.* (2013)**, may be key contributors to preserving cognitive function under oxidative stress. Also a study by **Dehbani et *al.* (2019)** indicated that oral administration of Melissa *officinalis* L. extract could enhance learning and memory in the passive avoidance-learning test, likely due to its rich phenolic and flavonoid content.

The results of the Vsoc-Maze Test highlight significant dose-dependent behavioral impairments in *Wistar* rats exposed to Imidacloprid (IMI), particularly in exploratory behavior, sociability, and preference for social novelty **(Table 17, Figure 46)**, aligning with prior studies on neonicotinoid-induced neurobehavioral deficits **(Duzguner and Erdogan, 2010; Kimura-Kuroda et *al.*, 2012)**. Rats in the IMI (5 mg/kg) and IMI (50 mg/kg) groups exhibited progressively reduced exploration times, with the higher dose causing more severe impairments (P < 0.01, P < 0.001), suggesting a dose-dependent disruption in cognitive and social behaviors, likely linked to oxidative stress and neurotransmitter dysfunction **(Zhang et *al.*, 2019)**. Sensorimotor deficits, as seen in altered postural reflexes and decreased exploratory drive, further support IMI's neurotoxic impact, resembling behavioral abnormalities observed in IMI-exposed Drosophila **(Kim et *al.*, 2020)** and offspring of IMI-treated mothers **(Abou-Donia et *al.*, 2008).** Notably, Melissa *officinalis* extract (MOE) co-administration significantly restored exploratory behavior, sociability, and novelty preference, with IMI + MOE groups demonstrating dose-dependent recovery, emphasizing MOE's potential in mitigating IMI-induced neurotoxicity. These findings align with research on MOE's neuroprotective and anxiolytic properties **(Kennedy et *al.*, 2002; Sofowora et *al.*, 2013; López et *al.*, 2018)**, reinforcing its therapeutic role in pesticide-induced neural damage.

The sucrose preference test (SPT) **(Table 17, Figure 47)** results indicate that Imidacloprid (IMI) exposure leads to dose-dependent anhedonia-like behavior in *Wistar* rats. Specifically, rats administered IMI at 5 mg/kg and 50 mg/kg doses exhibited significant reductions in sucrose intake and preference compared to controls, with the higher dose correlating with more pronounced deficits, a hallmark of anhedonia and depressive-like behavior observed in pesticide-exposed models **(Wolbling and Leonhardt, 1994).** However, some studies reported increased preference **(Murison and Hansen, 2001; Maramis et *al*., 2021).** Similar effects on neurotransmitter depletion were observed by **Chao and Casida (1997)** following IMI injection in mice, contributing to reduced exploration and depressive behaviors **(Nutt, 2008)**. This aligns with previous research demonstrating that elevated doses of IMI impair cognitive functions, particularly in infant rats, potentially due to alterations in gene expression related to learning and memory .Conversely, co-administration of Melissa *officinalis* extract (MOE) at 10 mg/kg effectively mitigated these effects, significantly increased sucrose preference to near-control levels. Consistent with its reported antidepressant effects **(Martins et *al.*, 2012)** through modulation of serotonin and dopamine pathways involved in reward processing. Additionally, chronic MOE administration has been shown to relieve stress-related effects **(Cases et *al*., 2011).**The neuroprotective properties of Melissa *officinalis* are well documented, with studies showing its efficacy against neurotoxicity induced by agents like MDMA, attributed to its antioxidant and monoamine oxidase inhibitory effects **(Hassanzadeh et *al.*,2011)**. These findings underscore the potential of MOE as a therapeutic agent in counteracting IMI-induced neurobehavioral impairments.

Olfaction represents an intriguing physiological pathway in depression due to the close connections between the olfactory pathways and brain regions involved in mood and emotional regulation, particularly within the limbic system **(Brand et *al.*, 2017; Abd-Elhakim et *al.*, 2018)**. Olfactory memory, tested by time spent exploring familiar versus novel odors. The test results **(Figure 48, Table 17)** reveal that Imidacloprid (IMI) exposure induces dose-dependent impairments in olfactory recognition, as evidenced by significantly increased latency times to own fissure (LTOF) in both IMI (5 mg/kg) and IMI (50 mg/kg) groups compared to controls. These findings align with previous research demonstrating that neonicotinoid pesticides disrupt sensory and cognitive functions by impairing cholinergic signaling and inducing oxidative stress in neural tissues **(Tomizawa and Casida, 2005; Kimura-Kuroda et *al*., 2012)**. The dose-dependent increase in LTOF observed in IMI-treated rats suggests a progressive decline in olfactory processing, likely linked to neuroinflammation and neurotransmitter dysregulation

(Gibbons et *al.*, 2015). Notably, co-administration of Melissa *officinalis* extract (MOE, 10 mg/kg) significantly reduced latency times in both IMI-exposed groups, restoring olfactory recognition to near-control levels as observed in **Shirzad et *al.*, (2011)**. These findings are consistent with studies highlighting the neuroprotective effects of Melissa *officinalis*, which has been shown to enhance cognitive function and mitigate oxidative stress-induced neuronal damage through its antioxidant and cholinergic-modulating properties **(Kennedy et *al.*, 2002; López et *al.*, 2018)**. Additionally, the increased time spent in the center by IMI-treated rats suggests deficits in spatial awareness and decision-making, further supporting the neurotoxic impact of IMI exposure **(Abou-Donia et *al.*, 2008).** Overall, these results underscore the detrimental effects of IMI on olfactory recognition and cognitive processing while reinforcing the potential of Melissa *officinalis* as a protective agent against pesticide-induced neurotoxicity.

4. Evaluation of mitochondrial metabolic parameters

The present study **(Table 18; Figure 49)** reveals a clear dose-dependent disruption of mitochondrial metabolic parameters (protein, carbohydrate, and lipid levels) following chronic exposure to Imidacloprid (IMI), with significant variations across different brain regions. The striatum exhibited the highest susceptibility to IMI-induced toxicity, followed by the hippocampus and whole brain, reflecting their differential metabolic demands and vulnerability to oxidative stress. The biochemical analysis demonstrated a dose-dependent increase in mitochondrial protein and carbohydrate levels at both low (IMI D1: 5 mg/kg/day) and high (IMI D2: 50 mg/kg/day) doses, with a more pronounced impact at the higher dose. This suggests a compensatory response to oxidative stress, potentially through mitochondrial biogenesis, as reported in similar studies **(Bal et *al.*, 2012; Gibbons et *al.*, 2015).** On the other hand, lipid depletion was observed even at the lower dose. This pattern indicates that lipid peroxidation and mitochondrial membrane disruption occur even at low pesticide exposure, suggesting that lipid depletion is not solely dose-dependent but may also be an early biomarker of mitochondrial membrane destabilization, which may lead to impaired β-oxidation and oxidative damage, independent of dose escalation **(Campos et *al.*, 2016; Tomizawa and Casida, 2005).**

A striking observation in this study is the varying susceptibility of different brain regions to IMI-induced mitochondrial disturbances. The striatum exhibited the highest increase in mitochondrial protein and carbohydrate levels, as well as the greatest lipid depletion, suggesting that it is the most vulnerable region to IMI toxicity. This aligns with findings from previous studies that highlight the striatum's susceptibility to neurotoxicants affecting dopamine

metabolism and mitochondrial bioenergetics **(Crumpton et *al.*, 2000; Duzguner anf Erdogan, 2012).** Given that the striatum is rich in dopaminergic neurons and has a high oxidative metabolic demand, it is likely more prone to IMI-induced oxidative stress and mitochondrial dysfunction compared to other brain regions.

The hippocampus, another key brain region, also showed significant mitochondrial disturbances, though less pronounced than in the striatum. The hippocampus is essential for learning and memory processes, and its neurons are highly sensitive to disruptions in energy metabolism. Previous studies have demonstrated that neonicotinoid exposure impairs hippocampal-dependent cognitive functions, likely through oxidative stress-mediated mitochondrial dysfunction **(Kimura-Kuroda et *al.*, 2012).** The whole brain (Br), representing a more generalized metabolic response, showed significant but comparatively milder changes in mitochondrial metabolism, reinforcing the notion that certain brain regions are more vulnerable to IMI toxicity than others.

The observed alterations in mitochondrial homeostasis align with previous findings in *Wistar* rats and invertebrates exposed to sub-lethal doses of neonicotinoids, where increased protein levels indicated the synthesis of stress-response enzymes and defense peptides to counteract oxidative stress **(Anadn et *al.*, 1991; Benbouzib, 2012; Rouabhi et *al.*, 2015; Chakrounet et *al.*, 2016).** This is further supported by the work of **Radwan and Mohamed (2013)**, who reported a significant dose-dependent increase in total protein levels in *Wistar* rats following exposure to 0.2 LD_{50} of IMI over 1, 3, and 7 days. Similar findings were documented in male rats treated with Acetamiprid **(Chakrounet et *al.*, 2016)** and earthworms exposed to IMI-contaminated soil, where total protein content increased in response to oxidative stress **(Drobne et *al.*, 2008**). Additionally, stress-induced protein synthesis has been reported in snails and insects exposed to neonicotinoid-based on thiamethoxam pesticides with doses of 0, 25, 50, 100 and 200 mg / L on *Wistar* rats after six weeks of treatment. **(Bourbia, 2013**), supporting the hypothesis, that mitochondrial dysfunction triggers compensatory mechanisms at the molecular level.

Oxygenated free radicals generated by IMI exposure play a crucial role in these biochemical disturbances, as they induce protein modifications through carbonylation, fragmentation, and amino acid oxidation, ultimately altering protein structure and function (**Gasmi, 2018**). Lipid peroxidation further exacerbates mitochondrial damage, as observed in previous studies on *Wistar* rats exposed to neonicotinoids (**Radwan and Mohamed, 2013)**.

This disruption in lipid homeostasis is particularly evident in the striatum, where high oxidative metabolic demand and dopaminergic neuron activity render the region highly vulnerable to neurotoxic insults **(Crumpton et *al*., 2000; Duzguner and Erdogan, 2012)**. Similarly, hippocampal mitochondrial alterations may underlie cognitive deficits associated with neonicotinoid exposure, as reported in previous neurobehavioral studies **(Kimura-Kuroda et *al*., 2012)**.

In contrast, Co-administration of Melissa *officinalis* extract (MOE) at 10 mg/kg/day showed partial neuroprotection against IMI-induced mitochondrial alterations, particularly by reducing carbohydrate accumulation and preventing excessive lipid depletion. However, this protective effect was not uniform across all brain regions. The hippocampus and striatum benefitted the most from MOE treatment, suggesting that these regions are more responsive to antioxidant interventions, possibly due to their higher baseline metabolic activity and oxidative stress vulnerability.

Despite the observed protective effects, mitochondrial protein, carbohydrate, and lipid levels in MOE-treated groups did not fully return to control values, suggesting that while MOE mitigates the biochemical alterations caused by IMI, it does not completely reverse mitochondrial dysfunction. This finding is in line with previous research indicating that while plant-based antioxidants can alleviate pesticide-induced oxidative stress, complete recovery often requires additional interventions **(Campos et *al*., 2016; Ranjbar et *al*., 2021).**

The antioxidant role of MOE, largely attributed to its vitamin C content, plays a crucial role in maintaining proteins, lipids, and enzymes in their functional state by reducing metal ions and scavenging free radicals **(Adimi, 2018)**. These findings are consistent with previous reports on oxidative stress protection, where MOE mitigated lipid degradation and β-oxidation disturbances induced by environmental toxins **(Radwan and Mohamed, 2013)**. While MOE exhibited strong neuroprotective potential, mitochondrial protein, carbohydrate, and lipid levels did not fully return to control values, suggesting that additional interventions may be required for complete restoration of mitochondrial integrity.

Overall, this study highlights that IMI-induced mitochondrial dysfunction is both dose-dependent and region-specific. While IMI D2 produced the most severe biochemical alterations, lipid depletion was evident even at lower doses, suggesting that different mitochondrial parameters respond to IMI toxicity through distinct mechanisms. The striatum

was the most affected brain region, followed by the hippocampus and whole brain, indicating that certain regions are more vulnerable to pesticide-induced mitochondrial stress. Melissa *officinalis* extract provided partial neuroprotection, particularly in the hippocampus and striatum, but was not sufficient to fully restore mitochondrial homeostasis.

5. Evaluation of redox status markers in brain mitochondria

Detrimental effects resulting from exposure to xenobiotics, including pesticides, have been associated with the induction of oxidative imbalance in both human and animal models **(Asghari et *al*., 2017; Wang et *al*., 2017).** Alterations in oxidative balance, characterized by an upsurge in reactive species, a decline in radical scavenging systems, or a combination of both, can compromise cellular integrity. Cellular antioxidant systems play a crucial role in mitigating oxidative stress and include non-enzymatic antioxidants like glutathione (GSH) and enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST), among others. Collectively, these antioxidants work to reduce the oxidative state within cells **(AYDIN, 2019).**

Our study **(Table 19)** shows that chronic exposure to Imidacloprid (IMI) at both 5 mg/kg/day (IMI D1) and 50 mg/kg/day (IMI D2) induced significant, dose-dependent reductions in mitochondrial antioxidant defenses and increased oxidative stress across different brain regions in *Wistar* rats, The depletion of GSH, GPX, SOD, CAT, and GST levels, coupled with elevated MDA, suggests that IMI disrupts mitochondrial redox homeostasis. A significant regional heterogeneity was observed in response to oxidative stress induced by IMI. The hippocampus exhibited the most pronounced disturbances, particularly a significant increase in MDA (5.10 ± 0.007 nmol/mg in IMI D2, $P < 0.001$), indicating exacerbated lipid peroxidation. This heightened vulnerability could be explained by the hippocampus's richness in phospholipid membranes, which are particularly susceptible to oxidation, as well as its high mitochondrial density, making this region more prone to alterations in redox metabolism **(Bhardwaj et *al*., 2010)**.

Although significantly affected by IMI, the striatum appeared more resilient compared to the hippocampus, showing slightly less pronounced reductions in GSH, SOD, CAT, and GST. This finding aligns with the results of **El-Morsy et *al.* (2022)**, who observed a differentiated regional response to oxidative stress induced by insecticides, suggesting an intrinsic variability in antioxidant capacity depending on brain regions. Similarly, **Saito et *al*. (2023)** demonstrated

region-specific susceptibility to neurotoxins, supporting our observation that hippocampal neurons are particularly sensitive.

IMI exposure likely induces its neurotoxic effects through the generation of reactive oxygen species (ROS), impairing mitochondrial function, and triggering neuronal apoptosis, as previously suggested by **Gibbons et *al.* (2015)**. Glutathione S-transferase (GST), a member of the phase II detoxification enzyme family, is critical in protecting cellular macromolecules from reactive electrophiles by catalyzing the conjugation of glutathione (GSH) to a wide range of endogenous and exogenous electrophilic chemicals **(Townsend and Tew, 2003).** GST activity is regulated by various parameters, including the type of xenobiotic, its concentration, duration of exposure, and species being studied **(Oruç and Üner, 2000).** Our biochemical analysis demonstrated a highly significant increase ($P \leq 0.01$) in GST enzymatic activity in the group treated with Imidacloprid at 50 mg/kg, aligning with studies that associate GST upregulation with cellular stress responses **(Di Monte et *al*., 1992; Mikolić et *al*., 2018; Lonare et *al*., 1999)**, the decline in GST activity highlights a compromised detoxification pathway, which may increase cellular susceptibility to electrophilic compounds and lipid peroxidation byproducts.

Our findings also highlight a substantial and dose-dependent reduction in catalase (CAT) activity, suggesting an indirect elevation of hydrogen peroxide (H_2O_2) levels in both mitochondria and cytosol. Catalase, a key antioxidant enzyme responsible for decomposing hydrogen peroxide into water and oxygen, showed a marked decline in IMI-exposed groups, particularly in the hippocampus. This reduction indicates an accumulation of hydrogen peroxide, further exacerbating oxidative stress and neuronal damage. The decline in GPx and CAT activities results in an accumulation of H_2O_2, generating hydroxyl radicals (OH°) via the Fenton reaction **(Cory-Slechta et *al*., 2005)**. While some studies report an increase in CAT and GPx activity following neonicotinoid exposure **(Kati´c et *al*., 2021)**, our findings reveal a significant decrease in GPx activity in treated rats, likely due to depletion of cellular antioxidant defenses against ROS. Additionally, Imidacloprid metabolism via cytochrome P450 produces substantial ROS concentrations, possibly exceeding the neutralization capacity of endogenous antioxidant enzymes, thereby confirming oxidative stress induction.

Additionally, MDA serves as the primary compound formed during oxidative degradation of polyunsaturated fatty acids in cell membranes, generating toxic aldehydes harmful to the organism **(Maiza et *al*., 2011).** Our results indicate a highly significant increase ($p \leq 0.001$) in

MDA levels in IMI 50-treated rats, suggesting lipid peroxidation and oxidative damage, which aligns with findings by **Ahmed et *al.* (2021)** and **Kapoor et *al.* (2010).** This elevated lipid peroxidation compromises the integrity of nerve cell membranes, disrupts mitochondrial function, and may lead to cell death **(Di Mauro et *al.*, 2006; Carole and Harvé, 2011)**. Increased MDA levels indicate lipid peroxidation, a hallmark of oxidative stress-mediated neuronal damage. The regional variation in susceptibility could be attributed to differences in mitochondrial density, enzymatic activity, and neuroanatomical function, with the hippocampus being more prone to oxidative damage due to its high metabolic demand and synaptic plasticity.

The correlation analysis between biochemical parameters reveals a strong inverse relationship between MDA and GSH levels ($r = -0.85$, $P < 0.001$), suggesting that GSH consumption is directly proportional to oxidative damage intensity. Similarly, the negative correlation between MDA and enzymatic antioxidant activities (SOD, CAT, GST) ($r = -0.78$, $P < 0.001$) indicates that the reduction in enzymatic antioxidant capacity amplifies lipid damage.

However, Co-administration of Melissa *officinalis* extract (MOE) significantly restored antioxidant enzyme activities (including CAT and GST, GSH, GPX, SOD,) and reduced lipid peroxidation MDA ($P < 0.001$) across all brain regions, highlighting its neuroprotective potential. The bioactive compounds in MOE, particularly its high phenolic and flavonoid content, likely contribute to its antioxidative effects by scavenging free radicals, enhancing mitochondrial defense mechanisms, and restoring enzymatic detoxification pathways. These findings reinforce the dose-effect relationship of IMI toxicity and highlight MOE's capacity to counteract oxidative damage, consistent with studies on plant-derived antioxidants mitigating pesticide-induced neurotoxicity **(Albasher et *al.*, 2020).**The regional variation in response suggests that brain structures differ in their susceptibility to oxidative damage, likely due to differences in mitochondrial density, enzymatic activity, and neuroanatomical function. Further research is needed to elucidate the precise molecular mechanisms underlying these protective effects. These effects align with studies by **Zarifkar et *al.* (2014),** who reported improvements in antioxidant markers and a decrease in cerebral oxidative stress following treatment with polyphenol-rich extracts, such as those of Melissa *officinalis*.

Our findings confirm that chronic exposure to Imidacloprid induces significant mitochondrial oxidative stress in different brain regions, with a clear dose-effect relationship and increased susceptibility of the hippocampus. The interactions between various redox

markers suggest a neurotoxic mechanism involving impaired antioxidant defenses and increased oxidative lipid damage. The administration of Melissa *officinalis* effectively attenuated these disturbances, suggesting its therapeutic potential against neonicotinoid-induced neurotoxicity.

6. Mitochondrial apoptosis parameter

Mitochondria, essential cellular organelles, play a crucial role in energy production by generating adenosine triphosphate (ATP) through oxidative phosphorylation. Their involvement in various cellular processes underscores their significance, and any disruption to their functions can have profound consequences. Notably, mitochondrial damage is associated with a decline in ATP production, emphasizing their pivotal role in maintaining cellular energy balance **(Cassarino and Bennett, 1999).**

The present study demonstrates a clear dose-dependent effect of IMI on mitochondrial function in different brain regions of *Wistar* rats, with higher doses (50 mg/kg/day) inducing significantly greater neurotoxic effects than lower doses (5 mg/kg/day). The observed mitochondrial swelling, decreased oxygen consumption, and increased permeability confirm the dose-dependent mitochondrial dysfunction induced by IMI. These findings align with previous studies, such as those by **Miao et *al.* (2012**), which reported a dose-dependent increase in oxidative stress and mitochondrial impairment following pesticide exposure. Similarly, previous research on neonicotinoid toxicity has shown that chronic exposure leads to neuronal apoptosis and neurodegeneration, with higher doses exacerbating oxidative stress and mitochondrial damage **(Gibbons et *al.*, 2015).**

Mitochondrial swelling, an indicator of membrane integrity disruption, significantly increased under IMI exposure. IMI D1 (5 mg/kg/day) led to a moderate increase ($P \leq 0.05$), whereas IMI D2 (50 mg/kg/day) caused a highly significant increase ($P \leq 0.001$), reflecting more severe mitochondrial alterations. Simultaneously, mitochondrial oxygen consumption was significantly reduced depending on IMI dose. This decrease suggests impairment in the electron transport chain, likely due to increased oxidative stress, as observed in previous studies on environmental neurotoxins **(You et *al.*, 2023).**

A significant aspect of this study is the differential impact of IMI on various brain regions. The hippocampus exhibited the highest susceptibility to IMI-induced mitochondrial

dysfunction, followed by the striatum, while the whole brain showed comparatively less damage. Mitochondrial swelling was significantly higher in the hippocampus under IMI D2 **(**Δ+77.5% vs. control, $P \leq 0.001$), whereas in the striatum, the increase was more moderate (Δ+70.7%, $P \leq 0.001$).This regional variability may be attributed to differences in mitochondrial density, antioxidant defense mechanisms, and metabolic activity. The hippocampus, being highly active in energy metabolism and synaptic plasticity, is particularly vulnerable to oxidative stress and mitochondrial dysfunction **(Gandhi and Abramov, 2012).** This aligns with **Milatovic et *al.* (2011),** who demonstrated that neurotoxic agents disproportionately affect the hippocampus, leading to cognitive and behavioral impairments.

The striatum, although affected, displayed relatively greater resilience compared to the hippocampus. This could be due to its distinct neurochemical environment and compensatory mechanisms that may buffer oxidative damage to some extent. Previous studies have also reported regional variability in mitochondrial response to toxins, with the hippocampus being more affected due to its higher glutamatergic activity and oxidative metabolism **(Gómez et *al.*, 2007).**

Mitochondrial alterations induced by IMI may be linked to oxidative stress induction and disruption of mitochondrial membrane potential. IMI is known to generate excessive production of reactive oxygen species (ROS), leading to lipid membrane oxidation, activation of mitochondrial permeability, and ultimately, apoptosis via cytochrome c release and caspase-3 activation **(Ge et *al.*, 2015).**

IMI-induced mitochondrial swelling suggests alterations in mitochondrial membrane integrity, leading to increased permeability transition pore (mPTP) opening. This phenomenon is associated with calcium dysregulation, loss of membrane potential, and initiation of apoptotic cascades **(Kroemer et *al.*, 2007).** The observed decline in oxygen consumption across all brain regions further confirms mitochondrial respiratory chain impairment and disrupt the electron transport cycle, which is a hallmark of neurotoxicity induced by environmental toxins **(Duarte-Hospital et *al.*, 2022).** Furthermore, the increased mitochondrial permeability suggests that IMI disrupts calcium homeostasis, leading to mitochondrial depolarization and cytochrome c release, ultimately triggering apoptosis. This aligns with previous findings on pesticide-induced neurotoxicity, where mitochondrial permeability transition was implicated in neuronal cell death **(Anadón et *al.*, 2020).**

Correlation analysis between different parameters revealed a strong relationship between mitochondrial swelling and membrane permeability ($r = 0.85$, $P \leq 0.001$), suggesting that increased swelling is an early marker of mitochondrial membrane integrity loss.

Furthermore, the decrease in mitochondrial respiration was strongly correlated with increased swelling (Δ -30% O_2 consumption; Δ+70% swelling, $r = -0.88$, $P \leq 0.001$), supporting the hypothesis of energy uncoupling and respiratory chain dysfunction under toxic stress.

The strong correlation between mitochondrial swelling, decreased oxygen consumption, and increased permeability highlights the interdependence of mitochondrial functions. The loss of mitochondrial membrane potential due to swelling disrupts ATP synthesis, leading to impaired respiration and increased permeability transition. This cascade ultimately results in neuronal apoptosis, further exacerbating neurotoxicity. The hippocampus showed the most significant changes across all mitochondrial parameters, reinforcing its vulnerability to IMI-induced damage. The protective effects of MOE were also most pronounced in the hippocampus, suggesting a potential region-specific efficacy of antioxidant therapy.

Our findings align with previous research on neonicotinoid-induced neurotoxicity. Studies have shown that chronic exposure to IMI leads to oxidative stress, mitochondrial dysfunction, and cognitive deficits in rodents **(El Din et *al.*, 2023).** The structural integrity of the mitochondria is severely impaired as a result of these processes, leading in mitochondrial dysfunction. As established in the research conducted to **(Gasmi et *al*., 2017),** this malfunction is accompanied by a decrease in the rate of oxygen consumption.

Furthermore, scientific studies have demonstrated that exposure to Imidacloprid can induce mitochondrial dysfunction in mammalian cells, particularly in the forebrain region of the brain. Additionally, this exposure has been linked to the triggering of apoptosis, or programmed cell death, in forebrain cells. As described in **(Bal et *al.*, 2012).** Alterations in mitochondrial complexes can lead to the generation of free radicals. This process results in the depolarization of the mitochondrial membrane, activating voltage-dependent receptors, facilitating the influx of calcium into the cell, and initiating cell death pathways. Over time, the cumulative effect of these processes may lead to the development of neurodegenerative diseases and cognitive deficits **(Trushina and Murray, 2007**).

Studies have demonstrated that treatment with Neonicotinoid (NN) pesticides results in an elevation of mitochondrial membrane permeability **(Gasmi, 2018; Annabi et *al*., 2019).** Furthermore, when bats were exposed to IMI (Imidacloprid), there was a considerable increase in neuronal apoptosis in the hippocampus CA1 area and the medial entorhinal cortex **(Wu et *al*., 2020).** This apoptotic pathway is begun by an increase in the permeability of the mitochondrial outer membrane, which results in the release of numerous proteins, including cytochrome C, in the cytoplasm**. (Wang and Youle, 2009)**

The dose-dependent and region-specific effects observed in this study further corroborate earlier findings, demonstrating that higher IMI doses exacerbate mitochondrial impairment, particularly in the hippocampus.

The process of mitochondrial swelling, often attributed to calcium overload, is widely acknowledged as a precursor to the generation of nitric oxide (NO) and the activation of xanthine oxidase. This activation contributes to the production of superoxide anion, which subsequently inactivates NADH dehydrogenase, NADH oxidase, and ATPase. Ultimately, this cascade of events results in mitochondrial oxidative stress **(Zhang and Darley**, **2000**)**.** The escalation in mitochondrial swelling is facilitated by the disruption of voltage-dependent giant pores influenced by reactive oxygen species (ROS). This disruption causes a substantial influx of water, as well as Ca^{2+} and Na^{+} ions, through the mitochondrial membranes **(Henine et *al*., 2016).**

Moreover, the protective role of plant-based antioxidants against pesticide-induced neurotoxicity has been widely documented. Studies on resveratrol and curcumin have shown similar neuroprotective effects through their antioxidant and anti-apoptotic properties **(Zaky et *al*., 2017).** The present study expands on this by demonstrating the efficacy of MOE in mitigating IMI-induced mitochondrial dysfunction, adding to the growing body of evidence supporting plant-derived neuroprotectants. Melissa extract not only enhances acetylcholine production but also aids mitochondria in energy production, preventing oxidative damage. It contributes to increased brain oxygen consumption and promotes dopamine synthesis. In summary, the extract demonstrates remarkable virtues, extending to the repair of damaged neurons **(Mona et *al*., 2014; Gao et *al*., 2014).**

Co-treatment with Melissa *officinalis* extract (MOE) significantly mitigated IMI-induced mitochondrial dysfunction, as evidenced by reduced mitochondrial swelling, restored oxygen

consumption, and stabilization of membrane permeability. Mitochondrial swelling was significantly reduced in the IMI + MOE groups compared to IMI-treated groups alone ($P \leq 0.05$ and $P \leq 0.01$). Similarly, mitochondrial respiration improved under MOE treatment, particularly in the hippocampus (+25.6%, $P \leq 0.001$).These protective effects can be attributed to MOE's rich content of polyphenols and flavonoids, which exhibit strong antioxidant and anti-inflammatory properties **(Elmorsy et *al.*, 2022).** MOE's ability to scavenge reactive oxygen species (ROS) likely plays a crucial role in preserving mitochondrial integrity and function.

Previous studies have reported similar protective effects of plant-derived antioxidants against pesticide-induced neurotoxicity. For instance, quercetin, a flavonoid found in various medicinal plants, has been shown to prevent mitochondrial dysfunction by enhancing antioxidant defenses and modulating apoptotic pathways **(Albasher et *al.*, 2020)**. The neuroprotective potential of MOE observed in this study is consistent with these findings, further supporting its role as a therapeutic agent against pesticide-induced neurodegeneration.

7. Assessment of brain lysosomal stability by determination of neutral red retention time (NRRT)

Lysosomal membrane stability is highly sensitive to various environmental stressors in a dose-dependent manner, making it a valuable indicator for assessing both the type and intensity of stress. Neutral red, a cationic and lipophilic dye, is widely used as a probe due to its ability to diffuse through cell membranes **(Lowe et *al.*, 1992)**. Exposure to stressors can lead to reduced lysosomal membrane fluidity **(Myers et *al.*, 1991)**. Furthermore, a decline in lysosomal stability is often accompanied by morphological alterations, including an increase in lysosomal size and the fusion of organelles **(Moore et *al.*, 1987; Lowe et *al.*, 1981)**.

The assessment of cerebral lysosomal stability using the Neutral Red Retention Time (NRRT) assay **(Table 20 ; Figure 53)** revealed significant alterations among the experimental groups, highlighting the neurotoxic effects of Imidacloprid (IMI) and the protective potential of Melissa *officinalis* extract (MOE). Absorbance values measured at 540 nm were used to monitor lysosomal integrity at 24 h and 48 h post-exposure. In the control group, lysosomal stability remained constant, providing a baseline reference. In contrast, exposure to IMI at 5 mg/kg (IMI D1) induced a slight increase in absorbance at 24 h, indicating increased lysosomal activity, followed by a marked decrease at 48 h, suggesting lysosomal destabilization and the onset of cell death. A higher dose of IMI (50 mg/kg, IMI D2) resulted in a more pronounced

increase in absorbance at 24 h, followed by a sharp decline at 48 h, indicative of severe lysosomal impairment and massive apoptosis. These results align with findings by **Topal et *al.*, (2017),** who demonstrated that IMI induces significant oxidative stress and disruption of cellular membrane structures, promoting lysosomal permeability and the release of hydrolytic enzymes.

Conversely, the administration of MOE alone (10 mg/kg) maintained absorbance values comparable to the control group, demonstrating no cytotoxic effect on cerebral lysosomes. The combination of MOE with IMI D1 initially showed values similar to the IMI D1 group alone, but significant protection was observed at 48 h, where absorbance remained higher, indicating reduced lysosomal destabilization. The protective effect of MOE was even more pronounced in the IMI D2 + MOE group, where the absorbance drop observed with IMI D2 alone was attenuated, suggesting that MOE mitigates lysosomal damage and reduces the neurotoxicity of high-dose IMI. These findings are consistent with those of **Dastmalchi et *al.*, (2009),** who demonstrated the protective impact of natural antioxidants on cerebral cellular structures by limiting membrane lipid oxidation and maintaining intracellular homeostasis.

Microscopic observations corroborated these findings **(Figures 54, 55, 56, 57 and 58)** revealing morphological alterations in cells exposed to IMI, including a "rounding-up" phenomenon and leakage of the neutral red dye into the cytoplasm, indicating membrane rupture. Time-dependent analysis confirmed these trends, highlighting a progressive loss of lysosomal integrity over time following IMI exposure. A similar study by **Topal et *al.*, (2017)** demonstrated that chronic exposure to neonicotinoids leads to increased lysosomal membrane permeabilization, facilitating the release of hydrolytic enzymes and thus accelerating the apoptotic process in neurons.

Furthermore, the analysis of lysosomal pH variations **(Figures 59 (A, B))** showed significant alkalinization in IMI-treated groups, more pronounced at the 50 mg/kg dose, indicating severe lysosomal dysfunction. In contrast, lysosomal pH remained stable in the MOE group, while in IMI + MOE groups, a reduction in alkalinization was observed, confirming the protective role of Melissa *officinalis* against IMI-induced lysosomal homeostasis disruption. These observations are supported by the work of **Dastmalchi et *al.*, (2009),** who demonstrated that polyphenol-rich extracts, such as Melissa *officinalis*, stabilize lysosomal membranes by reducing oxidative stress and modulating ion pump activity responsible for intracellular pH regulation.

These results suggest that IMI induces dose-dependent lysosomal toxicity in the brain, leading to membrane instability and cellular apoptosis, while Melissa *officinalis* extract exerts a neuroprotective effect by preserving lysosomal integrity and limiting cellular damage. Overall, these findings highlight the potential role of Melissa *officinalis* as a neuroprotective agent against neonicotinoid-induced neurotoxicity and pave the way for new therapeutic strategies targeting lysosomal stability and oxidative stress modulation in neurodegenerative disorders.

8. Histopathological examinations

The histopathological findings from our study provide critical insights into the neurotoxic effects of Imidacloprid (IMI) and the potential neuroprotective role of Melissa *officinalis* extract (MOE) in *Wistar* rats. As observed in **Table 21** and **Figure 60**, prolonged exposure to IMI induces dose-dependent neuronal damage, with severe neuronal damage observed at higher doses. While co-administration with MOE demonstrates a mitigating effect on neurotoxicity.

The control group exhibited normal neuronal architecture with no evidence of degeneration or inflammation, confirming the absence of endogenous neurotoxicity under standard conditions. However, rats exposed to low-dose IMI (IMI D1, 5 mg/kg/day) exhibited significant disorganization of nerve fibers, neuronal loss, vacuolization, and mild glial cell infiltration, indicating early signs of neurotoxicity and inflammation. These observations align with previous studies demonstrating the neurotoxic potential of IMI through oxidative stress, mitochondrial dysfunction, and disruption of neuronal integrity. These effects worsened at The high-dose IMI group (IMI D2, 50 mg/kg/day), where severe histopathological alterations such as early-stage cerebral necrosis, edematous changes in the brain parenchyma, and localized neuronal death were observed. The observation of "regular port spaces" suggests the presence of dilated perivascular spaces or mild edema, indicative of neuroinflammation and toxic-induced damage. The presence of “minimal foci of cerebral necrosis” suggests that oxidative stress, mitochondrial dysfunction, or excitotoxicity via overstimulation of nicotinic acetylcholine receptors contributed to neuronal apoptosis, as previously reported in pesticide-induced neurotoxicity studies **(Bal et *al.*, 2012; Duzguner and Erdogan, 2010).**

These findings are consistent with previous research indicating that prolonged exposure to neonicotinoids, particularly IMI, triggers oxidative stress, mitochondrial dysfunction, and neuroinflammation. **Duzguner and Erdogan (2010)** demonstrated that IMI exposure elevates

lipid peroxidation levels while reducing antioxidant enzyme activity, highlighting oxidative stress as a critical mechanism underlying its neurotoxic effects. Similarly, a 90-day oral toxicity study on female rats administered IMI at doses of 5, 10, and 20 mg/kg body weight per day revealed necrosis of Purkinje cells, dendritic loss, and granule depletion in the cerebellar granular layer compared to controls **(Bhardwaj et *al.*, 2010**). Furthermore, evidence suggests that neonicotinoids contribute to neurodevelopmental toxicity, as reflected in the abnormal microglial profiles observed in the hippocampus of neonatal mice **(Nakayama et *al.*, 2019).**

Similarly, **Bal et *al,* (2012)** demonstrated that pesticide exposure induces oxidative stress and neuronal damage, particularly in the hippocampus and cerebral cortex, supporting the idea that neuroinflammation plays a critical role in neurotoxicity. Similarly, our study found that high doses of Imidacloprid (IMI) resulted in significant histopathological damage, including early signs of cerebral necrosis (D2c) and edematous remodeling of brain tissue (D2 MC), reinforcing the link between oxidative stress and pesticide-induced neuronal injury.

In contrast, treatment with Melissa *officinalis* extract (MOE) at 10 mg/kg/day demonstrated neuroprotective properties. Histopathological analysis of rats treated with MOE alone revealed a regular cerebral parenchyma without signs of degeneration or inflammation, indicating the absence of cytotoxic effects. Furthermore, when co-administered with IMI, Melissa *officinalis* significantly reduced neurotoxicity. In the IMI D1 + MOE group, less vacuolization and glial infiltration were observed, demonstrating a moderate neuroprotective effect. The IMI D2 + MOE group also showed partial neuroprotection, with reduced tissue necrosis and edema compared to the IMI D2 group. The observation of “MD1C parenchyme cérébral régulier” in co-treated groups further supports the hypothesis that Melissa *officinalis* preserves neuronal integrity and mitigates IMI-induced damage. These findings are in agreement with previous research showing that Melissa *officinalis* has strong antioxidant and anti-inflammatory properties, helping to neutralize reactive oxygen species (ROS) and protect against neuronal apoptosis **(Zeraatpishe et *al*., 2011; Ghasemi et *al*., 2014)**.

These findings are consistent with previous studies that have reported the neuroprotective potential of Melissa *officinalis* in experimental models of neurodegeneration. The observed effects may be attributed to the plant's rich composition of polyphenols, flavonoids, and terpenoids, which possess strong antioxidant properties, capable of scavenging free radicals and reducing oxidative stress-induced neuronal damage. Furthermore, the anti-inflammatory effects

of MOE likely contribute to its protective role by modulating microglial activation and reducing inflammatory cytokine production.

Overall, the results emphasize the neurotoxic potential of IMI and the importance of regulating its use to prevent brain damage, particularly in agricultural and residential environments. The observed neurotoxicity raises concerns about the long-term impact of neonicotinoid exposure on brain health, including potential implications for neurodegenerative disorders. Additionally, the findings highlight the therapeutic potential of Melissa *officinalis* as a neuroprotective agent, suggesting its possible application in strategies aimed at reducing pesticide-induced neurotoxicity.

9. Determination of brain neurotransmitter levels and brain AchE activity

The cholinergic neurotransmitter system plays a crucial role in regulating cognitive functions such as learning, memory, and attention, and its impairment has been associated with the onset of various neurodegenerative disorders **(Maurer and Williams, 2017; Hampel, 2018).**

Neonicotinoids, synthetic derivatives of nicotine, are designed to target nicotinic acetylcholine receptors (nAChRs) in insects **(Tomizawa and Casida, 2003).** However, their interaction with these receptors in mammals can lead to disruptions in the cholinergic system, particularly by altering acetylcholinesterase (AChE) activity. The findings from **Table 22** and **Figures 61** indicate that chronic exposure to Imidacloprid (IMI) significantly affects AChE activity across different brain regions, which may contribute to cognitive dysfunction. Interestingly, the administration of Melissa *officinalis* extract (MOE) appears to modulate these effects, suggesting its potential role in counteracting neonicotinoid-induced neurotoxicity and preserving cognitive function.

The findings on AChE activity provide compelling evidence of the dose-dependent neurotoxic effects of IMI, which appears to significantly disrupt key neurotransmitter functions. Higher IMI doses (IMI D2, 50 mg/kg/day) lead to a very highly significant decrease ($p \leq 0.001$) in AChE activity across the total brain, hippocampus, and striatum, while lower doses (IMI D1, 5 mg/kg/day) also result in highly significant reductions ($p \leq 0.01$). Notably, the striatum's heightened sensitivity to AChE inhibition observed in our study is noteworthy at higher doses, which aligns with this region's critical role in motor coordination and its impairment could lead to motor deficits and behavioral disturbances, potentially indicating a susceptibility of motor

functions to IMI-induced toxicity.Similar findings have been reported in studies examining the effects of other neurotoxicants on the striatum, highlighting its vulnerability to chemical exposures that disrupt cholinergic signaling.

These findings are consistent with previous research indicating that neonicotinoids, including IMI, substantially disrupt cholinergic neurotransmission by inhibiting AChE activity. This inhibition impairs the breakdown of acetylcholine, leading to excessive neural excitation and increased oxidative stress, which are key factors in neurotoxicity **(Duzguner and Erdogan, 2010)**.

Comparable studies, such as those by **Slotkin et *al*., (2017),** have demonstrated that prolonged exposure to neonicotinoids in animal models disrupts neurochemical pathways, impacting both cognitive and motor functions. This aligns with the significant AChE reduction observed in the striatum in our study, likely resulting from oxidative damage and impaired neurotransmission in regions critical for movement and coordination. Furthermore, research indicates that Imidacloprid (IMI) at 20 mg/kg/day inhibits AChE activity in *Wistar* rats, leading to acetylcholine accumulation, excessive nAChR activation, and increased ion influx **(Bhardwaj et *al*., 2010; Banerjee et *al*., 2014; Kapoor et *al*., 2014; Vohra et *al*., 2014).** IMI also acts as a partial nAChR agonist, competing with acetylcholine **(Houchat et *al*., 2020; Park et *al*., 2021),** and binds to both high- and low-affinity sites on nAChRs **(Nagata et *al*., 1998).** Its acute toxicity, which manifests as tremors at high doses, has led to the establishment of a reference dose of 125 mg/kg/day for rodents **(Sheets, 2010).**

In contrast, the co-administration of MOE with IMI exhibits a protective effect, particularly evident at the lower IMI dose (IMI D1 + MOE), where AChE activity in the brain and hippocampus returns to non-significant levels relative to the control, indicating that MOE aids in restoring normal enzyme function in these cognitive regions. This protective effect is likely due to MOE's well-established antioxidant properties, as previous research on Melissa *officinalis* has highlighted its rich polyphenolic content, which effectively mitigates oxidative damage and neuroinflammation, thereby preserving neural function **(Soodi et *al*., 2014)**. However, the neuroprotective impact of MOE appears less pronounced in the striatum, as evidenced by significantly ($p \leq 0.05$) reduced AChE levels at IMI D1 and highly significant reductions ($p \leq 0.01$) at IMI D2, these results imply that MOE's antioxidant potential may not entirely counteract the neurotoxic effects of high-dose IMI in motor-related regions, potentially due to regional differences in MOE's efficacy or the severity of IMI-induced oxidative stress.

Overall, this study highlights the potential of MOE as a neuroprotective agent against pesticide-induced neurotoxicity, particularly in cognitive regions, while also enhancing acetylcholine production **(Mona et *al.*, 2014; Gao et *al.*, 2014)**. However, further investigations are needed to clarify the variability in regional variability protection, the precise antioxidant mechanisms involved, and how dosing adjustments might optimize MOE's efficacy in vulnerable brain areas, such as the striatum.

General Discussion

Neonicotinoids, particularly Imidacloprid (IMI), have been extensively studied for their neurotoxic effects, with accumulating evidence highlighting their impact on mitochondrial function, oxidative stress, and neurotransmission in mammals **(Tomizawa and Casida, 2005; Bhardwaj et *al.*, 2010).**

The present study investigated the neurotoxic effects of chronic Imidacloprid (IMI) exposure in *Wistar* rats while evaluating the neuroprotective role of Melissa *officinalis* L. (lemon balm) extract (MOE). The findings reveal a dose-dependent impact of IMI, with significant regional variations in toxicity, and provide compelling insights into the phytochemical properties of MOE and its ability to mitigate IMI-induced neurotoxicity. Additionally, the study elucidates the underlying protective mechanisms of MOE, highlighting its antioxidant potential. By integrating phytochemical characterization (Part One) with in vivo neurotoxicity assessments (Part Two), this discussion establishes a holistic understanding of how MOE counteracts IMI-induced toxicity.

The first part of our study focused on characterizing the aqueous extract of Melissa *officinalis* L., a widely used medicinal plant in Algeria, to identify its active constituents responsible for its therapeutic properties. The phytochemical screening confirmed that the extract is rich in polyphenols, flavonoids, and condensed tannins, which are known for their potent antioxidant activity **(Pourmorad et *al.*, 2006; Asadi et *al.*, 2010).** The results from antioxidant assays (DPPH, ABTS, CUPRAC, FRAP) demonstrated that lemon balm possesses significant free radical scavenging potential, comparable to synthetic antioxidants but with the added benefit of being a natural alternative with minimal toxicity risks **(Zheng and Wang, 2001).**

The pharmacological relevance of lemon balm extends beyond its antioxidant capacity, as its bioactive compounds have demonstrated neuroprotective and anti-inflammatory effects in previous studies **(Kennedy et *al.*, 2004; Miliauskas et *al.*, 2004).** These findings support the hypothesis that Melissa *officinalis* extract could serve as a therapeutic candidate against neurodegenerative conditions caused by oxidative stress and mitochondrial dysfunction.

Furthermore, the variations in bioactive compound yields observed in this study may be attributed to pedoclimatic factors and extraction techniques. A detailed structural analysis using X-ray diffraction (XRD), infrared spectroscopy (IR), and scanning electron microscopy (SEM) confirmed the presence of diverse bioactive molecules with potential pharmacological

applications. This in-depth characterization sets the foundation for in vivo applications, particularly in the context of neurotoxicity prevention.

The second part of this study evaluated the impact of chronic IMI exposure at two doses (5 mg/kg/day and 50 mg/kg/day) on various neurotoxic parameters in *Wistar* rats and assessed the protective potential of Melissa *officinalis* extract (10 mg/kg/day).

Our results confirm that IMI induces neurotoxicity in a dose-dependent manner, with higher doses (50 mg/kg/day) causing more pronounced damage than lower doses (5 mg/kg/day). The neurotoxic effects were particularly evident in mitochondrial function, as indicated by mitochondrial swelling, decreased oxygen consumption, and increased permeability. The depletion of mitochondrial antioxidants (GSH, GPX, SOD, CAT, and GST), disruptions in growth parameters, metabolic disturbances in protein, carbohydrate, and lipid levels, altered neurotransmitter levels, impaired lysosomal stability, and significant neurobehavioral and histopathological alterations. However, the severity of toxicity varied across different brain regions.

Notably, the striatum exhibited heightened sensitivity to IMI, particularly at higher doses, leading to substantial reductions in antioxidant defenses. This finding aligns with the striatum's critical role in motor coordination, suggesting that IMI toxicity may contribute to motor impairments. Similarly, the hippocampus, essential for learning and memory, showed significant inhibition, though MOE exerted a stronger protective effect in this region compared to the striatum.

Overall, the total brain antioxidant status was significantly compromised in a dose-dependent manner, reinforcing the link between oxidative stress and IMI-induced neurotoxicity.

1. Neurotoxicity Assessments of Imidacloprid

Oxidative stress is a key toxicological mechanism associated with neonicotinoid exposure, affecting various animal species **(Wang et *al*., 2018).**

Our study observed dose-dependent neurotoxic effects of chronic Imidacloprid (IMI) exposure, with higher doses (50 mg/kg/day) causing more severe symptoms such as lethargy, tremors, significant weight loss, and occasional seizures, consistent with previous research on pesticide toxicity **(Duzguner and Erdogan, 2010; Bal et *al*., 2012).** These symptoms are likely linked to IMI's impact on cholinergic neurotransmission and mitochondrial function. In

contrast, rats exposed to the lower dose (5 mg/kg/day) exhibited milder symptoms, such as reduced grooming, highlighting the dose-dependent nature of these effects. Despite the neurotoxic symptoms, no mortality was observed, indicating that while these doses cause neurotoxicity, they do not reach lethal levels.

Additionally, IMI exposure led to significant reductions in body weight, weight gain, and relative brain weight, with the higher dose causing the most pronounced effects. These reductions were attributed to oxidative stress and other chemical mediators, such as pro-inflammatory cytokines, which are consistent with findings in previous studies **(Carole and Harvé, 2011)**. A two-generation reproductive toxicity study also supported these results, showing similar effects on body weight in maternal rats and pups **(Gervais et *al.*, 2010)**.

Our study confirms that IMI exposure induces neurotoxicity in a dose-dependent manner, with the higher dose (50 mg/kg/day) causing more significant damage than the lower dose (5 mg/kg/day). This is reflected in the depletion of key mitochondrial antioxidants, including GSH, GPX, SOD, CAT, and GST, as well as notable disruptions in oxidative stress markers within the striatum and hippocampus. Similar findings were reported by **Kapoor et *al.* (2010)**, where subchronic dermal exposure to Imidacloprid in female rats resulted in reduced SOD and CAT activity along with increased lipid peroxidation. Additionally, **Ince et *al*. (2013)** demonstrated that oral administration of 15 mg/kg/day of Imidacloprid over 28 days significantly elevated MDA levels while reducing SOD and CAT activity in mice. Conversely, **EL-Gendy et *al*. (2010)** found that in male mice, the same dose of Imidacloprid led to a notable increase in antioxidant enzyme activity, including CAT, SOD, glutathione peroxidase, and GST. The increase in MDA levels suggests severe mitochondrial redox imbalance **(Kapoor et *al*., 2014; Slotkin et *al*., 2017).** These findings are consistent with previous research indicating that IMI exposure enhances oxidative stress by elevating reactive oxygen species (ROS) production and impairing mitochondrial integrity **(Vohra et *al*., 2014).**

The evaluation of mitochondrial metabolic parameters in this study revealed a dose-dependent disruption in protein, carbohydrate, and lipid levels following chronic exposure to Imidacloprid (IMI), with distinct variations across brain regions. The striatum displayed the highest susceptibility to IMI toxicity, followed by the hippocampus and whole brain, highlighting their differing metabolic demands and sensitivity to oxidative stress. Biochemical analyses showed a significant increase in mitochondrial protein and carbohydrate levels at both low (5 mg/kg/day) and high (50 mg/kg/day) IMI doses, with the higher dose exerting a more pronounced effect. This elevation suggests a compensatory mechanism, possibly linked to

mitochondrial biogenesis, in response to oxidative stress, as reported in previous studies **(Bal et *al.*, 2012; Gibbons et *al.*, 2015).** In contrast, lipid depletion occurred even at the lower dose, indicating that lipid peroxidation and mitochondrial membrane disruption can arise at minimal pesticide exposure levels. This suggests that lipid depletion may serve as an early biomarker of mitochondrial instability, leading to impaired β-oxidation and oxidative damage, irrespective of dose escalation **(Tomizawa and Casida, 2005; Campos et *al.*, 2016).** These findings emphasize the critical role of mitochondrial metabolism in pesticide-induced neurotoxicity and the potential for early metabolic alterations to signal broader mitochondrial dysfunction.

Our findings indicate that IMI exposure impairs mitochondrial function by reducing oxygen consumption, increasing swelling, and altering membrane permeability, ultimately leading to mitochondrial dysfunction **(Cestonaro et *al.*, 2023).** These results highlight mitochondria as primary targets of IMI-induced cytotoxicity. Previous studies have shown that Imidacloprid exposure induces mitochondrial dysfunction in mammalian cells, particularly in the forebrain, and is associated with apoptosis in forebrain neurons **(Lin and Beal, 2006).** Disruptions in mitochondrial complexes contribute to free radical generation, leading to mitochondrial membrane depolarization, activation of voltage-dependent receptors, calcium influx, and initiation of apoptotic pathways. The cumulative effect of these processes over time may increase the risk of neurodegenerative diseases and cognitive deficits **(Trushina and Murray, 2007)**.

Another major finding of this study is the significant inhibition of AChE activity, particularly in the hippocampus and striatum, following IMI exposure. Higher IMI doses (50 mg/kg/day) resulted in a highly significant ($p \leq 0.001$) reduction in AChE activity, whereas lower doses (5 mg/kg/day) also caused significant inhibition ($p \leq 0.01$). These findings align with previous studies demonstrating that IMI disrupts cholinergic neurotransmission by acting as a partial nicotinic acetylcholine receptor (nAChR) agonist, leading to excessive ion influx and neuronal overstimulation **(Banerjee et *al.*, 2014; Park et *al.*, 2021).** These findings align with previous research demonstrating that neonicotinoids impair cholinergic signaling and oxidative balance **(Duzguner and Erdogan, 2010)**.

Interestingly, the striatum exhibited heightened sensitivity to AChE inhibition, which may contribute to motor deficits and behavioral alterations observed in IMI-exposed rats **(Nagata et *al.*, 1998)**. This increased susceptibility suggests that IMI neurotoxicity preferentially affects brain regions associated with movement coordination and executive function.

Behavioral assessments revealed significant learning and memory impairments, which correlate with IMI-induced cholinergic disruption. Demonstrated that chronic exposure to imidacloprid (IMI) at both 5 mg/kg/day and 50 mg/kg/day over 90 days led to significant cognitive, emotional, and social impairments in rats, with the higher dose exacerbating these effects. Exploratory behavior declined by 65% in the open field test ($p < 0.01$), aligning with previous studies linking neonicotinoids to anxiety-like behaviors and cognitive dysfunction **(Gasmi et *al.*, 2017)**. Similarly, cognitive performance in the novel object recognition test dropped by 45% ($p < 0.05$), reinforcing the cognitive deficits associated with pesticide exposure. IMI also impaired spatial memory, as evidenced by prolonged escape latency in the Classic Labyrinth test, further supporting research on pesticide-induced memory decline **(Hassanen et *al.*, 2021)**. Additionally, IMI exposure also resulted in olfactory impairments and social deficits, social interaction scores in the Vsoc-Maze decreased by 50% at the higher IMI dose, suggesting disruptions in social behavior **(Zhang et *al.*, 2019)**, while a 30% reduction in sucrose preference indicated anhedonia, a marker of depressive-like states **(Patro et *al.*, 2021)**.

Histopathological analyses provided additional confirmation of neuronal damage, with marked cell degeneration, apoptosis, and neuroinflammation in the IMI-exposed groups.

Confirming the cytotoxic impact of IMI on brain tissues. Our findings align with those of **Soujanya et *al.*, 2013.** Who observed significant changes in brain sections of rats treated with 80 mg/kg body weight, including pronounced congestion in the cerebellum, degeneration of Purkinje cells with dendritic loss, vacuolation around neurons, and shrunken neurons by the 14th day of the experiment.

The lysosomal stability test further confirmed the cytotoxic effects of IMI, as evidenced by reduced Neutral Red Retention Time (NRRT), indicating lysosomal membrane destabilization**,** revealing significant changes in absorbance after 24 and 48 hours in treated groups, aligning with findings by **Sargent et *al.*, 2021,** who highlighted lysosomal dysfunction as a consequence of imidacloprid exposure. These results are consistent with previous studies by **Martinez-Gomez et *al.*, (2008)** and **Zhao et *al.*, (2011)**, examined the effects of Pyrethroid on lysosomal membrane fragility by evaluating the retention time of the neutral red probe within the lysosomal compartment of both control and treated hemocytes. Moreover, a qualitative assessment of lysosomal pH variations indicated a significant increase in pH in IMI-exposed groups, emphasizing the impact of chronic pesticide exposure on lysosomal function. When the cell dies or the pH gradient is reduced, the dye cannot be retained **(Filman et *al.*, 1975)**.

Microscopic observations further demonstrated morphological alterations in lysosomal structures, suggesting potential membrane disruption. This is consistent with the results reported by **Borenfreund and Puerner, (1984),** who demonstrated Maintaining a lower pH than that of the cytoplasm allows for dye retention within lysosomes, facilitating the differentiation between viable, damaged, or dead cells based on dye uptake and release dynamics.

This study provides strong evidence that chronic IMI exposure induces neurotoxicity through mitochondrial dysfunction, oxidative stress, cholinergic disruption, and neuronal apoptosis.

2. Protective Mechanisms of Melissa *officinalis* Against IMI Toxicity

MOE co-administration demonstrated a significant neuroprotective effect. Particularly at lower IMI doses (IMI D1 + MOE), given its strong antioxidant, anti-inflammatory, and neuroprotective properties, Compounds with antioxidant properties play a crucial role in deactivating and scavenging free radicals. They operate by either donating hydrogen atoms or chelating metals to counteract the effects of oxidants. **(Prakash et *al*., 2007).**

Melissa *officinalis* represents a promising natural alternative for mitigating pesticide-induced neurotoxicity. This protective action is attributed to MOE's rich polyphenolic content and antioxidant properties, as established in Part One. Additionally, the presence of flavonoid compounds, including rosmarinic acid, further contributes to the antioxidant capacity of M. *officinalis* L. **(Herodez et *al*., 2003).**

The key mechanisms underlying its neuroprotection include the reduction of oxidative stress through flavonoids, polyphenols, and condensed tannins, which neutralize reactive oxygen species (ROS) and restore mitochondrial antioxidant enzyme activity (GSH, GPX, SOD, CAT, and GST). Additionally, MOE inhibited mitochondrial dysfunction by mitigating IMI-induced swelling and permeability alterations, preserving ATP production and cellular energy metabolism. It also modulated neurotransmitter function by restoring AChE activity, preventing excessive nAChR activation and subsequent neurotoxic overstimulation. **(Beghoul et *al*., 2017).** It contributes to increased brain oxygen consumption and promotes dopamine synthesis. In summary, the extract demonstrates remarkable virtues, extending to the repair of damaged neurons **(Gao et *al*., 2014; Mona et *al*., 2014).**

Notably, IMI exposure resulted in significant alterations in growth parameters, including reduced body weight and organ mass. This was accompanied by metabolic disturbances, as mitochondrial protein, carbohydrate, and lipid levels were markedly decreased in a dose-dependent manner, indicating compromised bioenergetic function. MOE co-administration mitigated these effects, restoring metabolic balance and contributing to improved energy metabolism. These findings suggest that MOE not only protects neuronal integrity but also sustains overall physiological homeostasis, preventing IMI-induced metabolic disruptions.

Furthermore, MOE significantly improved neurobehavioral outcomes, with treated rats exhibiting fewer cognitive and motor impairments, suggesting that MOE's protective effects extend beyond biochemical markers to functional recovery. Notably, MOE significantly mitigated these neurobehavioral alterations, improving cognitive function, memory retention, and mood regulation, likely due to its neuroprotective properties, as observed in studies on rosmarinic acid's effects on neurotransmitter modulation **(Dastmalchi et *al.*, 2008)** and mood regulation pathways **(Martins et *al.*, 2012)**. These findings further support the potential of antioxidant-rich plant extracts in counteracting pesticide-induced neurotoxicity **(Sofowora et *al.*, 2013).**

Histological analysis revealed that IMI exposure led to neuronal degeneration, gliosis, and vacuolization in the hippocampus and striatum, whereas MOE co-administration reduced these structural alterations, preserving neuronal integrity. The lysosomal stability test further confirmed MOE's role in maintaining cellular homeostasis by preventing lysosomal membrane damage, a key factor in neuronal survival. These findings align with previous studies on other neurotoxicants, such as organophosphates, where antioxidant-rich plant extracts counteracted oxidative damage **(Banerjee et *al.*, 2014).** However, further research is needed to refine the dose-response relationship of MOE and assess its differential efficacy across brain regions.

3. Correlation between Parameters and Tests

The correlation analyses between biochemical, histological, mitochondrial, lysosomal, and neurobehavioral parameters, as well as growth and metabolic parameters, offer a comprehensive view of IMI-induced neurotoxicity and the neuroprotective effects of MOE in a dose-dependent and region-specific manner. AChE activity showed a strong negative correlation with oxidative stress markers (MDA) ($r = -0.85$, $p < 0.001$), confirming that IMI-induced oxidative stress significantly contributes to cholinergic dysfunction. This effect was

dose-dependent, with IMI D2 (50 mg/kg/day) causing a 42% greater reduction in AChE activity compared to IMI D1 (5 mg/kg/day). MOE co-administration restored AChE levels in a dose-dependent manner, particularly in the hippocampus, where AChE inhibition was reduced by 38% in the IMI D2 + MOE group compared to the IMI D2 group alone.

Mitochondrial dysfunction parameters, including oxygen consumption ($r = -0.78$, $p < 0.01$), swelling ($r = 0.81$, $p < 0.01$), and membrane permeability ($r = 0.75$, $p < 0.01$), showed strong correlations with behavioral impairments, reinforcing the link between mitochondrial energy failure and neurodegenerative symptoms. Regionally, the striatum exhibited heightened susceptibility to IMI toxicity, with mitochondrial swelling increasing by 65% at IMI D2 compared to controls, while the hippocampus showed a 47% increase. MOE administration mitigated these effects, reducing swelling by 40% in the hippocampus and 28% in the striatum.

In addition to mitochondrial dysfunction, the impact of IMI on growth parameters (body weight, organ weight, and growth rate) and mitochondrial metabolic parameters (protein, carbohydrate, and lipid levels) was observed. IMI-treated rats exhibited a significant reduction in body weight, with a 22% decrease at IMI D2 compared to controls ($p < 0.001$), as well as a 17% decrease in organ weights. Furthermore, mitochondrial metabolic dysfunction was evidenced by a significant depletion of protein ($r = -0.76$, $p < 0.01$), carbohydrate ($r = -0.71$, $p < 0.05$), and lipid levels ($r = -0.84$, $p < 0.001$) in both the hippocampus and striatum. MOE administration significantly reversed these effects, restoring protein levels by 28% in the hippocampus and 21% in the striatum, and carbohydrate and lipid levels by 25% and 30%, respectively.

Lysosomal stability was another critical factor, where Neutral Red Retention Time (NRRT) was significantly reduced in a dose-dependent manner ($r = -0.82$, $p < 0.001$). Histopathological scores correlated strongly with neurobehavioral deficits, with gliosis and neuronal vacuolization being more pronounced at IMI D2 ($p < 0.001$), leading to a 55% increase in motor dysfunction compared to controls. The hippocampus also exhibited neurodegenerative changes but with a slightly lower severity, correlating with a 43% increase in cognitive deficits. MOE co-administration improved lysosomal stability and reduced histopathological damage, with neuronal preservation rates reaching 67% in the hippocampus and 52% in the striatum.

The depletion of mitochondrial antioxidants (GSH, GPX, SOD, CAT, and GST) was significantly correlated with increased oxidative stress and neurotoxicity ($r = -0.88$, $p < 0.001$),

and MOE's ability to restore these antioxidants was linked to improved mitochondrial function and reduced histological and behavioral impairments. Additionally, variations in bioactive compound yields observed in this study may be attributed to pedoclimatic factors and extraction techniques. Structural analyses using X-ray diffraction (XRD), infrared spectroscopy (IR), and scanning electron microscopy (SEM) could further clarify the physicochemical properties of MOE and its cellular interactions.

These statistically significant correlations reinforce the interconnected nature of IMI-induced neurotoxicity and highlight the ability of MOE to alleviate damage through multiple protective pathways, including improvements in growth and mitochondrial metabolic parameters.

Conclusion and Perspectives

Conclusion and Perspectives

In an era where environmental toxins increasingly threaten human and animal health, the silent but insidious impact of neonicotinoid pesticides on the nervous system demands urgent attention. Imidacloprid (IMI), a widely used yet controversial compound, has been increasingly linked to severe neurotoxic effects. Despite its extensive agricultural use, the long-term consequences of chronic IMI exposure on mammalian brains remain underexplored, particularly in relation to mitochondrial stability, lysosomal integrity, and neurotransmitter homeostasis.

Our research investigates the neurotoxic effects of chronic Imidacloprid (IMI) exposure in *Wistar* rats, focusing on its dose-dependent impact on different brain regions. The study also explores the protective potential of Melissa *officinalis* L. (MOE), a medicinal plant known for its antioxidant and neuroprotective properties. The findings reveal that IMI exposure causes significant oxidative stress, mitochondrial dysfunction, neurotransmitter imbalances, histopathological and behavioral impairments, particularly at higher doses (50 mg/kg/day). The striatum and hippocampus were particularly vulnerable, with the striatum showing motor coordination issues and the hippocampus experiencing cognitive impairments. MOE administration provided partial protection, especially in the hippocampus, highlighting its potential to counteract some of the neurotoxic effects of IMI.

The first part of our research centered on the characterization of Melissa *officinalis* L. aqueous extract, to determine its bioactive compounds and therapeutic potential. Phytochemical analysis confirmed that the extract is rich in polyphenols, flavonoids, and condensed tannins**,** compounds known for their potent antioxidant properties**.** Antioxidant activity assays (DPPH, ABTS, CUPRAC, FRAP) demonstrated its potent free radical-scavenging abilities, comparable to synthetic antioxidants but with the advantage of being natural and low in toxicity.

Beyond its antioxidant profile, Melissa *officinalis* has demonstrated neuroprotective and anti-inflammatory effects, suggesting its potential as a therapeutic agent against neurodegenerative disorders associated with oxidative stress and mitochondrial dysfunction. Variability in bioactive compound yields observed in this study may be influenced by pedoclimatic conditions and extraction methods. Additionally, advanced structural analyses, including X-ray diffraction (XRD), infrared spectroscopy (IR), and scanning electron microscopy (SEM), confirmed the presence of diverse bioactive molecules with significant pharmacological applications.

These findings provide a strong foundation for the second part demonstrated that these antioxidant properties translate into in vivo protection against IMI-induced neurotoxicity, restoring mitochondrial function and neurotransmitter balance. This link underscores the pharmacological potential of MOE in mitigating pesticide-induced brain damage.

The second part of this study examined the neurotoxic effects of chronic Imidacloprid (IMI) exposure at two doses (5 mg/kg/day and 50 mg/kg/day) in *Wistar* rats, while also evaluating the neuroprotective potential of Melissa *officinalis* extract (MOE) at 10 mg/kg/day**.**

Findings confirmed that IMI induces neurotoxicity in a dose-dependent manner, with higher doses (50 mg/kg/day) causing more severe damage than lower doses (5 mg/kg/day). The toxicity was particularly evident in mitochondrial function, as reflected by mitochondrial swelling, reduced oxygen consumption, and increased permeability. Additionally, IMI exposure led to a depletion of mitochondrial antioxidants (GSH, GPX, SOD, CAT, and GST), metabolic imbalances in protein, carbohydrate, and lipid levels, neurotransmitter disruptions, histopathological alterations, lysosomal instability**,** and significant neurobehavioral deficits.

The severity of these toxic effects varied across brain regions, the striatum was especially vulnerable to IMI toxicity, particularly at higher doses, showing a marked decline in antioxidant defenses**,** which correlates with its key role in motor coordination**.** This suggests that IMI exposure may contribute to motor impairments. Conversely, the hippocampus, which is critical for learning and memory, also exhibited significant dysfunction, but MOE provided greater neuroprotection in this region compared to the striatum**.**

While this research provides critical insights into the neurotoxic effects of neonicotinoids and the protective role of antioxidant plant extracts, several avenues remain open for further exploration**:**

- **Extending Exposure Duration:** Investigate the effects of long-term or lifetime exposure to Imidacloprid (IMI) to better understand its cumulative impact on brain health.
- **Molecular Mechanisms:** Deeper exploration of molecular pathways, including gene expression and proteomic analysis, epigenetic studies could clarify how IMI causes neurotoxicity through oxidative stress, mitochondrial dysfunction and neuroinflammation.

- **Optimizing MOE Dosing:** Explore optimal dosing and administration methods for *Melissa officinalis* L. including potential improvements in delivery methods (e.g., nanoformulations, sustained-release systems) to enhance its effectiveness.
- **Comparative Studies:** Compare MOE with other antioxidants and pharmacological treatments for potential synergies.
- **Targeted Regional Neuroprotection:** Focus on brain-region-specific susceptibility to IMI toxicity, particularly in the hippocampus and striatum, and investigate other natural compounds for enhanced protection.
- **Advanced Behavioral and Cognitive Assessments:** Expanding behavioral tests to correlate biochemical changes with motor and cognitive impairments. (e.g., Rotarod Test, Electrophysiological Measurements)
- **Translational and Human Relevance:** Use alternative models like non-human primates or human cell cultures for greater relevance to human health.
- **Synergistic Effects of Multiple Stressors:** Study how IMI interacts with other environmental toxins to provide a clearer understanding of multi-toxicant exposure.

In conclusion, this research underscores the significant risks associated with chronic neonicotinoid exposure while highlighting the potential of natural antioxidants as protective agents. Future interdisciplinary approaches integrating toxicology, pharmacology, and neuroscience will be essential in advancing our understanding of environmental neurotoxicity and developing effective strategies for prevention and treatment.

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