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Evaluation and implementation of natural products to reduce the toxicity of some compounds contained in tobacco and cigarette smoke

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Abstract

This thesis evaluates the efficacy of natural products derived from *Juniperus phoenicea* (JPH) and *Juniperus oxycedrus* L. (JOX) berries, collected in northeastern Algeria, in reducing the toxicity associated with cigarette smoke compounds. Using advanced extraction and analytical methods, the study assesses the bioactivity of essential oils and ethanolic extracts through both in vitro and in vivo models.

Essential oils were extracted via hydrodistillation, while ethanolic extracts were prepared using ultrasound-assisted extraction (UAE) and conventional maceration. Chemical profiling by gas chromatography-mass spectrometry (GC-MS) and Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS/MS) identified major bioactive compounds including α-pinene, germacrene D, quinic acid, protocatechuic acid, and hesperidin.

In vitro assays revealed that JOX essential oil (JOX-EO) reduced nicotine levels with an IC50 of 16.8 µg/mL through a novel rapid extraction method, surpassing JPH oil. Cytotoxicity testing on A549 lung cancer cells demonstrated potent, dose-dependent inhibition by JOX-EO (IC50 = 15.9 µg/mL), outperforming the chemotherapy drug etoposide. Antioxidant capacity, measured by DPPH, FRAP, CUPRAC, and nitric oxide (NO) scavenging assays, was highest in UAE ethanolic extracts, correlating with elevated phenolic and flavonoid content. Notably, the NO scavenging activity presented an inverse concentration-response, showing stronger inhibition at lower doses.

UV-visible spectroscopy confirmed JOX-EO's ability to degrade polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke. In vivo, subchronic cigarette smoke exposure in rats followed by JOX-EO treatment restored antioxidant enzymes (GST, CAT, GSH-Px) and reduced malondialdehyde (MDA) levels. Chronic exposure studies showed that JOX extracts and essential oil facilitated partial recovery of body weight, organ function, and biochemical markers (ALT, AST, LDH), improved inflammatory and lipid profiles, without affecting blood glucose levels.

These results highlight JOX berries as a sustainable source of bioactive compounds with strong antioxidative, anticancer, and detoxifying properties, with extraction techniques significantly impacting their therapeutic potential. The findings support the development of JOX-based natural products for preventing and treating smoking related health hazards.

Keywords: Cigarette smoke toxicity, natural products, detoxification, inhibition power, *Juniperus oxycedrus,* Essential oils, Ethanolic extracts Antioxidant activity, and Cytotoxicity.

Résumé

Cette thèse examine l'efficacité des produits naturels extraits des baies de *Juniperus phoenicea* (JPH) et *Juniperus oxycedrus* L. (JOX), récoltées dans le nord-est de l'Algérie, pour atténuer la toxicité liée à la fumée de cigarette. Grâce à des méthodes avancées d'extraction et d'analyse, l'étude évalue l'activité biologique des huiles essentielles et des extraits éthanoliques via des modèles in vitro et in vivo.

Les huiles essentielles (HEs) ont été obtenues par hydrodistillation, tandis que les extraits éthanoliques ont été préparés par extraction assistée par ultrasons (EAU) et macération classique. La caractérisation chimique par chromatographie en phase gazeuse couplée à la spectrométrie de masse (CPG-SM) et Chromatographie Liquide-ionisation par Électrospray-Spectrométrie de Masse en Tandem (CL-IES-SM/SM) a révélé des composés bioactifs majeurs tels que l'α-pinène, le germacrène D, l'acide quinique, l'acide protocatéchuique et l'hespéridine.

Les essais in vitro ont démontré que l'HE de JOX réduisait la nicotine avec une Concentration inhibitrice à 50 % (CI50) de 16,8 µg/mL via une méthode d'extraction rapide innovante, surpassant celle de JPH. Les tests de cytotoxicité sur cellules cancéreuses pulmonaires A549 révèlent une inhibition dose-dépendante puissante par HE de JOX (CI50 = 15,9 µg/mL), dépassant l'efficacité du chimio thérapeutique étoposide. L'activité antioxydante, mesurée par DPPH, FRAP, CUPRAC et capture de l'oxyde nitrique (NO), était maximale pour les extraits obtenus par EAU, en corrélation avec une richesse phénolique et flavonoïde accrue. L'activité de capture du NO montrait une inhibition plus forte à faibles doses, suggérant une efficacité remarquable même à faible concentration.

La spectroscopie UV-visible a confirmé que HE de JOX dégrade efficacement les hydrocarbures aromatiques polycycliques (HAP) issus de la fumée. In vivo, le traitement des rats exposés subchroniquement à la fumée a restauré les enzymes antioxydantes (GST, CAT, GSH-Px) et réduit le stress oxydatif (malondialdéhyde). Les études chroniques montrent une récupération partielle du poids, une amélioration des fonctions organiques et des marqueurs biochimiques, ainsi qu'une modulation des profils inflammatoires et lipidiques, sans impact sur la glycémie.

Ces résultats confirment que les baies de JOX sont une source durable de composés bioactifs à fort potentiel antioxydant, anticancéreux et détoxifiant, avec un potentiel thérapeutique influencé par la technique d'extraction. Ces travaux encouragent le développement de produits naturels à base de JOX pour la prévention et le traitement des effets nocifs du tabagisme.

Mots clés : Toxicité de la fumée de cigarette, produits naturels, détoxification, pouvoir inhibiteur, *Juniperus oxycedrus*, huiles essentielles, extraits éthanoliques, activité antioxydante et cytotoxicité.

ملخص

تُقيّم هذه الرسالة فعالية المنتجات الطبيعية المستخلصة من ثمار العرعر الفينيقي (*Juniperus phoenicea*) والعرعر الشوكي (*Juniperus oxycedrus* L.) التي جُمعت من شمال شرق الجزائر في تقليل السمية المرتبطة بمركبات دخان السجائر. ومن خلال استخدام تقنيات استخراج وتحليل متقدمة، تم تقييم النشاط البيولوجي للزيوت العطرية والمستخلصات الإيثانولية باستخدام نماذج مخبرية (in vitro) وحيوانية (in vivo).

استُخرجت الزيوت العطرية بواسطة التقطير بالماء، بينما أُعدت المستخلصات الإيثانولية باستخدام الاستخلاص بالموجات فوق الصوتية (UAE) والتنقيع التقليدي. أظهر التحليل الكيميائي باستخدام كروماتوغرافيا الغاز-مطياف الكتلة (GC-MS) وكروماتوغرافيا السائلة - التأين بالرذاذ الإلكتروني - مطياف الكتلة الترادفي (LC-ESI-MS/MS) وجود مركبات حيوية رئيسية مثل ألفا-بينين، جيرماسرين D، حمض الكينيك، حمض البروتوكاتيكويك، والهيبيريدين.

كشفت الاختبارات المخبرية أن الزيت الأساسي للعرعر الشوكي (JOX-EO) خفض بشكل ملحوظ مستويات النيكوتين، حيث بلغ تركيز المثبط عند 50% 16.8 (IC50) ميكروغرام/مل باستخدام طريقة استخراج سريعة ، متفوقًا بذلك على زيت العرعر الفينيقي. كما أظهرت اختبارات السمية الخلوية على خلايا سرطان الرئة A549 تثبيطًا قويًا يعتمد على الجرعة بواسطة JOX-EO، حيث بلغ IC50 15.9 ميكروغرام/مل، متفوقًا على دواء العلاج الكيميائي إيتوبوسيد. وكانت القدرة المضادة للأكسدة، المقاسة بواسطة اختبارات DPPH وFRAP وCUPRAC وامتصاص أكسيد النيتريك (NO)، الأعلى في المستخلصات الإيثانولية المستخلصة بالموجات فوق الصوتية، وهو ما يرتبط بارتفاع محتوى المركبات الفينولية والفلافونويدية. ولاحظت الدراسة أن نشاط امتصاص أكسيد النيتريك أظهر علاقة عكسية مع التركيز، حيث كان التثبيط أقوى عند الجرعات المنخفضة.

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الكلمات المفتاحية: سمية دخان السجائر، المنتجات الطبيعية، إزالة السموم، قوة التثبيط، العرعر (*Juniperus oxycedrus,*)، الزيوت الأساسية، المستخلصات الإيثانولية، النشاط المضاد للأكسدة، والسمية الخلوية.

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Abbreviation and terminology list

Abbreviation	Designation

JOX	*Juniperus oxycedrus* L.
JPH	*Juniperus phoenicea*
EO	Essential oil
JOX-EO	*Juniperus oxycedrus* L. essential oil
JPH-EO	*Juniperus phoenicea* essential oil
JEE1	*Juniperus oxycedrus* ethaolic extract obtained by ultrasound-assisted extraction
JEE2	*Juniperus oxycedrus* ethaolic extract obtained by Conventional extraction
PAH	Polycyclic Aromatic Hydrocarbons
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
CUPRAC	Cupric Ion Reducing Antioxidant Capacity
NO	Nitric Oxide
WHO	World Health Organization
U. S	United States
USA	United States of America
UN	United Nations
UK	United Kingdom
FCTC	Framework Convention on Tobacco Control
SDGs	Sustainable Development Goals
LMICs	Low- and middle-income countries low- and middle-income countries
VTA	Ventral tegmental area
GABAergic	Receptors that bind to GABA (gamma-aminobutyric acid).
ROS	Reactive oxygen species
TSNAs	Tobacco-specific nitrosamines
VOC	Volatile organic compound
PM	Particulate matter
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
MAPK	Mitogen-Activated protein kinases
UDP	Uridine Diphosphate.
UAE	Ultrasound-assisted extraction
CE	Conventional extraction
TPC	Total phenolic contents
TFC	Total flavonoid contents
UHPLC	Ultrahigh performance liquid chromatography

SIL-30AC	A model name for an autosampler from Shimadzu, used in liquid chromatography systems to automatically inject samples.
CTO-10ASvp	A Shimadzu model of column oven used in chromatography to maintain and control the temperature of the column for consistent separation.
LC-30AD	A Shimadzu model of a high-performance liquid chromatography (HPLC) pump, providing solvent flow to the system.
RP-C18	Reverse Phase - Octadecylsilane (C18) column A common type of chromatographic stationary phase with an 18-carbon chain attached to silica, used for separating non-polar to moderately polar compounds
EC-C18	A specific type of C18 column often designed for end-capped or enhanced chromatography performance in reverse-phase liquid chromatography.
ODS-4	Octadecylsilane phase type 4 Another term for a C18 reverse-phase column used in HPLC.
ESI	Electrospray ionization
MRM	Multiple reaction monitoring
GC/MS	Gas chromatography and mass spectrometry
HP-5MS	A type of GC capillary column coated with 5% phenyl-methylpolysiloxane, widely used for its low bleed and suitability in mass spectrometry (MS) applications.
N6	Nitrogen gas with 99.9999% purity, which is very high purity
TIC	Total Ion Chromatogram
NaOH	Sodium Hydroxide
C1-C2	Concentration of Essential oil
Dx	D: Sample of nicotine, x: number of samples
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
HA	Antioxidant that can donate a hydrogen atom (H•)
ArOH	Hydroxyl group (-OH) attached to an aromatic ring (Ar).
SD	Standard Deviation
AOX	Antioxidant
$FeCl_3$	Ferric Chloride
$[K_3Fe(CN)_6]$	Potassium Ferricyanide
SNP	Sodium nitroprusside
NED	Naphthylethylenediamine dichloride
H_3PO_4	Phosphoric Acid
LOX	Lipoxygenase
XO	Xanthine Oxidase
BChE	Butyrylcholinesterase

units/well	the amount of an enzyme activity measure contained in an individual well of a microplate
15-LOX	15-lipoxygenase
FAHPs	Fatty acid hydroperoxides
A549 cell	A human lung carcinoma epithelial cell line widely used in biomedical research, especially in studies related to lung cancer, respiratory infections, and toxicology.
ATCC	American Type Culture Collection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl Sulfoxide
EU2010/63	European Union Directive 2010/63/EU on the protection of animals used for scientific purposes
WVS	well-ventilated space
C	Control group of the first part in vivo
COX	No smoker rats treated with JOX-EO
CPH	No smoker rats treated with JPH-EO,
CS	Withdrawal rats after 4 weeks of smoke exposure
STOX	Withdrawal smoker rats treated with JOX-EO
STPH	Withdrawal smoker rats treated with JPH -EO
CSG	Smoking control group exposed to cigarette smoke for 14 weeks.
EXD1	No smoker rats treated with JEE1 at 200 mg/kg/day for 15 days after 14 weeks
EXD3	No smoker rats treated with JEE1 at 800 mg/kg/day
JEO	No smoker rats treated with JOX-EO at 200 mg/kg/day for 15 days after 14 weeks
STEX1	Smoking group treated with JEE1 at 200 mg/kg for 2 weeks after withdrawing smoking
STEX2	smoking withdrawn group treated with JEE at 400 mg/kg for 2 weeks after withdrawing smoking
STEX3	smoking withdrawn group treated with JEE at 800 mg/kg for 2 weeks after withdrawing smoking
SOEO	smoking withdrawn group treated with JOX-EO at 200 mg/kg.
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
LDH	Lactate Dehydrogenase
HIV	Human Immunodeficiency Virus
NCDs	Non-communicable diseases
COPD	Chronic Obstructive pulmonary disease
AMD	Age-related macular degeneration

SHS	Secondhand smoke
SIDS	Sudden infant death syndrome
nAChRs	Nicotinic acetylcholine receptors
TRIG	Triglycerides
HDL	High-density lipoprotein cholesterol
LDL	Low-density lipoprotein cholesterol
CHOL	Total cholesterol
MDA	Malondialdehyde
GSH	Reduced glutathione
GST	Glutathione S-transferase
GSH-Px	Glutathione peroxidase
CAT	Catalase
LK	Left kidney
RK	Right kidney
WBC	White Blood Cells: Cells of the immune system involved in protecting the body against infections and foreign invaders. Its count indicates immune status
RBC	Red Blood Cells: Cells responsible for carrying oxygen from the lungs to the rest of the body and bringing carbon dioxide back for exhalation
HGB	Hemoglobin: The oxygen-carrying protein within red blood cells. Its level reflects the blood's capacity to carry oxygen
HCT	Hematocrit: The proportion (percentage) of blood volume occupied by red blood cells
MCV	Mean Corpuscular Volume: The average volume (size) of individual red blood cells
MCH	Mean Corpuscular Hemoglobin: The average amount (mass) of hemoglobin per red blood cell
MCHC	Mean Corpuscular Hemoglobin Concentration: The average concentration of hemoglobin in a given volume of packed red blood cells
PLT	Platelets: Small blood cell fragments involved in clotting and stopping bleeding
EDTA	Ethylenediaminetetraacetic Acid
Tris-HCl	Tris(hydroxymethyl)aminomethane Hydrochloride
TBA	Thiobarbituric acid
TBS	Tris-buffered saline
BHT	Butylated hydroxytoluene
TCA	Trichloroacetic acid
PBS	Phosphate-buffered saline
H_2O_2	Hydrogen Peroxide

Na_2HPO_4	Disodium hydrogen phosphate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
CDNB	1-chloro-2,4-dinitrobenzene
GDP	Gross Domestic Product.
AD	Anno Domini
BC	Before Christ
UV/UV-Vis	Ultraviolet and Ultraviolet-Visible
p-value	probability value: It indicates that the observed results are statistically significant at the 5% significance level.
CSE	Cigarette smoke exposure
µg QE/mL	Micrograms of Quercetin Equivalents per milliliter.
mg EQ/g ext	Milligrams of Equivalent Quercetin per gram of extract
mg EG/g ext;	Milligrams of Equivalent Gallic acid per gram of extract
v:v	Volume to Volume ratio

INTRODUCTION

Cigarette smoke constitutes a significant global public health challenge, with robust evidence linking it to severe health consequences and increased mortality. Smoking has been shown to meaningfully reduce life expectancy, with epidemiological data indicating that smokers live, on average, nearly eight years less than nonsmokers [1]. Moreover, indoor tobacco smoke not only degrades air quality but also poses serious health risks to non-smokers through secondhand smoke exposure, contributing to increased incidence of respiratory and cardiovascular diseases[2, 3]. The health burden of tobacco smoke is multifactorial, encompassing an elevated risk of heart disease[4], stroke, and lung cancer among both active smokers and individuals exposed passively [5].

The harmful effects of cigarette smoke are primarily due to its complex chemical makeup, which includes numerous toxicants such as nicotine, tar, and polycyclic aromatic hydrocarbons (PAHs). As demonstrated in extensive research, these compounds have been consistently associated with oxidative stress, DNA damage, and neurotoxicity [6-8]. This biochemical damage underscores the critical need to develop effective strategies that mitigate the toxicity induced by cigarette smoke.

Despite decades of tobacco-control policies and major progress in characterizing smoke toxicants, cigarette smoke exposure remains widespread, including involuntary exposure in indoor environments, and there is still no fully effective approach to prevent or attenuate the biological damage once exposure occurs. Existing interventions are primarily oriented toward cessation and avoidance; however, many individuals continue smoking or remain exposed to secondhand smoke, sustaining a significant burden of oxidative stress, inflammation, and organ dysfunction linked to smoke constituents such as nicotine and PAHs. In addition, many proposed protective agents have limitations related to safety, affordability, long-term tolerability, or limited mechanistic breadth, and preclinical evidence is often fragmented focused on a single assay, a single endpoint, or short exposure periods making translation and comparison difficult. These challenges highlight the need for accessible, biologically plausible, and multi-target strategies that can be rigorously assessed through integrated in vitro and in vivo models, using measurable biomarkers (oxidative stress, inflammatory markers, biochemical or hematological indices) and direct evaluation of smoke-component reduction.

In this context, the potential of natural products or medicinal plants, particularly essential oils from *Juniperus*, to counteract tobacco smoke toxicity is a promising avenue for public health research. Among *Juniperus* species, *Juniperus communis* stands out for its well-documented anti-inflammatory and antioxidant properties, thanks to its rich flavonoids, terpenes, and organic acids. A study on rats exposed to cigarette smoke showed that aerosols

of *Juniperus communis* essential oil improved tracheal and lung function by modulating bronchodilation and bronchoconstriction, reducing respiratory tissue damage, and suggesting its potential to protect against airway injury caused by cigarette smoke[9]. Both traditional use and modern research support its role in managing inflammation and oxidative stress[10-12]. Its essential oil, especially α-pinene, also shows strong anti-inflammatory effects.[13] Other *Juniperus* species available in Algeria, such as *Juniperus oxycedrus* L. and *Juniperus phoenicea*, may offer similar benefits, which this study aims to explore.

This thesis is original in that it proposes a comparative and stepwise framework applied to Algerian Juniperus species, combining rapid extraction with chemical and functional characterization of essential oils and a corresponding ethanolic extract, and validating activity through complementary in vitro screening and both short- and long-term in vivo cigarette-smoke exposure models. It focuses on smoke-relevant endpoints such as nicotine-reduction efficacy, PAHs degradation, cytotoxicity, antioxidant capacity, enzyme inhibition, and systemic biomarkers allowing prioritization of the most potent species and deeper mechanistic assessment in a locally grounded and methodologically comprehensive manner.

For this purpose, the present thesis investigates the bioactive potential of essential oils from the berries of these two species Juniperus phoenicea (JPH) and Juniperus oxycedrus (JOX), collected in northeastern Algeria (Tebessa province), to reduce cigarette smoke toxicity. Preliminary tests are conducted to identify the most potent specie, followed by comprehensive studies on its essential oil (EO) alongside the corresponding ethanolic extract.

Part 1 focuses on EOs from both species, employing in vitro methods to evaluate nicotine-reduction efficacy and cytotoxicity against lung cancer cells, alongside a one-month in vivo rat model exposed to cigarette smoke to measure oxidative stress biomarkers.

Part 2 analyzes the most potent species (identified in Part 1), combining UV spectroscopy to assess PAHs degradation by EO with multifaceted in vitro assays for antioxidant activity (DPPH, FRAP, CUPRAC, nitric oxide scavenging) and enzyme inhibition (butyrylcholinesterase, xanthine oxidase, lipoxygenase). A rigorous Fourteen weeks in vivo rat model further evaluates the ethanolic extract's protective effects against chronic smoke exposure, analyzing organ/body weight dynamics, hematological/biochemical parameters, inflammatory markers (e.g., C-reactive protein), glycemic status, and organ-specific metabolite levels.

Starting with an introduction and organized into three interconnected chapters, the thesis integrates rapid extraction techniques, multi-dimensional bioactivity assessments, and

translational in vitro-to-in vivo validation, ensuring a robust exploration of these natural compounds' therapeutic potential against smoke-induced damage.

The first chapter presents a detailed bibliographic review covering the toxicological impacts of tobacco use, the biological mechanisms underlying cigarette smoke-related diseases, and the diverse toxic substances present in cigarette smoke. It also explores the protective roles of medicinal plants, focusing on their bioactive constituents, extraction techniques, and the mechanistic basis of their biological activity in reducing toxicity.

The second chapter describes the research methodology, detailing the collection and preparation of the extraction procedures for EOs and ethanolic extracts, chemical analysis, and the experimental protocols for in vitro and in vivo assays designed to evaluate the extracts' capacity and efficacy in reducing cigarette smoke toxicity.

The third chapter presents and discusses the results from the chemical characterization and biological evaluations of the natural products investigated. It evaluates their effectiveness in reducing some toxic cigarette smoke components, their antioxidant and enzyme inhibitory activities, and the protective outcomes observed in animal models. These findings are analyzed in the context of existing scientific literature to underscore their relevance in developing natural therapeutic interventions for tobacco-related health issues.

This manuscript will end with a conclusion summarizing the most essential outcomes of this fascinating investigation.

CHAPTER I

THEORETICAL BACKGROUND

I. 1. TOBACCO SMOKE OVERVIEW

I. 1. 1. History of tobacco use

Tobacco has a long and complex history, and its origins can be traced back to ancient civilizations, with its origins tracing back to the indigenous peoples of the Americas [14]. The name "tobacco" was mistakenly applied to the plant by European explorers. The term tobacco, or tavaco, is the original cane pipe name used by Native Americans to smoke, not the plant itself [15].

Various names, including yetl, picietl, kutz, and sayri, have been known for the plant. As it spread to Europe, its nomenclature expanded, including petun, kohaba, Tsâla, herbe sainte, killikinnick, and American silver weed. Tobacco has been a constant companion in America for thousands of years, with Mayans in Central America burning and inhaling tobacco 2500 years ago in religious settings [15].

However, recent archaeological evidence could suggest otherwise. In 1993, researchers discovered nicotine concentrations in Peruvian mummies dating from 200-1500 AD, Egyptian mummies (1070 BC 395 BC), Sudanese skeletal tissue (5000-4000 BC and 400-1400 AD), and South Germany's Bell Culture (c. 2500 BC) [15]. These findings proved that, from thousands of years before the birth of Christ, tobacco usage was widespread throughout the world and in all civilizations. However, when European explorers like Columbus arrived in the Americas, they documented the natives using tobacco in pipes, cigars, and snuff, thus significantly contributing to the global spread of tobacco. Portuguese and Spanish sailors then helped spread different forms of tobacco use around the world. Tobacco has been an ever-present commodity in America, whether it is smoked, drunk, snuffed, or used in chewed [14].

During the 17th and 18th centuries, tobacco grew in demand and became a significant cash crop for European colonies in the Americas and the Caribbean [16]. This demand led to the widespread use of enslaved Africans to cultivate and harvest tobacco [16]. In the 18th century, tobacco imports into England reached 26 million pounds annually, with 60% re-exported to Europe [17]. Demand was driven by therapeutic beliefs, social rituals, and increasing tobacco addiction, making smoking an international ritual by the mid-19th century [16].

In the 16th century, tobacco was introduced to Africa by the Turks, who brought it to Egypt [18]. Tobacco cultivation and trade in Africa have been significant for over three centuries, with 33 countries growing over 500.000 tons of tobacco by 1993 [19]. The industry has been linked to slavery, with tobacco used to buy enslaved people and maintain the slave trade.

Africans' smoking habits today are influenced by their economic standing and local

norms. In cities, cigarette smoking has increased in popularity and taken over the more traditional pipe smoking [18]. In the past, smoking was exclusively associated with men, but the percentage of female smokers is rising. A growing number of African children and adolescents are also smoking [18].

The industrial revolution in the mid-19th century brought about a significant change in the tobacco industry. It led to the mass production and commercialization of tobacco, making it more affordable and accessible to the general population. Cigarette production also began during this time, further increasing tobacco consumption [20]. In the 20th century, tobacco use spread globally, with the tobacco industry heavily promoting its products through advertising campaigns. However, as the adverse health effects of tobacco use became more evident, governments started implementing regulations and tobacco control measures. The 1960s and 1970s saw public health campaigns and scientific research highlighting the harmful effects of tobacco, leading to a decrease in consumption in many developed countries [21]. Nevertheless, tobacco use continued to increase in developing countries where regulations and awareness were not as prevalent.

I. 1. 2. Prevalence of tobacco

The prevalence of tobacco use varies across different populations and studies. According to the WHO global report on trends in the prevalence of tobacco use 2000-2025, in 2020, it was 22.3% among those aged 15 and older, with 36.7% among men and 7.8% among women [22]. Age plays a significant role in tobacco consumption initiation. Globally, smoking prevalence declined from 22.7% in 2007 to 17% in 2021[23], a testament to the progress made in tobacco control. The U.S.'s tobacco use peaked in the mid-1960s but has since declined due to tobacco control interventions and antismoking efforts [24].

This decline, though promising, should not overshadow the need for continued vigilance and intervention. However, despite these efforts, the global prevalence of tobacco use remains high. With 1.18 billion people regularly smoking tobacco [25], 23% of adults worldwide smoke tobacco products, with the number of female smokers increasing [26]. In 2019, 1.14 billion smokers were consuming 7.41 trillion cigarette-equivalents of tobacco [27]. Tobacco use is linked to around 8 million deaths annually, with 7 million deaths attributed to direct tobacco use and an additional 1.2 million deaths among non-smokers exposed to secondhand smoke, and 80% are reported in low- and middle-income countries [28]. It kills millions of people worldwide, exceeding combined deaths from HIV, tuberculosis, and malaria [26], making it the leading risk factor for death among males. It is, therefore, crucial to continue monitoring and

addressing tobacco use through targeted interventions, public health campaigns, and regulatory measures to reduce its prevalence and associated health risks among smokers exposed to secondhand smoke.

I. 1. 3. Economic impacts of tobacco use

The economic impact of tobacco is not just significant, but it is a global crisis. The estimated annual cost of $1.85 trillion is a staggering 1.8% of the world's GDP [29]. This includes healthcare costs for tobacco-related diseases and lost productivity due to tobacco-related morbidity and mortality. Particularly alarming is the fact that tobacco use is most detrimental in low- and middle-income countries, contributing to poverty and hunger by reducing household spending on food and education.

The tobacco industry's impact is not limited to the economy and public health. It also causes significant environmental damage through soil degradation, water pollution, and deforestation, further undermining sustainable development[30]. This underscores the need for comprehensive solutions that address both the economic and environmental aspects of tobacco use.

Despite these costs, the industry spends billions on marketing and promotion, with the most prominent companies spending over $8.2 billion in the U.S. in 2019 [31].

The costs of tobacco use are high, both economically and in terms of public health. Higher tobacco taxes are an effective way to reduce consumption and recover these costs. However, it is equally clear that current tax levels in most countries are inadequate. This underscores the urgent need for higher tobacco taxes as a policy measure.

The WHO Framework Convention on Tobacco Control (FCTC) has been included in the UN Sustainable Development Goals (SDGs) to strengthen tobacco control efforts in low- and middle-income countries (LMICs) [32]. However, concerns have been raised that tobacco control could be overlooked among 168 other sustainable development goals, given low attention to it in multiple countries' reviews [32].

The FCTC 2030 project, initiated in 2016 and supported by Australia, Norway, and the UK, aims to accelerate tobacco control to achieve the SDGs. The project challenges the false notion that tobacco is a net plus for the economy, which is often embraced by decision-makers and society. The project includes country-led investment cases, estimating tobacco use's health and economic burden, and evaluating tobacco control measures' return on investment [32].

I. 1. 4. Tobacco smoke toxicity

This section delves into tobacco smoke toxicity by analyzing its impact on health, the underlying biological processes that cause disease, and the various types of toxic substances found in the smoke.

I. 1. 4. 1. Health impacts of tobacco use

Tobacco use has wide-ranging and serious consequences for health, significantly increasing the risk of numerous diseases and contributing greatly to premature death. The following sections outline the different types of health impacts associated with tobacco use:

a) Mortality

Tobacco consumption accounts for an estimated 8 million deaths annually worldwide, ranking among the foremost causes of preventable mortality. This includes around 7 million deaths among smokers and an additional 1.2 million deaths due to exposure to secondhand smoke[1].

b) Non-communicable diseases (NCDs)

- **Cancer**

  Smoking is a principal risk factor for various cancers, particularly lung cancer, which constitutes approximately 80% of all cases[33]. Importantly, lung cancer remains a leading cause of death among both current and former smokers, with over half of these cases occurring in former smokers 40% of whom develop the disease more than 15 years after quitting[34]. It is also strongly linked to throat, oral cavity, esophagus, pancreas, kidney, and bladder malignancies [5, 35].

- **Cardiovascular diseases**

  Tobacco uses meaningfully contributes to cardiovascular pathology by elevating blood pressure, impairing blood flow, and promoting arterial plaque accumulation. These effects augment the risk of coronary artery disease, myocardial infarction, and stroke due to compromised vascular function [3, 36, 37].

- **Respiratory diseases**

  Cigarette smoking is a leading cause of chronic respiratory illnesses, notably chronic obstructive pulmonary disease (COPD), which includes emphysema and chronic bronchitis. It also worsens asthma by increasing the frequency and severity of attacks. In smokers with asthma, the inflammatory response tends to be more intense, resulting in persistent symptoms that may continue even after quitting smoking [2, 38].

C) Mental health

A noteworthy association exists between tobacco use and mental health disorders. While some individuals with mental health disorders, such as anxiety and depression, use tobacco to self-medicate for temporary relief, but nicotine dependence often worsens these conditions over time, especially when smoking begins in adolescence [39, 40]. Nicotine's neurotoxic effects on the developing brain and its impact on nicotinic receptors contribute to mental health challenges. While withdrawal may initially intensify symptoms, long-term smoking cessation improves anxiety and depression, highlighting the importance of addressing psychiatric disorders to successfully overcome nicotine addiction [39, 41, 42].

d) Pregnancy and reproductive health

Maternal tobacco use elevates the likelihood of adverse pregnancy outcomes such as miscarriage, preterm delivery, low birth weight, and stillbirth. It also impairs fetal neurodevelopment, leading to long-term sequelae in offspring. Furthermore, tobacco adversely affects fertility in both sexes, reducing conception rates [43, 44].

e) Diabetes

Smoking is associated with a higher risk of type 2 diabetes mellitus through mechanisms such as insulin resistance, impaired glucose regulation, and chronic inflammation. Individuals with diabetes who smoke face a two- to four-fold increased risk of cardiovascular disease compared to non-smokers. Additionally, smokers are 30-40% more likely to develop type 2 diabetes than non-smokers[45, 46].

f) Bone health

Tobacco uses significantly reduces bone mineral density, increasing susceptibility to osteoporosis and fractures, particularly in older adults. Smoking disrupts calcium absorption and bone remodeling by affecting hormones essential for bone health, such as parathyroid and sex hormones [47, 48]. Additionally, oxidative stress from smoking accelerates bone resorption, leading to a 25% higher overall fracture risk and nearly double the risk of hip fractures compared to non-smokers [49].

g) Oral health

Smoking is a key risk factor for periodontal disease, leading to inflammation, tissue damage, and tooth loss. Nicotine constricts blood vessels, reducing gum blood flow and impairing nutrient and oxygen delivery while suppressing immune defenses[50]. Consequently, smokers have a weakened immune response, hindering the body's ability to fight periodontal

pathogens[50, 51]. Additionally, smoking alters the oral microbiota, promoting the growth of bacteria linked to periodontal disease[50].

h) Vision problems

Tobacco smoke elevates the risk of age-related macular degeneration (AMD) and cataracts, leading causes of vision impairment and blindness. Oxidative damage induced by smoke constituents adversely affects retinal structure and lens clarity [52].

i) Skin aging

The toxicants in tobacco accelerate dermal aging by damaging collagen and elastin fibers, reducing skin elasticity and promoting premature wrinkling and loss of tone. Reduced cutaneous blood flow further compromises skin health and repair [53].

j) Immune system dysfunction

Smoking impairs immune function by disrupting leukocyte activity and weakening pathogen defense, increasing infection risk and delaying healing. It alters key immune cells such as T helper and natural killer cells and induces chronic inflammation. This immune dysfunction also contributes to the worsening of autoimmune diseases through immunomodulatory effects[54-56].

K) Secondhand smoke exposure

Exposure to secondhand smoke (SHS) poses serious public health risks, especially for children who are highly vulnerable. SHS exposure is linked to increased rates of sudden infant death syndrome (SIDS), respiratory infections, asthma, ear infections, and impaired lung growth in children, as well as coronary heart disease, stroke, and lung cancer in adult nonsmokers[57]

I. 1. 4. 2. Mechanisms by which cigarette smoke induces disease

Nicotine addiction is mediated through its interaction with the central nervous system, primarily targeting nicotinic acetylcholine receptors (nAChRs) within the mesolimbic dopaminergic pathway. Upon inhalation, nicotine rapidly crosses the blood-brain barrier and binds to α4β2 and other nAChR subtypes on dopaminergic neurons in the ventral tegmental area (VTA) [58, 59]. Repeated nicotine exposure leads to adaptations in reward and aversion circuits, influencing addiction severity [60]. Chronic nicotine exposure induces neuroadaptive changes, including receptor upregulation, desensitization, and synaptic plasticity alterations,

which lead to tolerance and withdrawal symptoms such as dysphoria, anxiety, and cognitive impairments upon cessation [61].

Moreover, nicotine modulates other neurotransmitter systems, including glutamatergic, serotonergic, and GABAergic circuits that influence mood and reinforcement [62]. In contrast, environmental triggers like specific places, sights, smells, or situations that a person has previously associated with nicotine use activate brain pathways, increasing cravings and the risk of relapse.

Beyond addiction, the harmful chemicals and carcinogens of cigarette smoke elicit disease primarily through inflammation and oxidative stress pathways. Irritants in the smoke provoke sustained inflammatory responses by activating immune cells and releasing pro-inflammatory cytokines, resulting in chronic tissue damage, particularly in the respiratory tract, contributing to conditions like COPD and lung cancer[63].

Concurrently, cigarette smoke generates excessive reactive oxygen species (ROS) that overwhelm endogenous antioxidant defenses, causing oxidative damage to lipids, proteins, and DNA. This oxidative stress drives cellular injury, apoptosis, and genetic mutations that facilitate carcinogenesis[64]. The synergistic effects of inflammation and oxidative damage compromise vascular endothelial function, accelerating atherosclerosis and elevating cardiovascular risk via enhanced platelet aggregation and thrombosis. Additionally, cigarette smoke dysregulates immune function by suppressing protective responses while perpetuating inflammatory cell recruitment, exacerbating tissue injury and remodeling, especially in pulmonary tissues.

These pathological processes extend systemically to disrupt hormonal and metabolic regulation, contributing to insulin resistance and type 2 diabetes. Together, the intertwined mechanisms of nicotine-driven addiction and smoke-induced inflammation and oxidative stress compose the fundamental pathological basis through which cigarette smoking exerts its broad and severe health consequences, reinforcing the imperative for comprehensive tobacco control and cessation strategies.

I. 1. 4. 3. Classes of compounds in cigarette smoke and their synthesis

Cigarette smoke toxic compounds are generated primarily through the processes of combustion and pyrolysis that occur during cigarette smoking[66]. A cigarette is composed mainly of shredded tobacco wrapped in paper, with a filter at the mouth end designed to reduce some particulate matter inhaled by the smoker. When a cigarette is lit, combustion takes place at the burning coal or "tip" where the temperature can reach up to approximately 900°C during a puff. Combustion is a high-temperature, oxygen-rich chemical reaction that fully oxidizes

components of tobacco, producing carbon dioxide, water vapor, and a range of combustion products, including many toxic gases[66].

Simultaneously, pyrolysis occurs in regions of the cigarette where oxygen is limited, either deeper inside the cigarette or during the smoldering phase between puffs. Pyrolysis is the thermal decomposition of organic material in the absence or near-absence of oxygen. It breaks down tobacco constituents into smaller, often volatile, molecules. This process produces a complex mixture of harmful chemicals, including polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNAs), aldehydes such as formaldehyde and acrolein, carbon monoxide, and numerous free radicals and reactive oxygen species[67].

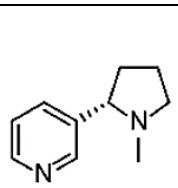
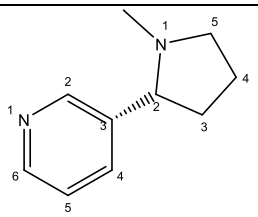
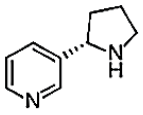
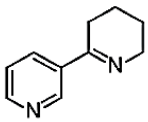
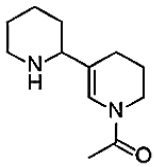
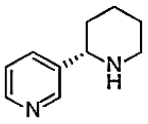
The cigarette's paper and filter influence airflow and combustion efficiency but cannot eliminate formation or inhalation of these harmful substances. Upon inhalation, this chemically complex aerosol delivers thousands of hazardous compounds directly to the respiratory tract and systemic circulation.

The combination of combustion and pyrolysis reactions is responsible for the generation of over 7,000 chemicals, many of which are known to be toxic and carcinogenic[68]. The composition can be broadly categorized into three main phases: gas phase, particulate phase, and vapor phase[69]. Here’s a detailed exploration of the key components:

a) Nicotine

Nicotine is the primary alkaloid and psychoactive component in cigarette smoke, responsible for addiction due to its stimulant effects on the central nervous system[70]. Table 1 summarizes the major alkaloids present in cigarette smoke, including their relative proportions and chemical structures. Nicotine is biosynthesized in tobacco plants through a complex pathway starting with the amino acids aspartic acid and nicotinic acid. This biosynthesis involves the conversion of asparagine into nicotinic acid, followed by multiple enzymatic reactions, including key methylation steps, that culminate in nicotine production. The presence of nicotine is central to the addictive properties of tobacco, driving continued use through its neuropharmacological impact[70].

Table 1: Alkaloid proportion and structure contained in cigarette smoke.

Major alkaloid 95%	 Nicotine  nicotine
Minor alkaloids 5%	 Nornicotine  Anabaseine  Ammodendrine  Anabasine

b) Major Carcinogenic Compounds

Polycyclic aromatic hydrocarbons (PAHs) and tobacco-specific nitrosamines (TSNAs) are key carcinogens in cigarette smoke known for their strong cancer-causing properties[67]. PAHs are formed during the incomplete combustion of organic material when hydrocarbons decompose at high temperatures, generating free radicals (Equ. 1) that recombine to create fused aromatic ring structures (Equ. 2) such as naphthalene and anthracene[71, 72].

$$RH \xrightarrow{\Delta,\,(a)} R^{\bullet} + H^{\bullet} \qquad (1)$$

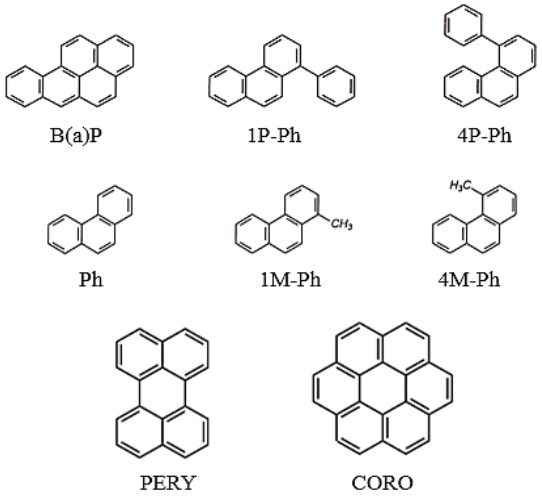
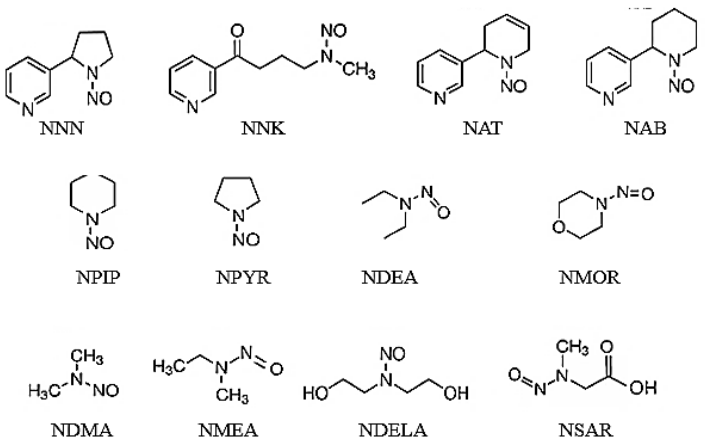
$$R^{\bullet} + R^{\bullet} \xrightarrow{(b)} PAH + H_2 \qquad (2)$$

R: Tobacco hydrocarbon (alkane, alkene, etc),
$R^{\bullet}$: Carbone-centred free radical,
$H^{\bullet}$: Hydrogen radical,
(a): Incomplete combustion,
(b): Recombination, cyclization.

TSNAs, on the other hand, are produced primarily during the curing process of tobacco, where nicotine and other alkaloids react with nitrites and nitrates from fertilizers. A typical reaction involves nicotine combining with nitrite to form TSNAs and water[73] as illustrated in the equation 3 below:

$$\text{Nicotine} + \text{Nitrite } (NO_2^-) \rightarrow \text{TSNAs} + H_2O \qquad (3)$$

Table 2: Chemical structure of some PAHs and TSNAs

PAHs	
TSNAs	

B[a]P (benzo[a]pyrene), 1P-Ph (1-phenylphenanthrene), 4P-Ph(4-phenylphenanthrene), Ph(phenanthrene), 1M-Ph (1-methylphenanthrene), 4M-Ph(4-methylphenanthrene) [74] CORO (coronene); PERY (perylene)[75], TSNAs [76], NNN (N-nitrosonornicotine), NNK (4-methylnitrosamino-1-3-pyridyl-1-butanone, NAT (N-nitrosoanatabine), NAB (N-nitrosoanabasine), NPIP (N-nitrosopiperidine), NPYR (N-nitrosopyrrolidine), NDEA (N-nitrosodiethylamine), NMOR (N-nitrosomorpholine), NDMA (N-nitrosodimethylamine), NMEA (N-nitrosomethylamin), NDELA (N-Nitrosodiethanolamine), and NSAR (N-nitrososarcosine).

c) Aldehydes

Formaldehyde and acrolein are aldehydes present in cigarette smoke, known for their irritant properties and associations with respiratory illnesses and carcinogenic effects[77].

formaldehyde is produced mainly during the combustion of organic matter. Acrolein is primarily generated by the pyrolysis of glycerol, which occurs naturally in tobacco or when glycerol is added as a sweetener[78]. The pyrolysis reaction can be represented as follows:

$$\text{Glycerol} \xrightarrow{\text{Heat}} \text{Acrolein} + H_2O \qquad (4)$$

d) Volatile organic compounds (VOCs)

Volatile organic compounds, such as benzene, toluene, and xylene, are organic chemicals that readily evaporate at room temperature and contribute significantly to air pollution and health hazards by causing respiratory irritation[79]. These compounds are formed during the incomplete combustion of organic materials in tobacco. High-temperature conditions cause fragmentation of complex hydrocarbons, leading to the generation of various VOCs[80].

e) Particulate matter (PM)

Tar is a complex mixture of solid particles and liquid condensates produced during tobacco combustion. It is a sticky substance that can coat the lining of the lungs, contributing to respiratory diseases and impaired lung function. Tar forms through the condensation of vaporized organic compounds generated in the high-temperature combustion process. As these vapors cool, they aggregate into particulate matter inhaled deep into the respiratory tract, where they accumulate and cause damage to lung tissue over time[81-83].

f) Heavy metals

Heavy metals such as lead (Pb), cadmium(Cd), arsenic(As), chromium (Cr), and nickel (Ni), which are toxic elements found in cigarette smoke, can cause serious health issues, including cancer and organ damage[84]. These metals are absorbed by tobacco plants from contaminated soil or introduced using fertilizers during cultivation[85].

g) Gases

Toxic gases such as carbon monoxide and hydrogen cyanide contribute to a wide range of health problems. CO is produced through the incomplete combustion of hydrocarbons and organic materials in tobacco[86], following reactions like:

$$C_xH_y + O_2 \rightarrow CO + H_2O + \text{Other products} \qquad (5)$$

Hydrogen cyanide forms primarily by the pyrolysis of nitrogen-containing compounds, especially proteins, during tobacco combustion[87], represented as:

$$\text{Proteins} \xrightarrow{\text{Heat}} HCN + \text{Various products} \qquad (6)$$

h) Phenolic compounds

Phenolic compounds such as catechol and cresol are harmful constituents of cigarette smoke known to irritate and damage the respiratory system. These compounds are formed through the thermal decomposition of lignin and cellulose, which are structural components of the tobacco plant. During combustion, the intense heat breaks down these complex plant polymers, producing phenolic compounds[88].

i) **Sugars and sweeteners**

Sugars like sucrose and corn syrup are added to tobacco products to improve flavor, but can produce harmful byproducts when combusted. During smoking, these sugars undergo thermal decomposition, generating toxic compounds such as acrolein that contribute to the adverse health effects of tobacco[89].

j) Additives and flavoring agents

Additives such as menthol and ammonia are used to enhance flavor and improve the smoking experience. Menthol can be obtained naturally or synthesized chemically, providing a cooling sensation, while ammonia is formed through chemical reactions involving nitrogenous bases and other compounds in tobacco. Ammonia also plays a role in increasing nicotine delivery, thereby intensifying addiction[90].

I. 2. Oxidative stress, inflammation, and the body's first line of defense

Oxidative stress and inflammation are fundamental biological responses central to maintaining homeostasis and protecting the body from harmful insults. Oxidative stress occurs when ROS generated during normal metabolic processes exceed the capacity of the body's antioxidant defense systems. These ROS, including free radicals, can damage cellular components such as lipids, proteins, and DNA, impairing cellular functions[91].

To prevent and control oxidative damage, the body relies on a robust first line of defense comprising enzymatic antioxidants like superoxide dismutase, catalase, and glutathione peroxidase, alongside non-enzymatic antioxidants such as vitamins C and E and glutathione. This system neutralizes ROS, maintains redox equilibrium, and supports cellular repair processes[92]. Simultaneously, the immune system orchestrates inflammatory responses to remove injured cells and pathogens and facilitate tissue healing[93].

Inflammation, when regulated, serves as a protective mechanism. Immune cells release cytokines and chemokines that coordinate the defense and repair[93]. However, excessive or chronic inflammation can contribute to tissue dysfunction and chronic disease. Importantly,

oxidative stress and inflammation are closely linked to ROS can activate inflammatory pathways, while activated immune cells generate more ROS, creating a feedback loop that amplifies cellular stress[94, 95].

I. 3. Impact of cigarette smoke and the protective role of medicinal plants

Medicinal plants have long been valued for their therapeutic properties, including their potential to mitigate the harmful effects of tobacco use. Tobacco smoke contains numerous toxic compounds that induce oxidative stress and inflammation, contributing to various diseases. Certain medicinal plants, rich in bioactive phytochemicals such as polyphenols, can counteract these damaging effects by enhancing antioxidant defenses and modulating inflammatory pathways. This makes them promising agents for protecting against tobacco-related health issues and aiding recovery from tobacco-induced damage (figure 2).

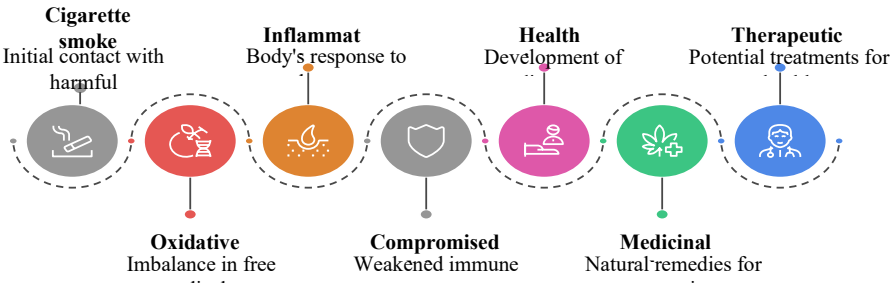


Figure1: Cigarette smoke impact and the protective role of medicinal plants.

Two notable examples are ginseng and turmeric. Ginseng contains saponins that activate the antioxidant pathway and inhibit inflammation, reducing oxidative damage and inflammatory responses induced by cigarette smoke[96]. Turmeric is rich in curcumin, known for its potent antioxidant and anti-inflammatory properties, which help scavenge free radicals and suppress pro-inflammatory mediators[97], offering protection against smoke-related lung injury and oxidative stress. These two examples underscore medicinal plants' significant potential in alleviating tobacco use's harmful effects. Moreover, the vast diversity of medicinal plants, rich in a wide array of bioactive phytochemicals, represents a fertile ground for ongoing and future research to develop effective interventions against tobacco-related health issues.

I. 4. Medicinal plants: bioactive constituents, extraction techniques, and mechanistic insights into biological activity

Medicinal plants have been valued across cultures for centuries due to their healing properties, forming the cornerstone of many traditional medicine systems. Their therapeutic effects arise from a broad spectrum of bioactive compounds, including alkaloids, flavonoids, terpenoids, phenolic acids, glycosides, tannins, saponins, coumarins, polysaccharides, lignans, steroids, fatty acids, anthraquinones, carotenoids, and quinones. These phytochemicals are typically extracted through various methods, from traditional approaches like maceration and infusion to modern techniques such as steam distillation, solvent extraction, cold pressing, and supercritical fluid extraction. Essential oils, rich in volatile terpenes obtained chiefly by steam distillation, exemplify the intricate chemical profiles contributing to aroma and medicinal properties.

The biological activities of these compounds play a critical role in the health benefits offered by medicinal plants. Notably, phenolic compounds, flavonoids, and others serve as potent antioxidants, neutralizing ROS and free radicals that cause oxidative stress, a key factor in aging and chronic diseases, through mechanisms like electron donation, metal ion chelation, and upregulation of endogenous antioxidant enzymes (e.g., superoxide dismutase, catalase)[98]. Beyond their use as antioxidants, many phytochemicals exert anti-inflammatory and cytoprotective effects by inhibiting pro-inflammatory mediators such as cytokines and prostaglandins and regulating signaling pathways including NF-κB and MAPK, thereby protecting tissues from damage caused by inflammation or toxins. Moreover, several plant-derived compounds modulate detoxification enzymes involved in phase I and phase II metabolism, such as cytochrome P450s, glutathione S-transferases, and UDP-glucuronosyltransferases, enhancing the body's ability to metabolize and eliminate harmful substances, reduce toxicity, and maintain cellular health[99, 100]. Integrating diverse chemical constituents with complex biological actions underscores medicinal plants' enduring importance and therapeutic potential in promoting physiological balance and preventing disease progression.

I. 4. 1. *Juniperus oxycedrus* L.

Juniperus oxycedrus, commonly known as prickly juniper or cade, is a Mediterranean evergreen shrub or small tree that thrives in rocky habitats from sea level upwards, including scrublands and open woodlands on sandy or rocky soils[101]. It is distinguished by sharp, needle-like leaves arranged in whorls of three, each typically displaying two white stomatal bands on their

surface an important feature for identification[102]. The plant produces reddish brown, berry like cones and belongs to the Cupressaceae family. Notable for its sharp-cedar characteristics, the species has historical ties to the ancient Greek concept of "cedar." *Juniperus oxycedrus* shows significant variability across its range, with subspecies such as *subsp. oxycedrus, subsp. badia*, and *subsp. macrocarpa*, whose distinctions are continually refined through ongoing research into their morphological, ecological, and phytochemical traits[102].

I. 4. 1. 1. Ecological significance

Juniperus oxycedrus L., widely distributed throughout the Mediterranean region, including Spain, Portugal, Italy, Greece, and North Africa, thrives in Mediterranean-plus seasonal climates that range from oceanic to continental conditions. It prefers rocky, steep, and well-drained soils where it often forms dense forests or scrublands, exhibiting a strong ability to colonize poor soils and act as a pioneer species within expanding edaphoxerophilous (soil-drought-tolerant) plant communities driven by the growth of rocky areas [103, 104]. Ecologically, *Juniperus oxycedrus* serves as a nurse plant facilitating the regeneration of woody species such as Quercus ilex by providing shade and enhancing soil conditions, a role especially critical in drought-prone and degraded environments [105, 106]. Its dense canopy also provides habitat and food for diverse wildlife, including birds, insects, and small mammals, contributing importantly to Mediterranean biodiversity [104, 106]. The species demonstrates notable adaptability to environmental stresses like drought, extreme temperatures, and parasitic infestations (e.g., Arceuthobium oxycedri); despite infestations potentially reducing its facilitative effects on other plants, *Juniperus oxycedrus* remains resilient, maintaining its vital ecological functions [103, 105].

In terms of conservation, *J. oxycedrus* is found within protected areas such as Lebanon's Horsch Ehden Natural Reserve and Spain's Arribes del Duero, highlighting its ecological importance[104, 105]. It is also part of the European Union's Natura 2000 network, underscoring its role in Mediterranean biodiversity preservation [104]. Despite its adaptability, the species faces threats from climate change, land-use changes, and overgrazing, which impair habitat quality and regeneration, especially in nutrient-poor soils[103, 106]. Conservation efforts focus on habitat protection, sustainable management, and international cooperation to secure long-term survival [103].

I. 4. 1. 2. Cultural and medicinal importance of *Juniperus oxycedrus* L.

Juniperus oxycedrus L. holds significant cultural and medicinal value in Mediterranean societies. Its essential oils and extracts have traditionally been employed to treat respiratory

infections, skin conditions, and digestive disorders due to their antimicrobial and anti-inflammatory properties [107, 108]. Beyond its medicinal use, the plant carries symbolic importance. It is often utilized in rituals and ceremonies for its aromatic qualities and perceived spiritual powers, representing longevity and resilience in harsh environments [105].

Phytochemically, *J. oxycedrus* is rich in bioactive compounds such as essential oils (notably α-pinene, sabinene, and limonene), flavonoids, terpenoids, tannins, and phenolic compounds, which confer potent antioxidant and anti-inflammatory effects that may aid in preventing and managing chronic diseases[107, 109, 110]. Its essential oils and extracts demonstrate robust antimicrobial activity against a broad spectrum of pathogens, including gram-positive and gram-negative bacteria as well as fungi, offering a promising natural alternative amid rising antibiotic resistance[108, 111, 112]. Furthermore, recent research highlights its anti-proliferative and cytotoxic effects on cancer scell lines such as lung and breast cancers, suggesting potential use in anticancer drug development[19, 113].

I. 4. 2. *Juniperus phoenicea*

Juniperus phoenicea, commonly known as the Phoenician juniper, is an evergreen species within the Juniperus genus of the cypress family (Cupressaceae). It typically grows as a shrub or small tree and is characterized by its scale-like adult leaves, a trait common to junipers. Primarily monoecious, it bears both male and female reproductive structures on the same plant, though it can occasionally be dioecious. *J. phoenicea* is part of a complex including *J. turbinata* and *J. canariensis,* species that share similar morphological features making visual differentiation challenging; however, they can be reliably distinguished through genetic markers such as isozymes and nuclear microsatellites[114].

Geographically, *J. phoenicea* is native to the western Mediterranean region, whereas J. turbinata has a wider circum-Mediterranean range, and *J. canariensis* is restricted to the Canary Islands and Madeira archipelago[114].

I. 4. 2. 1. Ecological significance

Juniperus phoenicea plays a vital role in Mediterranean and arid ecosystems due to its remarkable adaptability to harsh environments, including drought and poor soils[114]. This evergreen gymnosperm contributes significantly to soil stabilization and erosion prevention, particularly in mountainous and arid regions where it supports dense vegetation with associated sub-shrubs and annual plants [115]. As a key species within diverse plant communities, *J. phoenicea* provides critical habitat that sustains biodiversity[116, 117]. Its ecological resilience is further demonstrated by its ability to thrive on nutrient-poor limestone outcrops and withstand

browsing and human disturbance, underscoring its importance as a stable and adaptive component of Mediterranean ecosystems [115].

I. 4. 2. 2. Cultural and medicinal importance of *Juniperus phoenicea*

Juniperus phoenicea holds significant cultural importance across Mediterranean and arid regions, having been traditionally used for centuries in medicine and daily life. it is valued for its therapeutic properties, treating ailments such as digestive disorders, skin infections, and respiratory conditions through preparations like infusions and decoctions[118, 119]. Beyond medicinal uses, *J. phoenicea* carries symbolic meanings in many Mediterranean cultures, often associated with purification and protection rituals, while its durable wood has historically been employed in traditional construction and craftsmanship. Historically, it served as a source of fuel, food, and medicine, reflecting its integral role in shaping cultural and economic practices. Modern scientific research has validated many of these traditional claims, revealing *J. phoenicea's* essential oils possess notable anti-inflammatory, antibacterial, antioxidant, and antifungal activities, largely attributed to its bioactive constituents such as volatile oils, flavonoids, and terpenes, which underscore its medicinal value[120, 121].

CHAPTER II

MATERIAL AND METHODS

II. 1. PLANT MATERIAL

II. 1. 1. *Juniperus oxycedrus* L and *Juniperus phoenicea*.

The berries of *Juniperus oxycedrus* L. subsp. *oxycedrus* (JOX) and *Juniperus phoenicea* (JPH) (Figure 2) were collected from northeastern Algeria (Figure 3), specifically in the Djebel Doukane region of Tebessa province. The collection site is at 35°19'20"N latitude and 8°01'50"E longitude, at 1417 meters. Two separate harvests were carried out. The first, encompassing both JOX and JPH berries, occurred at the end of December 2020 for essential oil extraction; the second, involving only JOX berries, was conducted in May 2022 to obtain ethanolic extracts.

Figure 2: Collected *Juniperus oxycedrus* L. subsp. *oxycedrus* (JOX) and *Juniperus phoenicea* L. (JPH).

Dr. Soraya Hioun from the Laboratory of Applied Chemistry and Renewable Energies (LACRE) at Echahid Cheikh Larbi Tebessi University in Tébessa, Algeria, conducted the plant's identification.

Figure 3: Geographic map of the *J. oxycedrus* and J. *phoenicea* harvest region.

All harvested samples were carefully packaged in clean, non-contaminating sacks and dry boxes and transported in a manner that ensured their integrity. Each plant sample container was

properly labeled, with detailed records maintained for batch packaging. These records included the plant's scientific name, the specific plant part collected, the place of origin (collection site), the date of collection, and quantitative data.

For this study, the plant material needed to remain fresh. Therefore, the collected plant materials were stored at appropriately low temperatures, maintained below 4°C, to ensure optimal preservation.

II. 1. 2. Nicotiana tabacum

Tobacco, specifically Nicotiana tabacum, was extracted from RYM cigarettes (**Figure 4**), a brand produced in Algeria by the National Society of Tobacco and Alcohol (SNTA). Several unspecified varieties of black tobacco distinguish this kind of cigarette. The purpose of this study is to investigate this particular brand of tobacco and cigarette, which presents a serious health risk to Algerian smokers.

Figure 4: RYM cigarettes.

II. 2. EXTRACTION EXPERIMENTS

II. 2. 1. JOX and JPH essential oils distillation

The hydrodistillation technique using a "Clevenger" type device is a well-established method for extracting essential oils from plant material. It involves using heat and water to produce steam, which then passes through the plant material and condenses, separating the essential oils from the plant material.

The extraction of JPH and JOX berry essential oils (EOs) using hydrodistillation involves setting up a hydrodistillation apparatus (Figure 5), consisting of a two-liter glass flask, a heating mantle, a 50 cm vertical tube combined with a refrigerant, and a receiving bulb filled with

distilled water, ensuring all parts are clean and properly assembled. Then, 200 g of freshly ground JOX berries are weighed and placed in the flask, followed by the addition of distilled water (1000 mL). The flask is heated on the mantle, maintaining a gentle boil so that steam passes through the plant material, carrying the essential oil vapors. These vapors travel through the condenser, where they cool and condense into a liquid that separates into an aqueous phase (aromatic water) and an organic phase (EO). Due to its lower density, the essential oil rises to the surface and can be collected using a separatory funnel or by decanting. The collected oil is then dried with anhydrous sodium sulfate and stored in sealed brown glass bottles at 4°C until further use. The entire hydrodistillation process typically lasts around three hours to achieve optimal extraction [122].

Figure 5: Clevenger Apparatus used for Hydrodistillation

II. 2. 2. The extraction of JEE by ultrasound-assisted extraction (UAE)

To perform the UAE method, the following steps were taken: the extraction was carried out using an ultrasonic bath (YAXUN YX-2050) (Figure 6) with a power of 50 W and a thermostatic function to control the temperature. The UAE was conducted at a frequency of 40 kHz, with the temperature maintained between 22-30°C. These conditions were selected based on the available resources of the ultrasonic bath within the operational limits of the equipment, and aligned with the approaches documented in the literature for extracting phenolic compounds [123].

Approximately 50 g of the dry powder of JOX berries was mixed with 200 mL of the extracting solvent (200 g of plant is the total mass used), which was a 90% ethanol solution in water (v/v). The mixture was then sonicated for 40 minutes, followed by maceration for 24 hours at ambient temperature. The obtained extract was then evaporated to dryness under vacuum at 40°C and stored in sterile glass bottles at 4°C until further use.

Figure 6: Ultrasound-assisted extraction.

II. 2. 3. Conventional extraction (CE) of JOX ethanolic extract

Dry berries of JOX (200g) were extracted by exhaustive maceration (500 mL, 4 x 72 h) using ethanol (90%) as a solvent. The JOX extract was obtained after removing the extraction solvent at reduced pressure. The dried extract was kept in a brown bottle at 4°C before analysis.

II. 2. 4. Yield of essential oil and JOX ethanolic extracts

The yields of essential oil and ethanolic extracts of JOX were expressed in g relative to 200 g of dry vegetable matter; it was calculated according to formula (1):

$$\text{Yield (\%)} = \frac{\text{Amount of extracted oil or extracts (g)}}{\text{Amount of dry vegetal matter mass (g)}} \times 100 \qquad (01)$$

II. 3. EXTRACTS CHEMICAL COMPOSITION

II. 3. 1. Total phenolic contents (TPC)

The phenolic or polyphenol compounds in nearly all plant products possess an essential antioxidant power [123]. However, there is no method to accurately and simultaneously dose all the phenolic components in a non-purified plant extract. However, the phenolic content in total phenolic compounds (TPC) can be estimated via a colorimetric dosage using the Folin-Ciocalteu (FC) reaction. This reaction, composed of a mixture of phosphomolybdate and phosphotungstate, allows for quantifying the phenolic compounds in the plant extract [124].

- **Method**

The amount of phenolic compounds in the JOX extracts was verified according to Folin-Ciocalteau's method [124]. Briefly, the plant extracts were weighed appropriately and dissolved in EtOH (75 %) to a 5 mg/mL concentration. A calibration curve was established using gallic acid, dilutions. Then, the extracts and gallic acid dilutions were blended with 30 µL of Folin-Ciocalteau's reagent (diluted × 2, Sigma, USA) and 150 µL of sodium carbonate (3.5%) in microplate wells and then incubated at 40 ◦C for 30 min. Afterward, absorption was measured

at 765 nm at an ELISA microplate reader. The total phenolic contents of the JOX extracts were reported as gallic acid equivalent (mg/g extract).

II. 3. 2. Determination of total flavonoid contents (TFC)

Flavonoids are water-soluble, yellow, or colored polyphenolic compounds produced by plants. The aluminum chloride colorimetric method is used to determine the total flavonoid content in the JOX extracts [124]. This method's principle involves forming a yellow stable complex with the C-4 keto group and the C-3 or C-5 hydroxyl group of flavones and flavonols [125]. These complexes cause a shift in the absorption spectrum of the flavonoids, which can be measured spectrophotometrically. A standard curve is generated using a known flavonoid compound, like quercetin to quantify the total flavonoid content.

- **Method**

In summary, 25 µL of JOX extract or quercetin dilutions were combined with 75 µL 95% EtOH, 5 µL of 10% aluminum chloride reagent, 5 µL of 1 M sodium acetate, and distilled water. After 30 minutes of incubation at room temperature, the absorbance of the mixtures was measured at a wavelength of 415 nm using an ELISA microplate reader to determine the total flavonoid content. The results were reported as quercetin equivalent (mg Q /g ext) [124].

II. 3. 3. Estimation method

Quantification of TPC and TFC in two different dosages was determined using the equivalent concentration method, with results expressed in mg equivalent gallic acid and quercetin per gram of extract (mg EG/g ext; mg EQ/g ext). The values represent the mean of three replicates (n=3) ± standard deviation and were calculated using the formula:

$$X = \frac{C.V.DF}{W} \qquad (02)$$

where:

X= TPC or TFC,

C = Concentration (ppm),

V = Volume of sample solution (extract) (mL),

DF = Dilution factor of sample solution,

W = Sample weight (g).

II. 3. 4. LC-ESI-MS/MS analyses

A Shimadzu-Nexera model ultrahigh performance liquid chromatography (UHPLC) coupled with a tandem mass spectrometer was used to evaluate 53 phytochemicals quantitatively. The reversed-phase UHPLC was equipped with an autosampler (SIL-30AC model), a column oven (CTO-10ASvp model), binary pumps (LC-30AD model), and a degasser (DGU- 20A3R model). The chromatographic conditions were optimized to achieve optimum separation for 53 phytochemicals and overcome the suppression effects. Different coluımns such as Agilent Poroshell 120 EC-C18 model (150 mm×2.1 mm, 2.7 µm) and RP-C18 Inertsil ODS-4 (100 mm×2,1 mm, 2µm), different mobile phases (B) such as acetonitrile and methanol, different mobile phase additives such as ammonium formate, formic acid, ammonium acetate, and acetic acid, different column temperatures such as 25°C, 30°C, 35°C and 40°C were tried and applied until the optimum conditions were achieved. Consequently, the chromatographic separation was performed on a reversed-phase Agilent Poroshell 120 EC-C18 model (150 mm×2.1 mm, 2.7 µm) analytical column. The column temperature was set to 40°C. The elution gradient comprised eluent A (water+5 mM ammonium formate+0.1% formic acid) and eluent B (methanol+5 mM ammonium formate+0.1% formic acid). The following gradient elution profile was used: 20-100% B (0-25 min), 100% B (25-35 min), 20% B (35-45 min). Furthermore, the solvent flow rate and injection volume were settled as 0.5 mL/min and 5 µL, respectively.

The mass spectrometric detection was done using a Shimadzu LCMS-8040 model tandem mass spectrometer equipped with an electrospray ionization (ESI) source operating in negative and positive ionization modes. LC-ESI-MS/MS data were acquired and processed by LabSolutions software (Shimadzu). The MRM (multiple reaction monitoring) mode was used to quantify the phytochemicals. The MRM method was optimized to selectively detect and quantify phytochemical compounds based on screening specified precursor phytochemical-to-fragment ion transitions. The collision energies were optimized to generate optimal phytochemical fragmentation and maximal transmission of the desired product ions. The MS operating conditions were applied as follows: drying gas ($N_{2)}$ flow, 15 L/min; nebulizing gas (N_2) flow, 3 L/min; DL temperature, 250°C; heat block temperature, 400°C, and interface temperature, 350°C.

II. 3. 5. GC/MS analyses

The JOX berries EO obtained by hydrodistillation were analyzed by a team of expert researchers using gas chromatography and mass spectrometry (GC/MS). The analysis of this

EO was performed using a gas chromatograph "Hewlett Packard Agilent 6890" coupled with a quadrupole mass spectrometer "Hewlett Packard Agilent 5973". The GC/MS spectra were recorded for 139 minutes on a gas chromatograph with a split injector, equipped with a column (HP-5MS) with a length of 30 m and an internal diameter of 0.25 mm. The film thickness was 0.25 µm with a stationary phase composed of 5% Phenyl and 95% dimethylpolysiloxane. The carrier gas (Helium) used was N6 with a flow rate of 0.5 mL/min; the oven temperature was 60°C for 8 minutes with a heating rate of 2 °C/min and was then maintained at 250°C isothermal for 10 min. The mass spectrometer analysis mode was a TIC scan (From 30 to 550) with an ion trap and an electron ionization of 70 eV. The interface temperature was 270°C, and the source temperature was 230°C.

II. 4. Effects of essential oils on nicotine content

Nicotine is the main psychoactive compound in cigarettes and drives tobacco dependence, posing health risks to smokers and to non-smokers exposed through secondhand smoke [59]. Ongoing research seeks to reduce nicotine's harmful effects, including natural approaches for lowering nicotine levels. This study evaluates, in vitro, the effectiveness of selected junipers essential oils in reducing nicotine levels.

II. 4. 1. Nicotine extraction from tobacco

II. 4. 1. 1. Method selection and principal

Nicotine can be extracted from tobacco leaves using various methods, including acid-base extraction and maceration. These methods can produce different yields and nicotine contents, affecting the extracted nicotine's quality. Hydro-distillation method is preferred because it produces a purer nicotine extract and saves energy and solvents. However, the classic method is simple and easy to implement, but it produces nicotine that is far from pure. In this investigation, the objective and working conditions determine the hydro-distillation method, which effectively extracts nicotine because nicotine is a volatile alkaloid soluble in polar and nonpolar solvents and can be easily carried away by water vapor.

The principle of this method is that the tobacco leaves are brought into contact with a fixed base, such as sodium hydroxide, to convert the nicotine into a nicotine base. Then, the basic nicotine is carried away by the water vapor. After condensation, the nicotine separates from the aqueous part using a nonpolar organic solvent by decantation[126].

II. 4. 1. 2. Procedure

To perform this procedure, first weigh 10 g of tobacco from RYM cigarettes and prepare 200 mL of a 30% aqueous NaOH solution. Combine the tobacco and NaOH solution in a 500 mL flask. Then, subject the mixture to rotary evaporation under reduced pressure at 100 mbar and a temperature of 70 °C. Continue the distillation for 20 minutes to allow condensation, after which the distilled solution should be collected. To extract nicotine from the distilled solution, perform three successive washes using 25 mL portions of dichloromethane (DCM). After washing, add a small amount of anhydrous sodium sulfate to the organic phase to remove residual water and dry the solution. Finally, filter the dried organic layer and evaporate the filtrate under a vacuum to obtain a yellowish nicotine oil [126].

II. 4. 2. Analyzing extracted nicotine by spectrophotometry method

- **Extracted nicotine analysis**

UV-visible spectrophotometry is an analytical technique used to identify and quantify chemical compounds, particularly useful for analyzing nicotine, a key component of tobacco. The samples are fed into the spectrophotometer, exposing them to UV and visible light. The light absorption is then measured, and a spectrum is produced, showing the peaks and troughs of the light absorbance. This data is then compared to a standard nicotine solution to determine the nicotine concentration in the sample.

The light absorption is measured, and a spectrum is generated. The peak at 260 nm corresponds to the absorbance of nicotine, and its intensity is directly proportional to the nicotine concentration. This peak is then compared to the absorbance of a standard nicotine solution to determine the nicotine concentration[127].

II. 4. 2. 1. Sample preparation

The nicotine extract stock solution (20 µg/mL) (A) was prepared by dissolving 2 mg in distilled water and completing the volume to 100 mL. A solution (B) with a concentration of 12 µg/mL was generated from this solution. Stock solutions (C1-C2) were prepared by dissolving two µl of EOs (JOX and JPH) in 100 mL of acetonetric solution. The essential oils samples were prepared from stock solutions (C1-C2) and diluted to concentrations ranging from 3.8 to 19 µg/mL. These solutions and solution B were combined with the (v:v) ratio, thoroughly mixed, and then left at room temperature for 15 minutes before measuring the nicotine concentration.

II. 4. 2. 2. Nicotine dosage by UV spectrophotometry

The method of Al-Tamrah [128] was adopted for the dosage of nicotine with modifications. To a 5 mL test tube, introduce exactly 200 µL of potassium permanganate ($MgSO_4$, 2.08 x 10^{-3} M), followed by 400 µL of NaOH (5 M), swirl gently to mix, and add 3.4 mL of each sample, including the blank. Make up the volume to 5 mL with distilled water, shake well, and measure the absorbance at 610 nm after 180 seconds.

Remark: 3.4 mL is the volume of Dx solutions (solutions of nicotine with different EOs concentrations, the standard (solution B/acetonitrile, v:v), or the blank (acetonitrile/distilled water, v:v). A calibration curve using pure nicotine is prepared under the same conditions as the samples to be assayed, with different concentrations between 2 and 20 µg/mL. The following formula expresses the ability to reduce or inhibit nicotine:

$$\text{Inhibition } \% = \frac{\text{Abs C} - (\text{Abs S} - \text{Abs E})}{\text{Abs C}} \text{X100} \qquad (03)$$

where:

Abs C: Absorption of the control (the nicotine solution without the EO).

Abs E: Solution absorption when just the EO is present.

Abs S: The amount of nicotine solution that is absorbed in the presence of the EO.

The experiments were carried out in triplicate. We determined the IC50 value, which is the substrate concentration that causes the loss of 50% of the activity of the nicotine.

II. 5. EFFECT OF JOX-EO ON PAHS IN CIGARETTE SMOKE

II. 5. 1. Extraction and purification of PAHs

Extracting polyaromatic hydrocarbures (PAH) involves chromatographic and spectrophotometric methods. Waltz's method and Alice's base for extraction are used [129, 130]. The collected smoke is subjected to separation operations, non-miscible phase sharing, and liquid column chromatography to analyze the final products obtained through UV-Vis spectroscopy.

II. 5. 1. 1 Cigarette smoke collection

A hexane and ethanol (2:1, v:v) solution was used to extract chemicals contained in cigarette smoke, including PAHs. To allow flexibility in adjusting smoking parameters, mainstream cigarette smoke was generated at a rate of seven puffs per cigarette. The smoking process followed a modified version of the CORESTA puffing regime (Figure 7), where each puff lasted 2 to 3 seconds, with an interval of one puff every 30 seconds and a puff volume of 50 mL.

In this setup, smoke equivalent to 1073 puffs corresponding to approximately 150 cigarettes, was bubbled through 150 mL of (Hexane-ethanol) (2:1, v:v). The device maintained the fundamental principles of cigarette pyrolysis and combustion, enabling efficient recovery of the majority of chemicals present in cigarette smoke.

Figure 7: Cigarette smoke collection system.

II. 5. 1. 2. PAHs liquid-liquid extraction

In a separating funnel, 50 mL of distilled water was added to 150 mL of the organic cigarette smoke extract solution. After thorough stirring, the organic and aqueous phases were allowed to separate. The lower aqueous phase was then washed three times with 25 mL portions of hexane. The combined hexane extracts, containing the polycyclic aromatic hydrocarbons (PAHs), were collected and evaporated at 40°C.

II. 5. 1. 3. PAHs purification

Column chromatography was employed to purify the extracted PAHs. A suspension was prepared by mixing 20 g of silica gel with 250 mL of a hexane-dichloromethane (1:1, v:v) solvent mixture, which was then packed into a column with a diameter of 2 cm. The mobile phase, consisting of 250 mL of the same hexane-dichloromethane mixture, was eluted through the column at a flow rate of 30 mL every 25 minutes. Fractions of 8 mL each were collected in small, labeled brown glass vials. The collected fractions were subsequently analyzed using UV spectroscopy to identify the presence of PAHs.

II. 5. 2. Pre-treatment of PAHs with essential oil

All collected PAH fractions were divided into two portions: one served as the control, while the other was treated with the essential oil. To each 4 mL fraction, 2.5 µL of JOX-EO was added. The mixtures were then gently stirred for 15 minutes in the dark before being analyzed by UV-Vis spectrophotometry.

II. 5. 3. Spectrophotometric analysis

UV analysis was performed using a Shimadzu UV-1700 Pharmaspec spectrophotometer equipped with 10-mm quartz cuvettes. Spectra were recorded for each PAH fraction before and after treatment with JOX essential oil, which was dissolved in a hexane-dichloromethane mixture (1:1, v:v). The same hexane-dichloromethane mixture was used as the blank. Absorbance spectra were collected over the wavelength range of 200 to 400 nm.

II. 6. In vitro biological essays

II. 6. 1. Chemical tests determining antioxidant activity

This investigation focused on evaluating the antioxidant activity of JOX EO and extract using multiple established methods. The primary goal was to accurately measure their anti-radical properties through assays such as DPPH• free radical scavenging, ferric reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC), and nitric oxide (NO) scavenging activity. These techniques collectively assess the ability of the compounds to neutralize free radicals and reduce metal ions, providing a comprehensive profile of their antioxidant potential

II. 6. 1. 1. DPPH radical scavenging activity assay

- **Principle**

The DPPH• stable free radical is known for its electron delocalization, which results in a characteristic purple color with a maximum absorption peak around 517 nm. However, with the addition of a hydrogen donor, such as antioxidants, the DPPH• reacts and produces DPPH-H or DPPH-R, causing the violet color to disappear. The intensity of this color is directly related to the antioxidant concentration in the solution, making it a useful tool for determining the antioxidant capacity of different materials through spectrophotometry [131].

As illustrated in the Figure 8 below, in the presence of hydrogen donors (HA) in antioxidants, the DPPH• radical is reduced to a non-radical form DPPH-H (2,2-diphenyl-1 picrylhydrazine) and as a result, its initial color turns yellow, which leads to a decrease in its absorbance value.

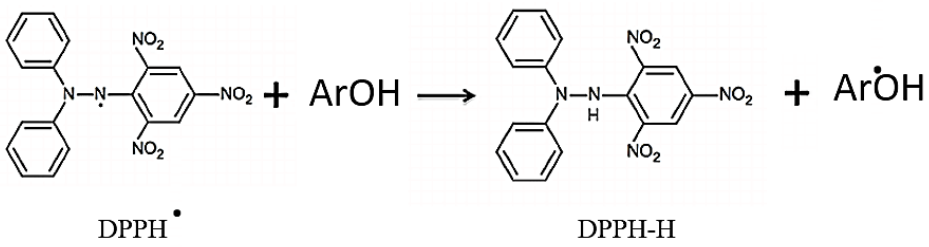


Figure 8: 2,2-diphenyl-1picrylhydrazyl radical (DPPH•) is reduced to DPPH-H by using the antioxidant phenol ArOH as a hydrogen donor.

- **Method**

To assess the stable DPPH radical scavenging activity, the described procedure [132] was applied. In a 96-well plate, 10 µL of the samples or reference dissolved in 96% EtOH were added, followed by 90 µL of a prepared DPPH solution in EtOH (1.5×10^{-4} M) using a reliable multichannel pipette (Eppendorf Research, Germany). After incubation at 37°C for 30 minutes, the remaining DPPH quantity was measured at 515 nm using spectrophotometry on an ELISA microplate reader (Molecular Devices, Spectramax i3, USA), and the results were compared to quercetin results, which served as a reference.

- **Data analysis for DPPH assays**

The DPPH radical scavenging activity of JOX extracts, EO, and the reference standard was evaluated and expressed as percent inhibition (I%) using the following formula:

$$I\,\% := \left(\frac{Ablank - Asample}{Ablank}\right) \times 100 \qquad (04)$$

A_{blank} indicates absorbance of the control reaction,

A_{sample} represents absorbance of the samples /references.

(The bioassays were) the measurements were carried out in triplicate, and the results were expressed as means ± standard deviations (SD).

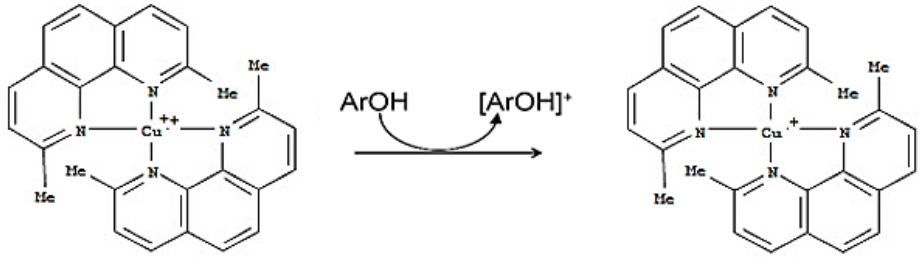
II. 6. 1. 2. Cupric-reducing antioxidant capacity (CUPRAC)

The CUPRAC assay measures total antioxidant capacity by reducing cupric (Cu^{2+}) to cuprous (Cu^{+}). It uses a ligand, neocuproine (Nc; 2,9-dimethyl-1,10-phenanthroline), to form a copper-ligand complex for absorbance measurement. This method applies to various matrices with lipophilic and hydrophilic antioxidants. The Cu^{2+}–neocuproine (Cu^{2+}–Nc) reagent, used as a chromogenic oxidizing agent, determines plant extracts' antioxidant capacity. The chromogenic oxidant reagent reacts with n-electron-reducing antioxidant substances (AOX),

yielding a Cu+ complex with a maximum absorption peak at 450 nm[133]. As shown in Figure 9, ArOH represents the antioxidant that donates electrons to reduce Cu^{2+}–Nc to the coloured Cu^{+}–Nc complex.

The following equation [132] illustrates the reaction between the Cu2+–Nc reagent and n-electron-reducing antioxidant compounds (AOX):

$n\mathrm{Cu(Nc)_2}^{2+}$ + *n*-electron reductant (AOX) $\rightleftarrows$ $n\mathrm{Cu(Nc)_2}^{+}$ + *n*-electron oxidized product + $n\mathrm{H}^{+}$



Copper (II) complex Copper (I) complex

Figure 9: Reaction scheme involved in Cupric Reducing Antioxidant Power (CUPRAC) assay. ArOH represents an antioxidant.

- **Method**

The CUPRAC of JOX extracts and the reference compound gallic acid were tested using this method [125]. A mixture of 25 µL of 10 mM copper (II) chloride, 25 µL of 7.5 mM neocuproin, 25 µL of ammonium acetate (1 M, pH 7), 25 µL of the extract being tested, and 100 µL of distilled water was prepared in a 96 well microplate. This mixture was then incubated for 30 minutes. At the end of the incubation, the absorbance was measured using an ELISA microplate reader at a wavelength of 450 nm. The findings were expressed as the mean ± S.D. of the absorbances from three parallel tests.

II. 6. 1. 3. Ferric reducing antioxidant power (FRAP) test

The FRAP test is a method based on a single electron transfer used to measure the antioxidant content in samples, measuring the reduction of ferric ions (Fe^{3+})-ligand complex to the intensely blue ferrous complex (Fe^{2+}) in acidic environments [133]. The test follows the protocol described by Oyaizu, renowned for its simplicity, speed, and reproducibility. In this assay, the presence of reducing compounds in the sample causes the reduction of ferric iron (Fe^{3+}) in potassium ferricyanide to ferrous iron (Fe^{2+}) under acidic conditions provided by trichloroacetic acid. Upon addition of $FeCl_3$, a blue complex known as Perl's Prussian blue forms, which can be quantitatively measured at 700 nm using UV-Vis spectrophotometry.

Higher absorbance values correspond to greater reducing power of the sample, directly reflecting its antioxidant capacity [134].

The principle of this test is based on the following chemical reactions [132]:

$$ArOH + Fe(CN)_6^{3-} \rightleftarrows Fe(CN)_6^{4-} + [ArOH]^+$$

$$Fe(CN)_6^{4-} + Fe^{3+} \rightleftarrows Fe\,[Fe(CN)_6]^-$$

ArOH represents a phenolic antioxidant, and $[ArOH]^+$ represents an oxidized phenolic antioxidant.

- **Method**

The ferric-reducing antioxidant power (FRAP) of samples was tested using the described method [125], where 10 µL of extracts and reference (quercetin) dissolved in ethanol were added to 25 µL of phosphate buffer (pH 6.6) and 25 µL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was pre-incubated for 20 minutes at 50 °C, after which 25 µL of 10% trichloroacetic acid, 85 µL of distilled water, and 17 µL of 0.1% $FeCl_3$ were sequentially added. The resulting solution was then incubated for an additional 30 minutes at room temperature, and the absorbance was measured at 700 nm using an ELISA microplate reader (Versamax tunable microplate reader, USA). The entire experiment was performed in triplicate to ensure accuracy and reproducibility.

II. 6. 1. 4. Nitric oxide (NO) scavenging activity

The principal method used to measure nitric oxide (NO) scavenging activity involves generating NO using sodium nitroprusside (SNP) and then measuring the reduction in NO levels using the Griess reagent. SNP spontaneously generates NO in an aqueous solution at physiological pH, producing nitrite ions through its interaction with oxygen. The Griess reagent, which contains sulphanilamide, naphthylethylenediamine dichloride (NED), and phosphoric acid, is then used to measure the nitrite levels spectrophotometrically at 546 nm. The test compound or plant extract is incubated with the SNP-generated NO, and the scavenging of NO by the test compound leads to a reduction in the nitrite levels, as measured by the Griess reagent. The percentage inhibition or scavenging of NO is calculated by comparing the absorbance of the test sample (Ae) to the control (Ac) through the following formula [125].

$$\text{NO Scavenged (\%)} = \frac{(Ac - Ae)}{Ac} * 100 \qquad (05)$$

- **Method**

The method used to assess the NO scavenging activity of JOX extracts was based on the following method [125]. In 96-well microplate wells, 60 µl of each sample and ascorbic acid (reference) dilutions were prepared and added to the wells along with 60 µl of sodium nitroprusside (10 mM). The mixture was incubated for 150 minutes at room temperature. Then 120 µL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride) was added to each well. The absorbance of the mixture was measured at 577 nm using an ELISA microplate reader. Experiments were done in triplicates, and the results were presented as average values with standard deviation.

II. 6. 2. Enzyme inhibitory activity assay

Enzyme inhibitors are essential for cellular regulation and pharmacology, as they control metabolic pathways and respond to environmental changes [135]. Cigarette smoke has complex effects on enzyme activity, inducing some and inhibiting others, impacting drug metabolism and effectiveness. Understanding these dynamics is crucial for drug design and therapeutic interventions. This study focuses on enzyme inhibition assays like Butyrylcholinesterase (BCHE), Xanthine Oxidase (XO), and Lipoxygenase (LOX) inhibitions to evaluate the enzyme inhibition potential of JOX extracts.

II. 6. 2. 1. Inhibition of cholinesterase activity

The Ellman method, a versatile and reliable tool, is known for its precision in measuring cholinesterase activity in various biological samples in vitro. Its principle is based on the use of the substrate acetylthiocholine or butyrylthiocholine and the chromogenic reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). When the cholinesterase enzyme (either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE)) hydrolyzes the substrate, it produces thiocholine, one of the products. The thiocholine then reacts with DTNB, producing a yellow-colored 5-thio-2-nitrobenzoate anion. The increase in absorbance of this yellow color at 412 nm is directly proportional to the rate of enzymatic hydrolysis, allowing for the quantification of cholinesterase activity [136]. The precision of this method instills confidence in its use, making it a valuable tool in the field of biochemistry and pharmacology.

- **Method**

The BChE inhibitory activity of the JOX extracts was evaluated using the Ellman et al. modified method [137] in a 96-well microtiter plate. The assay involved adding 140 µL of sodium phosphate buffer (0.1 mM, pH=8.0), 20 µL of the JOX extracts, ethanol (negative

control), the reference compound galantamine (positive control), and 20 µL of 0.2 BChE solution (0.003 units/well), followed by incubation at room temperature for 10 minutes. The reaction was initiated by adding 10 µL of 0.2 M butyrylthiocholine chloride as the substrate and 10 µL of DTNB. The absorbance of the solution was then measured at 412 nm. The percentage of inhibition (I%) was calculated by comparing the rates of enzyme reaction between the samples and the blank sample using the formula 06. The results were reported as IC50 values (µg/mL).

$$\text{I}\,(\%) = \left(\frac{\text{E}-\text{S}}{\text{E}}\right) X100 \qquad (06)$$

II. 6. 2. 2. Xanthine oxidase (XO) inhibition assay

The XO inhibition assay is a widely used method to evaluate the ability of compounds or extracts to inhibit the activity of the XO enzyme, which is a crucial enzyme involved in the catabolism of purines, catalyzing the stepwise oxidation of hypoxanthine to xanthine and then uric acid. The assay works by measuring uric acid's formation, the XO-catalyzed reaction's product, in the presence and absence of the test compound. Specifically, the assay involves preparing a reaction mixture containing the XO enzyme, the substrate xanthine, and a buffer solution, adding the test compound (e.g. plant extract, purified compound) to the reaction mixture, monitoring the increase in absorbance at 295 nm, which corresponds to the formation of uric acid over time [138, 139], and calculating the percentage inhibition of XO activity by the test compound compared to a control reaction without the inhibitor using the formula 07. The degree of XO inhibition is then used to determine the potency of the test compound, often expressed as the IC50 value.

$$\text{Inhibition}\% = 100 - \left[\left(\frac{\text{A1}}{A2}\right) X\ 100\right] \qquad (07)$$

A1= Absorbance of the sample solution

A2= Absorbance of the control solution

- **Method**

The XO inhibition assay was performed using the reported method [140]. 150 µL of 0.1 M phosphate buffer (pH 7.4), 10 µL of DMSO (as a negative control) or the JOX extracts, and 20 µL of the enzyme (0.003 units/well) were combined in the wells. After a 10-minute pre-incubation, 20 µL of xanthine (0.1 mM) was added as the substrate, and the absorbance at 295 nm was measured for 10 minutes using an ELISA microplate reader. The reference drug, allopurinol, was also included in the assay. All samples were tested in triplicate, and the results

were expressed as mean ± standard deviation (S.D.) of % inhibition from three independent experiments.

II. 6. 2. 3. Lipoxygenase (LOX) inhibitory activity assay

The soybean lipoxygenase inhibition assay was performed according to the procedure described previously by Jean-Paul Dzoyem [141]. This method is based on the 15-lipoxygenase (15-LOX) catalyzed reaction between oxygen and a polyunsaturated fatty acid, such as linoleic acid, which has a 1,4-diene structure. The enzyme converts the linoleic acid substrate into fatty acid hydroperoxides (FAHPs), which measure extracts' inhibitory activity against the LOX enzyme. In this procedure, the FOX reagent added, containing ferrous (Fe^{2+}) ions can oxidize FAHPs to ferric (Fe^{3+}) ions, which bind to the xylenol orange dye, producing a colored complex. The absorbance of this complex is measured at 560 nm[142], and the percent inhibition is calculated as the following formula, giving the extent to which the test extracts or compounds inhibited the 15-LOX enzyme activity:

$$In\% = \left[\frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}}\right] X100 \qquad (08)$$

- **Method**

The 15-LOX inhibition activity was measured spectrophotometrically. Tris-HCl buffer (60 mL; 50 mM, pH 7.4) was mixed with 10 µL of sample solution (diluted in DMSO) and 20 µL of LOX solution, then incubated at 25°C for 5 minutes. The reaction was initiated by adding 10 µL of linoleic acid (140 µM), and the mixture was incubated at 25°C for 20 minutes in the dark. The assay was terminated by the addition of 100 µL of FOX reagent [sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM), methanol/water (9:1)]. The absorbance was measured at 560 nm after 30 minutes at 25°C using an ELISA microplate reader. Reagent blanks, enzyme controls without plant extract, and the standard LOX inhibitor baicalein were used for comparison

II. 6. 3. Cytotoxicity of EOs against lung cancer cells

A549 cell lines for human lung cancer were obtained from the American Type Culture Collection (ATCC). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay colorimetric cell viability method was applied in determining the cytotoxicity of the essential oils against small lung cancer A549 cell lines as described previously [142]. The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% of heat-inactivated fetal bovine serum, kanamycin (100 µg/mL), and amphotericin B (5.6 µg/mL) and incubated overnight. Into each well of a 96-well microplate was introduced 1 µL aliquots of

EOs solutions (16-0.25 mg/mL in DMSO) in 99 µL of media containing 5 × 103 lung cancer cells. Wells without cancer cells served as the blank. The plate was incubated under 5% CO_2 atmosphere at 37 °C for 72 h. The medium was pipetted out, and 100 µL of MTT solution (0.5 mg/mL) was introduced into each well, followed by further incubation for 1.5 h. 100 µL of DMSO was added to each well to dissolve the MTT reduction product (formazan crystals). The absorbances of each well were measured using a spectrophotometer (Thermo Scientific Multiskan FC, Vantaa, Finland) at 540 nm. Etoposide was a positive control, while DMSO was the negative control. 1 µL of each dilution solution of etoposide (16-0.25 mg/mL in DMSO) was introduced to the corresponding wells with 99 µL of media. Final EO and etoposide serial concentrations ranged from 160-02.5 µg/mL. All measurements were done in triplicate, and results were reported as the mean ± SE of three parallel experiments. The percentage inhibition was calculated as given below.

$$\% \text{ inhibition of cell viability} = \left[1 - \frac{(Atest - Ablank)}{(Acontrol - Ablank)}\right] X\ 100 \qquad (09)$$

Where $A_{control}$ is the absorbance of the control (DMSO), A_{test} is the absorbance of the test sample, and A_{blank} is the absorbance of the cell-free wells.

Cytotoxicity was expressed as the concentration of oil inhibiting cell viability by 50% (IC_{50}).

II. 7. In vivo tests

II. 7. 1. Animals

The study used healthy adults male Wistar rats (Rattus norvegicus), obtained from the Pasteur Institute in Algiers, Algeria. Wistar rats are a widely used strain of laboratory rats and were the first animals to be standardized for use in cell biological and clinical medicine research.

After a 15-day acclimation period, the rats were divided into two works. Sixteen of the adult male rats were used to study the sub-chronic toxicity of cigarette smoke exposure, and the remaining rats were used to study chronic toxicity.

All animals were housed in groups (maximum six rats per group) in standard Makrolon cages lined with wood shavings under strict hygiene conditions. The rats were subjected to a 12-hour light/dark cycle, maintained at a room temperature of 19-25°C with a relative humidity of 40-60%. They were fed a standard laboratory diet (comprising corn 62%, soy 26%,

phosphate 1.6%, cellulose 1.0%, minerals 1.0%, and vitamins 1.0%) and had access to water ad libitum.

II. 7. 2. Ethical approval

The study was conducted using relevant institutional, national, and international guidelines, regulations, and legislation on the care of laboratory animals; every effort was made to decrease animal suffering and the number of animals used in research. Following the Guide for the Care and Use of Laboratory Animals established by the European Communities Council (EU2010/63) [143].

PART 01: EFFECT OF SUBCHRONIC CIGARETTE SMOKE EXPOSURE, JPH AND JOX EOS TREATMENT ON OXIDATIVE STRESS PARAMETERS

II. 7. 3. smoking system

With modifications, a simple smoking machine was developed according to the Whole-body cigarette smoke exposure system described by Lucas dos Reis Izolan [144]. This system was designed to ensure that rats exposed to smoking were subjected to a significant quantity of the toxicant compounds contained in cigarette smoke, just as an addicted smoker would consume these chemicals.

The cigarette smoke exposure system (Figure 10), comprises an exposure chamber, a vacuum pump, and a well-ventilated space (WVS). The vacuum pump and exhausters connected in the system force environmental air or cigarette smoke to move unidirectionally from the chamber to the WVS. The cigarette burns outside the exposure chamber. It is fixed in a movable copper tube to ensure a mix between air and cigarette smoke, puff by puff. The copper tube is connected to the exposure chamber via a plastic pneumatic quick coupler connector or silicone tubing. The exposure chamber is glass (50 × 30 × 30 cm) with two glass tubes (5 cm/15 cm, heights) on opposite sides. The vacuum pump produces a maximum of 15 L/min vacuum volume. The smoke is dragged to a WVS through a unidirectional airflow pushed by a fan.

Figure 10: smoking machine system

II. 7. 3. 1. Cigarette smoke exposure

The animal's tobacco smoke exposure setting follows the principle of the procedure outlined in the literature [145, 146] with slight modifications. The rats were kept in an exposure chamber with bedding made of wood shavings (Figure 11). Throughout the tobacco smoke exposure sessions, the rats were not confined. The smoker group was exposed to the smoke of four filtered commercial cigarettes each day for 30 days. Tobacco smoke is produced by burning filtered cigarettes; once the cigarette is lit; the suction is activated using a standardized smoking procedure (35 cm^3 puff volume, 1 puff per 30 sec, 2 s per puff). The rats were moved to the exposure cages just before the tobacco smoke exposure session and then placed back in their original cages.

Figure 11: Rat Smoking model display.

II. 7. 4. Drugs administration and animal investigation

Twenty-four rats weighing between 86 and 133 g were divided into six groups; each group contained four rats. The animals were randomly divided into control and experimental groups as follows:

A control group (C) received the vehicle (distilled water), while the COX and CPH groups were treated with JOX-EO oil and JPH-EO, respectively, administered by oral gavage at 200 mg/kg.

A cigarette smoke (CS) withdrawal group was observed after 4 weeks of smoke exposure. Additionally, two smoking withdrawal treatment groups were included: STOX, treated with JOX-EO, and STPH, treated with JPH -EO, both administered at 200 mg/kg for 2 weeks.

All drugs were administered orally by gavage, and rats were observed daily and weighed during the 4 weeks of cigarette smoking exposure and 2 weeks of treatment.

At the end of the experiment, rats were weighed and humanely sacrificed by decapitation, and the whole blood was collected in EDTA tubes for hematological analyses. Vital organs like the brain and lungs were immediately harvested and weighed to perform biochemical parameters indicative of oxidative stress.

II. 7. 5. Rats' and their relative organs weight

Throughout the experimental study, rats' bodies were weighed every two days, and the relative organ weights were determined using the following formula:

$$\text{Relative organ weight (\%)} = \frac{\text{absolute organ weight (g)}}{\text{body weight (g)}} \times 100 \qquad (10)$$

II. 7. 6. Hematological analysis

Using the automated hematology analyzer (Mindray, BC-31s), the following hematological parameters were measured: red blood cells, platelets, hemoglobin, hematocrit, and mean corpuscular volume.

II. 7. 7. Dosage of oxidative stress biomarkers

For each group, lung and brain samples were used to measure the levels of enzymatic and non-enzymatic biomarkers of oxidative stress, such as malondialdehyde (MDA), reduced glutathione (GSH), and the enzymatic activity of glutathione S-transferase (GST), glutathione peroxidase (GSH-Px), and catalase (CAT).

a) Dosage of reduced glutathione (GSH)

The method of Weckbecker and Cory (1988) [147] was chosen to measure reduced glutathione (GSH), their principle is based on measuring the absorbance of 2-nitro-5-mercapturic acid resulting from the reduction of acid 5 ,5-dithio-bis-nitrobenzoic acid (Ellman's reagent) by the (-SH) group of glutathione. Before the assay, it is necessary to protect the -SH groups of glutathione by deproteinizing the sample to keep only the specific thiol groups of

glutathione by adding 0.25% sulfosalicylic acid. The optical density is read at 412 nm by spectrophotometry.

- **Method**

500 mg of tissue is immersed with 20 mL of 0.2 M, EDTA solution. The mixture is placed on ice and homogenized using a porcelain pestle. The homogenate is then deproteinized by taking 800 µl of it and adding 200 µl of a 0.25% sulfosalicylic acid (SSA) solution. The mixture is vortexed, left for 15 minutes in an ice bath, and then centrifuged for 5 minutes at 1000 rpm. 500 µl of the supernatant is collected, and 1 mL of Tris-HCl+EDTA buffer (0.02 M, pH 9.6) is added. 25 µl of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) at 0.01 M, dissolved in absolute methanol, is added to the mixture. The mixture is allowed to stand for 5 minutes at room temperature and the absorbance (A) is measured at 412 nm.

The GSH concentration is calculated using the following formula:

$$GSH \text{ (nmol per mg Protein)} = [\frac{(Abs - Blank\ Abs)}{(Standard\ Abs - Blank\ Abs)XSC}]X\ \frac{1}{[\text{Proteins}]} \qquad (11)$$

Abs = Absorbance of the sample

Abs white = Absorbance of white

Standard Abs = Absorbance of GSH standard solution

SC = Known concentration of the GSH standard solution (in nmol/mL)

[Proteins] = Protein concentration of the sample (in mg/mL).

This formula makes it possible to relate the GSH concentration measured to the quantity of proteins present in the sample to express the results in nmol of GSH per mg of proteins.

b) Malondialdehyde (MDA)

The method of measuring malondialdehyde (MDA), according to Esterbauer et al. (1992) [148], allows for the indirect measurement of lipid peroxidation. The principle of this method is based on the formation of a pink-colored complex between MDA and thiobarbituric acid (TBA) that absorbs at 530 nm in an acidic and hot (100°C) environment.

- **Method**

100 mg of tissue is ultrasonically homogenized in 1 mL of 50 mM Tris-HCl buffer (pH 7.5). The homogenate is then centrifuged at 10,000 rpm for 10 minutes, and 375 µL of the supernatant is collected, it is mixed with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) and a solution of 20% trichloroacetic acid (TCA) containing 1% butylated hydroxytoluene (BHT) to prevent lipid oxidation. The mixture is centrifuged again at 1,000 rpm for 10 minutes. Subsequently, 400 µL of the supernatant is combined with 0.6 M HCl and a

Tris-thiobarbituric acid (TBA) solution (26 mM Tris, 120 mM TBA), vortexed thoroughly, and incubated in a water bath at 80°C for 10 minutes before measuring the optical density of the samples by spectrophotometry at 530 nm for the MDA assay.

c) Glutathione peroxidase (GSH-Px)

According to the method of Flohe and Gunzler (1984) [149], the enzymatic activity of GSH-Px was measured using hydrogen peroxide (H_2O_2) as a substrate. The principle of this method is based on the reduction of hydrogen peroxide (H_2O_2) by GSH, which is transformed into (GSSG) under the influence of GPx.

- **Method**

According to the described method [150], GSH-Px activity measurement involves homogenizing 1 g of tissue in 5 mL of phosphate-buffered saline (PBS) at pH 7.4 to obtain a homogenate, then collecting 0.2 mL of this homogenate into a tube with 0.4 mL of 0.1 mM reduced glutathione (GSH) and 0.2 mL of 0.067 M phosphate buffer at pH 7.8. The resulting mixture is then incubated at 25°C for 5 minutes, after which 0.2 mL of 1.3 mM hydrogen peroxide (H_2O_2) is added to start the enzymatic reaction. After 10 minutes, the reaction is stopped by adding 1 mL of 1% trichloroacetic acid (TCA), followed by placing the mixture on ice for 30 minutes and centrifuging it at 3000 rpm for 10 minutes. Afterward, 0.48 mL of the supernatant is mixed with 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), forming a colored compound whose optical density is measured at 412 nm after 5 minutes to determine GPx activity. This method relies on the enzymatic oxidation of GSH by GSH-Px in the presence of H_2O_2, with the resulting colorimetric change reflecting enzyme activity

d) Glutathione S-transferase (GST)

GST activity is determined according to the method of Habig et al. (1974) [151]. Its principle is based on the conjugation reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by GST. This reaction produces a new compound, 1-S-glutathionyl -2,4-dinitrobenzene, which absorbs light at 340 nm. Therefore, the enzyme's activity is quantified indirectly by measuring the absorbance of the product formed.

- **Method**

The samples are homogenized in 1 mL of phosphate buffer (0.1 M, pH 6) and then centrifuged at 14,000 rpm for 30 minutes. The recovered supernatant serves as the enzyme source. The assay involves reacting 200 µL of the supernatant with 1.2 mL of a mixture

containing the substrate (CDNB,1 mM) and the cofactor glutathione (GSH, 5 mM) prepared in phosphate buffer (0.1 M, pH 6) and ethanol. The absorbances are read for one minute, every 15 seconds, at a wavelength of 340 nm, using a blank containing 200 µL of distilled water instead of the supernatant.

e) Catalase CAT

The spectrophotometric assay of CAT activity follows the method of Cakmak and Horst (1991) [152], which measures the decomposition of H_2O_2 into water and oxygen by the CAT enzyme by monitoring the change in optical density at 240 nm as the H_2O_2 is consumed.

- **Method**

The described method allows for the measurement of the CAT enzymatic activity. The sample fragments are homogenized in 1 mL of phosphate buffer1 (0.1 M, pH 7.4). The homogenate is centrifuged at 9,000 rpm for 10 min, and the supernatant recovered will serve as a source of enzyme. The final reaction mixture has a volume of 3 mL and contains 100 µL of enzymatic extract, 50 µL of 0.3% H_2O_2 solution, and 2850 µL of 50 mM phosphate buffer at pH 7.2. The reaction is initiated by the addition of H_2O_2. The decrease in absorbance at 240 nm is recorded for 3 minutes using a spectrophotometer. The molar linear extinction coefficient (ε) of H_2O_2 at 240 nm is 39400 $\mu M^{-1}.cm^{-1}$. The instrument is calibrated in the absence of the enzymatic extract. The determination of the enzymatic activity is achieved by measuring the consumption of H_2O_2 over time using the Beer-Lambert law, which relates absorbance to H_2O_2 concentration.

PART 2: PERFORMED TESTS TO CONFIRM THE ABILITY OF JOX BERRIES (EO, JEE) TO REDUCE THE TOXICITY OF CIGARETTE SMOKE IN VIVO

II. 8. Chronic cigarette smoke exposure

In accordance with the same smoking exposure system (Figure 12), described above, the smoker rats' groups were exposed to the smoke of six RYM cigarettes each day for 14 weeks (One cigarette produces 12 mg of tar and 0.8 mg of nicotine). This was achieved using the same standardized smoking procedure with great caution.

Figure 12: Exhibition setup for chronic cigarette-smoking rat models.

II. 8. 1. Rat distribution and drugs administration

The rats weighing between 350 and 400 g were distributed into nine groups, with six animals per group. The groups were randomly divided into control and experimental groups as follows:

- **Control Groups**

1. CG: Control group, consisting of a healthy Rats not exposed to cigarette smoke

2. CSG: smoking control group exposed to cigarette smoke for 14 weeks.

- **Experimental Groups**

1. EXD1: treated with JEE at 200 mg/kg/day for 15 days after 14 weeks,

2. EXD3: treated with JEE at 800 mg/kg/day for 15 days after 14 weeks,

3. JEO: treated with JOX EO at 200 mg/kg/day for 15 days after 14 weeks,

4. STEX1: smoking group treated with JEE at 200 mg/kg for 2 weeks after withdrawing smoking (stop smoking after 14 weeks),

5. STEX2: smoking withdrawn group treated with JEE at 400 mg/kg,

6. STEX3: smoking withdrawn group treated with JEE at 800 mg/kg,

7. SOEO: smoking withdrawn group treated with JOX-EO at 200 mg/kg.

All drugs were orally administered.

Figure 13: Oral Administration Pathway for drugs.

II. 8. 2. Preparation of drugs

II. 8. 2. 1. JOX-EO emulsion

To prepare the JOX-EO emulsion, first mix the essential oil (5% of the total weight) with the aqueous phase composed of ethanol (5%) and distilled water (90%) in glass bottles. Then, agitate the mixture using an ultrasonic bath for 10 to 15 minutes until the emulsion is homogeneous and stable. The ethanol (5%) promotes uniform essential oil dispersion in the aqueous phase without additional emulsifiers.

To prevent an excessive temperature, rise that could degrade the components of essential oils, adjust the ultrasonic bath temperature to a comfortable level by cooling the ultrasonic bath water. Finally, administer this emulsion orally by gavage immediately once prepared. The emulsified form generally facilitates the absorption and bioavailability of essential oils.

II. 8. 2. 2. JEE dose

To prepare the JOX extract dose, dry JEE and 5% ethanol are used as the solvent. Prepare a quantity sufficient to reach 2 mL of the total volume for each dose (200, 400, and 800 mg/kg). 5% ethanol as a solvent for oral drug administration in animals is generally not highly toxic[153]. However, potential biological effects should be considered and controlled for this; the control group receives the same concentration of ethanol as the treatment group, ensuring the validity of the study results.

II. 8. 3. Collection of organs and blood samples

At the end of the 14- and 2-week treatment period, rats were sacrificed by decapitation under ether anesthesia. Whole blood samples were collected between 08:00 and 11:00 am, with one part collected in EDTA tubes for hematological analyses and the second part collected in heparinized bottles for biochemical and serology analysis. Immediately after blood collection, the vital organs were collected, weighed, and subjected to biochemical analysis.

II. 8. 4. Relative organ weight

During the 14-week experimental study, the rats' body weights were measured every two days. After sacrifice, the liver, lungs, kidneys, heart, and brain were collected and weighed in grams. The relative organ weight of each rat was calculated using the formula described in the first part of the sub-chronic smoking toxicity study, which involved dividing the weight of each organ by the animal's body weight and expressing the result as a percentage.

II. 8. 5. Glycemic marker dosage

The rat tail blood sampling protocol for measuring blood glucose levels involves the following steps: disinfect the tail with 75% alcohol before sampling, identify the caudal vein on the tail, and perform a puncture using a fine needle to collect a small amount of blood (about 1 µl) (Figure 14), by test strips to measure the blood glucose using a glucometer (Diagno-check), at the short time before the sacrifice of the rat, which was under ether anesthesia in order to avoid any movement, and stop the bleeding by applying cotton to the puncture site.

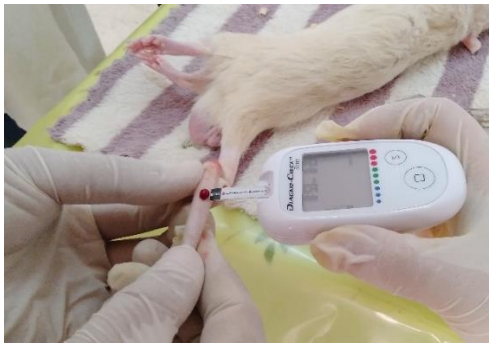


Figure14: Presentation of rat-tail blood sampling for glucose measurement using a glucometer.

II. 8. 6. Determination of hematological and biochemical parameters

The hematological components were determined using an automated hematology analyzer (Mindray, BC-6800), (Figure 15). The following parameters were measured: red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

The blood samples were collected in heparinized tubes, centrifuged at 3000 × g for 10 minutes, and the heparinized plasma was transferred into fresh plain sample tubes. The biochemical parameters were measured using an automated clinical biochemical analyzer (Mindray, BS-240 pro), (Figure 16), to perform the following tests:

1. Liver function tests

- Aspartate aminotransferase (AST)
- Alanine aminotransferase (ALT)

2. Lipid Profile

- Triglycerides
- Cholesterol
- Low-density lipoprotein cholesterol (LDL-cholesterol)
- High-density lipoprotein cholesterol (HDL-cholesterol)

3. Kidney Function Tests

- Creatinine
- Urea

4. Enzyme Marker

- Lactate dehydrogenase (LDH).

Figure15: Automated hematology analyzer (Mindray, BC-6800) (a), and automated clinical biochemical analyzer (Mindray, BS-240 pro) (b).

II. 8. 7. Assessment of C-reactive protein (CRP): an inflammatory marker

The CRP-latex slide agglutination test is a widely used, rapid, and straightforward method for detecting C-reactive protein (CRP) levels in blood serum. In this work, the dosage of CRP latex reagent for use with rat serum was carried out by using fresh rat serum obtained by centrifuging an EDTA tube containing rat blood. 50 µL of rat serum is put on the circle of the test slide, and 50 µL of CRP latex reagent is placed next to the serum (Figure 16). Mix the CRP latex reagent before use to ensure uniform particle suspension; use a disposable stirring stick to mix the serum and latex reagent over the entire test circle and gently shake the slide for 2 minutes. A positive result, indicated by visible agglutination, suggests a CRP level ≥6 mg/L, while a negative result, characterized by the absence of agglutination, indicates a CRP level <6 mg/L.

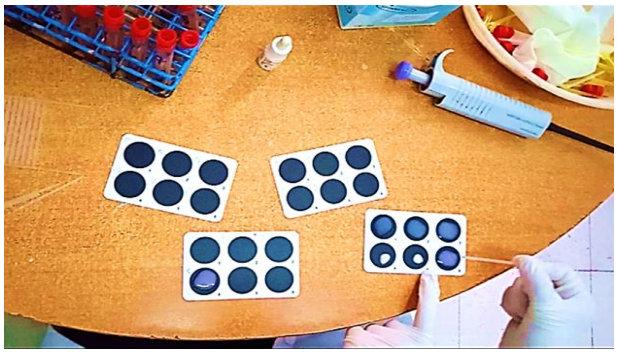


Figure 16: The CRP-latex slide agglutination test

II. 8. 8. Dosage of metabolites at the level of vital organs treated by JEE1

II. 8. 8. 1. Sample preparation

The first step involves the collection of 100 mg of tissue from each organ (brain, lung, liver, kidney, heart). Using an ultrasonicator, these samples are then crushed and homogenized in 2 mL of TCA at 20%. The homogenate is then centrifuged at 5000 rpm for 10 minutes. The supernatant (N°1) containing the glucose is recovered, while the pellet (N°1) containing the lipids and proteins is preserved.

II. 8. 8. 2. Carbohydrate dosage

The Duchateau and Florkin (1959) glucose dosage is performed by collecting 100 µL of the supernatant (N°1). This sample is added to 4 mL of anthrone reagent (150 mg of anthrone + 75 mL of concentrated sulfuric acid + 25 mL of distilled water). The mixture is then heated to 80°C for 10 minutes. The absorbance of the solution is measured at 620 nm using a spectrophotometer[154]. The concentration of glucose is calculated using a glucose standard curve.

II. 8. 8. 3. Lipid dosage

For the dosage of lipids, the method of Goldsworthy et al. (1972) [155] was carried out; 1 mL of a mixture of ether and chloroform (1:1) was added to the pellet (N°1). A second centrifugation at 5000 rpm for 10 minutes yields a supernatant (N°2) containing the lipids and a pellet (N°2) containing the proteins. 100 µl of the supernatant (N°2) are collected and added to 1 mL of sulfuric acid. The mixture is heated to 100°C for 10 minutes. After cooling, 2.5 mL of sulfophosphovanillin reagent is added. After 30 minutes of reaction in the dark, the absorbance is measured at 530 nm using a spectrophotometer. The concentration of lipids is calculated using a standard curve of table oil.

II. 8. 8. 4. Proteins dosage

Finally, the dosage of proteins by Bradford's method (1976) [156] is performed by adding 1 mL of NaOH (0.1 M) was added to the pellet (N°2) to extract the proteins. 100 µL of the extract is collected and added to 4 mL of Bradford reagent BBC. The absorbance is measured at 595 nm using a spectrophotometer. The concentration of proteins is calculated using a standard curve of bovine serum albumin.

II. 9. STATISTICAL ANALYSES

The results obtained were expressed as the average of three or four repetitions. Statistical analysis was carried out using GraphPad Prism 8 to calculate the mean ± SD for the measurements. The Holm-Sidak method with alpha = 0.05 was used to determine the statistical significance of the differences between the treated groups and the controls, with each row examined independently without assuming a constant SD. To better visualize the results, Excel 2018 was used to create graphs and histograms representing the data.

CHAPTER III

RESULTS AND DISCUSSION

III. 1. Extracts yield

The mass of EOs obtained after a 3-hour extraction using the Clevenger distillation technique was 1.185 g from 200 g of fresh JOX berries and 5 g from 200 g of fresh JPH berries. Additionally, the extracted masses of JOX ethanolic extracts were 49 g using ultrasound-assisted extraction (UAE) (JEE1) and 32 g using conventional extraction (CE) (JEE2) from 200g of JOX berries, which had a moisture content of 2.4%. The extraction yield results are as follows:

$$\text{Yield\%} = \frac{\text{Amount of extracted oil or extracts (g)}}{\text{Amount of vegetal matter mass (g)}} \text{x}100 \qquad (01)$$

Table 03: Extraction yield of extracts.

Extract	Method	Time (h)	Yield %
JOX-EO	HD- Clevenger	3	**00.59**
JPH-EO	HD- Clevenger	3	**02.50**
JEE1	UAE	24	**24.50**
JEE2	CE	72	**16.00**

III. 1. 1. Extraction methods

The extraction of JOX and JPH berries using various methods reveals in the efficiency and nature of the obtained compounds. The distillation-Clevenger method yielded 0.59% JOX-EO and 2.50% JPH-EO, whereas ethanolic extracts obtained by ultrasound-assisted extraction (UAE, JEE1) and conventional extraction (CE, JEE2) from JOX reached yields of 24.50% and 16.00%, respectively. This analysis highlights the implications of these results for extraction techniques and product characteristics.

The distillation-Clevenger technique, widely used for isolating volatile oils, produced a relatively low yield due to its specificity for essential oils. In contrast, ultrasound-assisted extraction (UAE) achieved a substantially higher yield by using ultrasound waves to disrupt plant cell walls and enhance compound solubility in ethanol, improving the efficiency of volatile and non-volatile phytochemical extraction. Conventional extraction yielded noticeably less than UAE while producing a significant amount of ethanolic extract. This traditional method relies on solvent soaking and lacks the mechanical enhancement provided by ultrasound, which limits its extraction efficiency relative to UAE.

The differences in obtained yield can be attributed not only to the extraction method but also to the nature of the recovered compounds. Essential oils generally occur in small amounts because they represent only the volatile fraction of the plant material, whereas ethanolic extracts include a broader range of constituents such as phenolics, flavonoids, terpenoids, and other

soluble metabolites. The higher yield recorded for UAE suggests that this method is more effective for maximizing extract recovery, while the lower yield of hydrodistillation remains expected for essential oil isolation. Thus, yield should be interpreted in relation to the target compounds, since a higher yield does not necessarily indicate a higher concentration of volatile bioactive constituents.

III. 2. Chemical composition

In addition to the impressive yields of ethanolic extracts, evaluating their quality and chemical composition is equally important.

III. 2. 1. TPC and TFC result

The ethanolic extracts JEE1 and JEE2 were analyzed for their total phenolic content (TPC) and total flavonoid content (TFC). The results are presented in Table 04.

Table 04: Total phenolic content (TPC) and total flavonoid content (TFC) of JOX ethanolic extracts JEE1 and JEE2. Values are expressed as mean ± SD.

Extract	TPC (mg GAE/g DW)	TFC (mg QE/g DW)
JEE1	20.07 ± 2.34	15.96 ± 1.25
JEE2	03.26 ± 3.49	00.54 ± 0.28

The results indicate notable differences in TPC and TFC across various extraction techniques, such as UAE and CE. The TPC values for the ethanolic extracts JEE1 and JEE2 are reported as 20.07 ± 2.34 and 3.26 ± 3.49, respectively. This substantial variation illustrates that the UAE significantly enhances the extraction of phenolic compounds compared to CE. Similarly, the TFC values are 15.96 ± 1.25 and 0.54 ± 0.28, respectively, further emphasizing the efficiency of UAE in yielding flavonoids.

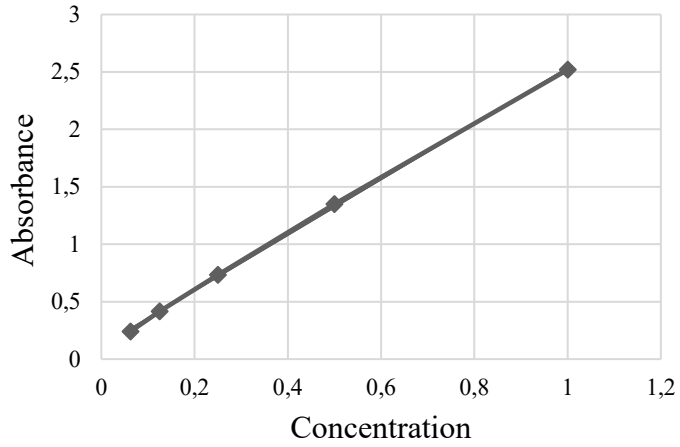


Figure 17: Calibration curve of Gallic acid (Y=2.4199 X + 0.1131; R^2=0.9995): to assess total phenolic content in JOX extracts.

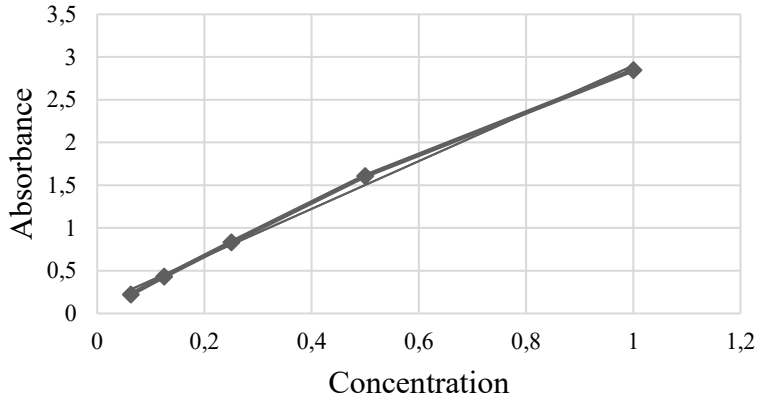


Figure 18: Calibration curve of quercetin (Y = 2.7935 X + 0.1061; R^2=0.9959): to assessing total flavonoids content.

III. 2. 2. GC-MS result

The chemical composition of the **JOX-EO** from fresh berries includes a variety of compounds analyzed through gas chromatography and mass spectrometry. The result is established in Table 05.

Table 05: Chemical composition of JOX-EO as determined by GC-MS Analysis.

N°	Compound	RT	KI	% composition
1	Tricyclene	09.58	922	00.0778
2	α-thujene	09.90	927	00.0298
3	**α-Pinene**	**10.64**	**940**	**57.4936**
4	Camphene	11.17	948	00.2186
5	Thuja-2,4(10)-diene	11.50	954	0.0858
6	Sabinene	12.67	973	0.0775

7	β-Pinene	12.89	977	0.8988
8	**β-Myrcene**	**14.17**	**998**	**7.9658**
9	δ-3-Carene	15.10	1011	0.0148
10	α-Terpinene	15.58	1018	0.0393
11	o-cymene	16.21	1027	0.0933
12	**D-Limonene**	**16.49**	**1031**	**1.0986**
13	γ-Terpinene	18.57	1060	0.0458
14	Terpinolene	20,66	1089	0.1740
15	p- cymenene	20.90	1093	0.0755
16	Perillene	21.67	1104	0.0378
17	exo -fenchol	22.76	1119	0.0271
18	α-Campholenal	23.45	1128	0.2358
19	camphor	24.50	1143	0.0001
20	cis-Sabinol	24.56	1144	0.6449
21	Pinocarvone	25,97	1163	0.0785
22	Borneol	26.50	1171	0.1176
23	p-Mentha-1,5-dien-8-ol	26.76	1174	0.2217
24	α -Terpineol	27.28	1182	0.1386
25	γ-Terpineol	28.40	1197	0.4257
26	verbenone	29.36	1211	0.1823
27	neoiso-Dihydro carveol	30.46	1228	0,0751
28	Bornylacelate	34.60	1289	0.1052
29	α-Cubebene	38.88	1353	0.6309
30	α-Ylangene	40.23	1373	0.0482
31	α-Copaene	40.56	1378	0.1861
32	β-Cubebene	41.50	1392	0.0592
33	ß-Elemene	41.69	1395	0.0570
34	ß-longipinene	42.26	1403	0.0313
35	Longifolene	42.36	1405	0.0390
36	(E)-Caryophyllene	43.36	1421	0.7963
37	β-Gurjunene	43.95	1431	0.1065
38	trans-α-Bergamotene	44.39	1438	0.0644
39	cis-Muurola-3,5-diene	44.89	1446	0.0241
40	trans-Muurola-3,5-diene	45.09	1449	0.0104
41	α-Caryophyllene	45.53	1456	0.8264
42	(E)-β-Famesene	45.93	1463	0.2112
43	cis-Muurola-4(14),5-diene	46.12	1466	0.0406
44	γ-Muurolene	46.78	1476	0.0601
45	**Germacrene D**	**47.52**	**1488**	**8.9852**
46	trans-Murrola-4(14),5-diene	48.26	1500	0.0278
47	α-Muurolene	48.48	1504	0.3704
48	δ-Amorphene	48.82	1510	0.0930
49	(Z)-γ-bisabolene	49.03	1513	0.0379
50	**δ-cadinene**	**49.40**	**1520**	**1.7340**
51	**Zonarene**	**49.91**	**1528**	**1.4463**

52	trans-cadina-1,4-diene	50.38	1537	0.0692
53	α-cadinene	50.69	1542	0.1635
54	α-Calacorene	51.02	1547	0.0961
55	(E)-Nerolidol	52,43	1572	0.3889
56	Caryophyllene oxide	53.28	1586	0.2196
57	salvial-4(14)-en-1-one	53.94	1597	0.2105
58	cedrol	54.49	1607	0.0435
59	Humulene epoxide Il	54.83	1613	0.2589
60	9,10-dehydro-Isolongifolene	55.22	1620	0.5996
61	1-epi-Cubenol	55.97	1634	0.1258
62	γ-Eudesmol	56.26	1639	0.0630
63	Caryophylla-4(12),8(13)-dien-5-ol	56.56	1644	0.0292
64	α-muurolol	56.86	1650	0.5219
65	thujapsan-3one	57.13	1654	0.2243
66	Eudesma-4(15),7-dien-1ß--ol	59.34	1694	0.4578
67	Rosa-5,15-diene <ent->	71.57	1933	0.0590
68	**Manooloxide**	**74.57**	**1996**	**1.5474**
69	Phyllocladene	75.52	2016	0.1384
70	Abietatriene	77.54	2060	0.3379
71	Abietadiene	78.72	2086	1.0348
		TOTAL		93.1549

RT: Retention Time
KI: (Kovats) Retention Index
% Composition: percentage composition of components.

GC-MS analysis of the essential oil extracted from JOX berries revealed the presence of various compounds, including monoterpenes, sesquiterpenes, and diterpenes. The major compounds identified were α-Pinene (**57.49**%), Germacrene D (**8.99**%), β-Myrcene (**7.97**%), δ-cadinene (**1.73**%), Manooloxide (**1.55**%), Zonarene (**1.45**%), D-Limonene (**1.10**%), and (E)-Caryophyllene (**0.80**%).

III. 2. 3. LC-ESI-MS/MS result

The chemical composition of the ethanolic extracts JOX JEE1 and JEE2, analyzed by liquid chromatography-mass spectrometry (LC-MS), showed the presence of phenolic compounds in the following table.

Table 06: Chemical compounds in JEE1 and JEE2 via LC-ESI-MS/MS.

No	**Analytes**	**JEE2**(mg Analyte/g Ext)	**JEE1**(mg Analyte/g Ext)
1	**Quinic acid**	**171.835**	**89.718**
2	Fumaric aid	N.D.	N.D.
3	Aconitic acid	N.D.	N.D.
4	**Gallic acid**	**00.040**	**00.080**

5	Epigallocatechin	N.D.	N.D.
6	**Protocatechuic acid**	**00.280**	**01.535**
7	Catechin	N.D.	N.D.
8	Gentisic acid	N.D.	N.D.
9	Chlorogenic acid	N.D.	N.D.
10	**Protocatechuic aldehyde**	**00.085**	**00.111**
11	Tannic acid	N.D.	N.D.
12	Epigallocatechin gallate	N.D.	N.D.
13	Cynarin	N.D.	N.D.
14	4-OH Benzoic acid	N.D.	N.D.
15	Epicatechin	N.D.	N.D.
16	Vanilic acid	N.D.	N.D.
17	Caffeic acid	N.D.	N.D.
18	Syringic acid	N.D.	N.D.
19	Vanillin	N.D.	N.D.
20	Syringic aldehyde	N.D.	N.D.
21	Daidzin	N.D.	N.D.
22	Epicatechin gallate	N.D.	N.D.
23	Piceid	N.D.	N.D.
24	p-Coumaric acid	N.D.	N.D.
25	Ferulic acid-D3-IS[h]	N.A.	N.A.
26	Ferulic acid	N.D.	N.D.
27	Sinapic acid	N.D.	N.D.
28	Coumarin	N.D.	N.D.
29	Salicylic acid	N.D.	N.D.
30	Cyranoside	N.D.	N.D.
31	Miquelianin	N.D.	N.D.
32	Rutin-D3-IS	N.A.	N.A.
33	Rutin	N.D.	N.D.
34	isoquercitrin	N.D.	N.D.
35	**Hesperidin**	**00.137**	**00.114**
36	o-Coumaric acid	N.D.	N.D.
37	Genistin	N.D.	N.D.
38	Rosmarinic acid	N.D.	N.D.
39	Ellagic acid	N.D.	N.D.
40	**Cosmosiin**	**00.070**	**00.123**
41	Quercitrin	N.D.	N.D.
42	Astragalin	N.D.	N.D.
43	Nicotiflorin	N.D.	N.D.
44	Fisetin	N.D.	N.D.
45	Daidzein	N.D.	N.D.
46	Quercetin-D3-IS	N.A.	N.A.
47	Quercetin	N.D.	N.D.
48	**Naringenin**	**00.005**	**00.012**
49	Hesperetin	N.D.	N.D.
50	Luteolin	N.D.	N.D.

51	Genistein	N.D.	N.D.
52	Kaempferol	N.D.	N.D.
53	**Apigenin**	**N.D.**	**00.016**
54	**Amentoflavone**	**00.006**	**00.066**
55	Chrysin	N.D.	N.D.
56	Acacetin	N.D.	N.D.

N.A: not applicable; **N.D**: not detected; **IS**: internal standard.

The chemical composition of JOX ethanolic extracts by LC-MS analysis showed the presence of phenolic compounds such as quinic acid, gallic acid, protocatechuic acid, protocatechuic aldehyde, hesperidin, cosmosiin, naringenin, and amentoflavone.

The comparison of JOX ethanolic extracts, JEE1, and JEE2 reveals notable differences in their chemical compositions as analyzed by LC-MS. JEE2 exhibits a significantly higher concentration of quinic acid (171.84 mg/g) compared to JEE1 (89.72mg/g), while JEE1 contains more protocatechuic acid (1.54 mg/g vs. 0.28 mg/g), cosmosiin (0.12 mg/g vs. 0.07 mg/g), and amentoflavone (0.07 mg/g vs. 0.01 mg/g). Both extracts have low levels of gallic acid, with JEE1 at 0.08 mg/g and JEE2 at 0.04 mg/g, and JEE1 has higher naringenin content (0.01 mg/g vs. 0.005 mg/g). Hesperidin is slightly more concentrated in JEE2 (0.14 mg/g vs. 0.11 mg/g). These variations suggest that JEE1 may offer more excellent benefits regarding certain phenolic compounds, while JEE2 is particularly rich in quinic acid.

These results highlight the differences in the chemical composition of the ethanolic extracts obtained by different extraction methods, which can significantly affect their quality and potential applications.

III. 3. IN VITRO ESSAYS

III. 3. 1. Effects of the tested EOs on the nicotine concentration reduction

The study involves a method of extraction, using qualitative analyses of nicotine extracted from tobacco and quantitative analyses to determine how JPH and JOX essential oils modulate nicotine content.

III. 3. 1. 1. Identification of extracted tobacco nicotine

Following the steps outlined in the previous section for extracting nicotine, 20 mg was recovered. According to nicotine's organoleptic properties, the extract obtained from tobacco leaves ensure these properties, namely, a hygroscopic and colorless to pale yellow oily liquid.

The presence of nicotine was also confirmed by obtaining a yellow-green color on the TLC plate after adding potassium permanganate. As already mentioned, the reaction of nicotine

with potassium permanganate in the presence of sodium hydroxide forms a complex of yellow-green color.

The characteristic magnitude of nicotine is λ_{max}= 260 nm, which makes it possible to identify nicotine in solution by its UV spectrum. However, the spectrum obtained from the extracted nicotine was compared with a reference spectrum (B) of pure nicotine [157] (Fig.19). According to the spectroscopic result obtained, we see that the two spectra, that of the reference and that of the extracted and diluted nicotine, have the same appearance, as well as the exact value of λ_{max} (spectrum a, Fig 19). Furthermore, in the UV-visible range, we did not observe any other peaks, which confirms the absence of different compounds and guarantees the purity of the extract obtained.

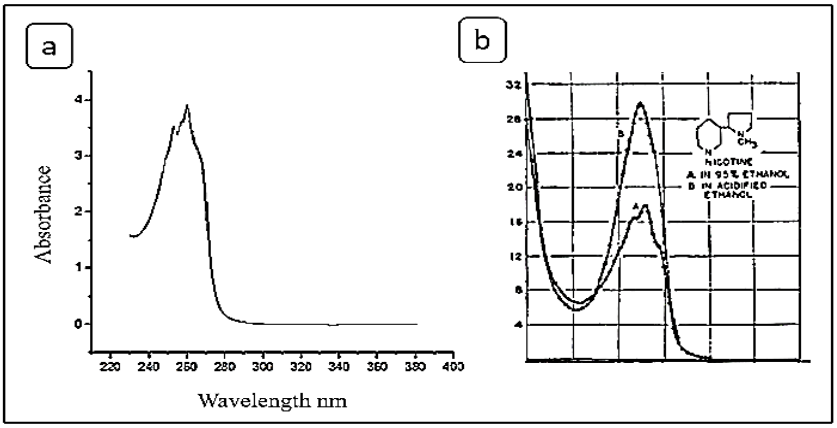


Figure 19: Nicotine spectrum: (a) represents the extracted nicotine spectrum; (b) represents the pure nicotine spectrum (in ethanol, 95%)[157].

Nevertheless, it is not possible to say that only (S)-(-)-nicotine is obtained because tobacco contains three different isomers of nicotine: the two enantiomers (S)-(-)-nicotine and (R)-(+)-nicotine, as well as the analog nor-nicotine[157]. It is worth recalling that according to Clayton et al., the (S)-(-)-nicotine is the most dominant compound in tobacco[158].

III. 3. 1. 2. Assessment of nicotine reduction

The results were carried out in triplicate; the mean and standard deviations are shown in Table 7. The concentration of nicotine (µg/mL) is determined by reference to the calibration graph Figure 20.

Table 07: Spectroscopic determination of nicotine extracted from tobacco and treated with EOs.

ABS of control	Conc. of nicotine in control solution µg/mL	Conc. (µg/mL)	Absorbance of samples in different concentration of EOs			
			JOX	JOX + N	JPH	JPH + N
0.319 ± 0.048	7.979 ± 1.268	19	0.1700 ± 0.010	0.305 ± 0.011	0.156 ± 0.008	0.309 ± 0.005
		15.2	0.1250 ± 0.006	0.289 ± 0.004	0.114 ± 0.009	0.307 ± 0.005
		11.4	0.0900 ± 0.006	0.278 ± 0.008	0.081 ± 0.009	0.309 ± 0.007
		7.6	0.0710 ± 0.003	0.306 ± 0.009	0.065 ± 0.012	0.316 ± 0.006
		3.8	0.0630 ± 0.004	0.305 ± 0.008	0.050 ± 0.009	0.315 ± 0.011
		1.9	0.0006 ± 0.005	0.310 ± 0.009	0.005 ± 0.004	0.318 ± 0.007

To evaluate the nicotine content, Tamrah's principle is followed, which says that nicotine reacts with potassium permanganate in the presence of sodium hydroxide to generate a green product owing to permanganate reduction to manganite [127]. In particular, concerning the protocol, we have made modifications for the following reasons.

Firstly, Temara requires that nicotine, sodium hydroxide, and potassium permanganate be combined and heated to produce the green product. However, in this study, heating was not employed since acetonitrile was added to facilitate interaction between the EOs and the nicotine, because nicotine cannot be dissolved in an organic solvent that cannot be combined with reagents.

Secondly, acetonitrile was used instead of alcohol because alcohols oxidize rapidly with permanganate. In contrast, acetonitrile takes a long time, so skipping the heating step avoids speeding up the reaction.

As a result, different concentrations of potassium permanganate and sodium hydroxide in the presence of nicotine and acetonitrile generated the manganese or green product faster without heating. Another consideration is the influence or interaction of the EOs with the reagents.

After repeating the procedure by replacing the nicotine with the investigated essential oils, a blue color appeared over time. There was also absorption at 610 nm, as seen in Table 07. Consequently, the absorbance at 610 nm for the samples containing essential oils and nicotine was not the absorbance of the green product resulting from the effect of nicotine alone. Nevertheless, EOs also influenced it. For this reason, solutions with different concentrations of essential oils compared to solutions containing essential oils and nicotine were used to eliminate the absorbance of the first solutions after reaction with the reagents.

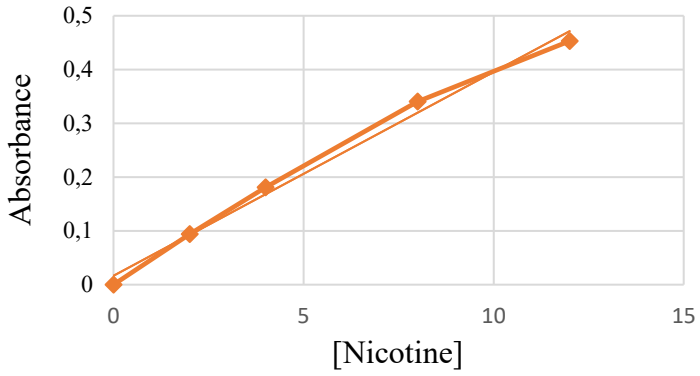


Figure 20: Nicotine concentration calibration graph (Absorbance = 0.0379 [nicotine] + 0.0166), with a correlation factor $R^2 = 0.991$ and concentrations in µg/mL.

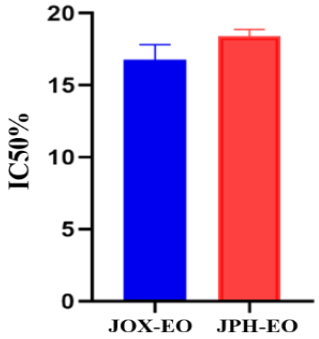


Figure 21: IC_{50} % of nicotine treated by JPH and JOX EOs.

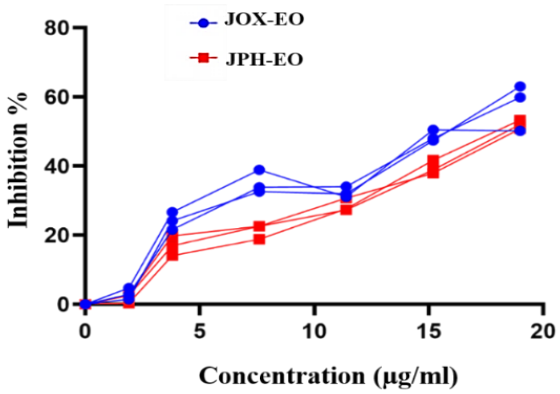


Figure 22: Inhibition percentage of nicotine by JOX-EO and JPH-EO.

This procedure's primary aim is to evaluate essential oils' ability to reduce nicotine levels rather than precisely quantify nicotine concentration due to the assay time constraints. According to the data presented in Figures 21 and 22, both JOX-EO and JPH-EO demonstrated notable nicotine reduction effects. JOX-EO showed a decrease in nicotine concentration from the initial 7.979 ± 1.268 µg/ml, exhibiting the highest inhibitory activity with an IC50 value of 16.782 ± 1.0434 µg/ml. Similarly, JPH-EO showed potent nicotine inhibition, with an IC50 of 18.40 ± 0.46 µg/ml. These results suggest that while both essential oils effectively reduce nicotine levels, JOX-EO is slightly more potent in inhibiting nicotine.

This reduction is likely due to direct chemical interactions between the essential oils' bioactive compounds and nicotine molecules. These interactions may involve binding, complex formation, or chemical modification of nicotine, reducing its detectable concentration in the assay. Since the test was performed, in vitro, enzymatic or metabolic effects were excluded,

indicating that the observed nicotine reduction results primarily from physicochemical interactions within the experimental setup.

III. 3. 2. Evaluation of JOX-EO's effectiveness in reducing PAHs

The extraction of polycyclic aromatic hydrocarbons (PAHs) from RYM cigarette smoke was primarily conducted using a method that traps PAHs in the smoke through a hexane and ethanol solvent mixture. This combination was effective because PAHs are generally lipophilic and poorly soluble in water; the non-polar hexane and polar ethanol enhance the dissolution of PAHs, especially those with lower molecular weights that are more volatile and soluble in organic solvents. Ethanol, acting as a co-solvent, improves the solubility of PAHs, facilitating their extraction, particularly for complex molecular structures that may be less soluble in non-polar solvents alone[130].

In the second step, liquid-liquid extraction was performed by adding distilled water to the hexane-ethanol solution containing the PAHs, followed by agitation to promote phase contact. The polar ethanol is miscible with water, aiding in the dissolution of certain polar compounds and influencing the solubility of PAHs in the organic phase. This method allows for efficient recovery of PAHs while preserving their chemical integrity due to the combination of ethanol's polarity and hexane's non-polarity. After separation, the lower phase was washed three times with hexane to extract any remaining PAHs. The hexane phases were then collected and evaporated at a temperature not exceeding 40°C to prevent degradation of the compounds.

The extracted fractions can be easily analyzed using chromatographic methods such as column and thin-layer chromatography, facilitating the effective separation and identification of PAHs.

III. 3. 2. 1. Interpretation PAHs result

Six fractions were collected after the evolution of the chromatographic column monitored by TLC (A, B…F). The PAHs' UV spectra were obtained for each standard or treated solution from those treated with JOX-EO and analyzed by UV spectrophotometry. The spectra are illustrated in Figure 23.

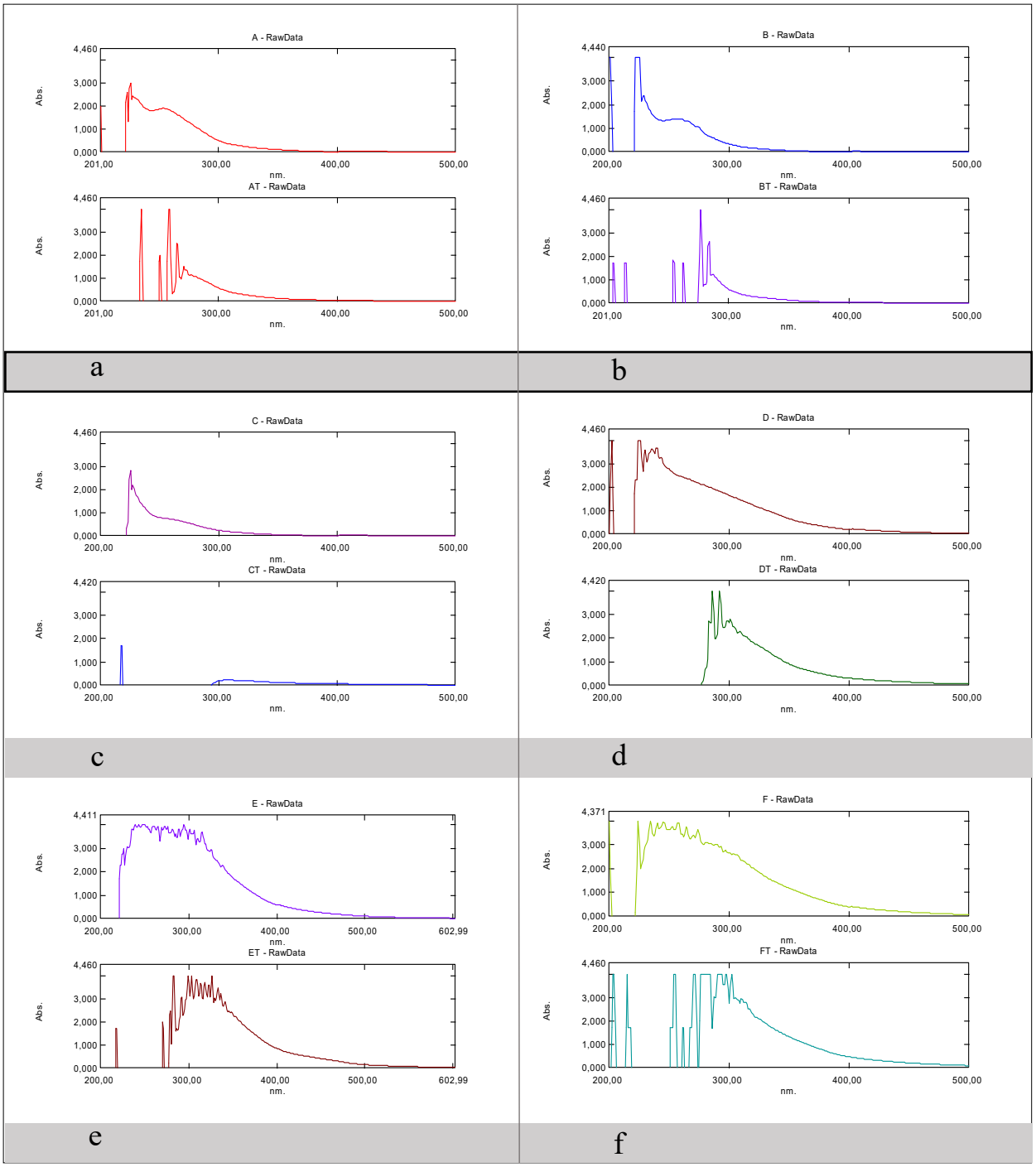


Figure 23: PAHs treated with JOX-EO with their control solutions (Control fractions: A, B, C, D, E, and F. Treated fractions with JOX-EO: AT, BT, CT, DT, ET, and FT).

As previously noted, tobacco smoke has a complex and highly reactive composition. The high temperatures generated during cigarette combustion initiate pyrolytic processes that produce radical species. These radicals can interact with various compounds present in the smoke, leading to the formation of new chemical substances[159] . According to the

abbreviated list of harmful and potentially harmful constituents published by the FDA, these constituents span multiple chemical classes, including polycyclic aromatic hydrocarbons.

As a preliminary test and to see if the essential oils directly interact with PAHs or not, or if they have the ability to inhibit PAHs, this investigation was conducted. PAHs spectra are higher homologs of benzene, so their appearance depends on the number of aromatic rings and their arrangement in the molecule. These spectra are more structured for heavier compounds [160, 161]. In general, the absorption range of the PAH family is between 200 and 460 nm [160].

The UV spectrum analysis of fraction A reveals the existence of three chemicals, which aligns with the observations made during the TLC analysis. From Fig. 23 (a), three intense peaks at 223 and 226 nm and between 228 and 230 nm are distinguished, confirming that these substances contain light PAHs and another broad peak between 245 and 325 nm. For fraction B, the TLC showed that it has at least two substances, and the spectrum in Fig. 23 (b) indicates that these substances contain at least one light PAH. It also has two strong peaks, one at 224 nm and the other at 229 nm, and a broad peak between 245 and 330 nm. The TLC of fraction C showed a single spot, probably a single substance. The spectrum in Fig. 23 (c) also indicates a light PAH because it has a strong peak at 224 nm and a broad peak between 250 and 300 nm. In the other fractions, solution D contains a single spot, as distinguished by TLC, so it is possibly a unique substance, while at least two substances are present in fractions E and F. The spectra (Fig. 23.d, e, and f) of the last three fractions show they are most likely heavy PAHs because they are more structured or stand out in the 225-400 nm range.

After analyzing the spectra of the previously treated fractions with the essential oil of JOX and superimposing them with those controls, a clear and well-marked difference, either in the chemical displacement, the intensity of the peaks, or the general appearance of the spectrum, was observed. Moreover, in the near UV range between 200 and 300 nm, additional thin peaks of varying intensity appeared with a hyperchromic effect for fractions A and B.

According to Olivier Thomas and Marine Brogat, a hyperchromic effect is observed when aromatic rings are less condensed [161]. As a result, the appeal of their spectra had undergone a complete transformation, indicating the appearance of new chemicals aside from PAHs. For fraction C, a hypsochromic effect was observed in the pic (Fig. 23. C), and the disappearance of the large band, which probably means that the PAH has been transformed into another product by degradation similar to the fine pic that appeared, may indicate the presence of acyclic dienes in s-trans form. In fraction D, a bathochromic effect was seen on the peaks (Fig. 23.d). This could be because the number of rings increased, conjugation increased, or other

substitutions appeared. Similarly, for fractions E and F, a bathochromic effect was observed in specific peaks, with the appearance of other fine peaks in the region between 200 and 275 nm (Figs. 23.e and f). These peaks, which are more intense in the spectrum of fraction F, indicate the transformation of heavy PAHs with the formation of other products. It can be concluded from the shape of the obtained spectra that there is an interaction between the PAHs and the essential oil of *J. oxycedrus L*. This interaction may generate other products that differ from the PAHs or the latter's degradation; however, identifying these new products will require additional high-performance separation and identification techniques.

III. 3. 3. Biological activities

III. 3. 3. 1. Antioxidant activity

In this work, the different antioxidant activity tests, DPPH, FRAP, CUPRAC, and nitric oxide, were employed to evaluate the antioxidant capacity of JOX extracts due to their unique mechanisms and the specific types of antioxidants they measure. Each method provides distinct insights into the antioxidant potential, and the combination of these antioxidant assays is crucial for a comprehensive understanding of JOX extracts' antioxidant profile. Each method targets mechanisms such as hydrogen atoms and single-electron transfer [133]. A low correlation between results from different assays can limit the full antioxidant potential [162]. Some antioxidants may react differently in one assay due to differences in redox potentials; for this reason, it was necessary to use multiple methods to accurately characterize the antioxidant capacity of JOX extracts in this research.

a) DPPH radical scavenging activity

The DPPH assay is widely used to assess plant extracts and compounds' free radical scavenging activity. It measures the reduction of DPPH radicals, which can be quantified by a color change from purple to yellow. The IC50 value, representing the concentration needed to scavenge 50% of radicals, compares antioxidant activity.

Based on the results in Table8, the ethanolic extract JEE1 obtained by UAE exhibited the highest DPPH radical scavenging activity, with an IC50 value of 0.224 ± 0.008 mg/mL. This suggests that JEE1 contains a higher concentration of antioxidant compounds than the JOX-EO and ethanolic extract JEE2 obtained by conventional extraction (CE).

The JOX-EO showed the lowest DPPH scavenging activity, with an IC50 value of 4.14 ± 0.35 mg/mL. This could be due to the different composition and concentration of antioxidant compounds in the essential oil compared to the ethanolic extracts. In contrast, the ethanolic

extract JEE2 exhibited only 9.38 ± 3.83 % inhibition at 0.5 mg/mL, suggesting a limited antioxidant activity compared to JEE1.

Quercetin, used as a reference compound, exhibited the highest DPPH inhibition, with an IC50 value of 0.01 ± 0.001 mg/mL. This is expected, as quercetin is a well-known and potent antioxidant compound.

these findings highlight the superior antioxidant capacity of the ethanolic extracts, particularly JEE1, over the JOX-EO and JEE2.

b) CUPRAC

The cupric ion reducing antioxidant capacity assay measures the reducing power of antioxidant compounds in JOX extracts by reducing Cu(II) to Cu(I), forming a colored complex with neocuproine. This reducing power is quantified using a calibration curve using gallic acid as a standard Figure 24.

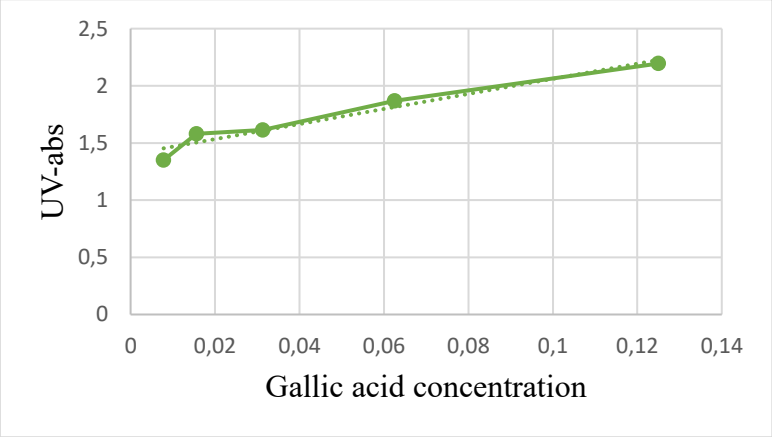


Figure 24: Gallic acid calibration curve (y =6.5937x+1.4018; R^2 = 0.9514) for CUPRAC assay for determining reducing the power of JOX extracts.

The CUPRAC assay revealed differences in the reducing power of the tested JOX extracts. Based on the calibration curve (Table 08), JOX essential oil (OXEO) at 5 mg/mL showed no detectable reducing activity. In contrast, as shown in Table 08, JEE1 at 0.625 mg/mL exhibited measurable activity, with an absorbance equivalent to 0.10 ± 0.06 mg GAE/mL, indicating the presence of reducing power. Under the same conditions, JEE2 at 0.625 mg/mL did not show detectable activity. These findings suggest that the UAE-derived extract (JEE1) possesses greater reducing power than both JOXEO and JEE2, highlighting the influence of the extraction method on the antioxidant potential of JOX extracts.

c) FRAP

The obtained results exhibited the existence of reductones, which can break off free radical chain reactions by donating an electron or a hydrogen atom, and are frequently associated with the reducing power [163]. In the provided results (Table 8), the reducing power of JOX extracts was evaluated using a quercetin calibration curve.

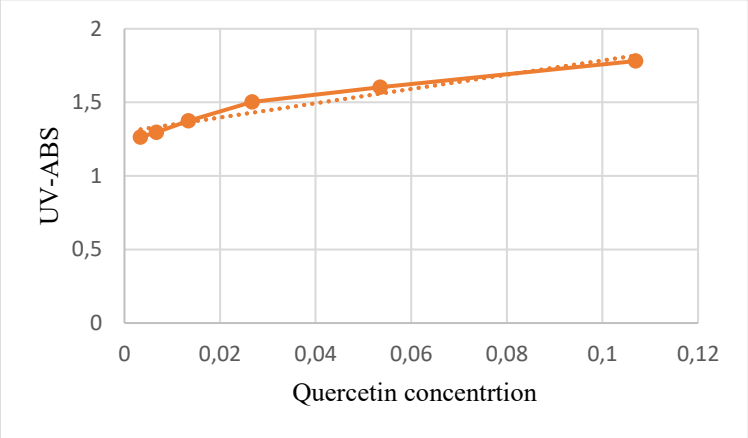


Figure 25: Quercetin calibration curve (y = 4.8448x + 1.3001; R^2 = 0.9359) for FRAP assay.

These results indicate that JEE1 exhibited the highest reducing power among the tested JOX extracts, with a value of 0.103 ± 0.004 µg QE/mL at 267 µg/mL, suggesting a greater presence of reductones or compounds with electron-donating ability. In comparison, the essential oil showed a lower but detectable reducing power of 0.022 ± 0.006 µg QE/mL at 2.11 mg/mL. By contrast, JEE2 showed no important activity, with a value of 0.002 ± 0.002 µg QE/mL at 267 µg/mL. Overall, these findings show that JEE1 has the strongest ferric-reducing capacity, while the essential oil exhibits weak activity and JEE2 is essentially inactive under the tested conditions. The reducing power measured by the FRAP assay reflects the ability of the extracts to donate electrons and reduce oxidized intermediates, and the use of the quercetin calibration curve allows this activity to be expressed in quercetin equivalents for standardized comparison.

Table 08: DPPH, FRAP, and CUPRAC test results for antioxidant activity.

JOX Extract	DPPH (IC50; mg/mL)	Reducing Power	
		CUPRAC (mg GAE/mL)	FRAP(µg QE/mL)
JOX-EO	4.14 ± 0.35	NA (at 5mg/mL)	0.022 ± 0.006 (at 2.11mg/ml)
JEE1	0.224 ± 0.008	0.10 ± 0.06 (at 0.625 mg/ml)	0.103 ± 0.004 (at 267µg/mL)

JEE2	In 0.50 mg/mL I=9.38 ± 3.83 % ~9.4%	NA (at 0.625 mg/mL)	0.002 ± 0.002. NA (at 267µg/mL)
Quercetine	0.01 ± 0.001	/	/

NA : no active, Mean ± SD of Triplicate Measurements.

d) Nitric oxide scavenging activity

A highly reactive and unstable free radical, NO, is involved in several physiological and pathological processes. Efficient scavenging of nitric oxide (NO) is essential to prevent its reaction with superoxide, which produces peroxynitrite, a highly reactive oxidant that can cause extensive cellular damage. This rapid, diffusion-controlled reaction leads to the formation of peroxynitrite, which oxidizes and nitrates various biomolecules, thereby contributing to oxidative and nitrosative stress in cells. Such stress plays a critical role in the development of numerous diseases, including cardiovascular and neurodegenerative disorders[164-166].

Table 09: NO results of ethanolic extracts JEE1 (UAE), JEE2 (CE), and JOX-EO compared to Ascorbic acid (AA) at varying concentrations: mean ± SD of triplicate measurements.

samples	**Concentration mg/mL**	**NO/Antioxidant Properties**		
		Inhibition %	**IC50; µg/mL**	**IC70; µg/mL**
JEE1	01.25	07.89 ± 1.48	204.00 ± 9.00	30.00 ± 18.00
AA	00.50	81.47 ± 2.27	153.00 ± 9.00	225.00 ± 09.00
JEE2	01.25	22.67 ± 0.19	N.A	N.A
JOX-EO	09.88	07.27 ± 2.53	N.A	N.A

The evaluation result of NO scavenging activity revealed that ethanolic extract JEE1 (UAE) at 1.25 mg/mL exhibited 7.89 ± 1.48 inhibition. Still, its IC50 of 204.00 ± 9.00 µg/mL and notably low IC70 of 30.00 ± 18.00 µg/mL suggest that its activity increases at lower concentrations, indicating a promising potential for higher efficacy.

In contrast, ethanolic extract JEE2 (CE) demonstrated better performance with 22.67 ± 0.19% inhibition at the same concentration, but it does not show the same capacity for increased activity at lower doses. The JOX-EO showed the least activity, achieving only 7.27 ± 2.53% inhibition at 9.88 mg/mL. As a benchmark, AA at 0.5 mg/mL exhibited a significant 81.47 ± 2.27% inhibition, with an IC50 of 153.00 ± 9.00 µg/mL and an IC70 of 225.00 ± 9.00 µg/mL.

These findings suggest that while ascorbic acid is a potent NO scavenger, JEE1 has a relatively low NO scavenging activity at 1.25 mg/mL; its activity increases significantly at lower concentrations. The IC50 and IC70 values indicate that JEE1 can achieve much higher inhibition of NO (50-70%) at 6-43 times lower concentrations than 1.25 mg/mL.

The NO scavenging activity of JEE1 is inversely proportional to its concentration, as the concentration decreases, the inhibition activity increases, as shown in Figure 26. This suggests that JEE1 may be a potent NO scavenger at lower doses. The JEE1 may be a more effective natural alternative due to its ability to enhance activity at lower concentrations.

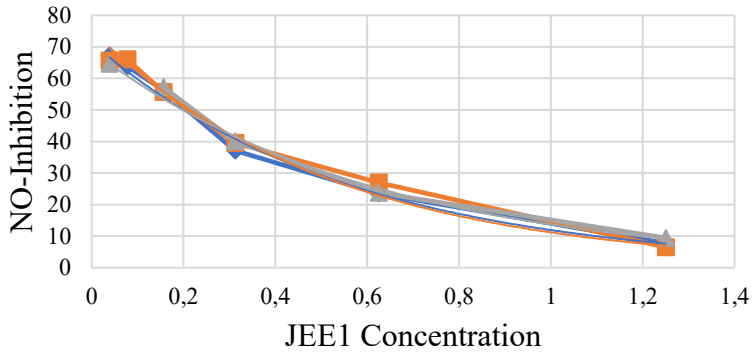


Figure 26: Effect of JEE1 concentration on the percentage of NO inhibition (three replicates).

The relationship between the concentration of JEE1 and its nitric oxide scavenging activity suggests that higher concentrations may contain inhibitory compounds that compete with active polyphenols, such as protocatechuic acid and naringenin, for binding sites. As the concentration of JEE1 decreases, these inhibitory compounds may also diminish, allowing the active polyphenols to exert their antioxidant effects more effectively. Additionally, lower concentrations may enhance the bioavailability of these compounds, facilitating their interaction with nitric oxide. Our research indicates that the increased NO scavenging activity at lower concentrations results from a delicate balance between synergistic and antagonistic interactions among the extract's constituents. This underscores the intricate and fascinating dynamics of natural extracts and their antioxidant potential, a field ripe for further exploration and understanding.

This study used various antioxidant activity tests to assess the antioxidant capacity of JOX extracts. It highlighted that reducing power alone does not necessarily indicate the overall antioxidant capacity, as other mechanisms like radical scavenging and metal chelation also play a role.

Therefore, the results underscore the exceptional DPPH radical scavenging activity of the ethanolic extract, particularly JEE1, obtained through UAE, compared to JOX-EO and JEE2. The CUPRAC assay results indicate that JEE1 exhibits the highest reducing power, followed by JOX-EO. Conversely, JEE2 shows the least reducing power, suggesting it may contain fewer reductones or compounds with weaker reducing abilities. This unique characteristic of JEE1,

as a potent NO scavenger at lower doses, underscores its potential as a more effective natural alternative due to its ability to enhance activity at lower concentrations.

The first comparative analysis of chemical compositions between JEE1 and JEE2 underlines that the higher concentrations of specific bioactive compounds in JEE1, such as protocatechuic acid, cosmosiin, and naringenin, along with its superior TPC and TFC, likely contribute to its more effective antioxidant activity. In contrast, while JEE2 has a higher concentration of quinic acid, its overall antioxidant capacity appears lower, as evidenced by its reduced TPC and TFC.

The outcomes underscore the importance of specific phytochemicals in determining plant extracts' antioxidant potential and highlight UAE's efficiency in extracting potent antioxidants. Further investigation into the individual antioxidant compounds present in these samples could provide deeper insights into their antioxidant properties.

III. 3. 3. 2. Enzyme inhibitory activity

This study involved the execution of enzyme inhibition assays, including butyrylcholinesterase, xanthine oxidase, and lipoxygenase. The results of assessing the tested JOX extracts' ability to inhibit these enzymes are presented in means ±SD for four measurements.

a) Inhibition of butyrylcholinesterase (BCHE) activity

The BChE inhibition activity of ethanolic extracts JEE1 (UAE) and JEE2 (CE), JOX-EO, and the reference compound galantamine hydrobromide (GAL-h) was evaluated. The results are summarized in Table 10.

Table 10: Butyrylcholinesterase inhibition activity by JEE1, JEE2, OXEO, and GAL-h.

JOX extracts	**BCHE Inhibition Activity ($IC_{50\%\ \mu g/ml}$)**	
	µg/ml	**Inhibition %**
JOX-EO	048.72 ± 002.56	50.00 ± 0.00
JEE1	110.62 ± 002.23	50.00 ± 0.00
JEE2	500.00 ± 000.00	42.12 ± 6.04
GAL-h	045.46 ± 000.44	50.00 ± 0.00

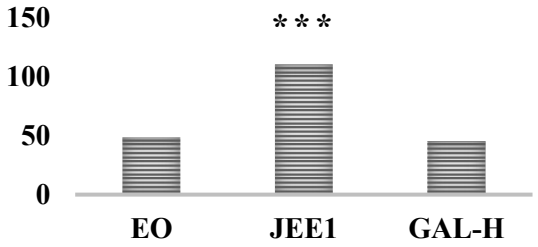


Figure 27: IC50 of JEE1, JOX-EO and GAL-h BCHE activity.

With its potent BChE inhibition demonstrated by an IC50 value of 48.72 ± 2.56 µg/mL, the JOX-EO emerges as a promising candidate for treating Alzheimer's disease.

The ethanolic extract JEE1, while showing a moderate BChE inhibition with an IC50 value of 110.62 ± 2.23 µg/mL, still demonstrates significant inhibitory activity against BChE. This reassures us of its potential in the field of medicinal chemistry.

For ethanolic extract JEE2, the inhibition was reported as 42.12% at a concentration of 500 µg/mL since an IC50 value was not provided.

The reference compound GAL-h, a known BChE inhibitor, exhibited an IC50 value of 45.46 ± 0.44 µg/mL. This is comparable to the essential oil, indicating that the JOX-EO has a potency similar to that of the drug galantamine.

b) Xanthine oxidase (XO) inhibition

The JOX extracts JEE1 (UAE), JEE2 (CE), OXEO, and the reference medication allopurinol were assessed and compared for their ability to inhibit xanthine oxidase. Following is an account of the findings.

Table 11: XO inhibition activity by JOX extracts, JEE1, JEE2, OXEO, and Allopurinol.

samples	XO inhibition activity	
	µg/ml	Inhibition %
JOX-EO	184.71 ± 6.75	50.00 ± 0.00
JEE1	234.43 ± 13.90	50.00 ± 0.00
JEE2	250.00 ± 0.00	24.46 ± 0.89
Allopurinol	00.78 ± 0.08	50.00 ± 0.00

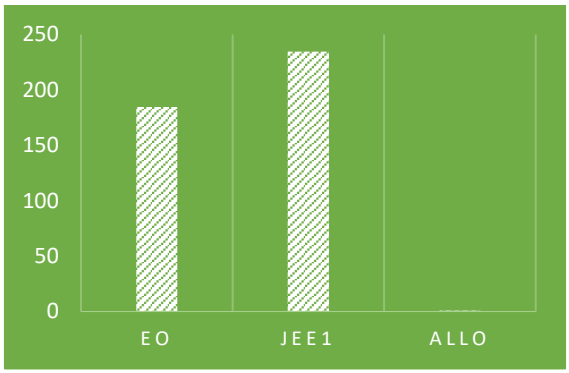


Figure 28: IC50 of XO activity of JEE1, JOX-EO (EO), and Allopurinol(allo).

The IC50 value represents the concentration required to inhibit 50% of the XO enzyme activity. A lower IC50 indicates more potent XO inhibition.

Allopurinol, the positive control, exhibited the most potent XO inhibitory activity with an IC50 of 0.78 ± 0.08 µg/mL. This is consistent with its clinical use as a first-line treatment for gout due to its ability to effectively reduce uric acid production by inhibiting XO.

The JOX-EO showed the most potent XO inhibition among the test samples with an IC50 of 184.71 ± 6.75 µg/mL, suggesting that the essential oil contains compounds that can effectively suppress XO activity, albeit less potent than allopurinol.

The ethanolic extract JEE1 had an IC50 of 234.43 ± 13.90 µg/mL, indicating moderate XO inhibitory potential compared to OXEO. In contrast, JEE2 exhibited only 24.46% inhibition at 250 µg/mL, demonstrating weaker XO inhibition than JEE1 and OXEO.

The results suggest that the essential oil and ethanolic extracts, particularly JEE1, contain bioactive compounds with XO inhibitory properties. Further investigation is needed to identify the specific compounds responsible for the observed activity and to optimize their XO inhibition potential.

Comparing the IC50 values, the JOX-EO and ethanolic extracts are less potent than allopurinol. However, they may still have therapeutic applications, especially in combination with allopurinol or as alternative treatments for individuals who cannot tolerate allopurinol's side effects.

Additional studies are required to assess these natural XO inhibitors' safety, efficacy, and mechanism of action in vivo and to determine their potential as complementary or alternative therapies for hyperuricemia and gout.

c) Lipoxygenase (LOX) inhibitory activity

The LOX inhibitory activity assay results for the ethanolic extracts and essential oil compared to baicalein reveal varying degrees of effectiveness in inhibiting the enzyme. Table 12 and Figure 33 show detailed results and the obtained IC50 values.

Table 12: JOX extracts, JEE1, JEE2, JOX-EO, and the baicalein's LOX inhibitory activity assay.

samples	LOX inhibition activity ($IC_{50\%\ \mu g/ml}$)	
	µg/ml	Inhibition %
JOX-EO	65.33 ± 2.91	50.00 ± 0.00
JEE1	229.96 ± 4.07	50.00 ± 0.00
JEE2	357.00 ± 0.00	23.12 ± 4.27
Baicalein	31.40 ± 3.71	50.00 ± 0.00

The outcomes reveal that while baicalein remains the most potent inhibitor with an IC50 of 31.4 ± 3.71 µg/mL, the EO and ethanolic extracts demonstrate varying degrees of inhibitory effects, with the JOX-EO showing a moderate IC50 of 65.33 ± 2.91 µg/mL, followed by JEE1 at 229.96 ± 4.07 µg/mL. This value suggests that JEE1 is less effective than the JOX-EO in inhibiting LOX activity, as a higher concentration is required to achieve the same level of inhibition.

Although the exact IC50 value for JEE2 is not provided, it showed 23.12 ± 4.27% inhibition at 357 µg/mL. This indicates that JEE2 has a relatively low inhibitory effect on LOX, as it requires a higher concentration to achieve only a modest level of inhibition.

These findings suggest that the JOX-EO and ethanolic extract JEE1, despite being less effective than baicalein, still hold potential as natural inhibitors of LOX, which could be beneficial in developing anti-inflammatory therapies.

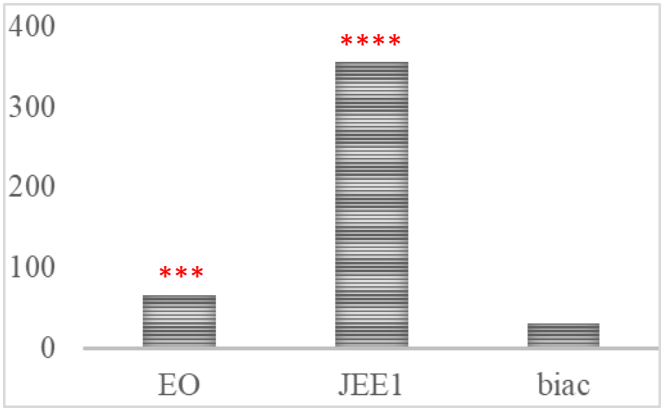


Figure 29: IC50 of JEE1, JOX-EO and baicalein's lipoxygenase inhibitory activity.

The results indicate that JOX-EO exhibits a greater efficacy as a natural inhibitor of specific enzymes, namely butyrylcholinesterase, xanthine oxidase, and lipoxygenase, compared to ethanolic extracts. However, while EO is more effective than ethanolic extracts, they are still less potent than certain reference compounds.

The pronounced effectiveness of JOX-EO is largely due to its specific terpene constituents, notably α-pinene, germacrene D, β-myrcene, δ-cadinene, and manooloxide. These compounds exhibit a stronger inhibitory effect on the enzymes butyrylcholinesterase (BCHE), xanthine oxidase (XO), and lipoxygenase (LOX) compared to the polyphenols present in the ethanolic extracts JEE1 and JEE2. This enhanced enzyme inhibition underscores the essential oil's distinctive bioactive profile, which may offer superior therapeutic potential in certain applications, particularly in targeting these enzymes.

Inhibition of BCHE, XO, and LOX is especially beneficial in the context of reducing cigarette smoke toxicity and its associated health risks. as mentioned before Smoking impacts nAChRs, with receptor dysfunction linked to neuroinflammation and the progression of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease[167, 168]. Smokers demonstrate elevated cerebrospinal fluid biomarkers of neurodegeneration, including β-amyloid 42 (Aβ42) and tumor necrosis factor alpha (TNFα), reflecting increased oxidative stress and neuroinflammatory responses[169]. Notably, BChE is concentrated near β-amyloid plaques, implicating it in AD pathology[170, 171]. The inhibition of BChE by extracts from JOX, especially JOX-EO, may attenuate smoking-related neuroinflammation given BChE's role in inflammatory and neurodegenerative mechanisms. Furthermore, in other studies, monoterpenes such as alpha-pinene have demonstrated effective inhibition of equine serum BChE in vitro, suggesting a potential neuroprotective mechanism [172]. The resut od this studie suggests that JOX-EO is an effective inhibitor of BChE and could potentially be used to treat Alzheimer's disease.

XO catalyzes the production of reactive oxygen species (ROS), contributing to oxidative stress[173], which is exacerbated by cigarette smoke exposure and implicated in cardiovascular and pulmonary diseases.Similarly,15-LOX metabolize arachidonic acid to bioactive lipids such as 15-hydroxyeicosatetraenoic acid (15-HETE), which plays a key role in airway inflammation and remodeling [174]. This enzyme facilitates the migration and activation of immune cells, including eosinophils and T cells, thereby sustaining chronic pulmonary inflammation[174]. Smoking elevates tissue factor expression via pathways involving 15-LOX products, contributing to inflammatory diseases like atherosclerosis and airway inflammation[175].

By targeting these enzymes, JOX-EO reduces oxidative stress and inflammation induced by cigarette smoke, potentially protecting against cellular damage and slowing disease progression. Thus, natural inhibitors like α-pinene in JOX-EO represent a promising approach to counteract smoking-related pathologies, including chronic obstructive pulmonary disease (COPD), cardiovascular disorders, and neurodegeneration.

III. 3. 3. 3. Cytotoxicity of JOX-EO

Smoking cigarettes is a major risk factor for lung cancer since some of the cigarette smoke components are carcinogenic. The potential cytotoxicity of the JPH and JOX berries' EOs against human lung adenocarcinoma cell lines (A549) was evaluated and reported as percentage cell viability compared to the control and IC50 in Figure 30.

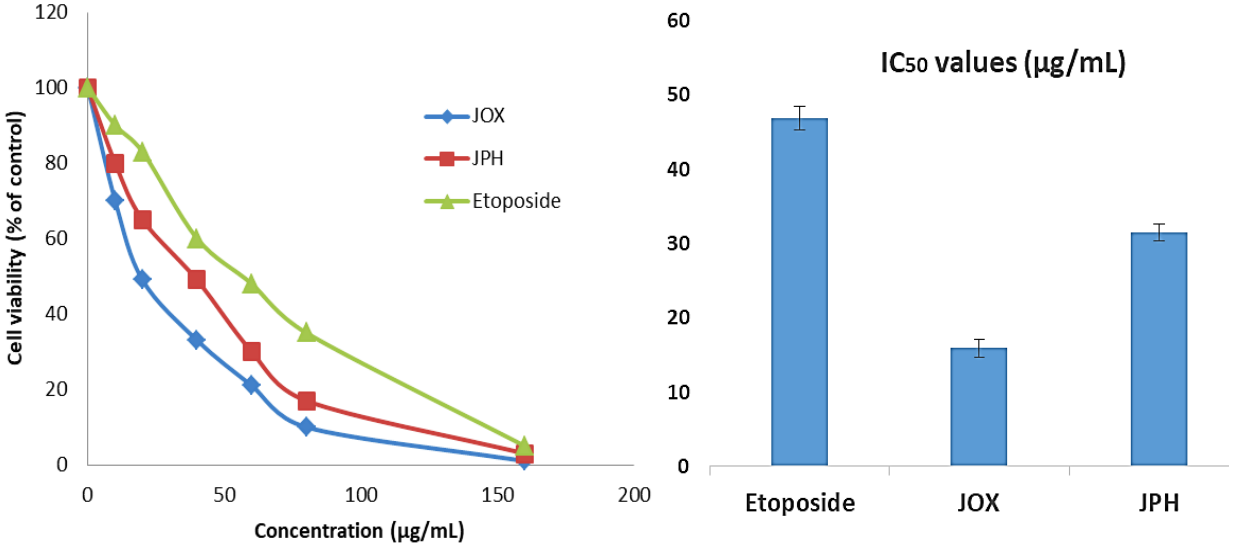


Figure 30: Cell viability inhibition and cytotoxicity of the JPH and JOX EOs against A549.

The MTT viability assay was used to evaluate the cytotoxicity of essential oils from JPH and JOX berries against A549 lung cancer cells.

This assay measures the metabolic activity of cells, which is indicative of their viability, after a 24-hour treatment with varying concentrations of the EOs. Both EOs demonstrated a concentration-dependent decrease in cell viability. The IC50 for JPH was 31.5 ± 1.11 µg/mL, indicating moderate cytotoxicity, while JOX showed a more substantial effect with an IC50 of 15.88 ± 1.25 µg/mL. Consequently, both EOs were more effective than the standard chemotherapy drug etoposide, a commonly used drug in lung cancer treatment, which exhibited an IC50 of 46.9 ± 1.55 µg/mL (Figure 30).

Cell viability represents the number of healthy cells surviving post-treatment, serving as a critical indicator of the potential anticancer efficacy of therapeutic agents. As concentrations of the EOs increased, cell viability decreased, with JOX-EO showing higher potency than JPH-EO. The cytotoxicity is likely due to the chemical composition of the oils.

The JOX-EO is rich in α-pinene, β-myrcene, and α-amorphene. α-Pinene is notable for its potent antioxidant, anticancer, and genotoxic effects, alongside respiratory infection treatment capabilities [176, 177]. Its anticancer activity involves inhibiting reactive oxygen species (ROS) production, preventing lipid peroxidation, and downregulating inflammatory mediators [178]. Furthermore, α-pinene suppresses metastatic lung colonization and induces apoptosis through mitochondrial membrane potential disruption, ROS generation, caspase-3 activation, DNA fragmentation, and phosphatidylserine exposure, reducing tumor growth in animal models [179-182]. Importantly, α-pinene selectively targets cancer cells without toxicity to normal cells, underscoring its therapeutic promise [181]. It modulates oxidative stress biomarkers and enhances antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase [183].

The notable anticancer effects of JOX-EO may also derive from its considerable β-myrcene content, which has demonstrated antioxidant, anti-inflammatory, and anticancer activities, including inhibition of tumor cell proliferation and migration [184-186].

EOs and their bioactive compounds often act synergistically, enhancing the efficacy of chemotherapeutic agents[187, 188]. The potential of EOs for improving the effectiveness of existing treatments offers optimism about their future use. Thus, JOX-EO shows significant potential in reducing harmful toxicants inhaled from cigarette smoke and boosting endogenous antioxidant defenses, suggesting its value in lung cancer prevention and adjunct therapy.

III. 4. IN VIVO OUTCOMES

Part 01: Subchronic CSE

III. 4. 1. Oxidative stress parameters

Cigarette smoke exposure (CSE) is a major contributor to oxidative stress. The first section of this animal study demonstrated that oxidative stress parameters were impacted by sub-chronic exposure to cigarette smoke, which is four weeks of exposure. The following main results drawn from this search include how oxidative stress indicators are affected by subchronic CSE after the withdrawal of smoke and the assessment of the effect of JPH and JOX EOs treatment on the oxidative stress markers in smoking cessation.

III. 4. 1. 1. Body and relative organ weights

Table 13 gathers the calculated relative organ weight (brain and lung body weight ratios) of the smoking-withdrawn rats treated with JOX-EO for 2 weeks. The results represent the mean ± SD of 4 determinations.

Table 13: Effect of EOs on Wister rats' relative organ (brain and lung) weight.

Groups		Final body weight (g)	organ weight (g)		Relative organ weight% %	
			brain	lung	brain	lung
Control groups	C	130.50 ± 15.19	1.56 ± 0.08	0.92 ± 0.09	1.23 ± 0.09	0.71 ± 0.02
	COX	148.00 ± 07.03	1.63 ± 0.06	0.93 ± 0.04	1.10 ± 0.02	0.63 ± 0.03
	CPH	154.00 ± 06.37	1.76 ± 0.04	0.97 ± 0.01	1.14 ± 0.02	0.63 ± 0.03
	CS	161.50 ± 0 5.78	1.64 ± 0.04	1.03 ± 0.01	1.01 ± 0.04	0.63 ± 0.02
Treated groups	STOX	152.00 ± 14.35	1.67 ± 0.03	1.06 ± 0.07	1.12 ± 0.07	0.69 ± 0.02
	STPH	152.75 ± 14.38	1.62 ± 0.03	0.99 ± 0.08	1.09 ± 0.12	0.65 ± 0.01

The results show that the brain and lung body weight ratios of smoking-withdrawn rats in the negative control and EOs-treated groups at a dose of 200 mg/kg for two weeks were not significantly different from those of the control rats ($P > 0.05$). This implies that JPH and JOX EOs treatment does not induce any adverse effects on organ weight relative to body weight during smoking withdrawal. This lack of significant difference suggests that EOs may maintain organ homeostasis and could be a viable option for managing withdrawal symptoms without compromising organ health.

Additionally, the similarity in organ weight ratios between the negative control and treated groups indicates that smoking withdrawal itself may not lead to significant changes in these specific organ metrics after one month of smoking, which could reflect a resilience in the physiological response of the rats.

III. 4. 1. 2. Hematological analysis

The hematological parameters (RBC, PLT, HB, HCT, and MCV) of Wistar rats given oral JPH and JOX EOs for two weeks are presented in Table 14. Every hematological evaluated parameter was contrasted with the control rats' values.

Table 14: Hematological parameters.

Red cell count	**Control**	**STOX**		**STPH**		**CS**		**COX**		**CPH**	
	Mean ± SD	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value	Mean ± SD	P value
RBC (10^12/L)	8.57±0.25	8.55±0.12	0.8648	11.35±0.48	0.0005	12.27±1.21	0.0010	6.50±0.00	0.0003	7.25±0.46	0.0024
PLT (10^9/L)	1235.50±8.81	1226.75±4.03	0.1209	1293.50± 8.06	0.0006	1515.25±104.36	0.0017	1210.50±1.29	0.0013	1198.75±6.85	0.0005
HB (g/dL)	15.45±1.00	14.70±0.31	0.2055	14.66±0.13	0.1726	9.15± 1.65	0.0006	13.15±0.50	0.0065	15.00±0.54	0.4626
HCT %	44.00±7.61	49.25±0.95	0.2203	44.00± 4.39	>0.999	29.25±9.77	0.0547	45.25±2.63	0.7668	44.75± 3.09	0.8612
MCV (fL)	55.75±1.70	55.62±1.10	0.9062	45.75± 2.50	0.0005	43.50±2.38	0.0001	49.90±2.16	0.0054	55.15±0.87	0.5543

Hematological analyses often show that smoking elevates RBC, HB, HCT, MCV, MCH, WBC[189, 190] .

In this study, rats exposed to cigarette smoke for one month exhibited increased RBC and PLT counts. In contrast, HB, HCT, and MCV levels decreased, possibly due to reduced carbon monoxide exposure during the 15-day smoking cessation period. Typically, HCT and MCV increase as a response to anoxia from carboxyhemoglobin formation during smoking [190]. Low MCV values observed in the COX (control treated with JOX-EO), CS (smoke-exposed), and STPH (smoking group treated with JPH-EO) groups may indicate microcytic anemia, often linked to iron deficiency or other illnesses [190].

These findings suggest that JPH-EO did not significantly mitigate hematological risks associated with smoking. Conversely, the STOX group (smokers treated with JOX-EO) had hematological parameters comparable to non-smokers, indicating a potential antioxidant-driven protective effect from JOX-EO.

III. 4. 1. 3. Results of oxidative stress biomarkers (OSB)

The assessment of the effect of JPH and JOX EOs treatment on the intracellular levels of oxidative stress markers in the lung and brain tissues. The results showed in Figures 31 and 32 were *($P < 0.01$), **($P < 0.001$), and ***($P < 0.0001$).

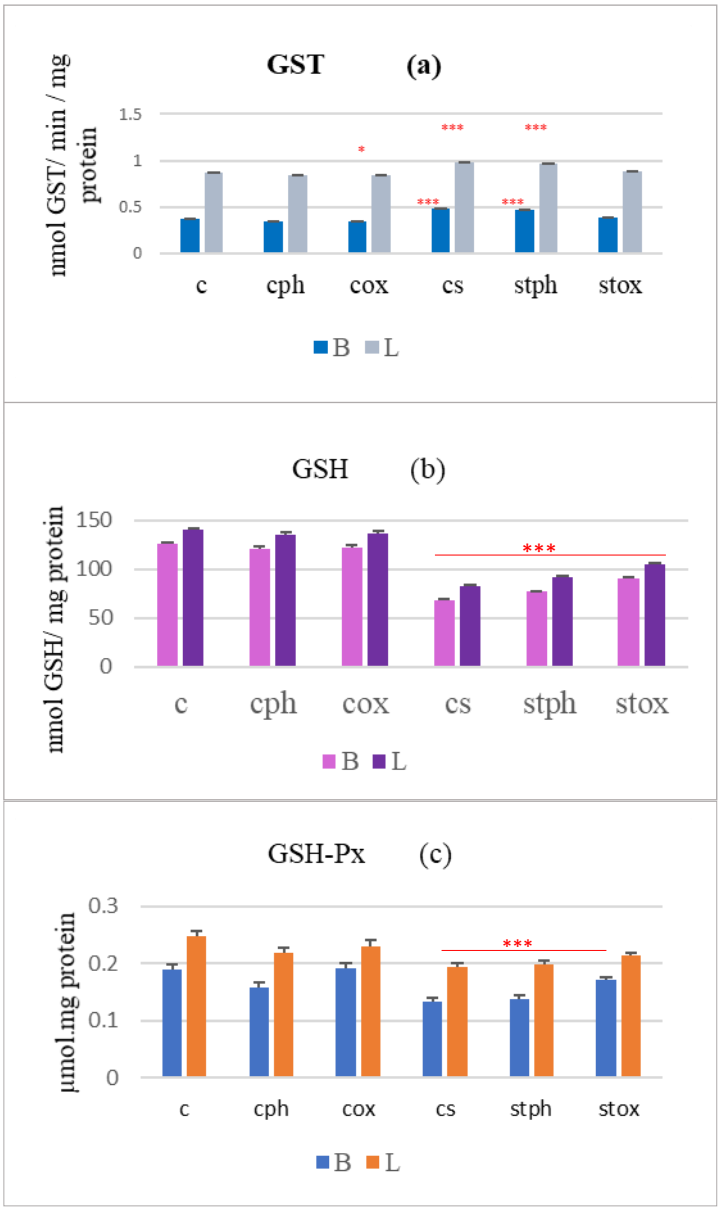


Figure 31: Impact of JOX-EO and JPH-EO treatment on intracellular levels of (a) glutathione-S-transferase GST, (b) glutathione GSH, (c) glutathione peroxidase GSH-Px, in lung and brain tissues of no smoker and smoking cessation rats relative to the control groups.

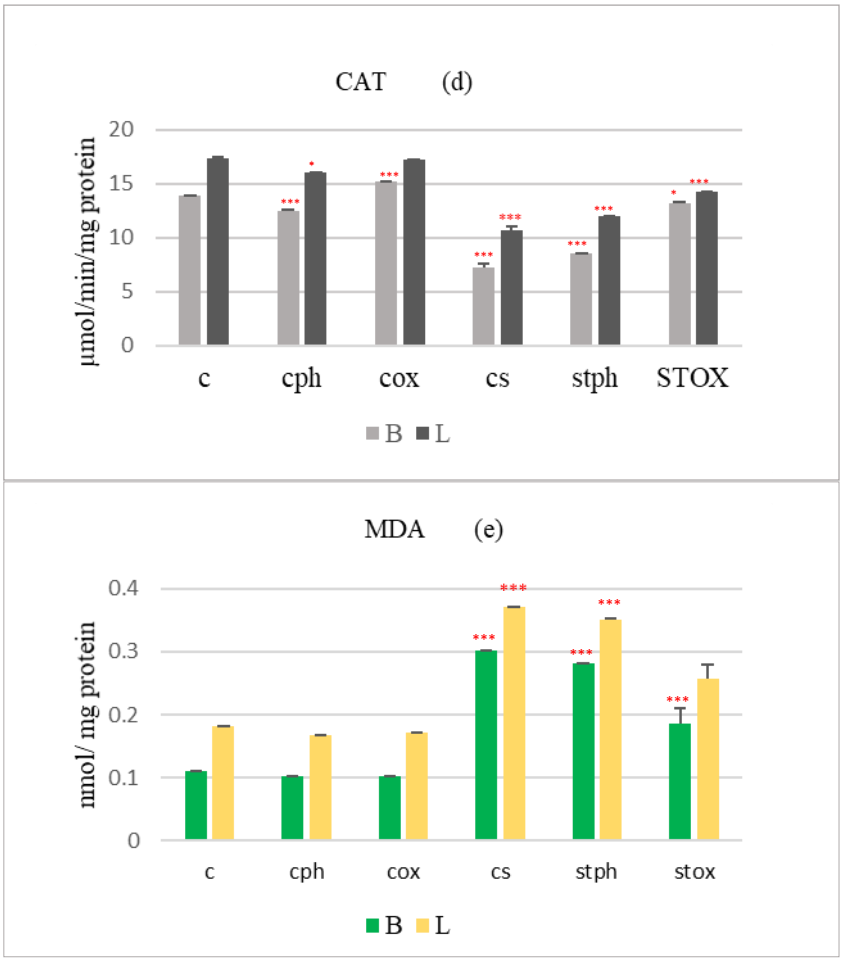


Figure 32: (d) catalase CAT, and (e) malondialdehyde MDA levels in the lung (L) and brain (B) following two weeks of treatment by the JOX-EO and JPH-EO in smoking cessation rats relative to the control groups.

Antioxidant defense systems in tissues like the lungs and brain protect cells from ROS damage. Enzymatic antioxidants such as SOD, GSH-Px, and CAT neutralize free radicals by converting them into harmless water and oxygen[192]. GSTs, CYPs, and non-enzymatic antioxidants like reduced GSH play vital roles in detoxification and maintaining redox balance [193]. However, an imbalance favoring ROS produces oxidative stress and lipid peroxidation, producing MDA, which damages cells and contributes to disease. Cigarette smoke, rich in oxidants and ROS-generating compounds like nicotine and PAHs, significantly induces oxidative stress by damaging biomolecules such as lipids, proteins, and DNA [194].

In this study, EOs improved antioxidant status in rat lung and brain tissues after cigarette smoke exposure, as evidenced by modulation of oxidative stress markers, including GSH-Px, CAT, GST, GSH, and MDA. Previous research highlights that smoking decreases GSH levels in lung cells, predisposing them to disease[195]. Decreased GSH in smoking-withdrawn rats

treated with EOs suggests partial mitigation of oxidative stress, with JOX-EO potentially modulating oxidative damage rather than directly increasing endogenous antioxidants like GSH. The reduction in GST following EO treatment may reflect interference with detoxification pathways or metabolic modulation rather than increased oxidative stress. Meanwhile, the elevated GST in untreated smoking-withdrawn rats appears to be a compensatory metabolic response to detoxify accumulating oxidative products, helping restore homeostasis. This finding underscores the potential of JOX-EO to restore homeostasis, providing reassurance and confidence in its therapeutic potential.

Notably, GST and MDA levels decreased in smoking rats treated with JOX-EO compared to untreated smokers, indicating a reduction in toxin-induced oxidative stress. JOX-EO demonstrated more antioxidative effects than JPH-EO at the same dosage. Despite a 15-day smoking cessation, oxidative stress markers GSH, GSH-Px, and CAT remained low while GST and MDA remained elevated, suggesting prolonged damage that requires extended recovery time. The superior antioxidant capacity of JOX-EO supports faster restoration of red blood cell counts and improved oxidative status in vivo, corroborating previous in vitro data showing higher antioxidant activity of JOX-EO compared to JPH-EO. This promising result underscores the potential of essential oils, derived from medicinal plants, as a therapeutic intervention for mitigating cigarette smoke-associated diseases and the neuropsychological impairments related to smoking cessation and nicotine withdrawal.

III. 4. 2. Ability of JOX berries (JOX-EO, JEE) in reducing the toxicity of cigarette smoke in vivo

Conducting the study on the ability of JOX extracts to reduce the toxicity of cigarette smoke offers several key benefits, including the assessment of organ health through relative weight measurements, analysis of blood and biochemical parameters to understand the inflammatory response, and evaluation of glycemic index and metabolites at vital organ levels. This research aims to validate previous findings regarding the protective effects of JOX extracts against oxidative stress, potentially leading to dietary recommendations for smokers. While the study is well-designed to reveal the extract's efficacy, its sufficiency will ultimately depend on factors such as sample size, control groups, and the duration of exposure, which are crucial for establishing a causal relationship between JOX consumption and reduced toxicity from cigarette smoke.

III. 4. 2. 1. Changes in rat weights over 18 weeks

Figure 33 displays histograms summarizing the findings from an 18-week study tracking weight changes in rats. The study involved two groups: experimental smoker rats and a control group of non-smoker rats. Weight measurements were taken weekly throughout three key phases: an initial adaptation period, a phase involving exposure to cigarette smoke, and a smoking withdrawal phase during which JOX extracts treatment was administered. Each data point represents the average weight of five rats per group, providing insight into how smoking, withdrawal, and therapy influence weight variations over time.

The first histogram (Fig. 33; I) demonstrates that the control group (C) of rats showed a steady and continuous increase in body weight throughout the entire 18-week study period. This pattern is consistent with normal growth expected in healthy rats, implying that the absence of harmful agents like cigarette smoke permits standard physiological development and weight gain. Results are reported as the mean of five weekly measurements for each group.

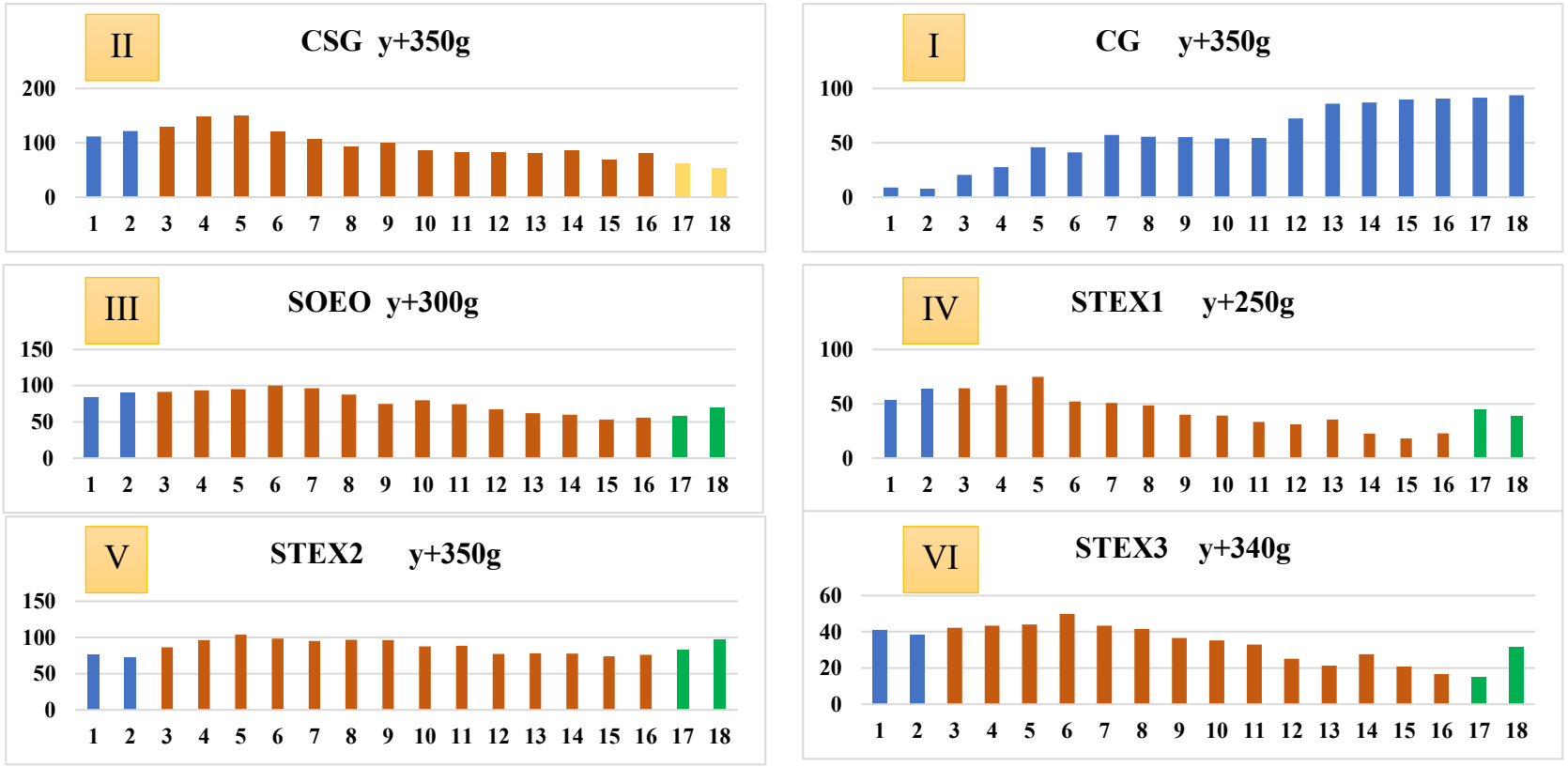


Figure 33: Weight changes in experimental smoker rats groups with control over 18 Weeks (1-2: adaptation period; 3-16: exposure to cigarette smoke; 17-18: withdrawal from smoking and treatment with JOX extracts), y + x (g): the original weight of rat.

In sharp contrast, the second histogram (Fig. 33; II) illustrates the weight progression of the cigarette smoke group (CSG). At first, these rats gained weight during the adaptation phase and the initial three weeks of smoke exposure. However, their weight declined significantly beginning at week 5 and continuing to the experiment's conclusion. This trajectory supports previous findings that smoking can induce weight loss [195]. While altered metabolism and reduced appetite are commonly cited explanations, a related study found that fluctuations in peripheral appetite-regulating hormones (leptin and ghrelin) do not fully account for weight changes in smoking and cessation models in rats [196]. In this investigation, rats were fed a controlled amount equivalent to 10% of their body weight daily to prevent obesity.

Significantly, all groups, including the smoke-exposed rats, consumed their full rations, suggesting that appetite suppression was not the primary cause of weight loss. Instead, nicotine's well-documented effect of suppressing weight gain, independent of food intake, likely played a central role, with no notable compensatory weight increases observed during nicotine cessation [197]. This explains the persistence of weight loss even during the withdrawal period in the smoker group.

It is also noteworthy that all smoking groups initially experienced weight gain during the first weeks of smoke exposure. This phenomenon may result from multiple interrelated factors. Nicotine's early physiological effects, combined with experimental stress, might promote energy storage and thus increase weight during adaptation. Additionally, environmental stressors such as changes in housing or discomfort caused by smoke exposure could lead rats to increase food consumption as a coping response. Moreover, nicotine-induced metabolic changes may not be fully activated during the early exposure period, causing temporary weight gain while the organism adjusts. As the study progressed, nicotine's metabolic effects, such as elevated fat utilization and increased energy expenditure, became more pronounced, leading to weight stabilization or loss, which is typical of nicotine's impact on body weight [198].

The remaining histograms illustrate the effects of oral administration of JEE1 during the withdrawal phase; the third histogram (Fig 33; III, SOEO treatment (JOX-EO, 200mg/Kg)) shows that while the rats initially gained weight during adaptation and the first four weeks of smoke exposure, they experienced a decline in weight until week 16. However, a slight increase in weight was observed during the last two weeks of withdrawal when treated with essential oil. This suggests that the essential oil had a mild restorative effect on weight during withdrawal, although insufficient to counteract the prior weight loss fully.

The fourth histogram (Fig 33; IV, STEX1 group (JEE1, 200mg/kg)) indicates a similar pattern, with initial weight gain followed by a decline. Notably, there was a significant weight

increase in the first week of withdrawal but a slight decrease in the second week. This suggests that while the ethanolic extract had a positive initial impact, its efficacy may wane.

The STEX2 group (Fifth histogram, Fig. 33; V) reflects a pattern of weight gain during adaptation and early exposure, followed by a decline. However, unlike STEX1, this group with 400mg/Kg of JEE1 showed a significant increase in weight during the withdrawal phase, indicating a potentially more effective treatment than 200 mg/Kg.

The STEX3 group (Sixth histogram, 800mg/kg, Fig. 33; VI) mirrors the trends observed in the previous groups, with initial weight gain followed by a decline. However, this group also showed significant weight recovery during withdrawal, suggesting that the extract JEE1 in 800mg/Kg may be effective in mitigating some of the weight loss associated with smoking cessation, but is not as effective as in 400mg/Kg. The weight variation observed between the final week of smoking and the last week of withdrawal and treatment (the second week post-withdrawal) offers essential insights into the efficacy of different JEE1 dosages. The smoker group (CSG) exhibited a substantial weight loss of 28.6 g, whereas the STEX2 group (400 mg/kg) showed a significant weight gain of 20.6 g, and the STEX3 group (800 mg/kg) gained 15 g. These results underscore the superior effectiveness of the 400 mg/kg dose of JEE1 in counteracting smoking-related weight loss. The dose-response relationship suggests that the 400 mg/kg dosage achieves a more optimal balance, yielding the most pronounced benefits in weight maintenance during withdrawal, making it a more effective treatment than the higher 800 mg/kg dose.

The trends in these histograms clearly illustrate the adverse effects of cigarette smoke on weight regulation in rats, with significant weight loss in the smoking group. The treated groups exhibit varying degrees of weight recovery during withdrawal, demonstrating that JOX-EO and JEE1 help alleviate smoking-associated weight loss.

III. 4. 2. 2. Hematological results

The levels of hematological parameters in Wistar rats following smoking cessation across different treatment groups (no-smoker, smoker, and treated groups) are presented in Tables 15 and 16. Each evaluated hematological parameter was compared with the control rats' values using P values for statistical analysis.

Table 15: Hematological parameters levels of no-smoker groups after experiment.

Red cell count	unit	**No smoker groups**						
		CG	**EXD1**		**EXD3**		**JEO**	
		Mean ± SD	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**
WBC	10^9/L	12.90 ± 00.56	10.40 ± 000.78	0.0107	10.13 ± 000.57	0.0038	10.47 ± 01.00	0.0212
RBC	10^12/L	08.02 ± 00.31	08.05 ± 000.68	0.9536	08.07 ± 000.75	0.9257	07.87 ± 00.57	0.5931
HGB	g/Dl	15.67 ± 00.47	15.23 ± 001.16	0.5811	15.73 ± 001.10	0.9279	14.80 ± 01.00	0.2463
HCT	%	36.63 ± 00.90	39.10 ± 002.66	0.2034	39.93 ± 002.67	0.1122	39.03 ± 03.58	0.3230
MCV	Fl	46.47 ± 01.10	48.60 ± 001.02	0.0696	49.57 ± 002.31	0.1042	47.87 ± 01.01	0.1803
MCH	pg	19.50 ± 00.20	18.90 ± 000.35	0.0602	19.50 ± 000.82	> 0.9999	19.37 ± 00.55	0.7136
MCHC	g/L	419.33 ± 12.86	404.33 ± 027.23	0.4369	400.33 ± 010.41	0.1175	403.67 ± 19.43	0.3088
PLT	10^9/L	743.33±59.07	636.67±231.74	0.4829	637.33±244.49	0.5059	642.67±32.88	0.0614

Table 16: Hematological parameters after smoking cessation across smoker and treated groups.

Red cell count		**Smoker groups**									
		CSG		**STEX1**		**STEX2**		**STEX3**		**SOEO**	
	Unit	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**
WBC	10^9/L	**03.70 ± 1.13**	**0.0002**	16.70 ± 1.93	**0.0305**	13.27 ± 2.10	0.7853	09.73 ± 1.78	**0.0423**	12.97 ± 1.85	0.9551
RBC	10^12/L	07.60 ± 0.33	0.1765	07.37 ± 0.05	**0.0221**	07.10 ± 0.85	0.1517	08.15 ± 0.79	0.8047	08.12 ± 0.16	0.6651
HGB	g/Dl	1480.60 ± 0.60	0.1326	15.00 ± 0.5	0.1686	14.50 ± 2.07	0.3944	17.60 ± 2.30	0.2270	16.13 ± 0.31	0.2242
HCT	%	44.43 ± 4.31	**0.0374**	36.57 ± 1.66	0.9542	38.03 ± 4.88	0.6505	42.30 ± 2.72	**0.0267**	40.07 ± 2.63	0.0989
MCV	Fl	54.40 ± 2.29	**0.0057**	49.60 ± 1.91	0.0696	56.10 ± 9.35	0.1512	53.33 ± 3.18	**0.0241**	50.17 ± 1.91	0.0439
MCH	pg	20.20 ± 0.46	0.0724	20.00 ± 0.82	0.3621	20.40 ± 0.61	0.0716	20.13 ± 1.16	0.4038	19.87 ± 0.25	0.1194
MCHC	g/L	370.00 ± 9.17	**0.0056**	410.33 ± 13.61	0.4520	369.00 ± 51.97	0.1788	377.67 ± 8.96	**0.0100**	396.33 ± 19.65	0.1651
PLT	**10^9/L**	658.33 ± 122.44	0.3397	620.67 ± 226.75	0.4158	624.67 ± 165.31	0.3066	641.00 ± 125.73	0.2710	801.00 ± 145.08	0.5584

Cigarette smoke significantly alters hematological parameters by increasing RBC, HGB, and WBC counts while affecting MCV and MCHC [188, 189]. Upon smoking cessation, improvements such as decreased WBC and enhanced oxygen transport are often observed; however, recovery varies depending on smoking history [191].

In this study (Tables 15 and 16), rats exposed (three month) to smoking (CSG) showed significantly reduced WBC and MCHC. Other parameters, RBC, HGB, MCH, and platelets, remained unchanged, though HCT and MCV were increased. These findings indicate that while cessation improves blood health, full normalization may require longer, consistent with previous reports of lasting hematological effects[189] [36].

Non-smoker treatment groups displayed improved hematological profiles, with EXD1 and EXD3 significantly reducing WBC counts, suggesting enhanced immune function. Among smoker withdrawal groups, STEX1 increased WBC but decreased RBC, possibly reflecting immune activation during withdrawal. STEX3 decreased WBC while increasing HCT and MCV. STEX2 showed no significant changes, highlighting the importance of dose optimization in JEE1 treatment for effective hematological recovery.

JEE1 demonstrates potential as an anti-inflammatory agent aiding recovery post-tobacco exposure. The stable hematological parameters in the JEO and SOEO groups further suggest the beneficial effects of JOX-EO treatment.

III. 4. 2. 3. Relative organ weights (ROW) and organ function biomarkers

The results from the 18-week experiment (Tables 17 and 18) provide valuable insights into the impact of various treatments on the ROW of the brain, lung, heart, liver, left kidney (LK), and right kidney (RK) in both control and smoker groups**.**

Table 17: Relative brain, lung, and heart weights across treatment groups.

Groups	**Relative organ weight %**					
	Brain	**P value**	**Lung**	**P value**	**Heart**	**P value**
CG	0.460 ± 0.024	/	0.818 ± 0.162	/	0.206 ± 0.025	/
CSG	0.488 ± 0.048	0.280	1.087 ± 0.128	**0.02**	0.327 ± 0.032	**< 0.001**
EXD1	0.639 ± 0.045	0.004	0.837 ± 0.100	0.81	0.238 ± 0.010	0.12
EXD3	0.631 ± 0.063	0.010	0.851 ± 0.103	0.89	0.244 ± 0.023	0.14
JEO	0.578 ± 0.142	0.270	0.837 ± 0.083	0.80	0.277 ± 0.017	0.02
STEX1	0.689 ± 0.029	**<0.001**	0.904 ± 0.240	0.52	0.330 ± 0.041	**< 0.001**
STEX2	0.458 ± 0.032	0.950	0.722 ± 0.256	0.50	0.220 ± 0.026	0.41
STEX3	0.506 ± 0.038	0.070	0.739 ± 0.198	0.51	0.235 ± 0.023	0.10

SOEO	0.531 ± 0.045	0.010	0.905 ±0.242	0.52	0.271 ± 0.065	0.07

a) Brain

Analysis of brain ROW (Table 17) showed no significant difference between the control group (C) and the withdrawing smoker group (CSG) ($p = 0.3$), indicating that smoking cessation alone did not alter brain weight. Similarly, the essential oil treatment group (JEO) also did not significantly differ from controls ($p = 0.3$), suggesting a minimal impact on brain ROW from this treatment. In contrast, treatment with the ethanolic extract JEE1 at 200 mg/kg (EXD1) and 800 mg/kg (EXD3) resulted in significant increases in brain ROW ($p = 0.004$ and 0.01, respectively). This increase likely reflects enhanced neuroprotective effects, supported by literature showing that natural extracts can promote brain growth through neurogenesis, improved blood flow, and antioxidant activity [199, 200]. Such mechanisms aid in maintaining brain function and plasticity, while nutritional components support overall brain health[201] [202]. However, increased brain ROW may raise concerns about vascular complications if linked with elevated platelet count[200].

Hematological parameters provide additional context to interpret brain ROW changes. Elevated WBC counts often indicate inflammation, while changes in hemoglobin and hematocrit affect oxygen delivery and the risk of ischemia[203]. In non-smoker rats, JEE1 treatment increased brain ROW alongside stable hematological profiles, including decreased WBC levels and normal HB, HCT, RBC, and PLT (Tables 15 and 16).This pattern suggests that quinic acid, the major compound in JEE1, may exerts neuroprotective and anti-inflammatory effects, promoting a healthier brain environment without adverse systemic inflammation.

Further, the withdrawal group treated with JOX-EO (SOEO) showed a significant increase in brain ROW compared to control ($p = 0.01$), indicating a pronounced effect when combined with smoking cessation. Similarly, the withdrawing smoker group treated with JEE1 at 200 mg/kg (STEX1) showed a highly significant increase in brain ROW ($p < 0.001$), but this was accompanied by a rise in WBC ($p = 0.03$), suggesting an inflammatory response. Other hematological measures remained within normal ranges. The CSG group exhibited a significant decrease in WBC, possibly reflecting reduced inflammation; nevertheless, the low WBC count ($3.7 \times 10^{\wedge 9}$/L) may indicate leukopenia and compromised immunity. These conflicting findings highlight the need to interpret treatment-related inflammatory responses carefully. In contrast, JEE1 at 400 mg/kg (STEX2) and 800 mg/kg (STEX3) showed no significant or only a marginal increase in brain ROW, with normal hematological parameters across these groups, indicating a favorable safety profile.

Together, these results underscore a complex interplay between treatment dose, brain weight, and systemic inflammation, with some doses promoting neuroprotective hypertrophy while others elicit inflammatory reactions that could offset benefits.

b) Lung

The lung analysis revealed a significant increase in lung ROW in the smoking cessation group (CSG) compared to controls ($p = 0.02$). This considerable finding and the notable reduction in WBC levels post-cessation suggest a partial recovery from inflammation. However, the persistently low WBC counts in actively smoking rats ($p = 0.0002$) signal ongoing immunosuppression and hematological dysfunction due to cigarette toxins like nicotine and benzene[204-205]. These toxic effects compromise lung health and contribute to lung weight changes, underscoring the importance and impact of our research in pulmonology and pharmacology.

When withdrawing smoker groups treated with JEE1 at various doses (200, 400, and 800 mg/kg), varying immune responses were observed. The 200 mg/kg group (STEX1) exhibited increased WBC counts ($p = 0.0305$), indicating immune activation. In contrast, the 400 mg/kg (STEX2) and 800 mg/kg (STEX3) groups had decreased WBC levels similar to or below controls ($p = 0.785$ and $p = 0.0423$, respectively), suggesting a potential anti-inflammatory effect at higher doses of JEE1. The potential of JEE1 at higher doses piques our interest in the future possibilities of this research.

In non-smoker rats, JEE1 at 200 mg/kg (EXD1) and 800 mg/kg (EXD2) did not significantly affect lung ROW ($p > 0.8$). Still, both doses resulted in significant WBC reductions ($p = 0.01$ and 0.0038), further supporting JEE1's anti-inflammatory properties. Additionally, the JOX-EO treatment groups (JEO and SOEO) showed no significant changes in lung ROW ($p = 0.8$ and 0.52, respectively), indicating a limited impact of JOX-EO on lung mass in non-smoking rats, but may distinctly affect lung weight during smoking cessation compared to the CSG group.

These findings underscore the damaging effects of smoking on lung and immune health, the partial recovery that follows cessation, and the potential of JEE1 treatment at higher doses and JOX-EO in modulating the immune system. This potential offer hope for the future of lung health research and the development of effective treatments for mitigating lung inflammation.

c) Heart

The results illustrated in Table 17, indicate that treatments with ethanolic extract at specific

doses had varying effects on heart ROW. The smoker withdrawal group (SG) exhibited a significant increase in heart ROW ($p < 0.001$), consistent with prior findings that chronic cigarette smoke exposure leads to increased relative heart and lung weights, alongside reduced body weight gain, as a compensatory response to toxic insults[208].

The JEO group demonstrated increased heart-relative organ weight among the treatment groups with a p-value of 0.02. In contrast, the withdrawal group treated with JOX-EO showed a non-significant increase ($p = 0.07$), suggesting its potential efficacy in improving heart health following cigarette smoke exposure compared to the CSG group.

Ethanolic extracts at 200 mg/kg (EXD1) and 800 mg/kg (EXD3) showed no significant differences compared to controls ($p = 0.12$ and $p = 0.14$, respectively). However, the withdrawing smoker group treated with the ethanolic extract JEE1 at 200 mg/kg (STEX1) demonstrated a significant increase in heart ROW ($p < 0.001$), mirroring the pattern observed in the untreated smoker withdrawal group. This suggests that at this dose, alterations related to smoke exposure may persist. Conversely, higher doses of JOXE at 400 mg/kg (STEX2) and 800 mg/kg (STEX3) did not produce significant changes in heart ROW ($p = 0.4$ and $p = 0.1$, respectively), indicating a potential for restoring heart weight toward levels seen in healthy controls. These findings support the idea that higher doses of JEE1 may mitigate the adverse effects of cigarette smoke on cardiac tissue.

d) Liver

The analysis of liver relative organ weight (ROW) (Table 18), along with serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Figure 34), revealed significant hepatic alterations in withdrawing smokers (CSG). This group exhibited a marked decrease in liver ROW ($P = 0.002$), a substantial increase in AST ($P = 0.001$), and a reduction in ALT ($P = 0.04$), indicating liver stress. Previous studies have reported that chronic cigarette smoke exposure in rats leads to a significant decrease in serum ALT and AST compared to non-smoker controls, consistent with clinical observations that smokers display elevated AST and ALT levels relative to non-smokers, reflecting hepatotoxic effects of smoking that may contribute to chronic liver disease upon prolonged exposure[209, 210]. Although smoking cessation is generally associated with normalization of liver enzymes [210], our data show that AST remains elevated and ALT decreases after two weeks of cessation, suggesting a longer recovery period may be required for full normalization in rats.

Treatment with JEE1 demonstrated dose-dependent effects on liver parameters. At 200 mg/kg (EXD1), no significant change was observed in liver ROW ($P = 0.25$), but AST increased

significantly (P = 0.005), while ALT decreased significantly (P = 0.002). A higher dose of 800 mg/kg (EXD3) resulted in a significant reduction in liver ROW (P = 0.03) and a more pronounced decrease in ALT (P = 0.0004). In the non-smoker group treated with JOX-EO (200 mg/kg), ALT decreased significantly (P = 0.001) without affecting liver ROW (P = 0.12), highlighting its potential in maintaining liver enzyme balance.

In the withdrawal group, STEX1 showed no significant effect on liver weight or ALT. Still, it caused a slight increase in AST (P = 0.04), suggesting a limited ability to support liver recovery post-cessation. Higher doses yielded more complex outcomes: 400 mg/kg (STEX2) significantly decreased liver ROW (P = 0.002) without affecting AST or ALT, whereas 800 mg/kg (STEX3) significantly increased ALT (P = 0.0003) and marginally elevated AST (P = 0.09). These results imply that while lower doses may offer hepatoprotective benefits, higher doses might elevate liver enzymes beyond levels seen in untreated withdrawal rats, emphasizing the importance of careful dose optimization for the therapeutic use of JEE1. Lastly, SOEO treatment did not significantly alter liver ROW, AST, or ALT levels, further supporting its potential liver-protective properties.

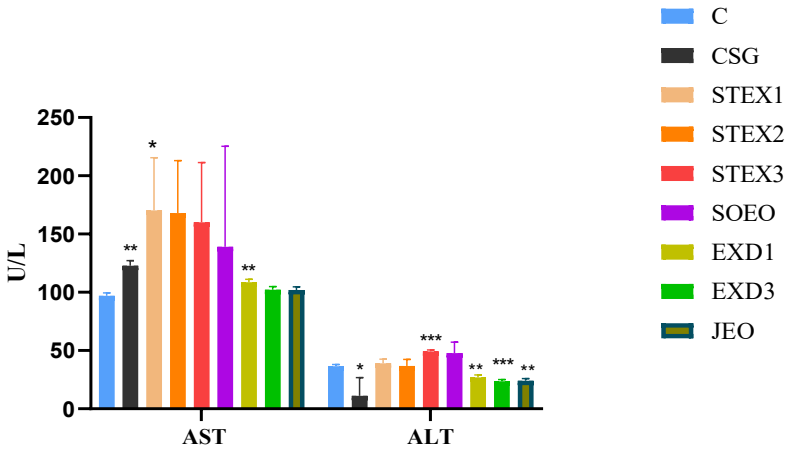


Figure 34: AST and ALT levels in experimental groups: (p>0.05 (ns), p < 0.05 *, p < 0.005**, p < 0.0005***).

Table 18: Relative liver, left kidney, and right kidney weights across treatment groups.

Groups	Relative organ weight %					
	Liver	P value	Left kidney	P value	Right kidney	P value
CG	3.266 ± 0.546	/	0.278 ± 0.015	/	0.288 ± 0.080	/
CSG	2.057 ± 0.199	**0.002****	0.276 ± 0.011	0.84	0.276 ± 0.027	0.75
EXD1	3.168 ± 0.502	0.25	0.301 ± 0.055	0.46	0.295 ± 0.072	0.97
EXD3	3.198 ± 0.228	0.030	0.309 ± 0.010	0.040	0.282 ± 0.039	0.90

JEO	3.483 ± 0.610	0.120	0.307 ± 0.039	0.260	0.278 ± 0.028	0.84
STEX1	3.766 ± 0.520	0.180	0.436 ± 0.096	0.007	0.412 ± 0.106	0.07
STEX2	2.126 ± 0.138	0.002	0.286 ± 0.027	0.580	0.332 ± 0.069	0.38
STEX3	2.648 ± 0.349	0.080	0.286 ± 0.022	0.460	0.266 ± 0.026	0.61
SOEO	3.319 ± 0.464	0.870	0.277 ± 0.029	0.940	0.307 ± 0.038	0.64

e) Kidney

The results (Table 18) indicate that there were no significant changes in the ROW of either the left or the right kidney in CSG compared to non-smokers.

Likewise, serum CREA and urea levels showed no significant differences between the withdrawing smokers and controls ($P > 0.4$), suggesting that smoking cessation leads to rapid stabilization of renal function. This is consistent with prior research showing smokers typically have elevated serum CREA, indicative of renal impairment[211], and a well-established link between smoking and progressive kidney dysfunction[212]. Thus, while smoking adversely affects kidney health over time, cessation appears to normalize kidney mass and key renal biomarkers, highlighting the potential for recovery.

Regarding JEE1 treatments, the low dose EXD1 (200 mg/kg) did not affect kidney ROW but significantly increased CREA ($P = 0.0005$) and urea ($P = 0.03$), indicating a notable impact on renal biomarkers. The higher dose of EXD3 (800 mg/kg) caused a significant reduction in left kidney ROW ($P = 0.04$). Still, there was no change in the right kidney ROW, with a non-significant increase in CREA and a significant rise in urea ($P = 0.01$), suggesting a complex dose-dependent effect on kidney function. Treatment with JOX-EO in the non-smoker group produced no significant changes in kidney weights or renal biomarkers, a pattern also observed for SOEO in withdrawing rats treated with JOX-EO.

Among withdrawal groups, STEX1 (200 mg/kg) induced a significant increase in left kidney ROW ($P = 0.007$) and urea levels ($P = 0.002$) but not creatinine ($P = 0.1$), whereas STEX2 (400 mg/kg) and STEX3 (800 mg/kg) did not significantly alter kidney weights or serum markers (CREA and urea p-values all > 0.05), indicating minimal renal impact at these doses. The absence of significant changes in CREA and urea at higher doses suggests that JEE1 is safe for kidney function in withdrawing smoker rats.

These findings imply that smoking cessation stabilizes renal parameters, and while lower doses of JEE1 may transiently elevate renal biomarkers, higher oral doses, and JOX-EO treatment do not adversely affect kidney function, supporting the safety and therapeutic potential of JOX extracts in this context.

Table 19: Serum creatinine and urea levels in treated, withdrawing smoker rats.

Experiments groups	CREA (mg/L)	P value	UREA (g/L)	P value
CG	6.54 ± 0.06	/	0.22 ± 0.03	/
CSG	5.67 ± 1.61	0.4053	0.26 ± 0.12	0.6130
STEX1	7.09 ± 0.54	0.1519	0.36 ± 0.01	**0.0015**
STEX2	6.90 ± 0.78	0.4687	0.34 ± 0.08	0.0777
STEX3	7.40 ± 1.26	0.3027	0.39 ± 0.13	0.1013
SOEO	6.89 ± 0.69	0.4233	0.23 ± 0.07	0.7676
EXD1	7.23 ± 0.08	**0.0005**	0.28 ± 0.01	**0.0303**
EXD3	6.38 ± 0.53	0.6336	0.30 ± 0.02	0.0128
OXEO	6.52 ± 1.06	0.9837	0.29 ± 0.04	0.0686

III. 4. 2. 4. Glycemic (GLY) marker

This study evaluated GLY levels among different treatment groups of rats (Figure 35 and Table 07), comparing these results to the control group (C).

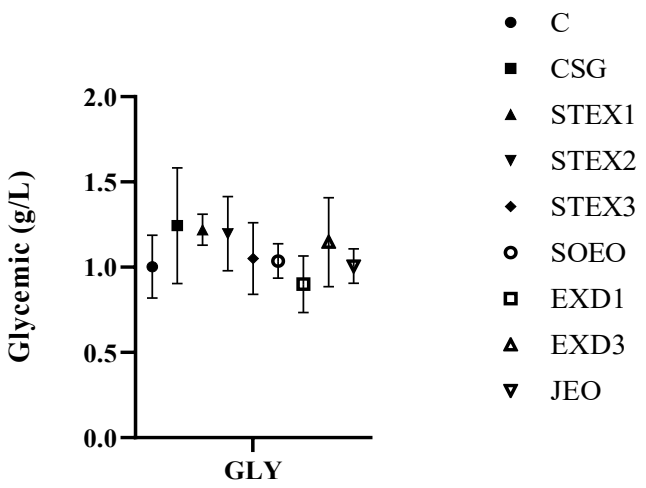


Figure 35: Comparative analysis of glycemia for each experimental group.

In both non-smoking rats and those withdrawn from smoking, treatment with the ethanolic extract JEE1 and JOX-EO demonstrated the ability to maintain or improve glycemic levels compared to controls. The 200 mg/Kg dose of JEE1 lowered blood glucose, while the 800 mg/Kg dose produced moderate but statistically non-significant improvements, both remaining close to control values. Essential oil treatments consistently preserved glycemic levels similar to the positive control. Rats undergoing withdrawal and treated with JEE1 exhibited moderate glycemic regulation, with the highest dose (800 mg/Kg) producing the most pronounced beneficial effect. These results suggest that JEE1 and JOX-EO do not adversely affect blood glucose and may offer therapeutic benefits after smoking cessation without a negative impact in managing glycemic levels.

III. 4. 2. 5. C-reactive protein (CRP)

The results of C-reactive protein (CRP) levels across different rat treatment groups after 18-week experimental period are presented in Table 20.

Table 20: Mean agglutination score for each group, calculated by assigning numerical values to agglutination intensity (e.g., 0 = negative, 1 = +, 2 = ++, 3 = +++, 4 = ++++). Error bars indicate standard deviation.

Groups	mean agglutination score ± SD	Signification
C	2.667 ± 1.155	-
CSG	1.667 ± 1.155	No
EXD1	2.000 ± 0.000	No
EXD3	2.000 ± 1.000	No
JEO	1.333 ± 0.5774	No
STEX1	1.667 ± 1.155	No
STEX2	2.333 ± 0.5774	No
STEX3	3.000 ± 0.000	No
SOEO	1.000 ± 0.000	No

This preliminary study evaluated the effects of JEE1 and JOX-EO on systemic inflammation in rats undergoing smoking withdrawal, using C-reactive protein (CRP) as a biomarker. Consistent with previous research, CRP levels remain elevated for years after smoking cessation, indicating persistent inflammation[213]. In this study, remarkably, withdrawing smoker rats showed no significant difference in CRP compared to controls. Treatment with JEE1 resulted in non-significant reductions at lower doses, while higher doses in STEX tended to elevate CRP without statistical significance. Conversely, JOX-EO demonstrated a trend toward lowering CRP levels, although these changes were not statistically significant. The study highlights the importance of careful dosing considerations for therapeutic use. Due to the small sample size and the exploratory nature of the work, statistical analyses were performed using nonparametric methods (Kruskal-Wallis test), and results should be interpreted cautiously as hypothesis-generating. Despite limitations in detecting subtle effects, the study identified clear trends that lay the groundwork for further research. Follow-up studies with larger sample sizes are planned to confirm these findings and provide more definitive conclusions about the efficacy of these drugs in reducing CRP levels.

III. 4. 2. 6. Lactate dehydrogenase (LDH)

The analysis of lactate dehydrogenase (LDH) levels (Figure 36) regarding withdrawal smoking and treatment effects suggests that groups with P values closer to the positive control

may indeed represent beneficial drugs for reducing LDH level increases associated with smoking.

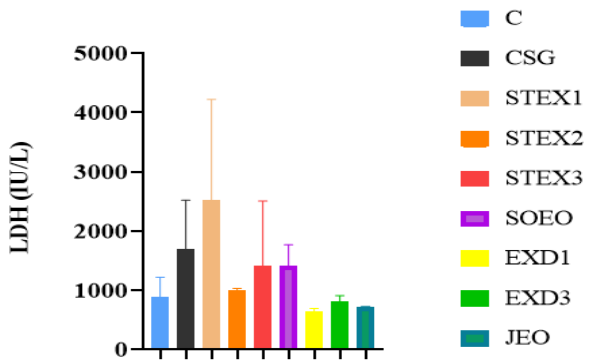


Figure 36: LDH analysis for each experimental group.

Lactate dehydrogenase (LDH) is a key enzyme released into the bloodstream during cellular damage and oxidative stress, making it an essential biomarker for tissue injury[214]. In this study, LDH levels in withdrawing smokers showed no significant elevation compared to controls ($p > 0.05$). Treatment with ethanolic extract JEE1 at various doses did not significantly reduce LDH levels in withdrawing smokers ($p > 0.05$). Similarly, JOX-EO treatment demonstrated a trend toward normalizing LDH levels closer to those of the non-smoker control group, but this change was not statistically significant.

These findings suggest that while LDH is a valuable biomarker for smoking-induced oxidative damage, the effects of JEE1 and JOX-EO on LDH levels during smoking withdrawal were not significant within the parameters of this study. Further research with larger sample sizes or longer treatment durations may be necessary to clarify their potential protective effects on tissue damage during smoking cessation.

III. 4. 2. 7. Lipid profiles

Lipid profiles comprising triglycerides (TRIG), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), and total cholesterol (CHOL) are essential markers for assessing cardiovascular and metabolic health. It is well established in the literature that smoking induces dyslipidemia, typically causing increased LDL and triglycerides alongside reduced HDL, thereby elevating cardiovascular risk [215].

In the current investigation, following 18 weeks of treatment, there were no significant changes in HDL, LDL, or total cholesterol levels among any of the groups (Figure 37), indicating that these lipid parameters were largely unaffected by smoking, cessation, or the tested treatments within the study period. However, triglyceride levels exhibited significant

variation; expectedly, withdrawing from smoking was associated with a significant decrease in triglycerides compared to controls, which may reflect metabolic adaptations occurring during smoking or withdrawal phases. The relationship between smoking cessation and triglyceride levels has been explored in various studies, indicating that quitting smoking is associated with a significant decrease in triglycerides compared to controls. While smokers generally exhibit higher triglyceride levels, cessation leads to a gradual reduction[216].

Treatment with the ethanolic extract JEE1 at both low and high doses significantly reduced triglyceride levels. In contrast, treatment with JOX-EO generated an even more pronounced decrease.

The study also identified limitations in using the Friedewald formula for calculating LDL cholesterol in contexts of low total cholesterol and triglycerides, yielding negative LDL values.

In conclusion, while HDL, LDL, and CHOL did not show significant differences across treatment groups, various treatments significantly affected triglyceride levels. Further investigation is warranted to explore these findings and their implications for understanding lipid metabolism in response to smoking and treatment interventions.

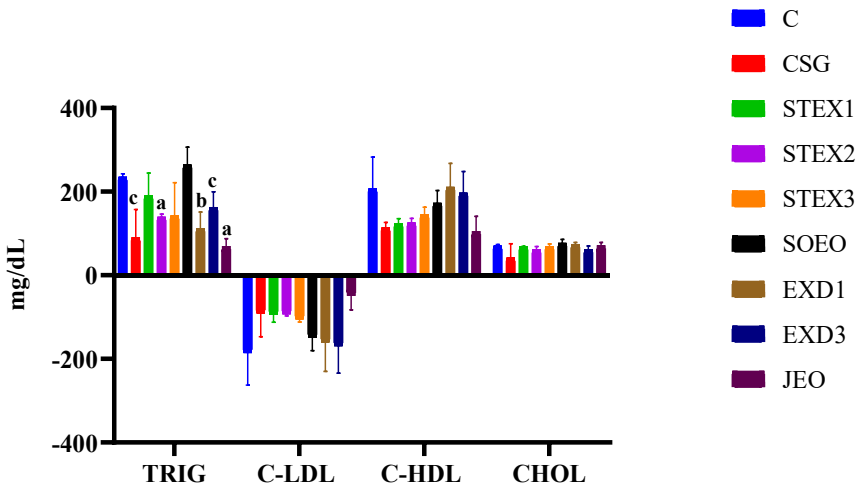


Figure 37: Influence of JOX extracts and smoking on lipid profiles: levels of triglycerides (TRIG), cholesterol HDL, cholesterol LDL, cholesterol (CHOL), and across experimental groups. (P=0.0001; a), (P<0.01;b), and (P<0.05;c).

III. 4. 2. 8. Metabolite analyses

Metabolic analyses of rats' total lipids, proteins, and carbohydrates are crucial for understanding various physiological and pathological conditions. The histograms (1, 2, and 3) summarize the findings from this study on these metabolic components.

a) Carbohydrate levels

Carbohydrates are essential for brain and lung function, yet their metabolic regulation affects these organs differently. In the brain, glucose is the primary energy source for supporting cognition and neurotransmitter synthesis; however, excessive consumption of refined carbohydrates can impair cognitive function and promote neuroinflammation, while insufficient glucose disrupts focus and overall brain performance[217-219]. In contrast, carbohydrates in the lungs support mucus integrity and antioxidant defenses[220]. Still, high-carbohydrate diets may worsen respiratory conditions such as COPD and asthma by increasing carbon dioxide production and oxidative stress[221]. Smoking disrupts carbohydrate metabolism by causing systemic insulin resistance[222], hyperinsulinemia, and oxidative stress, which lead to tissue-specific carbohydrate depletion, particularly in metabolically active organs like the brain and lungs.

This study measured total carbohydrate levels in lung and brain tissues to assess the impact of smoking cessation and treatment with the ethanolic extract JEE1 on metabolic status as illustrated in Figure 38.

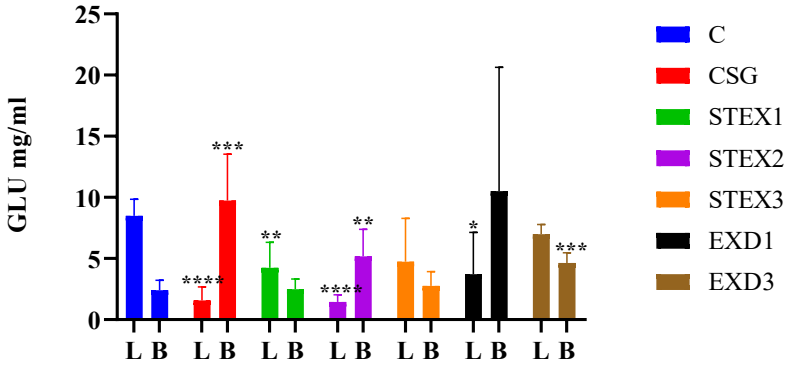


Figure 38: Carbohydrate levels in lung and brain tissues across different groups treated by JEE1.

Withdrawing smoker rats (CSG) exhibited a pronounced and highly significant reduction in lung carbohydrate levels ($P < 0.0001$), indicating that smoking severely impairs lung energy metabolism. This depletion likely compromises cellular function and increases susceptibility to oxidative stress, persisting through the initial weeks following smoking cessation. In contrast, brain carbohydrate levels significantly increased in this group ($P = 0.001$), indicative of a compensatory or protective metabolic adaptation aiming to offset the energy deficits induced by withdrawal.

Treatment with a low dose of JEE1 (EXD1, 200 mg/ml) caused a significant lung carbohydrate decrease ($P = 0.01$) without a notable change in the brain ($P = 0.07$), suggesting that at this lower dose, JEE1 may intensify lung carbohydrate depletion, potentially by modulating local metabolic pathways or oxidative stress, whereas brain metabolism remains comparatively stable. Conversely, a higher dose of JEE1 (EXD3, 800 mg/ml) maintained reduced lung carbohydrate levels ($P = 0.04$). Still, it resulted in a significant elevation of brain carbohydrates ($P = 0.001$), implying that higher concentrations of the extract promote cerebral carbohydrate metabolism or energy reserve replenishment. This observation reveals a dose-dependent, tissue-specific effect of JEE1, where brain tissue benefits metabolically from increased dosage despite ongoing pulmonary energy deficits.

In the smoker groups treated with JEE1, varied metabolic outcomes were observed. STEX1 showed significant lung carbohydrate depletion ($P = 0.002$) but stable brain carbohydrate levels ($P = 0.8$), indicating partial brain metabolic stability amid lung stress. STEX2 exhibited significant decreases in both lung ($P < 0.0001$) and brain carbohydrate levels ($P = 0.02$), suggesting more extensive metabolic impairment or inadequate brain compensation. STEX3 maintained lung carbohydrate reduction ($P = 0.04$) with no significant changes in brain metabolism ($P = 0.55$) compared to the control group.

These results demonstrate that smoking cessation and JEE1 treatment elicit complex, organ-specific effects on carbohydrate metabolism. The lungs consistently show carbohydrate depletion during the withdrawal and treatment phases, reflecting ongoing oxidative and metabolic stress. In contrast, the brain exhibits variable responses, including compensatory carbohydrate increases, particularly at higher JEE1 doses or after cessation alone, which may represent neuroprotective or restorative metabolic adaptations.

Meaningfully, JEE1 treatment during withdrawal partially restored lung carbohydrate levels, remaining below control but higher than untreated withdrawing smokers, suggesting that antioxidants protect or reestablish metabolic balance by reducing oxidative damage and preserving critical carbohydrate stores. Brain carbohydrate levels were notably elevated in JEE1-treated rats, possibly reflecting an enhanced compensatory or protective metabolic response. Future research should aim to elucidate the molecular mechanisms underlying these differential effects and optimize dosing to maximize therapeutic efficacy across tissues affected by smoking and its cessation.

b) Lipid metabolism

This study provides novel insights into the organ-specific effects of smoking cessation and treatment with the ethanolic extract JEE1 on lipid metabolism in brain and lung tissues, addressing a gap in the literature where systemic dyslipidemia is well-documented but tissue level lipid changes remain poorly quantified. Smoking disrupts systemic lipid homeostasis by elevating total cholesterol, triglycerides, and LDL, contributing to oxidative stress and inflammation[214]. These systemic alterations likely induce lipid peroxidation and functional impairment at the organ level, particularly affecting the brain and lungs, where lipid metabolism is critical for maintaining cellular and physiological integrity.

Lipids constitute approximately 60% of brain dry weight and play pivotal roles in forming cell membranes, myelin sheaths, and lipid rafts, which orchestrate neurotransmission, synaptic plasticity, and neuronal development. Disruptions in brain lipid metabolism can impair neuronal signaling and are implicated in neurodegenerative and psychiatric disorders[223, 224]. In the lungs, lipids are essential components of pulmonary surfactant that reduce surface tension to maintain airway patency, optimize gas exchange, and modulate immune responses and membrane stability[225]. Thus, proper lipid metabolism supports both neural and respiratory health.

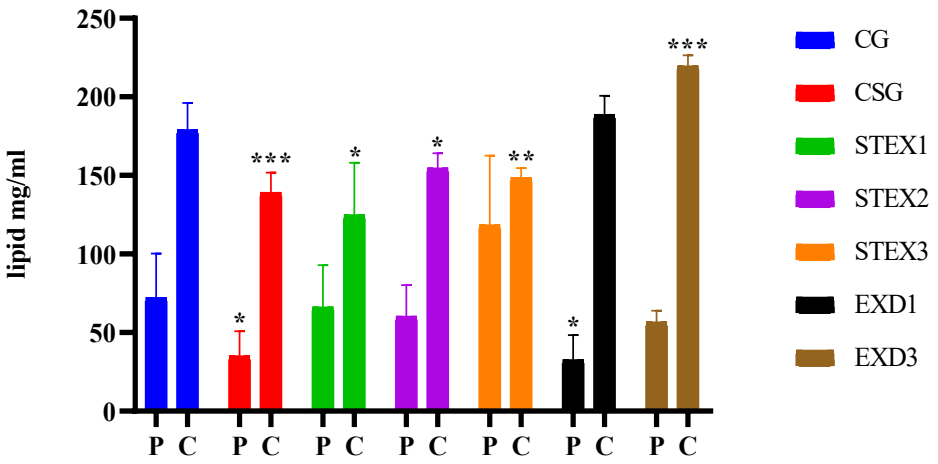


Figure 39: Total lipid levels in lung (L) and brain (B) tissues among different treatment groups.

The findings of this study are significant as they reveal the effects of smoking and treatment on lipid and protein levels in rats. The results show that withdrawing smoker rats (CSG) exhibited significant reductions in total lipid levels in both lungs ($P = 0.02$) and brain tissues ($P = 0.001$) compared to controls. This underscores the detrimental impact of smoking on tissue-specific lipid reserves and metabolic function. The depletion likely reflects enhanced

lipid peroxidation driven by oxidative stress, contributing to neurodegeneration and surfactant impairment previously linked to smoking-related disorders.

The potential of the ethanolic extract JEE1 in modulating tissue lipid levels is a key finding of this study. The results show that in non-smoker rats, the lower dose (200 mg/ml, EXD1) caused a significant decrease in lung lipids ($P = 0.01$) without affecting brain lipids ($P = 0.3$), suggesting a selective impact on pulmonary lipid metabolism. Contrastingly, the higher dose (800 mg/ml, EXD3) increased brain lipids significantly ($P = 0.0003$), indicating a dose-dependent stimulation of lipid accumulation or synthesis in brain tissues.

In withdrawing smoker rats treated with varying doses of JEE1, lung lipid levels remained largely unchanged across doses ($P > 0.05$), while brain lipid levels significantly decreased at all doses (200 mg/ml STEX1: $P = 0.005$; 400 mg/ml STEX2: $P = 0.01$; 800 mg/ml STEX3: $P = 0.002$). This reduction does not indicate that JEE1 exacerbates brain lipid depletion. Instead, it suggests that the treatment does not fully restore brain lipid levels during recovery from smoking-induced damage

c) Total protein levels

This study highlights the critical roles of proteins in brain and lung tissues and their differential responses to smoking cessation and JEE1 treatment. Proteins such as synaptophysin in the brain regulate synaptic plasticity and neuroglial interactions, which are vulnerable to disruption by smoking-induced oxidative stress, contributing to neurodegeneration[227, 228]. In the lungs, surfactant proteins (SP-A, SP-D) maintain airway stability and immunity, while smoking promotes protease-antiprotease imbalances that drive emphysema and chronic inflammation[229]. Using the Bradford method, we found that withdrawing smoker rats exhibited significant increases in protein levels in both lungs ($P = 0.045$) and brain tissues ($P = 0.01$), suggesting enhanced protein synthesis or retention during withdrawal.

Treatment with the ethanolic extract showed organ- and dose-specific effects. In non-smokers, lower doses (EXD1, 200 mg/ml) significantly increased lung protein levels ($P = 0.0005$), indicating a strong positive effect, potentially aiding lung repair or defense mechanisms. In contrast, brain protein changes were not significant. Higher doses (EXD3, 800 mg/ml) also elevated lung proteins ($P = 0.03$) but had a limited impact on brain proteins. Among withdrawing smokers treated with the extract, protein level changes were mostly non-significant across doses in both tissues, with only marginal lung increases at the lowest dose (STEX1, $P = 0.08$). This suggests that smoking-related damage may modulate tissue

responsiveness to the extract, particularly in the brain, which opens up intriguing possibilities for further research and understanding.

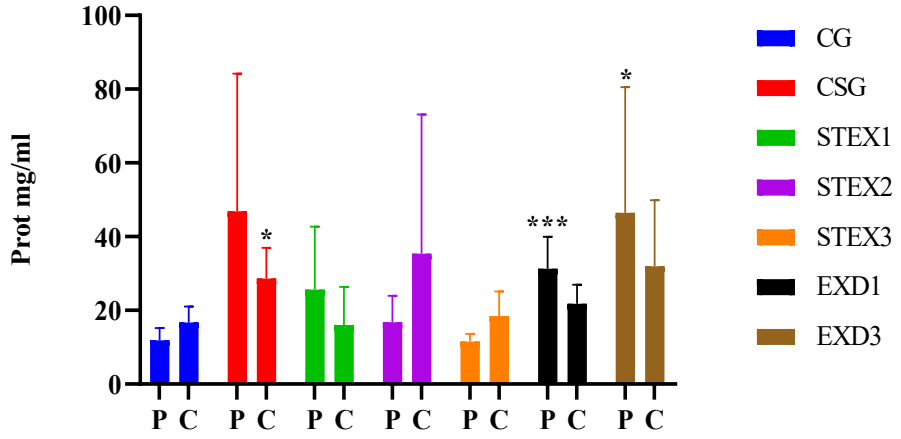


Figure 40: Total protein levels in lung and brain tissues from different treatment groups.

These findings demonstrate that the ethanolic extract significantly enhances lung protein levels at lower doses, potentially counteracting smoking-induced damage, while its influence on brain protein metabolism is less pronounced. The differential protein responses between lung and brain tissues underscore the distinct molecular pathways governing their function and recovery after smoking exposure. Further research is needed to elucidate the mechanisms by which the extract modulates protein synthesis and degradation in these organs, with implications for developing targeted therapies for smoking-related tissue injury.

CONCLUSIONS

This study investigated the efficacy of *Juniperus oxycedrus L.* (JOX) and *Juniperus phoenicea* (JPH), natural products, particularly their essential oils and JOX ethanolic extract, in targeting the complex toxicity of compounds found in tobacco and cigarette smoke. The research provided new and remarkable insights into these extracts' chemical composition, bioactivity, and potential applications in mitigating smoking-related harms.

Harvesting berries instead of leaves offers distinct environmental and economic advantages, promoting plant health and sustainability, a crucial factor for large-scale use.

Among extraction methods, ultrasound-assisted extraction provided higher ethanolic extract yield enriched in bioactive compounds renowned for their potent antioxidant properties; the EOs extracted via distillation contained powerful terpene components like α-Pinene.

Juniperus oxycedrus L. essential oil (JOX-EO) effectively reduced cigarette smoke toxins. It significantly decreased nicotine concentration in vitro. Direct chemical interactions between the EO bioactive compounds and nicotine molecules primarily drive this nicotine reduction.

UV spectroscopic analysis showed JOX-EO's interactive effects on PAHs, highlighting its potential to transform or degrade these harmful smoke constituents chemically. This multi-modal detoxifying capacity implies JOX-EO can neutralize several toxic components of tobacco smoke.

JOX extracts have shown promising antioxidant capabilities through various assays, and ultrasound-assisted extracts outperform conventional ones. This antioxidant activity is significant, especially in cigarette smoke-induced oxidative stress. Additionally, JOX-EO has demonstrated multi-target enzyme inhibition, which could pave the way for therapeutic effects against inflammation and oxidative pathology caused by smoking.

Beyond its roles in detoxification and antioxidant activity, JOX-EO exhibited notable anticancer effects, particularly against lung adenocarcinoma cells. This potent activity is primarily attributed to its abundant α-pinene and β-myrcene, compounds known for their antioxidant, anti-inflammatory, and selective cytotoxic properties that target cancer cells while sparing normal tissues. JOX-EO's capacity to reduce lung cancer cell viability and trigger apoptosis underscores its potential as a natural therapeutic agent with multiple benefits. This extends its usefulness beyond merely mitigating tobacco smoke effects to a promising candidate for cancer treatment and prevention.

This investigation selected JOX over JPH to enable more comprehensive in vitro and in vivo evaluation of JOX’s properties, particularly its interactions with PAHs. Although JPH essential oil also demonstrated the ability to reduce nicotine levels and exert cytotoxic effects

against lung adenocarcinoma cells, its efficacy was lower than that of JOX, supporting the choice of JOX as the primary focus of this study.

The in vivo study was conducted in two parts. The first part demonstrated that JPH and JOX essential oils (EOs) effectively enhanced antioxidant defenses and reduced oxidative stress in lung and brain tissue of rat which were under sub-chorionic exposition of cigarette smoke for one month. Despite persistent oxidative damage after smoking cessation, JOX-EO showed superior antioxidative effects compared to JPH-EO, facilitating faster recovery of oxidative balance and red blood cell counts. These results support the potential therapeutic use of JOX-EO to mitigate cigarette smoke-induced oxidative damage and related health impairments.

The second part involved extensive in vivo studies confirming the potential of JOX as an EO and an ethanolic extract. Rats which were under chronic exposition to cigarette smoke for three months, exhibited typical toxicity signs such as weight loss, altered organ weights, disrupted blood parameters, and organ damage. Treatment with JOX-EO and the ethanolic extract for two weeks after smoking cessation led to normalization or improvement of hematological and biochemical markers. Notably, the ethanolic extract (JEE1) reverses smoke-induced weight loss most effectively. Additionally, JOX extracts supported the recovery of carbohydrate, lipid, and protein metabolism in brain and lung tissues, indicating tissue-specific reparative actions. However, some dose-dependent biochemical and inflammatory responses suggest the need for careful dosing. While this study's results are encouraging, it is essential to acknowledge its inherent limitations. These limitations underscore the crucial need for further research in this area, which could revolutionize our approach to smoking-related health issues and is of utmost importance to the audience.

Many perspectives are envisaged, as the interaction products between JOX-EO and PAHs were inferred via spectral changes, but exact molecular entities remain unidentified. Advanced analytical techniques such as GC-MS, LC-MS/MS, or NMR are needed to characterize these transformations and elucidate detoxification mechanisms.

The dose-dependent effects observed in organ biomarkers and inflammation markers underscore the importance of defining safe and effective dosing ranges. Moreover, long-term toxicological studies are necessary to rule out adverse effects, especially at higher doses.

Although the experimental approach was evaluated mainly in vitro and in vivo, human clinical trials should validate efficacy, pharmacokinetics, safety, and optimal delivery forms (e.g., air diffusers and supplements) for practical application.

Furthermore, incorporating JOX-EO in air purification devices, respirator filters, or topical preparations requires formulation science to ensure stability, bioavailability, and user acceptability.

Beyond tobacco smoke toxicity, JOX extracts may have potential against other oxidative or inflammatory diseases, suggesting broader therapeutic and commercial uses. This potential opens up a new and intriguing area of research, piquing the audience's interest.

In brief, JOX natural products, mainly their essential oil and ethanolic extract, demonstrate multifaceted bioactivities instrumental in reducing oxidative and toxic burdens from cigarette smoke. By reducing nicotine and altering harmful PAHs, boosting antioxidant defenses, anti-lung cancer effects, and supporting organ recovery, JOX extracts represent a promising natural strategy for mitigating tobacco-related health risks. With further research addressing current limitations, JOX could find valuable roles in public health interventions, smoking cessation aids, and environmental decontamination efforts, contributing to improved well-being and sustainable natural product utilization.

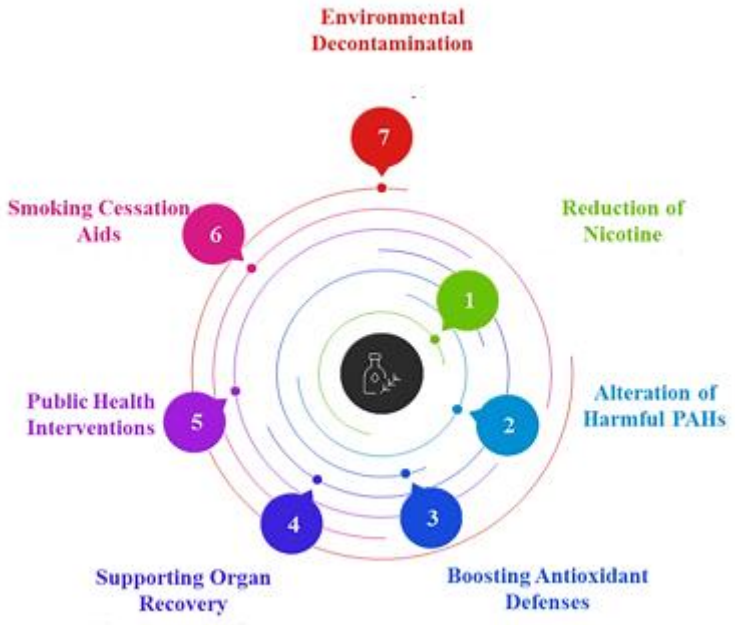


Figure 41: Multifaceted benefits of JOX natural product

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