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PREFACE

This dissertation is submitted for the degree of Doctorate of Sciences at University of Mentouri Constantine 1. The research described herein was conducted under supervision of Professor Amar Zellagui between October 2014- November 2022, at laboratory of Biomolecules and Plant Breeding, Faculty of Exact Sciences, Nature and Life Sciences, University of Larbi Ben Mhidi, Oum El Bouaghi and during trainings at different research centers in Algeria and Turkey.

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Boulechfar S, Akbulut Z, Tepe HD, Zellagui A, Aktas RG, Bensouici C, Doyuk F, Khattabi L, Demirel G, Lahouel M. **LC–MS/MS analysis, antioxidant and anticancer effects of phenolic‑rich extracts from Algerian propolis: a comparative study**. *J Food Meas Charact.* 2022; 17:564–575. https://doi.org/10.1007/s11694-022-01652-3

ABSTRACT

This study aimed to investigate the chemical composition and the biological properties of propolis collected from different regions of Algeria. The total bioactive content, antioxidant, anti-enzymatic, antimicrobial, toxicity and anticancer effects were evaluated. The chemical composition of propolis extracts and essential oils (EOs) was also analyzed. The results demonstrated the richness of propolis extracts in phenolic and flavonoid compounds. Antioxidant tests (DPPH, ABTS, alkaline DMSO, CUPRAC, ferric reducing power and β-carotene-linoleic acid tests) revealed the strong radical scavenging, ion reducing and lipid peroxidation inhibition abilities of the extracts, whereas EOs were the least active. Anti-enzymatic assays against acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and α-glucosidase showed that all propolis extracts possessed a potent inhibitory effect on α-glucosidase better than acarbose and revealed the ability of extracts to inhibit BChE more effectively than AChE. However, the tested oil was more effective on AChE than BChE. The antimicrobial assay indicated that all extracts were mainly active against Gram-positive bacteria and yeast. The inhibition of bacterial growth by propolis extracts and EOs was found to be through bactericidal or bacteriostatic mechanism, whereas the inhibition of yeast growth was through fungicidal or fungistatic mode. The toxicity test against brine shrimp larvae indicated that propolis extracts possess moderate toxic properties. In addition, anticancer effect of propolis extracts on HepG2 human hepatocellular carcinoma cells (HepG2) determined using CCK-8 assay revealed the strong cytotoxic activity of the extracts against HepG2 with IC_{50} values ranged from 12.22±0.05 to 60.39±1.82 µg/mL. The GC-MS analysis of three EOs allowed the identification of a total of 112 compounds, in which α-pinene, limonene, *trans*-pinocarveol, α-terpinenyl acetate and δ-Cadinene were common between the three oils. The LC-MS/MS analysis of propolis extracts that showed potent anticancer effect revealed the richness of these extracts in phenolic acids such as caffeic and ferulic acids, and flavonoids such as kaempferol, apigenin and quercetin. These findings indicate the importance of Algerian propolis as a source of bioactive principles for the development of pharmaceutical products.

Keywords: Propolis, GC-MS, LC-MS/MS, antioxidant, cholinesterase, α-glucosidase, antimicrobial, anticancer, toxicity.

RÉSUMÉ

Cette étude visait à étudier la composition chimique et les propriétés biologiques de la propolis collectée de différentes régions d'Algérie. Le contenu en substances bioactifs totaux, les effets antioxydants, anti-enzymatiques, antimicrobiens, toxiques et anticancéreux ont été évalués. La composition chimique des extraits de propolis et des huiles essentielles (HEs) a également été analysée. Les résultats ont montré la richesse des extraits de propolis en composés phénoliques et flavonoidiques. Les tests de l'activité antioxydante (DPPH, ABTS, DMSO alcalin, CUPRAC, pouvoir réducteur ferrique et β-carotène-acide linoléique) ont révélé les fortes capacités de piégeage des radicaux, de réduction des ions et d'inhibition de la peroxydation lipidique des extraits, alors que HEs étaient les moins actives. Les tests anti-enzymatiques contre l'acétylcholinestérase (AChE), la butyrylcholinestérase (BChE) et la α-glucosidase ont montré que tous les extraits de propolis possédaient un puissant effet inhibiteur sur l'α-glucosidase mieux que l'acarbose et ont révélé la capacité des extraits à inhiber BChE plus efficacement que l'AChE. Cependant, l'huile testée était plus efficace sur l'AChE que sur la BChE. Le test antimicrobien a indiqué que tous les extraits étaient principalement actifs contre les bactéries Gram-positives et les levures. L'inhibition de la croissance bactérienne par les extraits de propolis et HEs s'est avérée être par un mécanisme bactéricide ou bactériostatique, alors que l'inhibition de la croissance des levures était par le mode fongicide ou fongistatique. Le test de toxicité contre les larves d'artémias a indiqué que les extraits de propolis possèdent des propriétés toxiques modérées. De plus, l'effet anticancéreux des extraits de propolis sur les cellules de carcinome hépatocellulaire humain HepG2 (HepG2) déterminé à l'aide du test CCK-8 a révélé la forte activité cytotoxique des extraits contre HepG2 avec des valeurs IC_{50} allant de 12,22 ± 0,05 à 60,39 ± 1,82 µg/mL. L'analyse GC-MS de trois HEs a permis l'identification d'un total de 112 composés, dans lesquels l'α-pinène, le limonène, le *trans*-pinocarvéol, l'acétate d'α-terpinényle et le δ-cadinène étaient communs aux trois huiles. L'analyse LC-MS/MS d'extraits de propolis qui ont montré un puissant effet anticancéreux a révélé la richesse de ces extraits en acides phénoliques tels que les acides caféique et férulique, et en flavonoïdes tels que le kaempférol, l'apigénine et la quercétine. Ces résultats indiquent l'importance de la propolis Algérienne comme source de principes bioactifs pour le développement de produits pharmaceutiques.

Mots clés : Propolis, GC-MS, LC-MS/MS, antioxydant, cholinestérase, α-glucosidase, antimicrobien, anticancéreux, toxicité.

ملخص

هدفت هذه الدراسة إلى التحقق من التركيب الكيميائي والخصائص البيولوجية للعكبر التي تم جمعها من مناطق مختلفة من الجزائر. تم تقييم المحتوى النشط بيولوجيًا الكلي ، ومضادات الأكسدة ، ومضادات الإنزيم ، ومضادات الميكروبات ، والسمية ، وتأثيراتها المضادة للسرطان. كما تم تحليل التركيب الكيميائي لمستخلصات البروبوليس والزيوت الأساسية. أظهرت النتائج غناء مستخلصات البروبوليس بالمركبات الفينولية و الفلافونويدية. كشفت اختبارات DPPH و ABTS و DMSO القلوية و CUPRACوطاقة الارجاع الحديدي واختبارات حمض الكاروتين واللينوليك) عن قدرة المستخلصات القوية على الكسح الجذري وارجاع الأيونات ومنع بيروكسيد الدهون، في حين كانت الزيوت الأساسية هي الأقل نشاطًا. أظهرت الاختبارات المضادة للأنزيمات ضد أسيتيل كولينستراز (AChE)و بتيريل كولينستراز(BChE) و ألفا جليكوزيداز أن جميع مستخلصات البروبوليس لها تأثير مثبط قوي على ألفا جليكوزيداز أفضل من أكاربوز وكشفت قدرة المستخلصات على تثبيط BChE بشكل أكثر فعالية من .AChE ومع ذلك ، كان الزيت المختبَر أكثر فعالية على AChE من BChE. أظهر فحص مضادات الميكروبات أن جميع المستخلصات كانت فعالة بشكل رئيسي ضد البكتيريا إيجابية الجرام والخميرة. تباينت قيم MIC لمستخلصات البروبوليس والأكسدة بشكل عام في النطاق 0.0156 - 2 ميكروغرام / ميكرولتر. تم العثور على أن تثبيط نمو البكتيريا عن طريق مستخلصات البروبوليس والزيوت الأساسية تم من خلال آلية ابادة البكتيريا أوايقاف مؤقت لنمو البكتيريا، بينما كان تثبيط نمو الخميرة من خلال ابادة أوايقاف مؤقت لنمو الفطريات. أظهر اختبار السمية ضد يرقات الجمبري أن مستخلصات البروبوليس تمتلك خصائص سامة متوسطة. بالإضافة إلى ذلك ، كشف التأثير المضاد للسرطان لمستخلصات البروبوليس على الخلايا السرطانية الكبدية البشرية (HepG2) التي تم تحديدها باستخدام اختبار CCK-8 عن النشاط السام للخلايا القوي للمستخلصات ضد HepG2 بقيم IC_{50} تتراوح من 12.22 ± 0.05 إلى 60.39 ± 1.82 ميكروغرام / مل. سمح تحليل GC-MS لثلاثة زيوت طيارة من البروبوليس بتحديد ما مجموعه 112 مركبًا ، حيث كانت α-pinene و limonene و trans-pinocarveol و α-terpinenyl acetate و δ-Cadinene مشتركة بين الزيوت الثلاثة. أظهر تحليل LC-MS / MS لمستخلصات البروبوليس التي أظهرت تأثيرًا قويًا مضادًا للسرطان ثراء هذه المستخلصات في الأحماض الفينولية مثل أحماض الكافيين والفيروليك والفلافونويد مثل الكايمبفيرول والأبيجينين والكيرسيتين. تشير هذه النتائج إلى أهمية العكبر الجزائري كمصدر للمركبات النشطة بيولوجيًا لتطوير المنتجات الصيدلانية.

الكلمات المفتاحية: البروبوليس ، GC-MS، LC-MS / MS ، مضادات الأكسدة ، الكولينستراز ، ألفا جلوكوزيداز ، مضادات الميكروبات ، مضاد للسرطان ، السمية ،

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List of abbreviations

ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate
AChE: acetylcholinesterase
AD: Alzheimer's disease
ALA: α-lipoic acid
$AlCl_3$: Aluminum chloride
ATCC: American type culture collection
BACE-1: β-amyloid precursor cleavage enzyme
BChE: butyrylcholinesterase
CAT: Catalase
CCK-8: Cell Counting Kit-8
CERAC: Ceric ion reducing antioxidant capacity assay
Co Q10: coenzyme Q10
Cu: copper
CUPRAC: Cupric ion reducing antioxidant capacity
Cys: cysteine
DM: Diabetes mellitus
DMEM: Dulbecco's modified Eagle's medium
DMSO: Dimethyl sulfoxyde
DNA: Deoxyribonucleic acid
DTNB: 5,5'-Dithiobis(2-nitrobenzoic) acid
DPPH: 2,2'-diphenyl-1-picrylhydrazyl
FBS: fasting blood sugar
Fe: iron
FRAP: ferric reducing antioxidant power
GC-MS: Gas Chromatography – Mass Spectroscopy
GPx: glutathione peroxidase
GR: glutathione reductase
GSH: reduced glutathione
GSSG: oxidized glutathione
GST: glutathione transferase
HbA1c: hemoglobin A1c
HepG2: hepatocellular carcinoma human cancer cell line
H_2O_2: hydrogen peroxide
HOCl: hypochlorous acid
HOMA-β: Homeostasis model assessment of β-cell function
HOMA-IR: Homeostasis model assessment of insulin resistance
$HOO^{\bullet}$: hydroperoxyl radical
HRSA: Hydroxyl radical scavenging activity
IC_{50} : The half-maximal inhibitory concentration
IDDM: insulin-dependent diabetes mellitus
IKKβ: IκB kinase β
IR: insulin resistance
kg: Kilogram
$L^{\bullet}$: lipid radical
LA: Lipoic acid
LC–MS/MS: Liquid chromatography with tandem mass spectrometry
LH: fatty acid
$LOO^{\bullet}$: lipid peroxyl radical
MAO: monoamine oxidase
MBC: minimum bactericidal concentration
MDA: malondialdehyde
ME: methanolic extract
MFC: minimum fungicidal concentration
MIC: minimum inhibitory concentration
Mn: manganese
MPO: myeloperoxidase
$NADP^+$: nicotinamide adenine dinucleotide phosphate
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
NaOH: sodium hydroxide
NBT: nitroblue tetrazolium

NIDDM: noninsulin-dependent diabetes mellitus
NO•: nitric oxide
NO_2•: nitrogen dioxide
NOS: nitric oxide synthase
ORAC: Oxigen Radical Absorbance Capacity
$^1O_2$: singlet oxygen
$O_2^{\bullet-}$: superoxide radical anion
•OH: hydroxyl radical
$ONOO^-$: peroxynitrite
PKC: protein kinase C
PNP: *p*-nitrophenol
PNPG: *p*-nitrophenyl-α-D-glucopyranoside
Prx I–IV: peroxiredoxin I–IV
PUFA: polyunsaturated fatty acid
RI: retention index
RNA: Ribonucleic acid
RNS: reactive nitrogen species
RO•: alkoxyl radical
ROO•: peroxyl radical
ROOH: organic peroxide
ROS: reactive oxygen species
RS•: thiyl radical
RSKK: Refik Saydam Type Culture Collection
RSOH: sulfenic acid
RS(O)2SR: disulfide-S-oxide
RSS: reactive sulfur species
RT: retention time
Se: selenium
Se-OH: selenoles
SOD : Superoxyde dismutase
SZO: streptozotocin
TAC: total antioxidant capacity
TBARS: thiobarbituric acid reactive substances
T1DM : Type 1 diabetes mellitus
T2DM : Type 2 diabetes mellitus
TFC: Total flavonoid content
TPC: Total phenolic content
TRAP: radical-trapping antioxidant parameter
UFC : Unité Formant Colonie
UV-Vis : Ultraviolet/Visible
YPD: Yeast Peptone Dextrose
Zn: zinc

Introduction

Free radicals derived from oxygen, nitrogen and sulphur molecules in the biological system are highly active to react with other molecules due to their unpaired electrons. These radicals are produced during cellular metabolism and functional activities. However, overproduction of them can adversely affect various important classes of biological molecules such as nucleic acids, lipids, and proteins, thereby altering the normal redox status leading to increased oxidative stress and causing a variety of chronic diseases such as diabetes mellitus, neurodegenerative disorders and cancer (Lu *et al*., 2010; Phaniendra *et al*., 2015). In order to scavenge superfluous free radicals and maintain the balance of homeostasis in human body as well as accomplish the prevention and treatment of diseases, the consumption of antioxidants is necessary. However, synthetic antioxidants have toxic effects to some extents. Thus, the uptake of natural antioxidants is the first choice because natural antioxidants not only play an important role in the prevention and adjunctive treatment of diseases but also can avoid the adverse reactions to human health (Li *et al.*, 2014).

Type 2 diabetes mellitus (T2DM) is a metabolic disorder that causes permanent hyperglycemia due to the fact that insulin is not sensitive to glucose overloading (Taslimi *et al*., 2019; El Shafay *et al*., 2022). It is the most common type of diabetes throughout the world and it is projected that the prevalence of this disease will increase in the coming years (Ozcan, 2020). On the other hand, Alzheimer's disease (AD) is a lethal and neurodegenerative disease that arises with the symptoms such as loss of memory, cognitive disorders, and dementia and it is projected that the number of peoples influenced by the AD will increase in near future (Ozcan, 2020). One of the current approaches for T2DM and AD treatment is inhibition of the key enzymes. However, the available enzyme inhibitors are reported to have side effects including cytotoxicity, hepatotoxicity, gastrointestinal disturbances, and diarrhea. Therefore, the development and utilization of alternative and potentially more effective and less toxic substances are indicated to be very necessary (Zengin *et al*., 2015).

Hepatocellular carcinoma (HCC) is the fifth most common cancer type and the third leading cause of cancer-related death around the world, accounting for 75% to 85% of all primary liver cancer cases. The therapeutic approach for HCC depends on disease staging (Huang *et al*., 2020). Patients with early stage HCC are candidates for surgical resection, or radical therapy

(cryosurgery, liver transplantation or local ablation via percutaneous ethanol injection (PEI) or radiofrequency ablation (RFA) with 5-year survival rates of 41-74%. For intermediate stage HCC, patients benefit from hepatic artery chemoembolization (TACE), whereas at advanced stage, systemic chemotherapy is the only option (Huang *et al*., 2020; Rashid *et al*., 2022). However, systemic drugs have been reported to possess some significant side effects. Therefore, the development of an effective novel agents with reduced toxicity against normal cells is a priority to improve survival for advanced HCC (Bteich & Di Bisceglie, 2019; Huang *et al*., 2020).

Infectious diseases are still a major health concern, caused by microorganisms, such as bacteria, viruses, fungi, and parasites. Approximately 10 million people died of infectious diseases in 2016, accounting for one-fifth of all deaths worldwide (Furuse, 2018; Hemeg *et al*., 2020). One of the main causes of this problem is the increasing resistance of the microorganisms towards conventional antimicrobial agents. This global phenomenon, therefore, encourages the development of new agents that can effectively inhibit microbial growth. An alternative and very promising approach to overcome this issue might be the use of natural antimicrobial products (Gaziano *et al*., 2019).

Propolis, a bee natural product, is a plant-derived resinous substance that is metabolized by honeybees (*Apis mellifera*). It has been traditionally used as a therapeutic agent for millennia (Abutaha, 2020). It has been also reported to possess a wide range of pharmacological effects such antimicrobial, antioxidant, anticancer, anti-infammatory, antidiabetic and neuroprotective effects (Braakhuis, 2019, Pant *et al*., 2021). The broad spectrum of its biological activities is believed to be related to its chemical composition, which is significantly influenced by geographical location, climatic zones, flora, strength of bee colony and production season, which gives diversity and uniqueness to propolis of each country, state and zone (Pant *et al*., 2021). Propolis has been explored globally for its medicinal and nutritional properties and is widely used in medicine and cosmetics, as well as health foods (Xu *et al*., 2009; Sadhana *et al*., 2017). However, research over Algerian propolis are scarce. Up to now, only few data are available on the chemical composition and the functional properties of Algerian propolis. Since propolis from different regions may contain different bioactive compounds and could exhibit different biological activities, the current study, therefore, was conducted for the first time to investigate the chemical composition and functional properties of propolis collected from *Apis mellifera* hives located at different

Northesastern regions of Algeria. The regions are: Collo (Skikda), El Harrouch (Skikda), Bouteldja (El-Taref), Grarem (Mila), El-Menia (Constantine), Oum El Bouaghi (Oum El Bouaghi) and Mestaoua & Chelala mountains (Batna).

This study reports the antioxidant, enzyme inhibition, antimicrobial, anticancer and toxic potentials of Algerian propolis essential oils and extracts as well as their chemical composition. It is worth mentioning that there is no previous study on the chemical and the biological activities of propolis from these regions.

The thesis is structured in two parts. The first part presents a literature review, which is divided in two chapters. Chapter 1 is a revision of free radicals, their physiological and pathological roles as well as the various antioxidants systems. Chapter 2 is also a revision of different aspects of propolis regarding its origin, types, physical characteristics, chemical composition, traditional use and biological properties. The second part concerns the experimental and contains two chapters. The first chapter describes the techniques employed within the scope of this study, whereas the second chapter reports the findings and presents a discussion of the results.

Part 1: Literature review

Chapter I: Free Radicals and Antioxidants

I. Free Radicals and Antioxidants

I.1. Free radicals

I.1.1. General characteristics

A free radical can be defined as any molecular species capable of independent existence that contains one or more unpaired electrons. The presence of unpaired electron makes free radicals extremely reactive species having very low stability. So as short-lived particles, these free radicals tend to attack neighboring molecules in order to re-establish a stable state and structure. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants (Lobo *el al*., 2010; Kurutas, 2015; Sanjay & Shukla, 2021).

A wide variety of free radicals can be found in living systems. Most of them are, or arise from, reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS). ROS include oxygen-based free radicals, such as the superoxide radical anion ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$), organic peroxyl ($ROO^{\bullet}$) and hydroperoxyl ($HOO^{\bullet}$) radicals and other species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl). RNS comprise peroxynitrite ($ONOO^-$), nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$), while the most common RSS are thiyl radicals ($RS^{\bullet}$), sulfenic acids (RSOH), and disulfide-S-oxides (RS(O)2SR) (Lu *et al*., 2010; Galano, 2015). However, radicals derived from oxygen represent the most important class of radical species generated in living systems (Valco *et al*., 2007).

I.1.2. Generation of free radicals

The generation of ROS begins with the reduction of O_2 with NADPH to produce $O_2^{\bullet-}$, a precursor to most remaining reactive oxygen and nitrogen species (Figure 1). Subsequent dismutation of two molecules of $O_2^{\bullet-}$ catalyzed by SOD generates O_2 and H_2O_2. The latter in turn may undergo partial reduction to $^{\bullet}OH$ through the Fenton reaction or alternatively *via* the Haber-Weiss process (Figure 1). While H_2O_2 is more damaging to DNA, the $^{\bullet}OH$ is highly reactive and turns biomolecules into free radicals, thus perpetuating a free radical chain reaction. H_2O_2 may also be converted to the potent oxidant HOCl in the presence of the chloride ion (Cl^-), an omnipresent species. This transformation is catalyzed by the enzyme myeloperoxidase (MPO). Reaction of HOCl with H_2O_2 regenerates Cl^- and produces $^1O_2$ as yet another ROS. On the other hand, RNS such as $NO^{\bullet}$ are produced by the enzyme nitric oxide synthase (NOS) starting from the precursor L-arginine. $NO^{\bullet}$ functions as a superoxide quencher forming $ONOO^-$, a strong oxidant that reacts

indiscriminately with biological targets. Further, it may disintegrate into a pair of •OH and NO_2• radicals and cause damage through such species (Figure 1) (Moussa *et al*., 2019).

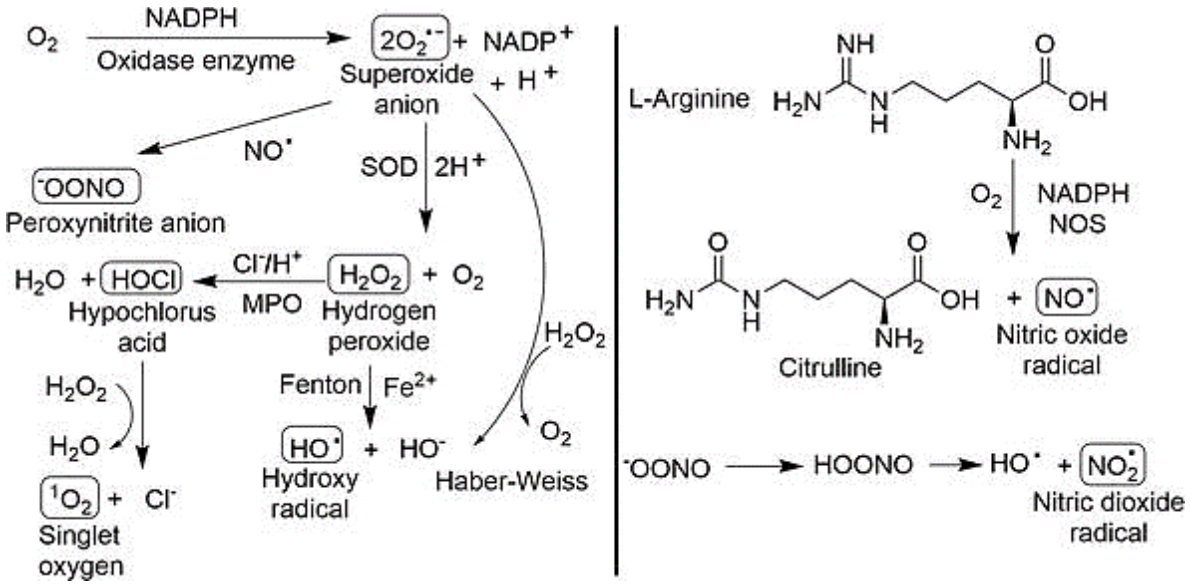


Figure 1. Generation of ROS and RNS in living species (Moussa *et al*., 2019).

I.1.3. Physiological roles of free radicals

At low or moderate concentrations, free radicals play several beneficial roles for the organism. They are needed to synthesize some cellular structures and used by the host defense system to fight pathogens. $O_2^{\bullet-}$, for instance, has been reported to serve as a cell growth regulator and can attack various pathogens inducing physiological inflammatory response. In addition, phagocytes synthesize and store free radicals, in order to be able to release them when invading pathogenic microbes have to be destroyed. Free radicals are also involved in a number of cellular signaling pathways. They play a key regulatory role in intracellular signaling cascades, in several cell types such as fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, and thyroid tissue. NO•, for example, is a signaling molecule participating in cellular and organ function as a neurotransmitter and a mediator of the immune responses. It is an important cell-to-cell messenger required for a proper blood flow modulation and involved in thrombosis. NO• is also involved in nonspecific host defense, required to eliminate intracellular pathogens and tumor cells. Another

physiological activity of free radicals is the induction of a mitogenic response (El-Bahr, 2013; Pizzino *et al*., 2017).

I.1.4. Pathological roles of free radicals

I.1.4.1. Oxidative damage to biomolecules

When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA). Oxidative stress can arise when cells cannot adequately destroy the excess of free radicals formed. In other words, oxidative stress results from an imbalance between formation and neutralization of ROS/RNS (Pham-Huy *et al*., 2008).

I.1.4.1.1. Oxidative damage to proteins

Proteins are major targets for attack by ROS predominantly by OH•, RO• and RNS causing damage. Hydrogen peroxide and superoxide radicals have weak effects on proteins except for proteins containing SH groups. (Engwa, 2018). Protein containing amino acids such as methionine, cysteine, arginine, and histidine seem to be the most vulnerable to oxidation (Lobo *et al*., 2010).

Proteins can be oxidatively modified by free radicals via various possible oxidative pathways such as oxidation of the protein backbone, formation of protein-protein cross-linkages, oxidation of amino acid side chains and protein fragmentation (Sitte, 2003). Besides, free radicals induced proteins oxidation can lead to changes in the protein's three-dimensional structure (Rao *et al*., 2011). Protein oxidation products are usually keto, aldehydes and carbonyls compounds Engwa, 2018).

The consequences of protein damage include loss of biological function of the protein, alteration of enzymatic activity and receptors, alteration of membrane transport and signal transduction mechanism, alteration of heat stability and increasing proteolysis susceptibility, which leads to aging (Sitte, 2003; Lobo *et al*., 2010; Engwa, 2018).

I.1.4.1.2. Oxidative damage to lipids

Lipids of cell membranes, especially the polyunsaturated fatty acid (PUFA) residues of phospholipids are more susceptible to oxidation by free radicals. The lipid peroxidation is initiated, when free radicals, particularly OH, attack and abstract hydrogen from a methylene groups (CH_2)

in a fatty acid (LH) which results in the formation of a carbon centered lipid radical ($L^{\bullet}$) (Rao *et al.*, 2011; Phaniendra *et al*., 2015). The lipid radical can react with O_2 to form a lipid peroxyl radical ($LOO^{\bullet}$). The resultant $LOO^{\bullet}$ undergo rearrangement via a cyclisation reaction to form endoperoxides, which finally form malondialdehyde (MDA) and 4-hydroxyl nonenal (4-HNA), the toxic end products of lipid peroxidation that cause damage to the DNA and proteins. These lipid peroxyl radicals can further propagate the peroxidation process by abstracting hydrogen atoms from the other lipid molecules (Phaniendra *et al*., 2015). The lipid peroxidation results in the loss of membrane functioning, for example, decreased fluidity, inactivation of membrane bound enzymes and receptors (Phaniendra *et al*., 2015).

I.1.4.1.3. Oxidative damage to DNA and RNA

Both ROS/RNS can oxidatively damage the nucleic acids. The mitochondrial DNA is more vulnerable to ROS attack than the nuclear DNA because of the lack of protective protein, histones of nuclear DNA and close locations to the ROS producing systems (El-Bahr, 2013; Phaniendra *et al*., 2015). However, RNA is more prone to oxidative damage than DNA, due to its single stranded nature, lack of an active repair mechanism for oxidized RNA, less protection by proteins than DNA and moreover these cytoplasmic RNAs are located in close proximity to the mitochondria where loads of ROS are produced (Phaniendra *et al.*, 2015).

ROS can interact with DNA to cause several types of damages which include double- and single-DNA breaks, modification of DNA bases, loss of purines (apurinic sites), DNA-protein cross-linkage, damage to the deoxyribose sugar and damage to the DNA repair system (Engwa, 2018). The modifications induced by ROS on RNA, however, include alteration of RNA structure, alterations of ribose, base excision, and strand break (Fimognari, 2015).

Hydroxyl radical is the most detrimental ROS that affects nucleic acids. It abstracts hydrogen atoms to produce a number of modified puine as well as pyrimidine base by-products such as 8-hydroxydeoxyguanosine, 8-hydroxydeoxyadenosine, thymine glycols, 5-hydroxy deoxycytidine in DNA and 8-hydroxyguanosine in RNA (Phaniendra *et al*., 2015; Fimognari, 2015; Engwa, 2018).

RNS, most importantly, peroxynitrite ($OONO^-$) interacts with guanine to produce 8-oxodeoxyguanosine and 8-nitroguanine. The latter is unstable and can be spontaneously removed,

resulting in the formation of an apurininc site. Conversely adenine can be paired with 8-nitroguanine during DNA synthesis resulting in a G-T transversions (Phaniendra *et al*., 2015).

DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Valko *et al*., 2006). Oxidative damage can alter RNA function and interfere with the interaction between RNA and other cellular molecules. As an example, oxidative damage to RNA produces the block of reverse transcription. Moreover, oxidation of mRNA leads to reduced translation efficiency and abnormal protein production and causes ribosome dysfunction (Fimognari, 2015).

I.1.4.2. Oxidative stress and human diseases

I.1.4.2.1. Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive impairments in cognitive functions. It is characterized by two histological traces in brain: β-amyloid peptide ($A\beta_{1-42}$) forming senile plaques as extracellular deposits; and hyperphosphorylated tau protein (P-tau) forming neurofibrillary tangles as intracellular deposits. These deposits are believed to lead to loss of neurons and synapses in the brain regions that are involved in cognitive functions and emotional regulation (Caruso *et al*., 2019; Peña-Bautista *et al*., 2019; Silva *et al*., 2019). The clinical evolution of AD can be divided in three phases: **1)** Preclinical AD, in which individuals conserve cognitive capacity but different biomarkers could be altered; **2)** Mild cognitive impairment (MCI), when affected individuals show the first symptoms of cognitive impairment, the most common of which being episodic memory loss; **3)** Dementia, when cognitive impairment affects the ability to carry out daily activities in an independent way, and individuals suffer from behaviour changes (Peña-Bautista *et al*., 2019).

Many risk factors have been associated with AD development such as increasing age, genetic factors, brain injuries, vascular diseases, diabetes, psychiatric factors, infections, and environmental factors (heavy metals, trace metals, and others) (Armstrong, 2019; Breijyeh & Karaman, 2020). Several experimental and clinical research in AD showed that oxidative damage also plays a pivotal role in neuron loss and progression to dementia (Pizzino *et al*., 2017).

The brain is sensitive to oxidative damage because of multiple reasons: 1) it is a postmitotic tissue with a high energy demand; 2) it is exposed to high oxygen concentration and throughput and utilizes about one-fifth of the oxygen consumed by the body; 3) it contains relatively low levels of antioxidants and related enzymes; 4) it is rich in polyunsaturated fatty acids, such as arachidonic acid and docosohexanoic acid, which are more prone to oxidation; and 5) it is abundant in iron, which accumulates in the brain as a function of age and can be a potent catalyst for oxidative species formation (Perry *et al*., 2008).

The brain membrane phospholipids are composed of polyunsaturated fatty acids, this organ is particularly vulnerable to free radical attacks. Their double binds allow the removal of hydrogen ions and increased lipid peroxidation, which is the most prominent feature in which degenerative change is most pronounced in the AD brain (Huang *et al*., 2016). In addition, the oxidation of proteins by free radicals may be significant in AD since it can affect enzymes critical to neuron and glial functions. This is the case for two enzymes especially sensitive to oxidative modification, that of glutamine synthetase and creatine kinase, which are markedly reduced in AD brains, reflecting the alteration of glutamate concentrations and enhancement of excitotoxicity, whereas oxidative impairment of creatine kinase may cause decreased energy metabolism in AD (Huang *et al*., 2016). In addition, phosphorylation is linked to oxidation through the microtubule-associated protein kinase pathway and through activation of the transcription factor nuclear factor-κB, thus potentially linking oxidation to the hyperphosphorylation of tau proteins. Protein oxidation is also capable of inducing advanced glycation end products as a post-translational modification of proteins that are formed when amino group of proteins react non-enzymatically with monosaccharides. Furthermore, oxidation of the brain can affect DNA, producing strand breaks, sister chromatid exchange, DNA-protein crosslinking, and base modification (Huang *et al*., 2016).

Several markers of oxidative damage of macromolecules have been described in association with the susceptible neurons in AD brains: 1) DNA and RNA oxidation is marked by increased levels of 8- hydroxy-2-deoxyguanosine (8OHdG) and 8-hydroxyguanosine (8OHG), 2) protein oxidation is marked by elevated levels of protein carbonyls in the frontal lobe, hippocampus and superior middle temporal gyrus of AD patients. Furthermore, it has been shown that nitrotyrosine and dityrosine cross-linked proteins are elevated 8-fold and 3-fold respectively in hippocampal and neocortical regions of AD brain compared to age-matched controls. Lipid

peroxidation is marked by higher levels of thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), 4- hydroxy-2-transnonenal (HNE), isoprostanes, and altered phospholipid composition. Modification to sugars is marked by increased glycation and glycoxidation (Perry *et al.*, 2008).

I.1.4.2.2. Diabetes

Diabetes mellitus (DM) is a group of metabolic disorders characterized by an elevated blood glucose level (hyperglycemia) resulting from defects in insulin secretion, insulin action or both (Aruoma *et al.*, 2006). DM can be divided into two types: insulin-dependent diabetes mellitus (IDDM) or type 1 and noninsulin-dependent diabetes mellitus (NIDDM) or type 2. Type 1 diabetes mellitus (T1DM) is most commonly occur in juvenile age that may result from a deficiency of insulin secretion due to the autoimmune destruction of pancreatic β-cell (Ullah *et al*., 2015; Arman *et al*., 2019). Type 2 diabetes mellitus (T2DM) occur in the adult age and obese individuals are more susceptible to such disease (Arman *et al*., 2019). In this type, there are certain mechanisms broken that keep regulation between tissue sensitivity to insulin, which consequently leads to impaired insulin secretion by the pancreatic β-cells and impaired insulin action through insulin resistance (Ullah *et al*., 2015). Patients with T2DM do not need insulin administration for their treatment and survival (Chandra *et al*., 2019). DM symptoms include polyphagia (increased hunger), polydipsia (increased thirst), polyuria (increase urination). Due to hyperglycemia malfunctioning and dysfunction of various organs such as heart (mainly myocardial infraction), kidneys (diabetic nephropathy), nerves (diabetic neuropathy), and eyes (diabetic retinopathy) usually occur (Khan *et al*., 2015). The risk factors for T1DM include genetic, infectious agents, dietary, psychosocial, socioeconomic, and environmental factors (Rewers *et al*., 2018). However, it has been reported that obesity, overweight, genetic component, sedentary life style and old age are possible relevant factors for T2DM (Zhang *et al*., 2020). Besides, Covid-19 infection could be a risk factor for both T1DM and T2DM (Rathmann *et al*., 2022; Wang et al., 2023).

Increasing studies suggest that oxidative stress plays a pivotal role in the pathogenesis and progression of diabetes. Oxidative stress was observed in experimental diabetes and has been found to play an important role in all cases of diabetes mellitus (particularly T2DM) and the pathogenesis of diabetic complications (Zhang *et al*., 2020). Nevertheless, the precise underlying mechanisms are not yet fully understood. T2DM is associated with increased oxidative stress

resulting from several abnormalities, including hyperglycemia, inflammation and dyslipidemia. In turn, elevated ROS can act as a second messenger and regulate the biological function of various proteins including IκB kinase β (IKKβ), protein kinase C (PKC) and Kelch-like ECH-associated protein 1 (Keap1) through interaction with cysteine residues (termed "redox sensors") of these proteins (Zhang *et al*., 2020). This dynamic modification of intracellular redox sensors by ROS is defined as redox modification, similar to other posttranslational modifications such as protein phosphorylation, acetylation, or ubiquitination, which plays an important role in the development of diabetes. Redox modification of these proteins can activate alternative downstream signaling pathways which play critical roles in impaired insulin secretion and insulin resistance, facilitating the development of diabetes and diabetic complications (Zhang *et al*., 2020).

Different studies have determined the levels of stress-related biomarkers in both type 1 and type 2 diabetes. In T2DM evidence of lipid peroxidation was observed with high plasma and urine isoprostane levels. MDA level results were also higher than in the normal subjects. Nitrotyrosine formation is increased in plasma of both types of diabetic patients while TRAP (radical-trapping antioxidant parameter) level is decreased. In addition, it has been proven that hyperglycemia independently increases 8-OHdG; a marker of DNA oxidation; levels in urine and plasma of patients with T2DM (Piconi *et al*., 2003).

I.1.4.2.3. Cancer

Cancer can result from abnormal proliferation of any of the different kinds of cells in the body. A tumor is any abnormal proliferation of cells, which may be either benign or malignant. A benign tumor remains confined to its original location, neither invading surrounding normal tissue nor spreading to distant body sites. A malignant tumor, however, is capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems (metastasis). Only malignant tumors are properly referred to as cancers, and it is their ability to invade and metastasize that makes cancer so dangerous. Whereas benign tumors can usually be removed surgically, the spread of malignant tumors to distant body sites frequently makes them resistant to such localized treatment (Cooper, 2000). As opposed to benign tumors, malignant cancers acquire metastasis, which occurs in part due to the down-regulation of cell adhesion receptors necessary for tissue-specific cell–cell attachment, and up-regulation of receptors that enhance cell motility. In addition, activation of membrane metalloproteases provides

a physical pathway for metastatic cancer cells to spread (Sarkar et *al.*, 2013). Both benign and malignant tumors are classified according to the type of cell from which they arise.

Most cancers fall into one of three main groups: carcinomas, sarcomas, and leukemias or lymphomas. Carcinomas, which include approximately 90% of human cancers, are malignancies of epithelial cells. Sarcomas, which are rare in humans, are solid tumors of connective tissues, such as muscle, bone, cartilage, and fibrous tissue. Leukemias and lymphomas, which account for approximately 8% of human malignancies, arise from the blood-forming cells and from cells of the immune system, respectively. Tumors are further classified according to tissue of origin (e.g., lung or breast carcinomas) and the type of cell involved. For example, fibrosarcomas arise from fibroblasts, and erythroid leukemias from precursors of erythrocytes (red blood cells) (Cooper, 2000). The characteristics of cancer cells include loss of contact inhibition, resistance to apoptosis, and insensitivity to cell growth arrest signals. Angiogenesis is a chief characteristic of cancer cells (Nourazarian *et al.*, 2014).

Many agents, including chemical compounds, smoking, unhealthy diet, viruses, bacteria, UV radiation and pollution have been found to induce cancer (Hassanpour & Dehghani, 2017; Fatima Zahra *et al*., 2021). In addition, oxidative DNA damage is one of those stimuli responsible for cancer development. Cancer can be driven and/or promoted by chromosomal abnormalities and oncogene activation determined by oxidative stress. Hydrolyzed DNA bases are common by-products of DNA oxidation and are considered one of the most relevant events in chemical carcinogenesis. The formation of such kind of adducts impairs normal cell growth by altering the physiological transcriptomic profile and causing gene mutations. Oxidative stress can also cause a variegated amount of modifications against DNA structure, for example, base and sugar lesions, DNA-protein cross-links, strand breaks, and base-free sites (Pizzino *et al*., 2017).

I.2. Antioxidants

I.2.1. Definition

Antioxidants are inhibitors of oxidation, even at small concentrations. They act as free radical scavengers, by reacting with the reactive radicals and demolishing them to become less active, less dangerous, and long-lived substance than those radicals that have been neutralized. Antioxidants may be able to neutralize free radicals via accepting or donating electron(s) to remove the unpaired status of the radical (Azat Aziz et al., 2019).

I.2.2. Classification and mechanism of action

Based on their activity, antioxidants can be categorized as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants work by breaking down and removing free radicals. They convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in presence of cofactors such as copper, zinc, manganese, and iron. Non-enzymatic antioxidants work by interrupting free radical chain reactions (Nimse & Pal, 2015).

I.2.2.1. Enzymatic antioxidants (Endogenous)

Enzymatic antioxidants involve superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and peroxiredoxin I–IV (Prx I–IV) (Pham-Huy *et al.*, 2008; Nimse & Pal, 2015).

SOD converts $O_2^{\bullet-}$ into H_2O_2 by successive oxidation and reduction of the transition metal ion at the active site in a "Ping-Pong" type mechanism. In humans, there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (Valco et al., 2006).

$$2O_2^{\bullet-} \xrightarrow{\text{SOD}} H_2O_2 + O_2$$ (Lawson *et al.*, 2017)

CAT is an enzyme located in a cell organelle called the peroxisome (Njuma et al. 2014). The enzyme is very efficient in the decomposition of H_2O_2 into water and molecular oxygen. It has been estimated that one molecule of CAT converts approximately six million molecules of H_2O_2 into water and oxygen each minute (Valco *et al*., 2006; Lawson *et al*., 2017).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

GPx are selenium-dependent glutathione peroxidases. These enzymes act by adding two electrons to reduce peroxides by forming selenoles (Se-OH). These selenoprotein GPx enzymes remove H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). The substrate for the catalytic reaction is either H_2O_2 or an organic peroxide ROOH (Pham-Huy *et al*., 2008; Lawson *et al*., 2017). Catalytic reactions can be described according to the following reactions:

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$ (Lawson *et al.*, 2017)

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$ **(**Lawson *et al.*, 2017)

GR, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power (Pham-Huy et al., 2008). GR protects red blood cells, hemoglobin, and cell membranes from oxidative stress by generating GSH (Bhattacharyya *et al*., 2014).

$$GSSH + NADPH \xrightarrow{GR} GSH + NADP^+$$ (Lawson *et al*., 2017)

Prx catalyze the reduction of H_2O_2 and various organic hydroperoxides (e.g., lipid hydroperoxide) to form water and alcohols, respectively, through the reactive cysteine (Cys) residues of the enzymes. Some peroxyredoxin have been shown to also reduce peroxynitrite ($ONOO^-$) (Li *et al*., 2020).

I.2.2.2. Non enzymatic antioxidants

Non enzymatic antioxidants are also divided into metabolic antioxidants and nutrient antioxidants (Pham-Huy *et al*., 2008; Rao *et al*., 2011).

I.2.2.1.1. Metabolic antioxidants (endogenous)

Metabolic antioxidants are produced by metabolism in the body, such as glutathione, melatonin, lipoic acid, coenzyme Q10 (Co Q10), uric acid, etc (Pham-Huy *et al*., 2008; Rao *et al*., 2011).

Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is a tripeptide and is the most abundant intracellular antioxidant protecting normal cells from oxidative injury due to its role as a substrate of ROS scavenging enzymes. In cells, glutathione is maintained in the reduced form (GSH) by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants. Glutathione functions as a nonenzymatic antioxidant through free radical scavenging in cells and serves as a cofactor for several enzymes, including GPx, GR, and glutathione transferase (GST) (Lobo *et al*., 2010; Azat Aziz *et al*., 2019).

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced in many organs including the pineal gland. Melatonin and its derivatives are considered as powerful direct free radical scavengers. The mechanisms by which melatonin detoxifies oxidants include single electron transfer, hydrogen transfer, and radical adduct formation. Besides direct scavenging of

ROS/RNS, melatonin also stimulates antioxidant enzymes, suppresses pro-oxidant enzymes, and improves mitochondrial function thereby reducing radical formation (Zhang & Zhang, 2014).

Lipoic acid (LA) or α-lipoic acid (ALA) is a short-chain fatty acid, composed of sulfur in their structure that is known for its contribution in the reaction that catalyzes the oxidative decarboxylation of α-keto acids, for example pyruvate and α-ketoglutarate, in the citric acid cycle (Azat Aziz *et al*., 2019). Both the oxidized and reduced forms of LA are powerful antioxidants whose functions include quenching of ROS, regeneration of exogenous and endogenous antioxidants such as vitamins C and E, and GSH, chelation of metal ions, reparation of oxidized proteins, etc (Golbidi *et al*., 2011).

Co Q10, also known as ubiquinone, is an endogenous lipid-soluble antioxidant that present in the inner membrane of mitochondria. It acts as a small electron carrier in the respiratory chain during oxidative phosphorylation. Co Q10, like other antioxidants inhibits certain enzymes involved in the formation of free radicals and thus attenuates oxidative stress and prevents the initiation and propagation of lipid peroxidation in cellular membranes (Samimi *et al*., 2019).

Uric acid is the most abundant aqueous antioxidant found in humans. It contributes for as much as two-thirds of all free radical scavenging activities in the plasma. It is a powerful scavenger of carbon centered radicals and peroxyl radicals in the hydrophilic environment. It loses, however, its radical scavenging activity within lipid membranes. Uric acid is an exceptional scavenger of $ONOO^-$ in the extracellular fluid. However, it requires the presence of ascorbic acid and thiols for the complete scavenging of $ONOO^-$ (Nimse & Pal, 2015).

I.2.2.1.2. Nutrient antioxidants (exogenous)

Nutrient antioxidants are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc (Pham-Huy *et al*., 2008; Rao *et al*., 2011).

Carotenoids are pigments that are found in plants and microorganisms. Their antioxidant activity arises primarily because of the ability of the conjugated double-bonded structure to delocalize unpaired electrons. This is primarily responsible for the excellent ability of β-carotene to physically quench $^1O_2$ without degradation, and for the chemical reactivity of β-carotene with

free radicals such as ROO•, •OH and $O2^{\bullet-}$. At sufficiently high concentrations, carotenoids can protect lipids from peroxidative damage. Generally, three mechanisms are proposed for the reaction of free radicals (ROO•, R•) with carotenoids: radical addition, hydrogen abstraction from the carotenoid and electron-transfer reaction (Valco *et al*., 2006).

Vitamin C or ascorbic acid is a water-soluble free radical scavenger (Nimse & Pal, 2015). It plays an important role in protection against oxidative stress on various tissues. Vitamine C acts directly to scavenge ROS and RNS generated during normal cellular metabolism. Its antioxidant mechanisms are based on hydrogen atom donation to lipid radicals, quenching of singlet oxygen, and removal of molecular oxygen. Scavenging aqueous radicals and regeneration of α–tocopherol from the tocopheroxyl radical species are also well known antioxidant mechanisms of ascorbic acid (Akbari *et al*., 2016).

Vitamin E functions as an essential lipid soluble antioxidant, scavenging hydroperoxyl radicals in lipid milieu (Traber & Stevens, 2011). It is a potent chain-breaking antioxidant that inhibits the production of reactive oxygen species molecules when fat undergoes oxidation and during the propagation of free radical reactions. It acts as the first line of defence against lipid peroxidation, protecting the cell membranes from free radical attack. Due to its peroxyl radical-scavenging activity, it also protects the polyunsaturated fatty acids present in membrane phospholipids and in plasma lipoproteins (Rizvi *et al*., 2014).

Minerals such as zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), and selenium (Se) are key components of enzymes with antioxidant functions and are designated as antioxidant micronutrients. Zn, Mn, and Cu are cofactors of superoxide dismutase. Fe is a component of catalase. Se is a major antioxidant in the form of selenoproteins that mitigates the cytotoxic effects of ROS. (Bhattacharyya et al., 2014).

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants (Kumar & Pandey, 2013). The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability. The B ring hydroxyl configuration is

the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to •OH, ROO• and ONOO– radicals, stabilizing them and giving rise to a relatively stable flavonoids radical (Kumar & Pandey, 2013). The mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; (2) scavenging ROS; and (3) upregulation or protection of antioxidant defenses (Kumar & Pandey, 2013).

Chapter II: Propolis

II. Propolis

II.1. Origin, characteristics and types

Propolis, also called bee glue, is a resinous natural mixture, collected by honeybees from different plant leaves, buds and exudates, partially digested by β-glycosidase from bees' saliva, and after that mixed with beeswax (Ristivojevic *et al*., 2015). The word propolis has Greek origin in which "Pro" means ''in front of" or ''at the entrance to" and "polis" means "community" or "city" which means hive defensive substance (Anjum *et al*., 2018). Bees use propolis on their hives as protection against predators and microorganisms, to repair damage, as a thermal isolator, and to build aseptic locals to prevent microbial infection of larvae (Carvalho *et al*., 2015). In addition, propolis prevents entry of water to the hive, which maintains constant humidity and also serves as a control over the air flow towards the hive (Anjum *et al*., 2018).

Propolis is collected by worker-bees, from numerous plant resinous secretions such as mucilage, gums, resins and lattices and also from leaf buds of different plant species like palm, pine, alder, poplar, beech, conifer and birch and then mixed with salivary and enzymatic secretions (Anjum *et al*., 2018). Although it is an animal product, most of the components of propolis, especially the active ones, come from plants (Da Cruz *et al*., 2022). Several bee species are capable of producing propolis, however, *Apis mellifera* is recognized as the main producer among all of them. Annually, from each *A. mellifera* hive, 100 to 300 grams of propolis can be extracted, which makes this species a very efficient producer (Da Cruz *et al*., 2022).

In terms of physical properties, propolis has variable consistency; it is hard and breakable when cold (at temperatures below 15 °C) but soft, flexible, and very sticky when warm (at temperatures in the range of 25–45 °C). The usual melting point for propolis is 60–70 °C, although it may be up to 100 °C for some samples. It has a typical odor and a bitter taste. The odor can vary from sample to sample, having a distinct flavor and an aromatic pleasant smell, some samples being odorless. The color varies from yellow, green to red and dark brown (Carvalho *et al*., 2015; Alanazi *et al*., 2021; Balica *et al*., 2021). Considering the complex structure of propolis, it cannot be used directly. Propolis is extracted commercially with suitable solvent. The most common solvents used for extraction are water, methanol, ethanol, chloroform, dichloromethane, ether, and acetone (Wagh, 2013).

Based on the plant source and the area of collection, numerous types of propolis has been described. Among them are poplar, birch, Brazilian green, Brazilian red, Clusia, Pacific, and Mediterranean. Poplar propolis is found in temperate zones (Europe, North America, and non-tropical regions of Asia) and the main botanical sources are the bud exudates of *Populus* species, mostly *Populus nigra* L. Birch propolis found in Russia is derived from birch buds. Brazilian green propolis, the most popular tropical propolis type, is originated from the leaves of *Baccharis dracunculifolia* D.C., whereas Brazilian red propolis is derived from the red resinous exudates at the surface and the branch orifice of *Dalbergia ecastophyllum* (L.) Taub. Clusia propolis is derived from resin exuded by the flowers of different *Clusia* species found in Cuba and Venezuela. Pacific propolis found in Taiwan, Okinawa and Indonesia originates from the fruits of *Macaranga tanarius* (L.) Müll. Arg. Mediterranean propolis, that seems to be originated from cypress. This type is found in Greece, Malta, Sicily, Turkey, and Algeria. (Ristivojevic *et al*., 2015; Balica *et al*., 2021).

II.2. Chemical composition

Propolis composition is strongly associated with its botanical and geographical origin among other factors; however, its overall percent composition remains almost unchanged. Resins and balsams (45–55%), waxes (8–35%), essential oils and aromatics (5–10%), fatty acids (5%), pollen (5%) and organic and mineral substances (5%) are the main compounds found in this product (Irigoiti *et al*., 2021).

More than 850 components have been identified in propolis samples from all over the world belonging to phenolic compounds (flavonoids as main constituents, phenolic acids, and their esters, phenylpropanoids), terpenes and terpenoids, ketones, aromatic aldehydes and alcohols, proteins, fatty acids, waxy acids, amino acids, hydrocarbons, steroids, stilbenes, sugars, vitamins, minerals, and enzymes (Balica *et al*., 2021; Irigoiti *et al*., 2021). The main chemical compounds from propolis are presented in table 1.

Table 1. Main chemical compounds present in the composition of propolis (Balica *et al*., 2021)

Chemical class	Compounds
Phenyl carboxylic acids and derivatives	Caffeic acid, caffeic acid phenethyl ester, cichoric acid, cinnamic acid, ferulic acid, *p*-coumaric acid, benzoic acid, salicylic acid, rosmarinic acid, chlorogenic acid, caffeoylquinic acid, vanillic acid, artepillin C, baccharin, drupanin
Flavonoids	Apigenin, kaempferol, pinobanksin, chrysin, tektochrisin, pinocembrin, galangin, quercetin, myricetin, rutin, rhamnetin, isorhamnetin, luteolin, naringenin, acacetin, baicalein, hesperitin, sakuranetin, formononetin, liquiritigenin, isalpinin, daidzein, genistein, eupatorin, hispidulin, propolins, prokinawan, isosativan, medicarpin, vestitol, nymphaeol, isonymphaeol
Terpenoids	Geraniol, nerol, bisabolol, guaiol, farnesol, linalool, limonene, eudesmol, terpineol, camphor, squalene, copaene, calarene, calamenene, caryophyllene, patchoulene, elemene, ferruginol, junicedric acid, pimaric acid, abietic acid, isocupressic acid, acetylisocupressic acid, communic acid, imbricatoloic acid, totarol, amyrin, amyrone, lupeol, lupenone, moretenol, ferutinin, teferin, germanicol, agarospirol, lanosterol, erythrodiol, cycloartenol, ambonic acid, mangiferonic acid, ambolic acid
Alkaloids	Demecolcine, papaverine, thebaine, morpholine, norlobeline, pagicerine, oreophilin
Amino acids	Aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, tryptophane, asparagine, cystine
Sugars and sugar alcohols	Xylose, galactose, mannose, glucuronic acid, lactose, maltose, melibiose, d-ribofuranose, d-fructose, d-gulose, talose, sucrose, d-glucose, erytritol, xylitol, inositol, d-glucitol
Aliphatic hydrocarbons, aliphatic acids	Eicosine, 1-octadecene, eicosane, heneicosane, docosane, tricosane, tetracosane, pentacosane, hexacosane, heptacosane, octacosane, nonacosane, triacontane, behenic acid, cerotic acid, lauric acid, linoleic acid, lignoceric acid, montanic acid, nonanoic acid, palmitic acid, oleic acid, stearic acid, behenic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, heptadecanoic acid, tetracosanoic acid, eicosanoic acid, hexacosanoic acid
Vitamins	B1, B2, B3, B5, B6, C, E
Minerals	Sr, Ba, Cd, Sn, Pb, Ti, Ag, Co, Mo, Al, Si, V, Ni, Mn, Cr, Na, Mg, Cu, Ca, Zn, Fe, K

II.3. Traditional uses

Propolis has long been used as a bactericidal, antiviral, and antifungal drug in folk medicine to treat inflammations in several body areas worldwide. It was used for skin regeneration, wound healing, and as local anesthetic. Propolis has also been advised in folk medicine for the treatment of purulent disorders, as it has been shown to improve wound healing and relieve many types of discomfort. Besides, craftsmen utilized propolis for no-health purposes such as windows sealer, impregnant for valuable timber objects, varnish and repairing instrument. The alternative and complementary medicine used different propolis-based preparations such as sprays, ointments, and powders (mainly consisting of tinctures and ethanolic extracts) for the treatment of colds, flu, bronchial asthma, and other human ailments such as gastric disorders (Hossain *et al*., 2022).

II.4. Biological activities

II.4.1. Antioxidant activity

Antioxidant capacity is one of the most important properties of propolis (Daleprane, 2013). Several investigations have validated the antioxidant potential of propolis by *in vitro* and *in vivo* tests. It has been reported that propolis exhibit significant ability of scavenging free radicals through DPPH (2,2'-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate), HRSA (Hydroxyl radical scavenging activity), ORAC (Oxigen Radical Absorbance Capacity) and superoxide anion radical scavenging assays (Socha *et al*., 2014; Castro *et al*., 2014; Segueni *et al*., 2017; Bouaroura *et al*., 2020; Turnia *et al*., 2020; Liaudanskas *et al*., 2021). The ability of propolis to reduce copper, ferric and ceric ions has been also demonstrated using CUPRAC (Cupric ion reducing antioxidant capacity), FRAP (Ferric reducing antioxidant power), reducing power and CERAC (Ceric ion reducing antioxidant capacity assay) methods (Socha *et al*., 2014; Segueni *et al*., 2017; Bouaroura *et al*., 2020; Bayram *et al*., 2020; Liaudanskas *et al*., 2021). In addition, propolis has been reported to inhibit lipid peroxidation in β-carotene-linoleic acid test system (Bouaroura *et al*., 2020).

The *in vivo* experiments revealed that the principal antioxidant mechanism of propolis includes the prevention of oxidative stress by increasing enzymatic and non-enzymatic antioxidants, as well as decreasing lipid peroxidation (Benguedouar *et al*., 2008; Boutabet *et al*., 2011; Brihoum *et al*., 2018). Benguedouar *et al*. (2008) studied the effect of propolis extract against mitochondrial oxidative stress induced by two anticancer drugs (doxorubicin and

vinblastin) in female wistar rat, using liver and heart mitochondria. The results showed that the pretreatment of rats with propolis extract (100 mg/kg/day) administered 4 days prior to doxorubicin (20 mg/kg) and/or vinblastin (2 mg/kg) injection, substantially reduced the peroxidative damage in myocardium and hepatic tissues and markedly restored the tissues catalase and SOD activities.

In human studies, Jasprica *et al*. (2007), investigated the influence of 30-day supplementation with powdered propolis extract on antioxidant enzymes such as SOD, GPx, CAT, and a lipid peroxidation marker MDA in healthy individuals. In the male group, after 15 days of propolis treatment, a 23.2% decrease in MDA level was observed, whereas after 30 days, a 20.9% increase in SOD activity was found. The propolis treatment had no effect on any of the studied parameters in women. The authors concluded that the effect of propolis was both time and gender dependent.

II.4.2. Antimicrobial activity

Antimicrobial efficiency is one of the most important properties of propolis (Siheri *et al*., 2017). Numerous studies confirmed the antimicrobial activity of propolis of different types against a wide spectrum of microorganisms (Wieczorek *et al*., 2022). Benhanifia *et al.* (2014) stated that propolis from Western Algeria were active against Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus*). Koru *et al.* (2007) reported that Turkish and Brazilian propolis were more effective against Gram-positive anaerobic bacteria than Gram-negative ones. Roh & Kim (2018) confirmed the inhibitory effect of Korean propolis on oral pathogenic bacteria (*Streptococcus mutans*, *Staphylococcus aureus*, and *Enterococcus faecalis*) and fungi (*Candida albicans*). In addition, the antifungal potential of various extracts of propolis have been examined against several yeasts, such as *Candida albicans, C. dubliniensis, C. glabrata, C. krusei, C. parapsisolis, C. tropicalis, Saccharomyces cerevisiae* as well as against molds, such as *Alternaria solani, Alternaria alternata, Aspergillus niger, Aspergillus ochraceus, Botrytis cinerea, Cladosporium spp., Fusarium solani, Fusarium oxysporum, Mucor mucedo, Penicillium digitatum, Penicillium expansum, Penicillium chrysogenum, Rhizopus stolonifera, Rhodotorula mucilaginosa* and *Trichophyton spp* (Ożarowski *et al*., 2022).

Various propolis extracts have been also found to exert substantial antiviral activity against several types of viruses such as *Herpes simplex* virus type 1 and 2 (HSV-1 and HSV-2), *Canine*

distemper virus, *Human rhinovirus* type 2, 3 and 4 (HRV-2, HRV-3 and HRV-4), *Influenza virus* type A and B, *Parainfluenza* virus, *Human immunodeficiency virus* (HIV), *Adenovirus*, *Coronavirus* 229E (Hossain *et al*., 2022; Silva-Beltran *et al*., 2022).

II.4.3. Antidiabetic activity

The antidiabetic effect of propolis has been widely studied. By *in vitro tests*, extracts of propolis from different regions of the world were found to inhibit the activity of α-glucosidase and α-amylase, enzymes responsible for the breakdown of carbohydrates into glucose (Popova *et al*., 2015; Taleb *et al*., 2020; Baltas, 2021; Uddin *et al*., 2022). In addition, studies in animal models have proven the antidiabetic potential of propolis. Taleb *et al*. (2020) investigated the effect of propolis on streptozotocin (SZO) induced Type 1 diabetes in male Wistar rats. The treatment with 30% or 15% propolis extract (at a dosage of 0.5 mL/100 g for 4 weeks) showed a decrease in blood sugar levels from 393 ± 192.7 to 154 ± 28.0 mg/dl and from 386 ± 141.1 to 331.5 ± 123.74 mg/dl, respectively. An improvement was also observed in both groups treated with propolis at the pancreatic, hepatic, and renal tissue levels. In another study, Laaroussi *et al.* (2020) investigated the preventive effect of propolis on Type 2 diabetes induced by D-glucose in male Wistar rats. The results showed that propolis was able to attenuate the Type 2 diabetes caused by a high-glucose intake. The authors concluded that the role of propolis involves prevention of hyperglycemia, insulinemia, HOMA-IR index (Homeostasis model assessment of insulin resistance), HOMA-β (Homeostasis model assessment of β-cell function), insulin sensitivity, pancreatic β-cell function and lipid profile.

In clinical trials, Fukuda *et al.* (2015) investigated the effectiveness of propolis in patients with Type 2 diabetes. The authors found that 226.8 mg/day of Brazilian green propolis for 8 weeks prevented the actions of hyperuricemia and dysfunction of renal glomerular filtrating function that commonly develop in patients suffering from diabetes mellitus. In another study, Afsharpour *et al.* (2019) evaluated the effects of propolis on the glycemic status, insulin resistance and antioxidant status in Type 2 diabetic patients. The Patients were given doses of 500 mg, three times a day (1500 mg), of propolis. After two month, the fasting blood sugar (FBS), two-hour postprandial glucose (2-hp), insulin, insulin resistance (IR), hemoglobin A_{1c} (HbA_{1c}) were significantly decreased in patients treated with propolis. Additionally, intake of propolis significantly increased the blood levels of total antioxidant capacity (TAC) and activity of GPx and SOD. The authors

concluded that propolis treatment can be helpful as a diet supplement in patients with Type 2 diabetes through improvement in glycemic status, reduction in insulin resistance and amelioration in antioxidant status.

II.4.4. Anti-Alzheimer activity

The anti-Alzheimer potential of propolis has been studied and proved by several authors. Propolis has been reported to inhibit some key enzymes implicated in the pathology of AD. It was shown to inhibit acetylcholinesterase and butyrylcholinesterase, enzymes responsible for catalyzing the hydrolysis of the neurotransmitter acetylcholine (Wang *et al*., 2016; Baltas *et al*., 2016; Bouaroura *et al*., 2020). It was also reported to inhibit the human β-amyloid precursor cleavage enzyme (BACE-1) that is responsible for initiating β-amyloid production (Wang *et al*., 2016). Besides, extracts, fractions and pure constituents of propolis have been demonstrated to inhibit monoamine oxidase (MAO) A and B (Chaurasiya *et al*., 2014), enzymes that contributes to the amyloid beta (Aβ) and neurofibrillary tangles aggregation, and cognitive destruction (Manzoor & Hoda, 2020).

Additionally, the neuroprotective effect of propolis was demonstrated in various animal studies. Nanaware *et al.* (2017) showed that extract of Indian propolis ameliorates β-amyloid induced memory deficits in rats. They found that propolis extract increased brain catecholamines concentration to improve memory, improved antioxidant defense system with diminishing MDA in the brain, inhibited AChE (acetylcholinesterase) activity and activated BDNF (brain derived neurotrophic factor) potential. In another study by Gao *et al*. (2017), propolis was found to reduce the neuronal damage induced by oxygen-glucose deprivation/reoxygenation (OGD/R) in mouse neuroblastoma N2a cells. Ethanolic extract of Brazilian propolis has been also reported to ameliorate cognitive dysfunction and suppress protein aggregations caused by hyperhomocysteinemia (Miyazaki *et al.*, 2015).

In a clinical study, propolis was found to be effective in improving cognitive functions such as memory, information processing, complex attention, and concentration in elderly Japanese (Asama *et al*., 2021).

II.4.5. Anticancer Activity

Propolis has been reported to exhibit cytotoxic and antitumor activities in both animal and cell line models. Turan *et al.* (2015) reported powerful cytotoxic effects of Turkish propolis against prostate adenocarcinoma (PC-3), hepatocellular carcinoma (HepG2), colon adenocarcinoma (WiDr),

cervix adenocarcinoma (HeLa) and mammary adenocarcinoma (MCF-7) human cancer cell lines. In the study by Salem *et al.* (2019), it was shown that Egyptian propolis exhibit good cytotoxic effects on several cell lines, in which the IC_{50} of propolis on EAC (Ehrlich ascites carcinoma), HCT-116 (colon cancer), MDA-231 (breast cancer), MCF-7, HeLa cells, was 11.38, 18.69, 41.63, 35.06, 44.60 µg/mL, respectively. The Egyptian propolis demonstrated also antitumor effects against EAC mice model by reducing tumor volume, count of viable tumor cells with a significant elevation in the life span as well as the mean survival time of mice.

Additionally, propolis extracts have shown apoptosis-promoting potential against diverse cancer cell lines such as HeLa, prostate adenocarcinoma, basophilic leukemia, and human breast (Elumalai *et al*., 2022). Azarshinfam *et al.* (2021) reported that Iranian propolis induced apoptosis in HT-29 cell line of colorectal cancer (CRC) by increasing Bax pro-apoptotic gene expression and decreasing Bcl-2 anti-apoptotic gene expression. In the study by Motomura *et al.* (2008), propolis was found to inhibit the proliferation of human leukemic U937 cells in a dose-dependent manner by inducing apoptosis and blocking cell cycle progression in the G2/M phase. Western blot analysis showed that propolis increased the expression of p21 and p27 proteins, and decreased the levels of cyclin B1, cyclin A, Cdk2 and Cdc2, causing cell cycle arrest. The results suggested that propolis-induced apoptosis was related to the selective activation of caspase-3 and induction of Bcl-2/Bax regulation. Kamiya *et al.* (2012) reported that Brazilian red propolis significantly reduced the viability of MCF-7 breast cancer cells through the induction of mitochondrial dysfunction, caspase-3 activity, and DNA fragmentation. Besides, propolis was found to promote MCF-7 cell apoptosis via endoplasmic reticulum stress.

II.4.6. Other activities

In addition to the above-mentioned activities, propolis has been reported to exhibit many other activities. In a cell based-model, Brazilian propolis was found to exert anti-inflammatory and anti-allergic activities (Conte *et al.,* 2022). Xool-Tamayo *et al.* (2020) reported that Mayan propolis demonstrated anti-inflammatory effect through reducing the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and increasing the anti-inflammatory cytokines (IL-10 and IL-4). In the study by Shukla *et al.* (2005), propolis was proved to exert hepatoprotective effect against carbon tetrachloride (CCl4) induced liver injury in rats. Propolis was also reported to possess wound healing property. The investigation carried out by Debbab *et al.* (2019) demonstrated the

ability of Algerian propolis to increase the wound healing rate and reduce the healing time. Besides, propolis from Bulgaria, Bangladesh and Nigeria demonstrated analgesic effect (Paulino *et al*., 2003; Tanvir *et al*., 2018; Ipav *et al*., 2022).

Part 2: Experimental

Chapter I: Materials and Methods

I.1. Propolis collection

Seven propolis samples, produced by honey bee *Apis mellifera*, were collected by beekeepers from apiaries located in different geographical regions of Algeria. The sites of sampling, geographic information of collection sites as well as the period of collection are presented in the Table 2. The samples were collected by scraping frames, walls and the entrance of the beehive. After removing impurities such as parts of plants and insects, crude propolis samples were kept in freezer and then the frozen propolis samples were powdered.

Table 2. Sampling sites, geographic information period of collection

Site of collection	Geographic information of collection site	Collection period	Propolis
Collo (Skikda)	37° 00′ 23″ North, 6° 33′ 39″ East Altitude 20 m Humid area	August 2014	
El Harrouch (Skikda)	36° 39′ 11″ North, 6° 50′ 11″ East Altitude 132 m Humid area	November 2014	
Bouteldja (El-Taref)	36° 30′ 10″ North, 8° 06′ 17″ East Altitude: 35 mm Humid area	September 2018	
Grarem (Mila)	36° 31′ 00″ North, 6° 20′ 00″ East Altitude: Min. 135 m Max. 1310 m Subhumid area	April 2015	
Menia (Constantine)	36°21′ North, 6°36′ East Altitude: 694 m Subhumid area	April 2015	
(Mestaoua & Chelala mountains) Batna	35°33′ North, 6°10′ East Altitude: 1048 m Semi- arid area	July 2018	

Oum el Bouaghi (Oum El Bouaghi)	35° 52′ 39″ North, 7° 06′ 49″ East Altitude : 891 m Semi- arid area	April 2013 September 2018	

I.2. Extraction of bioactive compounds

I.2.1. Extraction of phenolic compounds

The methanolic extract (ME) was obtained by the methodology described by Park & Ikegaki (1998) with slight modification. Air-dried powdered material (20 g) of propolis was extracted three times with 200 mL hydroalcoholic solution (80% Methanol, 20% Distillated water) for 72 h. After filtration, the filtrate was evaporated by rotary evaporator (under 50°C temperature) to obtain dry extract and stored under dry conditions at 4°C until analyzed.

I.2.2. Extraction of Essential oils

The extraction of essential oils was carried out in the same year of propolis samples collection. Briefly, 100 g of propolis (Propolis from Grarem, Oum El Bouaghi and Batna) were subjected to hydrodistillation using a Clevenger type apparatus for 3 h. The obtained oils were dried over anhydrous sodium sulphate and stored at 4°C.

I.3. Determination of total bioactive content of propolis methanolic extracts

1.3.1. Total phenolic content (TPC)

Total phenols were assayed by Folin-Ciocalteu method according to Singleton & Rossi (1965). Folin Cio-calteau reagent is formed from a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}). The blue color produced has a maximum absorption in the region of 765 nm and is proportional to the total quantity of phenolic compounds originally present (Anju *et al*., 2019).

Briefly, a 200 µL of diluted extract (0.5 mg/mL) was added to 1 mL of Folin–Ciocalteu reagent. After incubation in the dark for 4 min, 800 µL of 7.5% Na_2CO_3 was added. After incubation in the dark for 2 h, absorbance at 765 nm was read versus a prepared blank. The total phenol content of propolis extracts was expressed as micrograms of Gallic acid equivalents per milligram of extract (µg GAE/mg E) from a calibration curve with Gallic acid.

I.3.2. Total flavonoid content (TFC)

Total flavonoid content (TFC) was determined using aluminium chloride ($AlCl_3$) assay (Djeridane *et al*., 2006). In this method, $AlCl_3$ forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Besides, $AlCl_3$ forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. The reaction between $AlCl_3$ and flavonoids results in a yellow color, which can be quantifed using a spectrophotometer at an absorbance range of 410–437 nm. (Załuski *et al*., 2017; Nonglang *et al*., 2022). Briefly, 1 mL of extract solution (0.5 mg/mL) was added to 1 mL of 2% $AlCl_3$. After incubation in the dark for 10 min, the absorbance of the reaction mixture was measured at 430 nm with a UV/VIS spectrophotometer immediately. Quercetin was used as the standard for the calibration curve. Flavonoid content was expressed as μg of Quercetin equivalent (QE)/mg of extract.

I.4. GC-MS analysis of propolis essential oils (EOs)

The gas chromatography-mass spectrometry (GC-MS) measurements were performed on the following systems at conditions and parameters listed in the Table 3. EOPG (EO of propolis from Grarem) was chemically characterized using a Varian Saturn 2100 Ion Trap machine, whereas, EOPO (EO of propolis from Oum El Bouaghi) and EOPB (EO of propolis from Batna) were analyzed by using a Thermo Scientific TRACE 1310 / ISQ LT.

The volatile compounds were identified by comparing their retention time (RT), retention index (RI) or mass spectra with those of databases (Main library, TRLIB Library, Wiley 9 and NIST). The constituents were expressed as percentages from peak area normalization, assuming that the total injection was 100% of essential oil. The RI was calculated from retention times relative to that of *n*-alkane series.

Table 3. Measurement conditions and parameters of GC/MS

GC/MS	Varian Saturn 2100 Ion Trap MS	TRACE 1310/ ISQ LT
Column	DB-1 MS (30 m x 0.25 mm I.D., film thickness 0.25 μm).	TG-WAXMS (60 m × 0.25 mm I.D., film thickness 0.25 μm)
Carrier gas	Helium	Helium
Stationary phase	Dimethylpolysiloxane	Acid optimized Polyethylene Glycol (PEG)
Auto sampler	-	TriPlus RSH
Injection volume	0.2 μL	1 μL
Inlet	Injector temperature 250°C, Splitless mode (split ratio of 1:30), Flow mode (Flow rate 1.4 mL/min)	Injector temperature 230°C, Splitless mode (split ratio of 1:12), Flow mode (Flow rate 1.2 mL/min)
Oven	60°C (5 min), 4°C/min to 240°C (10 min)	60 to 230^{0}C at 4^{0}C/min
Detector	Quadrupole ion -trap MS, Electronic impact (EI) mode (70 eV), Transfer line temperature 250°C, Scan mode (scan range 28 to 650 m/z)	Quadrupole MS, Electronic impact (EI) mode (70 eV), Transfer line temperature 250°C, Scan mode (scan range 50–500 m/z)

I.5. Biological activities

I.5.1. Antioxidant activities

I.5.1.1. Scavenging ability

I.5.1.1.1. DPPH radical scavenging assay

The DPPH (2,2'-diphenyl-1-picrylhydrazyl) radical scavenging assay was conducted using the method of Blois (1958). It is based on reduction of the violet DPPH radical by the antioxidant via a hydrogen atom transfer mechanism to cause a change in the color to stable pale yellow DPPH molecules. The remaining violet DPPH radical is measured by a UV-Vis spectrophotometer at

approximately 515 – 520 nm to determine the antioxidant activity (Sirivibulkovit *et al*., 2018).

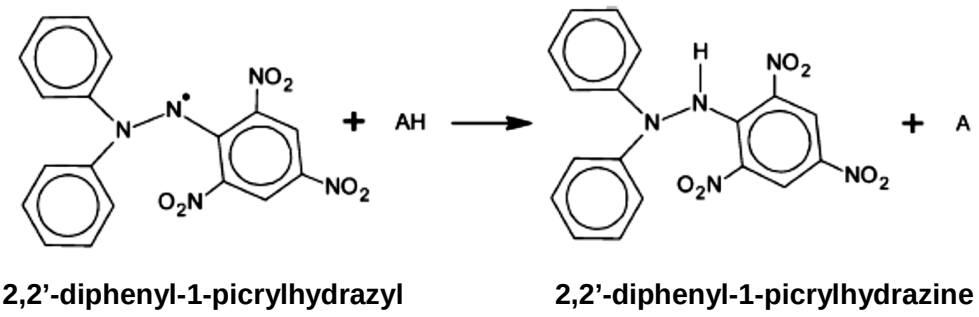


Figure 2. DPPH free radical's chemical structure and its reaction with a scavenger indicated by AH (Pyrzynska *et al*., 2013)

Briefly, 40 μL of sample solution was mixed with 160 μL of DPPH solution. The reaction mixture was incubated for 30 min at 25°C, and the absorbance was measured at 517 nm. The radical scavenging activity was calculated using formula as follows:

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

The results are expressed as IC_{50} value (μg/mL).

I.5.1.1.2. $ABTS^{\bullet+}$ cation radical scavenging assay

The $ABTS^{\bullet+}$ scavenging activity was done by the method of Re *et al.* (1999) with slight modifications. $ABTS^{\bullet+}$ is created by oxidation of ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) with potassium persulfate, resulting in a green–blue ABTS chromophore that was formed by losing an electron by the nitrogen atom of ABTS. In the presence of hydrogen-donating antioxidants, the nitrogen atom quenched the hydrogen atom, yielding the solution decolorization. This change in absorbance intensity can then be quantifed at an absorbance of 734 nm (Xiao *et al*., 2020; Nonglang *et al*., 2022).

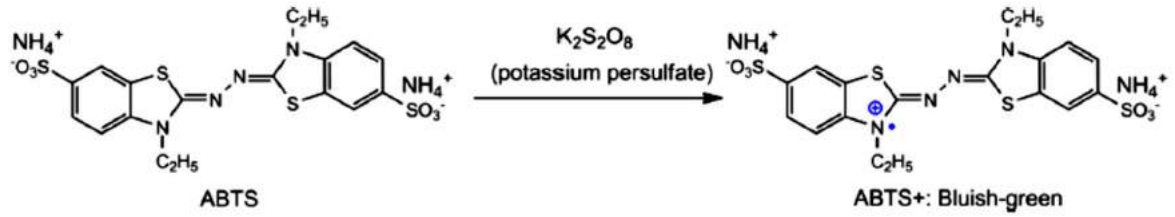


Figure 3. Formation of stable ABTS radical from ABTS with potassium persulfate (Xiao *et al.*, 2020)

Briefly, the $ABTS^{\bullet+}$ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days with storage in the dark at room temperature. Before usage, the $ABTS^{\bullet+}$ solution was diluted with ethanol to get an absorbance of 0.70±0.02 at 734 nm. Then, 160 µL of $ABTS^{\bullet+}$ solution were added to 40 µL of sample solution in methanol at different concentrations. After 10 min, the percentage inhibition at 734 nm was calculated. The scavenging capability of $ABTS^{\bullet+}$ was calculated using the following equation and the results were given as IC_{50} value.

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

I.5.1.1.3. Superoxide radical scavenging activity

The scavenging activity of extracts towards the superoxide radical ($O_2^{\bullet-}$) was measured in terms of inhibition of generation of $O_2^{\bullet-}$. The method was performed by using alkaline DMSO method described by Kunchandy & Rao (1990) with slight modification adapted at microplate-reader. Superoxide radical ($O2^{\bullet-}$) is generated by the addition of sodium hydroxide (NaOH) to air saturated DMSO. The generated superoxide remains stable in solution and reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature, which can be measured at 560 nm (Bendjabeur *et al*., 2018). Briefly, to the reaction mixture containing 40 µL of extract (or standard compound) at various concentrations and 130 µL of alkaline DMSO (100 mL DMSO containing,

20 mg NaOH in 1 mL distillated water), 30 µL NBT (1 mg/mL solution in distillated water) was added and absorbance was noted at 560 nm against blank samples. The decrease in the absorbance of reaction sample indicated the increase of superoxide anion scavenging activity. The percent inhibition of superoxide anion generation was calculated using the following formula:

$$I\% = \frac{Ac - As}{Ac} x100$$

I%: inhibition percentage, Ac: absorbance in the presence of the control. As: Absorbance in the presence of the sample.

The results are expressed as IC_{50} value (µg/mL).

I.5.1.2. Reducing ability

I.5.1.2.1. Cupric ion reducing antioxidant capacity (CUPRAC) assay

CUPRAC was determined according to the method developed by Apak *et al.* (2004). In this method, the CUPRAC reagent, bis(neocuproine)copper(II) chloride (Cu(II)-Nc), reacts with n-electron reductant antioxidants (AO) in the following manner:

$$\text{n Cu(Nc)}_2^{2+} + \text{n electron reductant (AO)} \leftrightarrow \text{n Cu(Nc)}_2^{+} + \text{n electron oxidized product} + \text{n H}^+$$

In this reaction, the reactive Ar–OH groups of polyphenols and other antioxidants are essentially oxidized to the corresponding quinones (Ar–O), and the light-blue colored Cu(II)-Nc is reduced to the orange-yellow colored $Cu(Nc)_2^+$ (Figure 4). The protons liberated in the reaction are neutralized by ammonium acetate aqueous buffer. It should be noted that the real oxidant is the $Cu(Nc)_2^{2+}$ species and not Cu^{2+} alone, since the standard redox potential of the Couple (II/I)-Nc is 0.6 V, much higher than that of the non-complexed couple Cu^{2+}/Cu^+ (0.17 V). The main antioxidants in foodstuffs and biological compounds have a redox potential corresponding to the range of 0.2–0.6 V, according to that of the redox couple Cu(II/I)-Nc (Ozyurek *et al*., 2011; Munteanu & Apetrei, 2021).

The method comprises mixing of 40 µL of sample solution with 60 µL of ammonium acetate aqueous buffer (pH 7), 50 µL of neocuproine alcoholic solution and 50 µL of a copper(II) chloride solution. After 60 min, the absorbance was read at 450 nm. The results were given as $A_{0.50}$, which corresponds to the concentration producing 0.50 absorbance.

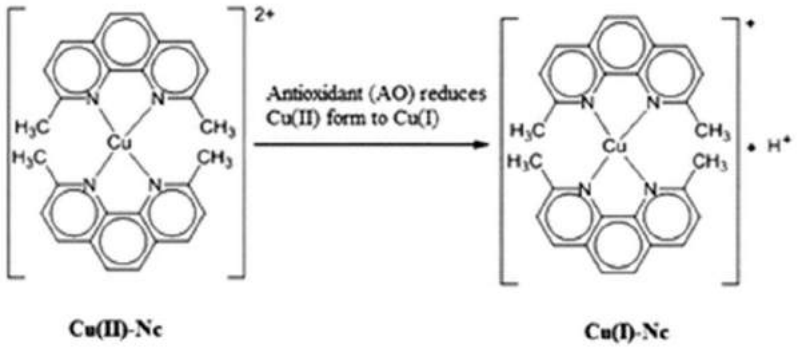


Figure 4. The CUPRAC reaction and chromophore: Bis(neocuproine) copper (I) chelate cation (Ozyurek *et al*., 2011)

I.5.1.2.2. Ferric reducing ability assay

The ferric reducing power was determined by the method of Oyaizu (1986) with slight modifications. Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each compound (Irshad *et al*., 2012; Bhalodia *et al*., 2013).

Briefly, sample solution (10 µL) were mixed with 40 µl sodium phosphate buffer (pH 6.6) and 50 µL of 1% potassium ferricyanide. The mixture was intensively shaken, then incubated at 50°C for 20 min. Thereafter, 50 µL of 10% trichloroacetic acid (w/v) was added and the resulted mixture was mixed with 40 µL distilled water and 10 µL of 0.1% ferric chloride. The absorbance was spectrophotometrically measured at 700 nm. Ascorbic acid was used as a positive reference compound. The results were given as $A_{0.50}$, which corresponds to the concentration producing 0.50 absorbance.

I.5.1.3. Lipid-peroxidation inhibitory activity

The lipid peroxidation inhibitory was determined by the β-carotene-linoleic acid test system (Marco, 1968). In this model, β-carotene undergoes rapid discoloration in the absence of an antioxidant because of the coupled oxidation of β-carotene and linoleic acid, which generates free

radicals. The linoleic acid free radical (formed upon the withdrawal of a hydrogen atom from one of its diallylic methylene groups) attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidised and partly broken down; subsequently the system loses its chromophore (Amensour *et al*., 2009). The addition of an antioxidant inhibit lipid peroxidation and thus delays β-carotene bleaching.

Briefly, β-carotene (0.5 mg) in 1 mL of chloroform and 25 µL of linoleic acid were dissolved in 200 µL of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 50 mL of distilled water saturated with oxygen, were added by vigorous shaking. The assay mixture, containing 160 µL β-carotene emulsion and 40 µL methanolic extract or EO, was incubated at 45°C. After 120 min, the decrease in the absorbance of β-carotene was measured at 470. The antioxidant activity was expressed as percent inhibition relative to the control using the following equation:

$$I\% = \left[1 - \frac{As0 - Ast}{Ac0 - Act}\right] x100$$

Where **I%** is the inhibition percentage, $\mathbf{A_{S0}}$ is the initial absorbance at time 0 in the presence of the sample, $\mathbf{A_{St}}$ is the absorbance at time 120 min in the presence of the sample, $\mathbf{A_{C0}}$ is the initial absorbance at time 0 in the presence of the control and $\mathbf{A_{Ct}}$ is the absorbance at time 120 min in the presence of the control.

The results are expressed as IC_{50} value (µg/mL).

I.5.2. Enzyme inhibitory properties

I.5.2.1. Cholinesterase inhibitory assay

The inhibition activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured by spectrophotometric method developed by Elman *et al.* (1961) with slight modification (Ozturk *et al*., 2011). AChE from electric eel and BChE from horse serum were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. 5,5'-Dithiobis(2-nitrobenzoic) (DTNB) acid was used for the measurement of the activity.

Briefly, in the 96-well plates, 150 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of sample solution dissolved in methanol at various concentrations, 20 µL of AChE (5.32 x 10^{-3} U) or BChE (6.85 x 10^{-3} U) solution were added to the wells. The mixture was shaken, then

incubated at at 25 °C for 15 min. Thereafter, 10 µL of DTNB (0.5 mM) was added to each well. The reaction was then initiated by the addition of 10 µL of acetylthiocholine iodide (0.71 mM) or butyrylthiocholine chloride (0.2 mM). The hydrolyses of these substrates were monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion, as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, respectively. The absorbance of the colored end-product was measured at 412 nm at 0 min and 15 min. Galantamine was used as a positive reference compound. The percentage inhibition was calculated using the following formula and the results were given as IC_{50} value (µg/mL) (Ozturk *et al*., 2011).

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

I.5.2.2. α-Glucosidase inhibitory assay

α-glucosidase inhibitory activity was conducted according to Lordan *et al*. (2013) with some modifications. In this assay, α -glucosidase catalyze the conversion of the substrate *p*-nitrophenyl-α-D-glucopyranoside (PNPG) to α-D-glucopyranoside and *p*-nitrophenol (PNP), as shown in the equation below. The yellow color of PNP is measured spectrophotometrically at 405 nm (Eertmans *et al*., 2014).

[PNPG + α-glucosidase] → α-D-glucopyranoside + PNP (yellow)]

Briefly, a volume of 50 µL of sample solution and 50 µL of 5mM PNPG solution prepared in phosphate buffer (pH 6.9) was mixed and incubated at 37°C for 10 min. Then, 100 µL of *α*-glucosidase solution (0.1 U/mL) prepared in phosphate buffer (pH 6.9) was added. The absorbance was mesured at 405 nm for 30 min at 10-min intervals. The *α*-glucosidase inhibitory activity was then calculated using the following equation and the results were given as IC_{50} value.

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

I.5.3. Antimicrobial activity

I.5.3.1. Test microorganisms

In vitro antimicrobial activity of propolis extracts and EOs was tested against eight human pathogens including three Gram-positive bacteria (*Bacillus cereus* RSKK 863, *Bacillus subtilis* RSKK 244 and *Staphylococcus aureus* ATCC 25923), four Gram-negative bacteria (*Escherichia coli* ATCC 11229, *Escherichia coli* O157:H7, *Salmonella enteritidis* ATCC 13076 and *Pseudomonas aeruginosa* ATCC 27853) and one yeast (*Candida albicans* ATCC 10231). Bacterial strains were cultured overnight at 37ºC in nutrient broth while yeast was cultured for 48 h at 30ºC in YPD (Yeast Peptone Dextrose) broth medium.

I.2.3.2. Preparation of propolis solutions

10 mg of propolis methanolic extracts and EOs were dissolved in 1 mL of Dimethyl sulphoxide (DMSO) to obtain a final concentration of 10 mg/mL. Then, the obtained solutions were sterilized by 0.45 µm Millipore filter and diluted in the growth medium to a desired concentration.

I.5.3.3. Disc diffusion assay

The disc diffusion method was used to determine the antimicrobial potential of the investigated extracts (Murray *et al*., 1995). The culture suspensions were adjusted by comparing with 0.5 McFarland. Then, a volume of 100 µL of suspension was spread on agar plates. Thereafter, sterile 6-mm-diameter filter discs (Whatman paper n°3) were placed on the inoculated plates and impregnated with 15 µL (150 µg/disc) of propolis extracts and EOs solutions. The treated petri dishes were kept at 4°C for 1 hour to enable prediffusion of the extracts and EOs into the agar. Finally, the inoculated plates were incubated at 37°C for 24 h for bacterial strains and 30°C for 48 h for yeast. Ampicillin (AM, 10 µg/disc), Kanamycin (K, 30 µg/disc) and Erythromycin (E, 15 µg/disc) were chosen as standard antibacterial while Fluconazole (FCA, 25 µg/disc) was chosen as standard antifungal. The results were obtained by measuring the diameter of growth inhibition zone surrounding the discs and expressed in mm.

I.5.3.4. Microdilution assay

The two-fold microdilution method was used to determine the minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal (MFC) concentrations according to the

protocol described by Koneman *et al.* (1997) with slight modification. The propolis extracts were added to each growth medium to obtain a final concentration of 4 µg/µL and diluted to 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.031 µg/µL in tubes, while the EOs were added to each growth medium to obtain a final concentration of 8 µg/µl and diluted to 4, 2, 1, 0.5, and 0.25 µg/µL in tubes. The total volume was 100 µL in each tube. 1.25 µL of each tested bacteria or yeast (adjusted to 0.5 McFarland) were inoculated into each tube. The content of the tubes was mixed and they were incubated at appropriate temperatures for 24 h and 48 h. The MIC value was defined as the lowest concentration of the extract and EO, which inhibited bacterial or fungal growth. MBC and MFC were determined by sub-culturing 5 µL of the test dilutions from each clear tube on solid growth medium and incubating for 24 h and 48 h at appropriate temperature. The lowest concentration that did not show bacterial growth was defined as the MBC value whereas the MFC value was determined as the lowest concentration with no fungal growth. The results are expressed as µg/µL.

I.5.4. Toxic effect

The toxicity of the extracts was evaluated *in vivo* using brine shrimp lethality assay according to Meyer *et al.* (1982) with slight modification. Briefly, 4 mg of each extracts was dissolved in 0.05% DMSO (50 µl DMSO, 950 µl of seawater) and diluted with seawater. Then, 20 μL of each extract dilution was incubated, for 24 h under lighting, with 180 µL of seawater containing 10 brine shrimp larvae (*Artemia salina* nauplii). Others (ten larvae) were placed in a mixture of 180 µL seawater and 20 μL of DMSO (0.05%) to serve as negative control, while potassium dichromate was used as the positive control. After 24 h, *Artemia salina* larvae were examined against a lighted background and the average number of survived larvae was counted. The percentage of mortality was calculated using the following equation:

$$Mortality\ \% = \left[\frac{Control - Survived}{Control}\right] x100$$

The results were given as LC_{50} value (μg/mL) corresponding to the concentration that led 50% lethality of the larvae.

I.5.5. Anticancer activity

I.5.5.1. Cell culture

The human hepatocellular carcinoma (HepG2) cell line was maintained in Dulbecco's modified

Eagle's medium (DMEM) supplemented with L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin at 37 C° in a humidified incubator with 5%CO_2. Cells were checked under Zeiss PrimoVert inverted microscope, and subculturing was performed when cells reached 80% confluency.

I.5.5.2. Cytotoxicity assay

The cytotoxicity of the extracts on HepG2 cells was determined by using Cell Counting Kit-8 (CCK-8) assay according to the manufacturer instructions. In the CCK-8 measurement, the dye of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4disulfophenyl)-2H-tetrazolium, monosodium salt] was reduced by dehydrogenase in cells to form a water-soluble orange-colored product (formazan). The amount of the produced formazan dye by cellular dehydrogenases is correlated with the number of living cells. Therefore, the cell viability can be simply estimated by recording the optical density (OD) of formazan at 450 nm (Cai *et al*., 2019).

Briefly, the cells were counted using a trypan blue solution. Next, 100 µL of cell suspension (1×10^4 cells per well) was plated into 96-well plate and incubated at 37°C in a CO_2 incubator (5%) for 24 h. Then, cells were treated with serial concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) of each extract (100 µL) and incubated for 72 h. Thereafter, cells were washed and 100 µL of fresh medium was added. Then, 10 µL of CCK-8 solution was added to each well and incubated at 37°C for 3 h. Absorbance at 450 nm was determined using a microplate reader. The cytotoxic activity was measured using the following equation and the results were given as IC_{50} value.

$$Cytotoxicity\ \% = 100\% - [\frac{Abs\ treated\ cells}{Abs\ untreated\ cells} x100]$$

I.5.5.3. Cell morphology analysis

The morphological changes in HepG2 cells exposed to increasing concentrations (3.125-200 µg/mL) of propolis extracts were investigated using inverted phase microscope (PrimoVert, Zeiss) at 40 X magnification and compared with control cells.

I.6. LC–MS/MS analysis of the potent extracts

The phenolic component of the extracts that showed good anticancer activity was analyzed by using an LC (Agilent 1260 Infinity) system coupled to a triple quadrupole mass spectrometer (Agilent 6420 Triple Quadrupole LC-MS) (Tepe & Doyuk, 2020). The chromatographic separation of the phenolic compounds was carried out on a C18 reversed-phase ODS column (25 x 4.6 mm x 5 μm). The injection volume of the standards and the samples was 2 μL. The mobile phase consisted of water/0.1% formic acid (eluent A), methyl alcohol (eluent B) at a flow rate of 0.4 mL/min. The elution conditions were as follows: : 2% B for 3 min, 25% B for 6 min, 50% B for 10 min, 95% B for 14 min, 2% B for 17.5 min. MS analysis was performed in both positive and negative ionization modes. The multiple reaction monitoring (MRM) mode was used to quantify the analytes. The LC-MS/MS data were collected and processed by Mass Hunter softwere (version B.07.01). The phenolic compounds of samples were identified by comparing their retention time, UV profile and mass spectra with those of authentic standards. All the phenolics detected were quantified using the calibration curves of corresponding standard solutions and the results were expressed as nanogram per milligram of dried propolis extract. The analytical parameters of LC-MS/MS method are presented in the Table 4.

Table 4. Analytical parameters of the LC-MS/MS method

Analyte	MRM Transition	RT (min)	Ionisation mode	LOD (µg/L)	LOQ (µg/L)	Calibration equation	R2	Linear range (µg/L)
Phenolic acids								
Gallic acid	168.9 -> 125.0	8.808	Negative	1,14	3,81	y = 8.888589 x + 138.496429	0.9977	25-1000
Protocatechuic acid	152.9 -> 108.9	10.59	Negative	0,34	1,12	y = 8.578239 x + 152.118840	0.9966	25-1000
3,4-Dihydroxyphenylacetic acid	167.0 -> 123.0	10.905	Negative	0,60	2,00	y = 6.667736 x + 72.831362	0.9972	25-1000
Chlorogenic acid	355.0 -> 163.0	11.786	Positive	0,64	2,14	y = 9.981107 x + 157.265757	0.9957	25-1000
3-Hydroxybenzoic acid	137.0 -> 93.0	12.854	Negative	1,99	6,64	y = 4.742547 x + 55.891367	0.9985	25-1000
4-Hydroxybenzoic acid	136.9 -> 93.1	12.114	Negative	0,95	3,16	y = 8.944440 x + 168.413400	0.9977	25-1000
2,5-Dihydroxybenzoic acid	152.9 -> 109.0	11.988	Negative	1,18	3,93	y = 6.906765 x + 198.953187	0.9961	25-1000
homovanillic acid	181.0 -> 137.1	12.642	Negative	14,82	49,40	y = 0.503363 x + 4.997721	0.9943	25-1000
Caffeic acid	179.0 -> 135.0	12.651	Negative	0,32	1,07	y = 21.967016 x + 617.835457	0.9949	25-1000
Syringic acid	196.9 -> 181.9	12.782	Negative	45,27	150,92	y = 0.556853 x + 3.179725	0.9933	25-1000
Verbascoside	623.0 -> 160.8	13.468	Negative	1,28	4,27	y = 9.449269 x + 200.698172	0.9949	25-1000
p-Coumaric acid	162.9 -> 119.0	13.802	Negative	0,37	1,23	y = 22.159790 x + 509.428301	0.9962	25-1000
Sinapic acid	222.9 -> 207.9	13.874	Negative	9,97	33,24	y = 2.546042 x + 43.401753	0.9976	25-1000
Ferulic acid	193.0 -> 134.0	13.934	Negative	2,49	8,31	y = 4.412759 x + 62.244173	0.9974	25-1000
Rosmarinic acid	359.0 -> 160.9	14.508	Negative	0,93	3,08	y = 10.368229 x + 115.743273	0.9984	25-1000
2-Hydroxycinnamic acid	162.9 -> 119.1	14.846	Negative	1,03	3,44	y = 16.730856 x + 268.444413	0.9973	25-1000
Flavonoids								
(+)-Catechin	289.0 -> 245.0	11.37	Negative	10,90	36,33	y = 2.683307 x + 55.906922	0.9962	25-1000
(-)-Epicatechin	291.0 -> 139.1	12.379	Positive	1,35	4,49	y = 3.146153 x + 92.391081	0.9983	25-1000
Taxifolin (dihydroquercetin)	303.0 -> 285.1	13.713	Negative	0,38	1,28	y = 31.203828 x + 1388.467333	0.9916	25-1000
Luteolin 7-glucoside	447.1 -> 285.0	14.273	Negative	0,52	1,75	y = 105.789564 x + 2363.901129	0.9970	25-1000
Hesperidin	611.1 -> 303.0	14.303	Positive	3,66	12,19	y = 4.754827 x + 76.915683	0.9963	25-1000
Hyperoside (quercetin-3-O-galactoside)	465.1 -> 303.1	14.489	Positive	0,39	1,30	y = 12.261241 x + 221.919701	0.9974	25-1000
Apigenin 7-glucoside	433.1 -> 271.0	14.74	Positive	0,59	1,95	y = 19.624698 x + 435.972923	0.9977	25-1000
Eriodictyol	287.0 -> 151.0	15.072	Negative	0,03	0,11	y = 25.220677 x + 671.022786	0.9959	25-1000
Quercetin	301.0 -> 151.0	15.571	Negative	1,57	5,23	y = 19.882317 x + 536.764350	0.9958	25-1000
Luteolin	287.0 -> 153.1	15.81	Positive	0,95	3,16	y = 10.540740 x + 439.171991	0.9901	25-1000
Kaempferol	285.0 -> 229.1	16.106	Negative	3,98	13,28	y = 4.314895 x + 88.708655	0.9933	25-1000
Apigenin	271.0 -> 153.0	16.245	Positive	0,64	2,13	y = 11.012899 x + 371.736509	0.9966	25-1000
Lignans								
Pinoresinol	357.0 -> 151.0	14.944	Negative	13,28	44,26	y = 0.323604 x - 2.751408	0.9916	25-1000
Other polyphenols						y = 15.066712 x + 260.210571	0.9975	
3-hydroxytyrosol	153.0 -> 123.0	10.268	Negative	0,43	1,45			25-1000
Pyrocatechol	109.0 -> 52.9	10.891	Negative	13,53	45,11	y = 0.321084 x + 2.262137	0.9954	25-1000
Vanillin	151.0 -> 136.0	13.071	Negative	5,57	18,55	y = 2.516488 x + 93.808330	0.9950	25-1000
Oleuropein	539.2 -> 275.1	14.607	Negative	0,23	0,78	y = 9.144321 x + 134.849555	0.9969	25-1000

I.7. Statistical analysis

Except LC-MS/MS, the results were illustrated as means ± standard deviation of three measurements. The IC_{50} and $A_{0.50}$ values were calculated by linear regression analysis. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism software (version 6.0.1). Results were considered statistically significant at $p<0.05$.

Chapter II: Results and Discussion

II.1. Extraction yield

The present study showed that the yield of extractions was influenced by the geographic origin of propolis samples (Table 5). The yield of propolis methanolic extracts varied between 9.50 – 39.00%, in which the highest yield was obtained by MEPM. However, the yield of propolis essential oils ranged from 0.09 to 0.61%, in which the best yield was observed with EOPO.

Table 5. Extraction yield

Sample	(ME) Extraction yield (%)	(EO) Extraction yield (%)
MEPC	20.50	/
MEPH	36.00	/
MEPT	33.30	/
MEPG	9.50	/
MEPM	39.00	/
MEPO	38.00	/
MEPB	34.10	/
EOPG	/	0.09
EOPO	/	0.61
EOPB	/	0.27

Abbreviations: ME: Methanolic extract. EO: Essential oil. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna. EOPG: Essential oil of propolis from Grarem. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from Batna.

Similarly, previous studies have reported variable ranges of extraction yield of propolis obtained from different areas. Kouadri *et al.* (2021) reported yields ranged from 14% – 37% of ethanolic extracts of propolis from Tipaza, Tébessa, El-Oued and Constantine regions. Rebiai *et al.* (2014) indicated yields of 7.26% and 24.13% of methanolic extracts of propolis from Ghardaïa and Khenchela. Belfar *et al.* (2015) studied propolis from Boumerdes, Mostaganem Bejaia and Ghardaia and found yields within the range 15.57 % – 41.10%. Moreover, Segueni *et al.* (2010) found yields of 0.03% and 0.11% of propolis essential oils from Mila and Jijel. Ayari *et al.* (2020) reported yields from 0.095% to 0.324% of Tunisian propolis essential oil. Hence, the results of the current study and the literature indicate clearly the influence of the geographical origin of propolis on the extraction yield.

II.2. Total bioactive content of propolis methanolic extracts

The TPC was measured using the Folin–Ciocalteu assay and the results were derived from a calibration curve (y = 0.0063x +0.0562, R^2 = 0.9897) of gallic acid (0–200 µg/mL) (Figure 5) and expressed as micrograms of Gallic acid equivalents per milligram of extract (µg GAE/mg E).

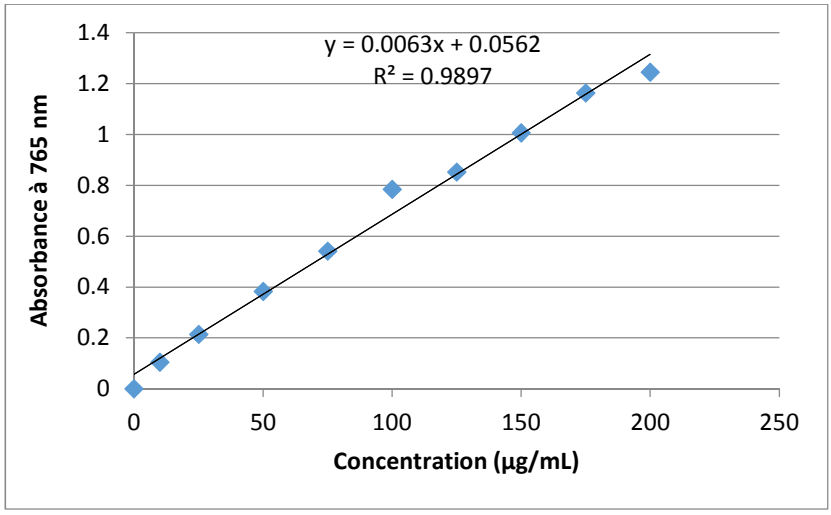


Figure 5. Standard curve of Gallic acid

The TFC was determined using aluminium chloride and the results were derived from the calibration curve (y = 0.0307x + 0.0702, R^2 = 0.982) of quercetin (0–30 µg/mL) (Figure 6) and expressed as µg of Quercetin equivalent (QE)/mg of extract.

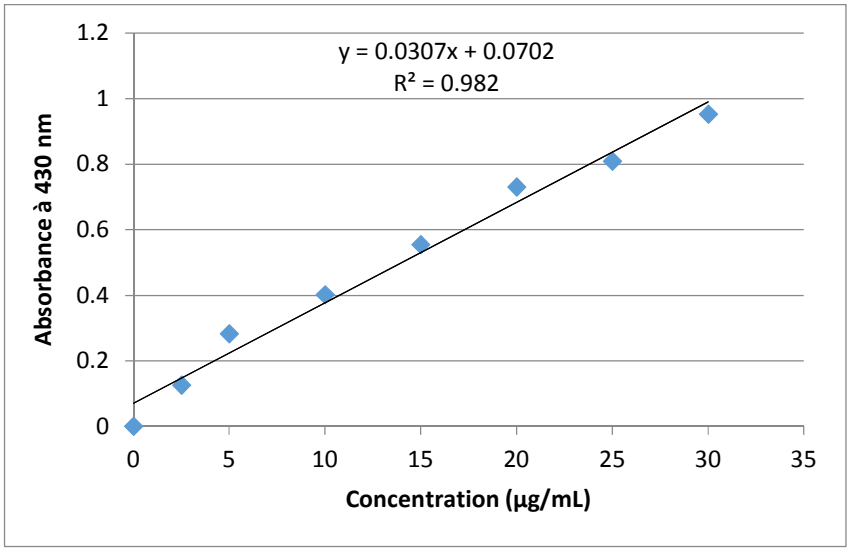


Figure 6. Standard curve of Quercetin

As inferred in Table 6, the quantitative estimation of total bioactive content showed significant difference ($p<0.05$) between the extracts with regard to TPC and TFC, except MEPC, MEPH and MEPT that showed no significant difference ($p>0.05$) between them regarding TFC. The phenolic levels of propolis extracts ranged from 32.85±3.26 to 561.99±3.50 µg GAE/mg E while the flavonoid levels were within the range 1.91±0.08 –76.98±0.26 µg QE/mg E. The highest amounts of TPC and TFC were recorded with MEPM, whereas, the lowest amounts were found in MEPB.

Table 6. TPC and TFC of propolis methanolic extracts

Sample	TPC (µg GAE/mg E)	TFC (µg QE/mg E)
MEPC	504.21±2.23^{c}	46.66±0.98^{d}
MEPH	524.95±2.54^{b}	47.31±2.54^{d}
MEPT	201.61±3.50^{f}	44.37±1.90^{d}
MEPG	279.72±2.07^{d}	60.43±0.65^{b}
MEPM	561.99±3.50^{a}	76.98±0.26^{a}
MEPO	270.62±1.91^{e}	54.35±0.20^{c}
MEPB	32.85±3.26^{g}	1.91±0.08^{e}

Note: Data are expressed as Mean±SD of three parallel measurements ($p<0.05$). The values with different superscripts (a, b, c, d, e, f or g) in the same columns are significantly different ($p < 0.05$).
Abbreviations: TPC: Total phenolic content is expressed as µg Gallic acid equivalent/mg of extract. TFC: Total flavonoid content is expressed as µg Quercetin equivalent/ mg of extract. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna.

Previous studies have described a variety of ranges for total phenolic and flavonoid content of propolis from different geographical origins. Nedji & Loucif-Ayad (2014) who studied propolis from other localities in Algeria found phenolic levels ranged between 100.90 and 257.40 mg GAE/g E. Béji-Srairi *et al.* (2020) reported phenolic amounts from 35 to 93.16 mg GAE/g of Tunisian propolis. Ozkok *et al*. (2021) reported phenolic content from 34.53 to 259.4 mg GAE/g of Turkish propolis. Jobir & Belay (2020) reported phenolic levels from 63.09±3.55 – 82.07±3.72 mg GAE/g of Ethiopian propolis. In regards to the flavonoid content, Algerian propolis was reported to contain flavonoids at levels of 58.99 – 91.44 µg QE/mg E (Nedji & Loucif-Ayad, 2014). Turkish propolis was reported to contain amounts ranged from 21.28 to 152.56 mg CE/g

while the Ethiopian propolis contained flavonoid levels from 17.26±0.35 – 24.42±0.53 mg QE/g E (Ozkok *et al*., 2021; Jobir & Belay, 2020).

Compared to these results, certain Algerian propolis samples possessed considerable total polyphenol and flavonoid contents. However, the observed difference in phenolic and flavonoid contents between propolis of the current study and the literature can be ascribed to many factors including the preferred regional plants visited by honeybees, geographical location, altitudes, seasons, processing methods and extraction solvents (Sorucu & Oruç, 2019; Bayram *et al*., 2019).

II.3. GC-MS Analysis of propolis essential oils

The GC-MS analysis of three volatile oils of propolis from Grarem (EOPG), Oum El Bouaghi (EOPO) and Batna (EOPB) allowed the identification of a total of 112 compounds: seventy-eight compounds for EOPG (93.52% of the total oil), thirty-three for EOPO (99.7% of the total oil) and twenty-five for EOPB (99.8% of the oil). The constituents of the oils are given in Table 7.

Table 7. Volatile components identified in EOPG, EOPO and EOPB

N°	Compounds	RI[a]	RI[b]	EOPG (%)	EOPO (%)	EOPB (%)
1	Dimethylvinylcarbinol		621	-	5.3	-
2	2-Buten-1-ol, 3-methyl		773	-	1.4	-
3	Santolina triene	908		0.33	-	-
4	α-Thujene	930		0.27	-	-
5	*α*-Pinene	939	942	**9.50**	3.5	**56.1**
6	2- *α*-Pinene		953	-	-	1.0
7	Camphene	954		1.70	-	-
8	Thuja-2,4(10)-diene	960		0.32	-	-
9	Sabinene	975		0.94	-	-
10	*β*-Pinene	979		**4.02**	-	-
11	α-Phellandrene	1002		0.94	-	-
12	α-Terpinene	1017		0.62	-	-
13	*Delta*-3-Carene		1021	-	-	1.8
14	Limonene	1029	1032	2.78	1.4	1.0
15	*o* -Cymene	1026	1051	0.55	-	0.9
16	γ-Terpinene	1059		1.51	-	-
17	Cymenene <meta->	1085		0.17	-	-
18	Terpinolene	1088		0.43	-	-
19	Linalool		1095	-	1.4	-
20	Heptenol acetate <(3Z)->	1099		0.64	-	-
21	*α*-Campholenal	1126		0.80	-	-
22	Verbenone		1128	-	-	2.2
23	Camphor		1132	-	-	0.9
24	*p*-Mentha-1,5-dien-8-ol	1181	1136	**23.69**	-	1.3
25	*trans*-Pinocarveol	1139	1142	0.66	2.0	2.7
26	*trans*-Verbenol	1144		0.66	-	-
27	Verbenol		1151	-	1.1	1.8
28	α-Fenchyl alcohol		1153	-	-	1.1

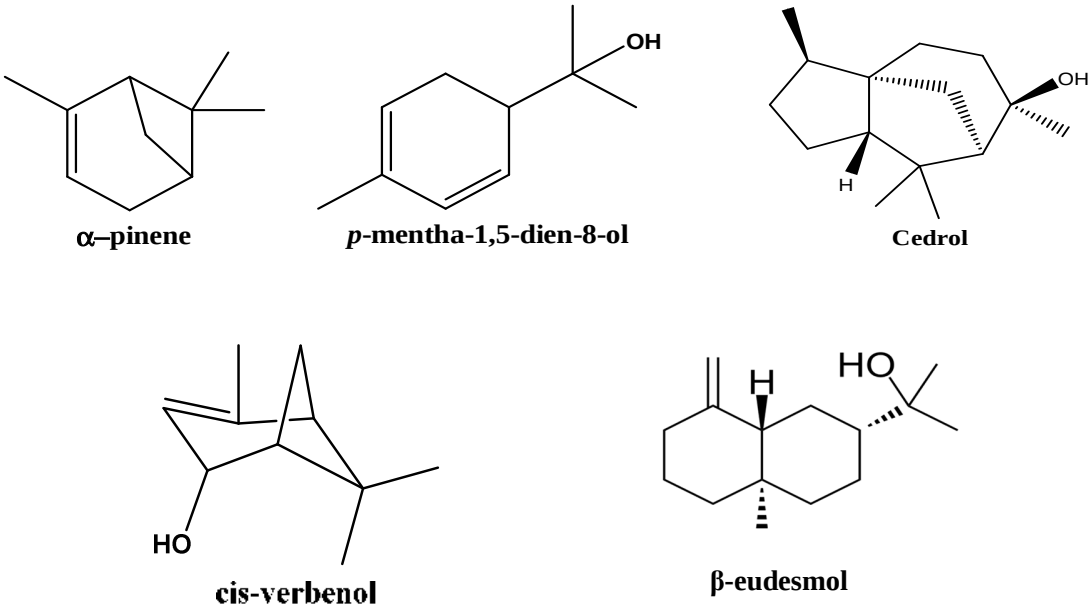
29	Pinocarvone	1164		0.62	-	-
30	Menthol <iso->	1182		1.22	-	-
31	Thuj-3-en-10-al	1184		0.66	-	-
32	Borneol		1187	-	1.7	-
33	*α*-Terpineol	1188		0.27	-	-
34	*cis*-Verbenol		1196	-	2.7	**6.0**
35	Myrtenol	1195	1198	0.25	-	1.0
36	Safranal	1196		0.30	-	-
37	Dihydro carvone <*trans*->	1200		1.45	-	-
38	1-Carveol		1211	-	-	1.0
39	*cis*-Carveol	1229		0.19	-	-
40	Ascaridole	1237		0.12	-	-
41	Carvone	1243		0.12	-	-
42	Phenylethyl acetate <2->	1256		0.01	-	-
43	Thujanol acetat <*iso*-3->	1270		0.23	-	-
44	*α*-Copaene	1376	1226	0.64	1.1	-
45	Bornyl acetate	1288	1281	**9.13**	1.2	-
46	*α*-Terpinenyl Acetate	1349	1336	2.69	1.4	0.8
47	*α*-Cubebene	1352		0.65	-	-
48	Isoledene	1374		0.45	-	-
49	*β*-Bourbonene	1385		0.67	-	-
50	*β*-Cubebene	1390		0.16	-	-
51	Sibirene	1399		0.19	-	-
52	*α*-Gurjunene	1409		0.54	-	-
53	*β*-Copaene	1432		0.55	-	-
54	Aromadendrene	1441	1391	0.35	2.2	-
55	*α*-Himachalene	1451		0.60	-	-
56	Alloaromadendrene	1460		0.45	-	-
57	Cadina-1(6),4-diene<*cis*->	1463		0.37	-	-
58	*γ*-Muurolene	1479		0.22	-	-
59	Germacrene D	1481		1.52	-	-
60	*α*-Amorphene	1484		1.03	-	-
61	*β*-Selinene	1490		2.04	-	-
62	Muurola-4(14),diene <*trans*->	1493		0.22	-	-
63	*γ*-Amorphene	1495		0,69	-	-
64	*δ*-Amorphene	1510		0.15	-	-
65	*β*-Eudesmene		1439	0.22	2.1	-
66	*γ*-Cadinene	1513	1448	0.69	1.6	-
67	Benzene,1-(1-formylethyl)-4-(1-buten-3-yl)-		1456	-	-	0.7
68	*α*-Guaiene		1461	-	1.8	-
69	Cyclohexene, 3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl		1476	-	1.4	**4.4**
70	Eremophilene		1479	-	2.3	-
71	*γ* -Gurjunene		1482	-	4.9	-
72	*trans*-Caryophyllene		1498	-	4.9	-
73	*α*-Muurolene	1496	1502	0.55	0.8	-
74	*α*-Campholene aldehyde		1506		-	3.0
75	Calamenene	1520		0.25		
76	Cyclohexanemethanol,4-ethenyl-*α*,*α*,4-trimethyl-3-(1-methylethenyl)-,[1R-(1α,3α,4α)]-		1532	-	1.9	-
77	*cis*-calamenene		1542	-	3.4	-
78	*α*-Calacorene	1545		0.21	-	-
79	*β*-Calacorene	1565		0.54	-	-
80	Spathulenol	1578		**3.47**	-	-
81	Caryophylene oxide	1582		0.89	-	-
82	Cedrol	1599	1548	0.39	**17.0**	-
83	*δ*-Cadinene	1523	1559	2.02	4.3	0.7
84	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	1609		0.39	-	-
85	Cubenol<1,10-di-epi->	1622		0.23	-	-
86	*γ*-Eudesmol	1632		0.19	-	-

87	Hexenyl phenyl acetate <(3Z)->	1634		0.10	-	-
88	*τ*-Cadinol	1640		0.09	-	-
89	Cubenol	1646	1586	0.08	1.3	-
90	*β*-Eudesmol	1649	1597	0.51	**7.7**	-
91	Guaiol		1621		3.8	-
92	Eudesmol <7-epi-α->	1663		0.95	-	-
93	Eudesma-4(15)7,dien-1β-ol	1688		1.12	-	-
94	Valencene		1715		1.4	-
95	*α-D*-Mannofuranoside, farnesyl-		1876		-	0.9
96	*n*-Nonadecane	1900		0.12	-	-
97	Manoyl oxide	1987		0.06	-	-
98	Abietatriene	2056		0.03	-	-
99	Heneicosane	2100		0.22	-	-
100	*τ*-Muurolol		2181	-	1.5	-
101	Bulnesol		2210	-	2.5	-
102	*α*-Eudesmol		2241	-	**6.7**	-
103	Totarol	2314		0.14	-	-
104	Octadecoxyethanol	2328		0.48	-	-
105	*n*-Tetracosane	2400		0.19	-	-
106	Glycerol 1-palmitate		2486		-	1.7
107	*n*-Pentacosane	2500		0.33	-	-
108	6,9,12,15-Docosatetraenoic acid,methylester		2510		-	1.1
109	Octaethylene glycol monododecyl ether		2631		-	1.5
110	Finasteride		2692		-	2.7
111	15,15'-*Bi*-1,4,7,10,13-pentaoxacyclohexadecane		3633		-	3.5
112	1-Heptatriacotanol		3949		1.9	-
	Total identified compounds (%)			**93.52**	**99.7**	**99.8**
	Monoterpenic hydrocarbons			23.80	4.9	59.9
	Oxygenated monoterpenes			43.07	12.9	21.7
	Sesquiterpenic hydrocarbons			15.75	30.9	0.7
	Oxygenated sesquiterpenes			8.58	42.4	-
	Ditertpenic hydrocarbons			0.03	-	-
	Hydrocarbures			0.86	-	-
	Others			1.23	8.6	17.5

Abbreviations: RI[a]: Retention Index of the volatile compounds analyzed by Varian Saturn 2100 Ion Trap machine; RI[b]: Retention Index of volatile compounds analysed by using Thermo Scientific TRACE 1310 / ISQ LT. EOPG: Essential oil of propolis from Grarem. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from batna.

Five compounds which are *α*-pinene, limonene, *trans*-pinocarveol, *α*-terpinenyl acetate and *δ*-Cadinene were common between the three oils but with different amounts. However, fifteen components were common between EOPG and EOPO, eight components were common between the EOPG and EOPB and eight others between the EOPO and EOPB. The major constituents found in EOPG were *p*-mentha-1,5-dien-8-ol (23.69%), *α*-pinene (9.50%), bornyl acetate (9.13%) and *β*-pinene (4.02%). The main constituents of EOPO were cedrol (17.0%), *β*-eudesmol (7.7%) and *α*-eudesmol (6.7%), whereas *α*-pinene (56.1%), *cis*-verbenol (6.0%) and cyclohexene,3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl (4.4%) were mainly detected in EOPB.

Monoterpenic hydrocarbons and oxygenated monoterpenes were the main constituents of EOPG and EOPB, whereas oxygenated sesquiterpenes and sesquiterpenic hydrocarbons were more abundant in EOPO. Ditertpenic hydrocarbons and hydrocarbures were found in trace amounts and were detected only in EOPG.



α–pinene ***p*-mentha-1,5-dien-8-ol** **Cedrol**

cis-verbenol **β-eudesmol**

Figure 7. Some of the main compounds identified in EOPG, EOPO and EOPB

The chemical profile of propolis volatiles has been little studied, especially Algerian propolis. Up to now, only one research carried out by Segueni *et al.* (2010) exists on Algerian propolis volatiles, in which the authors studied the EOs of propolis from El-Malha (Mila city, a sub-humid region), Benibelaîd and Kaous regions (Jijel, a humid region). The authors found that the main constituents of EO of propolis from El-Malha were 2-hexenal, myristic acid, linoleic acid and spathulenol, whereas isooctane, linoleic acid, undecane, myristic acid, hexadecane, *p*-cymene, palmitic acid and 4-terpineol were predominant in propolis from Benibelaîd. 2-hexenal, myristic acid, linoleic acid, carvacrol, α-cedrol and *p*-cymene were more abundant in EO of Kaous (Segueni *et al.*, 2010).

Compared to EOs of propolis from other regions of the world, there were some differences. Ayari *et al.* (2020) reported that Tunisian propolis essential oils were mainly dominated by

sesquiterpenes and diterpenes hydrocarbons. The major components were α-cedrol, manoyl oxide, manool, totarol, tricosane, and eicosane. El-Guendouz *et al.* (2018) reported that Moroccan propolis oils were predominantly sesquiterpene rich. Melliou *et al.* (2007) stated the predominance of terpenoids, especially of α-pinene in Greek propolis. Bankova *et al.* (2014) indicated that most EOs from European propolis were predominated by sesquiterpenes, followed by aromatic compounds, such as benzyl acetate, benzyl benzoate and benzyl alcohol. β-eudesmol was found to be the major constituent of propolis volatile oils from France, Hungary, Bulgaria and Northern Italy (Bankova *et al.* 2014).

It is interesting to note that the differences in the chemical composition, between the oils of the current study and those from the literature, depend on multiple factors such as the type of vegetation, climatic conditions and geographical location, among others (Kamatou *et al*., 2019).

II.4. Biological activities

II.4.1. Antioxidant activities

The antioxidant activity of the extracts and the EOs was evaluated *in vitro* using different methods. DPPH, ABTS and superoxide anion radical assays were used to assess the radical scavenging ability. CUPRAC and ferric reducing assays were used to assess the ability of the extracts to reduce copper and ferric ions, respectively. β-carotene/linoleic acid assay was used to evaluate the lipid peroxidation inhibition potential.

II.4.1.1. Scavenging ability

Regarding the scavenging activity, all the tested extracts showed good ability in this respect and inhibited DPPH, ABTS and $O_2^{\bullet-}$ radicals in a concentration-dependent manner (Figure 8). All the tested extracts had more ability to scavenge ABTS and $O_2^{\bullet-}$ than DPPH radicals. This could be explained by the fact that the capacity of extracts to react and quench different radicals is affected by various factors such as stereo-selectivity of the radicals, solubility of the extract in different testing systems, polarity of the solvent and functional groups present in the bioactive compounds (Mukherjee *et al*., 2011). The extracts were also found to be more effective in scavenging superoxide anion radical than the standard antioxidants, which could be related to their contents of phenolic compounds that have been recognized as powerful antioxidant agents, mainly due to their hydroxyl groups (Ozturk *et al*., 2007). Phenolic

compounds can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Manojlovic *et al*., 2012).

Among the extracts, MEPH had the highest capacity to trap DPPH with IC_{50} value (22.24±0.43 µg/mL) which was similar ($p>0.05$) to that of BHT (22.32±1.19 µg/mL), whereas MEPM exerted the most potent scavenging activity for ABTS and $O_2^{\bullet-}$ radicals with IC_{50} values of 5.81±0.48 µg/mL and 5.62±0.07 µg/mL, respectively (Table 8).

Table 8. Antioxidant activities of propolis methanolic extracts and essential oils

Sample	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	$O_2^{\bullet-}$ scavenging IC_{50} (µg/mL)	CUPRAC $A_{0.50}$ (µg/mL)	Ferric reducing $A_{0.50}$ (µg/mL)	Lipid peroxidation Inhibition IC_{50} (µg/mL)
MEPC	41.33±0.61^{d}	8.73±0.32^{e}	14.86±0.15^{d}	18.25±2.34^{d}	47.32±0.36^{e}	30.59±0.01^{e}
MEPH	22.24±0.43^{b}	7.60±0.32^{e}	9.25±0.25^{c}	11.83±0.12^{c}	69.53±2.93^{f}	12.06±0.01^{c}
MEPT	43.45±1.29^{d}	24.11±0.61^{g}	ND	43.82±1.45^{e}	13.63±0.30^{b}	ND
MEPG	72.08±0.43^{e}	10.08±0.11^{f}	17.54±0.52^{e}	47.76±0.03^{f}	97.50±3.54^{g}	47.76±0.03^{f}
MEPM	29.06±0.20^{c}	5.81±0.48^{c}	5.62±0.07^{a}	18.01±2.15^{d}	31.46±1.08^{c}	16.31±0.28^{d}
MEPO	42.02±1.15^{d}	6.99±0.16^{d}	6.19±0.24^{b}	15.98±1.10^{d}	40.14±0.42^{d}	17.58±1.98^{d}
MEPB	>200	90.92±1.46^{h}	ND	>200	150.08±4.98^{h}	ND
EOPG	ND	516.05 ±11.66^{i}	ND	ND	ND	198.01± 6.71^{g}
EOPO	>800	505.28±19.02^{i}	ND	351.52±14.71^{g}	>800	ND
EOPB	>800	>800	ND	651.11±15.40^{h}	>800	ND
BHT	22.32±1.19^{b}	1.29±0.30^{a}	85.30±2.08^{f}	9.62±0.87^{b}	>50	1.05±0.01^{b}
BHA	5.73±0.41^{a}	1.81±0.10^{b}	86.33± 3.53^{f}	3.64±0.19^{a}	8.41±0.67^{a}	0.90±0.02^{a}

Note: Data are presented as IC_{50} mean±SD (n=3) and $A_{0.50}$ mean±SD (n=3).The values with different superscripts (a, b, c, d, e, f, g, h or i) in the same columns are significantly different ($p < 0.05$).
Abbreviations: BHT: butylatedhydroxytoluene. BHA: Butylated hydroxyanisole. ND: Not determined. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna. EOPG: Essential oil of propolis from Grarem. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from Batna.

The strong antiradical potential of MEPH and MEPM could be linked to their TPC and TFC. Interestingly, in this study, the Pearson's correlation analysis indicated a strong positive relationship of TPC with DPPH (r = 0.7241), ABTS (r = 0.7328) and $O_2^{\bullet-}$ (r = 0.8340) scavenging activities. There was also a strong positive correlation between TFC and DPPH (r =

0.9004) and ABTS (r = 0.8964) scavenging effects. In contrast, weak positive correlation was found between TFC and $O_2^{\bullet-}$ (r = 0.1213) scavenging activity. This could be due to the fact that flavonoids are often bound to the sugar moieties forming glycosides, which were reported to be weaker scavengers on a weight basis than their aglycones or phenolic acids due to the inclusion of non-participating structures like sugars (Muzolf-Panek & Stuper-Szablewska, 2021).

The EOs also inhibited DPPH and ABTS radicals in a concentration-dependent manner, however, they were found to be less active than the extracts. Although many reports have stated the strong antioxidant properties of propolis essential oils (Naik & Vaidya, 2011; Ayari *et al*., 2020; Chi *et al*., 2020), EOs in this study did not show strong antiradical effects and this can be explained by their major constituents, the ones that have already had weak activity reported by the literature, such as cedrol, α-pinene and β-pinene (Emami *et al.*, 2011a). However, the overall antioxidant activity of EOs is usually the result of interaction between all components (Emami *et al*., 2011b).

Our results were coherent with those of literature that indicated different ranges of DPPH, ABTS and $O_2^{\bullet-}$ radicals scavenging effects. In DPPH scavenging activity, Piccinelli *et al.* (2013) indicated IC_{50} values ranged from 32.3±1.9 to 600.0 ± 15.6 µg/mL of Algerian propolis. Bouaroura *et al.* (2021) have also studied the antioxidant activity of Algerian propolis and found IC_{50} values within the range 10.03±0.26 – 17.00±0.24 µg/mL. Béji-Srairi *et al.* (2020) reported IC_{50} values ranged from 20.1 to 43 µg/mL of Tunisian propolis. Mercan *et al.* (2006) found IC_{50} values within the range 34.03 – 46.00 µg/mL of Turkish propolis. As for ABTS radicals scavenging activity, Bouaroura *et al.* (2021) reported IC_{50} values from 5.38 ± 0.35 to 95.07± 3.68 µg/mL of Algerian propolis. Béji-Srairi *et al.* (2020) reported values ranged from 244 – 616 µg/mL of Tunisian propolis. Regarding superoxide radical scavenging effect, Miguel *et al.* (2014) reported IC_{50} values ranged from 0.001±0.003 to 0.053±0.003 mg/mL of Portuguese propolis, while Ichikawa *et al.* (2002) indicated an IC_{50} value of 6.2 mg/mL of Brazilian propolis.

The noticeable differences in the results between propolis samples could be explained by the difference in the chemical composition, especially the phenolic compounds that are a major determinant of the antioxidant potential of propolis (Kurek-Górecka *et al*., 2022).

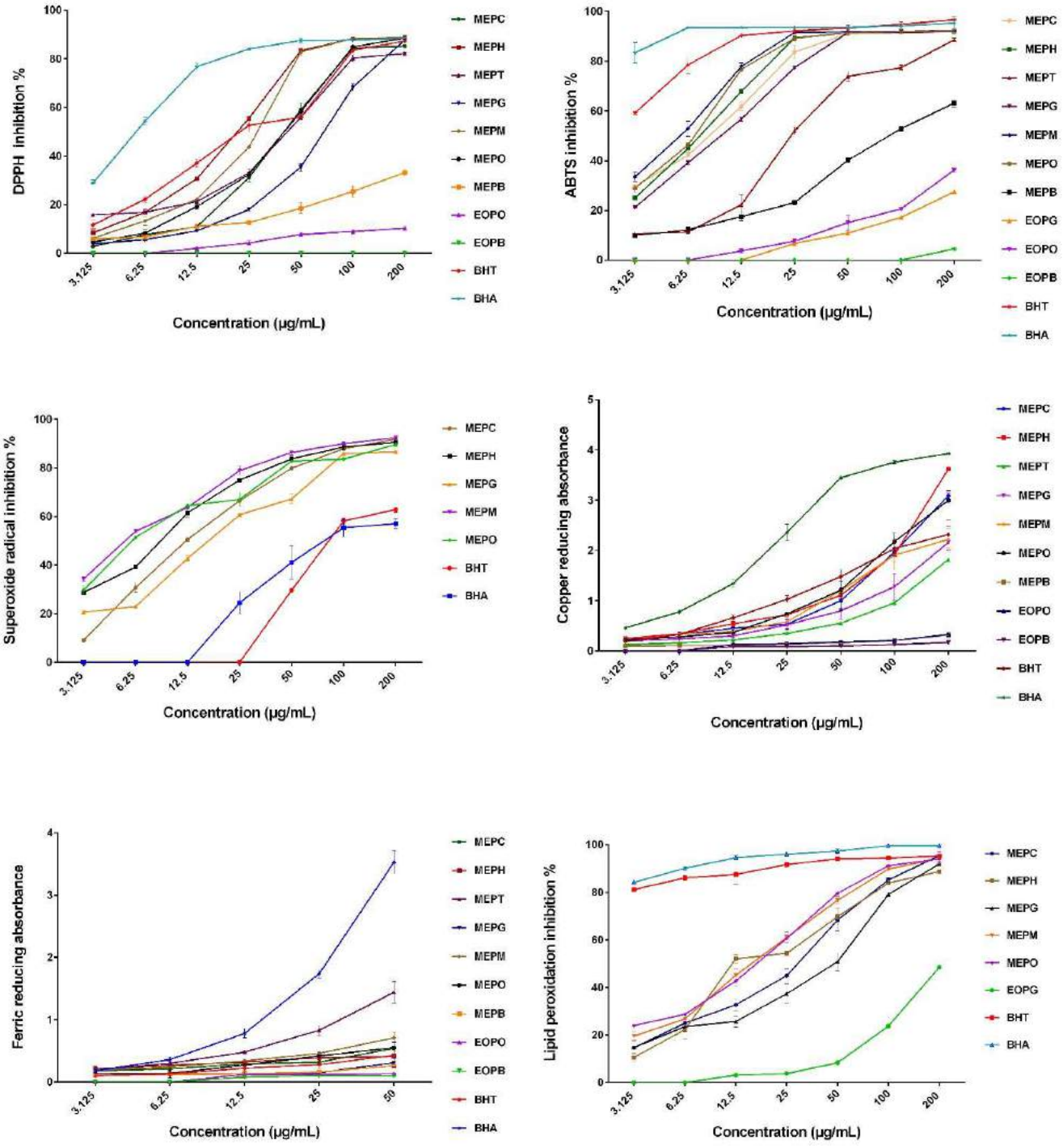


Figure 8. Concentration-dependent antioxidant activities of propolis methanolic extracts, essential oils and the standards

II.4.1.2. Reducing ability

The tested extracts and EOs possessed also reducing capacity and were found to be able to reduce copper and ferric ions in a concentration-dependent manner (Figure 8). The reducing power increased with increasing amount of the extracts. Most extracts and EOs showed higher efficiency in reducing copper ions than ferric ions. The highest copper ($A_{0.50}$= 11.83±0.12 µg/mL) and ferric ions ($A_{0.50}$= 13.63±0.30 µg/mL) reducing abilities were exhibited by MEPH and MEPT, respectively (Table 8). However, they were significantly ($p<0.05$) less active than the positive control BHA. The EOs exerted weaker reducing activities than the extracts. This suggested that phenolic compounds contributed significantly to the antioxidant abilities of the extracts (Muzolf-Panek & Stuper-Szablewska, 2021). Importantly, the copper ion reducing capacity was strongly correlated to TPC (r = 0.7750) and TFC (r = 0.6126). However, a weak correlation was found in case of ferric ion reducing activity with both TPC (r = -0.1907) and TFC (r = 0.08391). This could be explained by the fact that extracts can contain phenolic and non-phenolic compounds that can influence their antioxidant potential (Arro-Díaz *et al*., 2021).

Compared to the existing studies in the literature, our findings are comparable to the range of copper ions reducing (5.59±0.11– 93.92± 1.80 µg/mL) and ferric ions reducing (24.74±1.71 – 155.49±2.04 µg/mL) activities observed for Algerian propolis (Bouaroura *et al.*, 2021).

II.4.1.3. Lipid-peroxidation inhibitory activity

In regards to β-carotene assay, the tested extracts showed higher lipid peroxidation inhibition potential than the EOs and inhibited the bleaching of β-carotene in a concentration-dependent manner (Figure 8). Based on the IC_{50} values (Table 8), the samples can be classified in their effectiveness against β-carotene bleaching as follows: BHA > BHT > MEPH > MEPM > MEPO > MEPC > MEPG > EOPG.

Previous studies proved the lipid peroxidation inhibition potential of propolis from various geographic origins and reported variable effects (Kumazawa *et al*., 2004; Isla *et al*., 2009). In the current study, the extracts showed also variable effects, indicating the influence of geographic origin on the antioxidant activity of propolis. The IC_{50} values varied from 12.06±0.01– 47.76±0.03 µg/mL. Such results are closer to those reported by Bouaroura *et al.* (2021) who investigated the lipid peroxidation inhibition capacity of some Algerian propolis

methanolic extracts and indicated IC_{50} values ranged from 11.34±0.17 to 40.38± 0.39 µg/mL. However, our findings are better than those of Tunisian propolis (IC_{50}, 1300 – 2000 µg/mL) reported by Béji-Srairi *et al.* (2020).

In this study, a weak correlation was found between lipid peroxidation inhibition potential and TPC (r = -0.04886) and TFC (r = 0.2658). Such results are in agreement with that reported by Hatami *et al.* (2014). This may be explained by the fact that the β-carotene bleaching technique employs an emulsified system, so the activity depends on the substrate polarity. Apolar antioxidants can exhibit stronger antioxidative properties in emulsions because they concentrate at the lipid phase, whereas polar antioxidants remain in the aqueous phase and are thus less effective in th lipid protecting. (Nickavar & Esbati, 2012).

II.4.2. Enzyme inhibitory properties

II.4.2.1. Cholinesterase inhibitory activity

The anticholinesterase activity of propolis methanolic extracts (MEPC, MEPH, MEPT, MEPG, MEPM, MEPO and MEPB) and essential oils (EOPG and EOPB) was evaluated by using a combination of two complementary methods: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity assays. Overall, all propolis methanolic extracts, essential oils and galantamine (the standard drug) inhibited AChE and BChE activities in a concentration dependent manner (Figures 9 and 10) and most samples were more selective inhibitors of BChE than AChE enzyme. The same trend has been observed in a previous study on Algerian propolis (Bouaroura *et al.*, 2020), which can be explained by the fact that BChE enzyme can accept a wide range of substrates over AChE, because of its low substrate specificity (Orhan *et al.*, 2007).

The maximum AChE inhibition was observed at the final assay concentration of 200 µg/mL and the decreasing order of AChE inhibition percent was: galantamine (94.77 ± 0.34%) > MEPM (82.98 ± 2.10%) > MEPT (72.16±3.65%) > MEPO (62.84±1.80%) > EOPB (68.05 ± 3.68%) > MEPG (66.56 ± 0.10%) > MEPH (53.09 ± 0.74%) > EOPG (43.96 ± 1.28%) > MEPB (23.23 ± 2.77%) > MEPC (22.86 ± 2.85%). Based on IC_{50} value (Table 9), MEPT was the best inhibitor of AChE among the extracts with IC_{50} of 59.09±4.88 µg/mL while EOPB (IC_{50} =96.54 ± 1.79 µg/mL) was the most active essential oil against AChE. The potent AChE inhibitory activity of MEPT could be linked to its flavonoid content. Indeed, the Pearson's correlation

coefficient between the AChE inhibitory activity and TPC and TFC were 0.2421 and 0.7552, respectively, indicating flavonoid type substances as the main responsible for this activity (Arro-Díaz *et al*., 2021).

However, the BChE inhibition percent order was: MEPM (99.48± 2.71%) > MEPC (82.95± 0.42%) > galantamine (78.95± 0.58%) > MEPG (77.07± 0.89%) > MEPO (76.82± 3.19%) > MEPT (76.64± 0.47%) > EOPG (72.36 ± 0.82%) >MEPB (66.44± 1.86%) > MEPH (58.19± 2.97%) > EOPB (43.07± 1.26%). MEPM, followed by MEPG and MEPO were found to be the best BChE inhbitors with IC_{50} values better than that of galantamine (Table 9). This high activity of MEPM, MEPG and MEPO could be linked to their high content of flavonoids. Importantly, the BChE inhibitory activity was found to be strongly correlated with TFC (r = 0.6401) than TPC (r = 0.3786).

Table 9. Cholinesterase inhibitory activity of propolis methanolic extracts and essential oils

Extract	AChE inhibitory assay IC_{50} µg/mL	BChE inhibitory assay IC_{50} µg/mL
MEPC	>200	35.70 ± 1.06^{c}
MEPH	180.80 ± 3.56^{g}	44.04 ± 2.52^{d}
MEPT	59.09 ± 4.88^{b}	43.69 ± 1.35^{d}
MEPG	124.50 ± 2.46^{e}	20.30 ± 0.52^{b}
MEPM	71.29 ± 2.73^{c}	16.06 ± 0.85^{a}
MEPO	155.48 ± 1.67^{f}	33.57 ± 0.68^{c}
MEPB	>200	114.78 ± 2.07^{e}
EOPG	> 200	115.70 ± 6.59^{e}
EOPO	ND	ND
EOPB	96.54 ± 1.79^{d}	339.87 ± 13.38^{f}
Galantamine^{RC}	6.27 ± 1.15^{a}	34.75 ± 1.99^{c}

Note: Data are presented as IC_{50} mean±SD (n=3). The values with different superscripts (a, b, c, d, e, f or g) in the same columns are significantly different ($p < 0.05$).
Abbreviations: RC: reference compound. ND: Not determined. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna. EOPG: Essential oil of propolis from Grarem. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from Batna

In the current study, the samples exhibited variable AChE and BChE inhibitory activities, which is consistent with previous works. El-Guendouz *et al.* (2016) reported that Moroccan propolis samples exhibited antiacetylcholinesterase effect with IC_{50} values ranged from 0.002±0.051–3.555±0.051 mg/mL. Baltas *et al.* (2016) indicated that ethanolic extracts from Turkish propolis exerted acetylcholinesterase inhibitory ability with values ranged from 0.081 to 1.353 mg/mL. Abd El-Hady *et al.* (2016) found that Sudanese propolis possessed variable inhibitory activities against AChE with values ranged between 25.5– 91.7%. This variability in the cholinesterase inhibitory activities among propolis samples could be due to the difference in propolis composition, which is extremely variable and depended on the geographical and botanical origin (Chaillou *et al.*, 2009; Segueni *et al*., 2017).

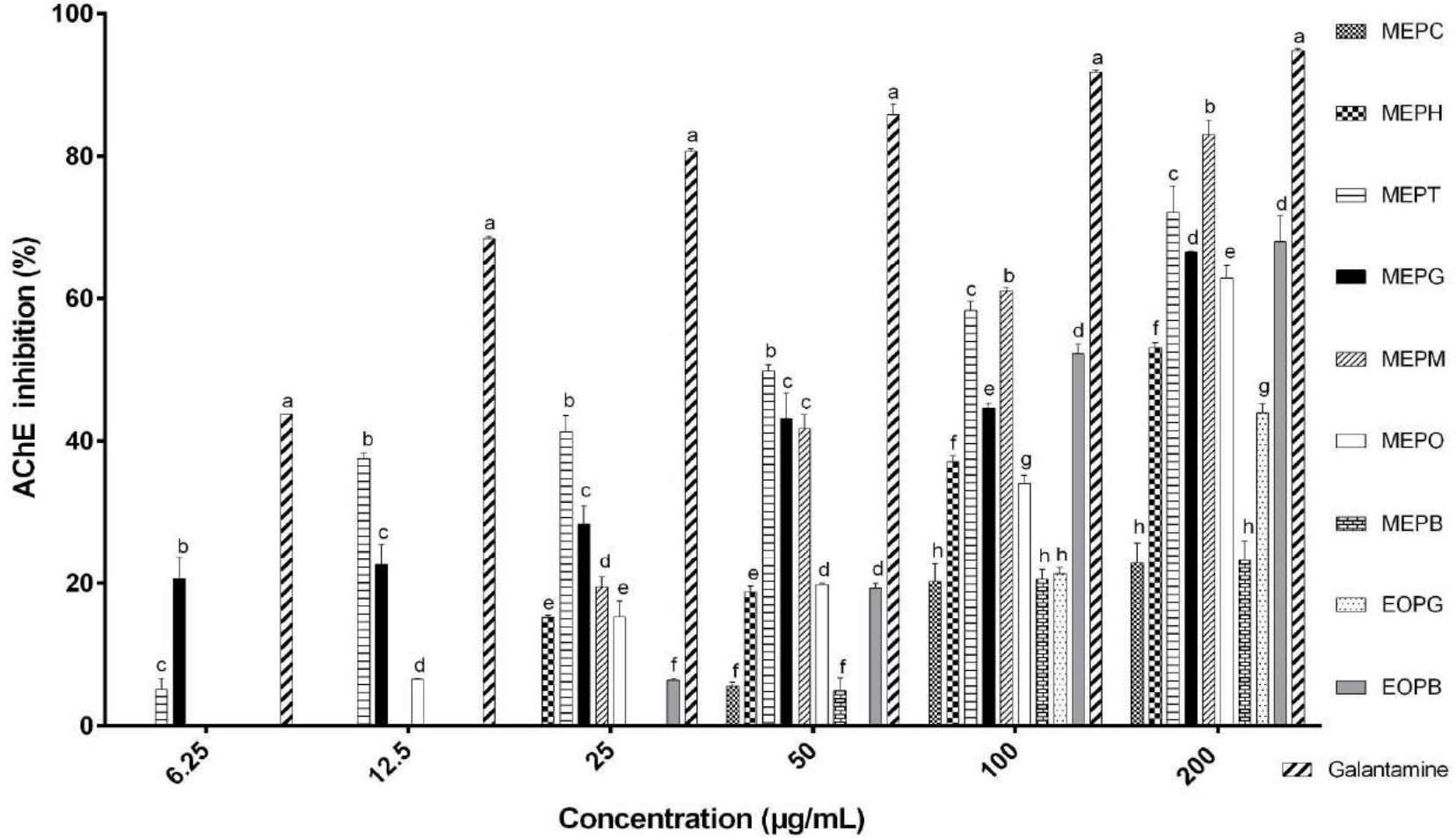


Figure 9. Dose-dependent inhibition of acetylcholinesterase by propolis methanolic extracts, essential oils and galantamine. Data are expressed as inhibition (%) mean ± SD (n= 3). Columns with different letters indicate statistically significant differences ($p<0.05$). Vertical bars represent the standard deviation.

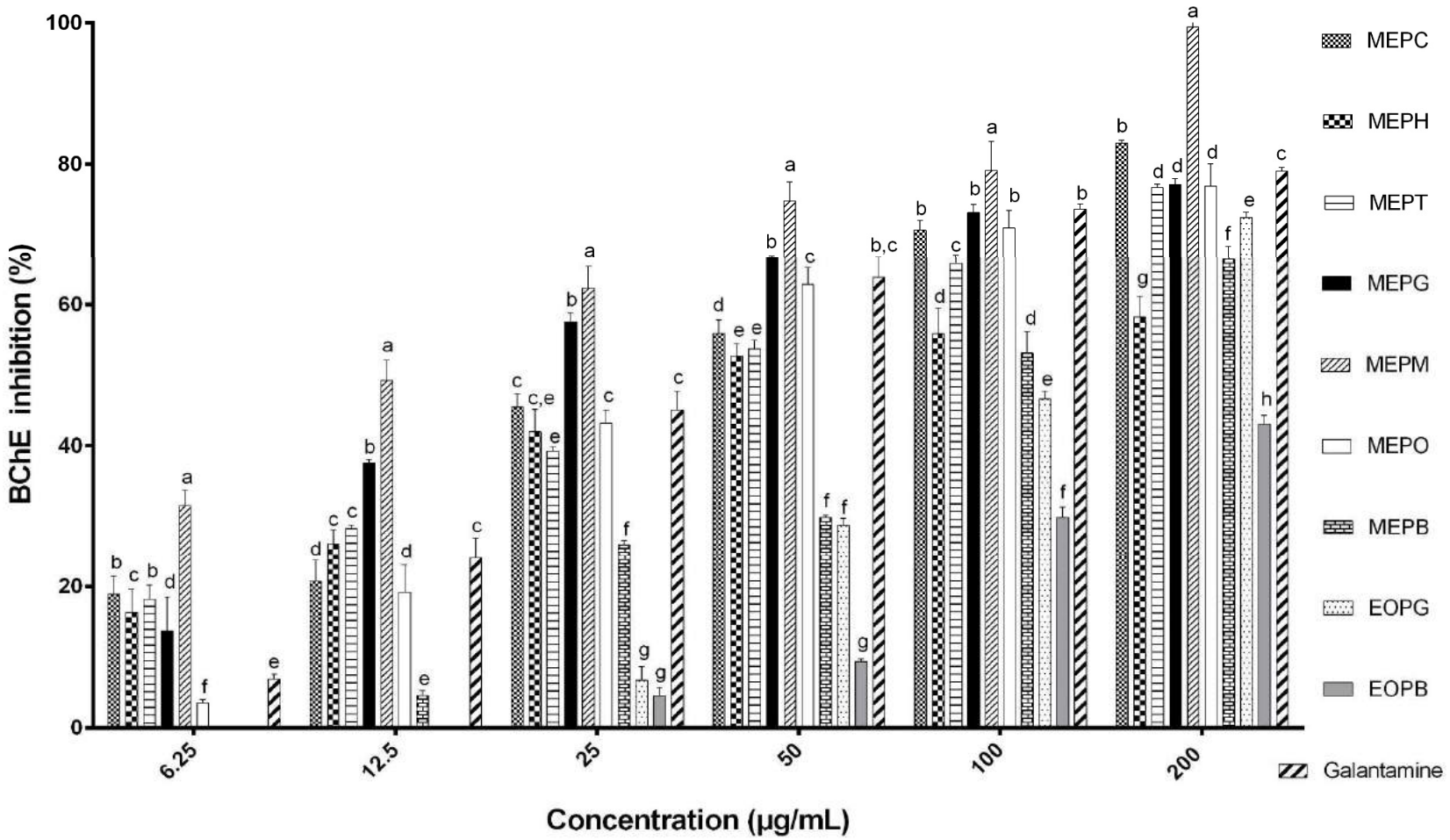


Figure 10. Dose-dependent inhibition of butyrylcholinesterase by propolis methanolic extracts, essential oils and galantamine. Data are expressed as inhibition (%) mean ± SD (n= 3). Columns with different letters indicate statistically significant differences ($p<0.05$). Vertical bars represent the standard deviation

II.4.2.2. α-Glucosidase inhibitory activity

The results of α-glucosidase inhibitory assay showed that all the tested extracts were able to inhibit α-glucosidase in a dose-dependent manner (Table 10). The maximum inhibition percentages (93.34±0.19%, 85.65±0.66%, 83.15±1.31%, 76.07±0.51%, 71.83±0.97%) were reached at the final assay concentration (250 µg/mL) of MEPC, MEPM, MEPO, MEPH and MEPG, respectively. Acarbose, however, reached 80.19±1.66% at a concentration of 1250 µg/mL.

Table 10. α-glucosidase inhibitory activity of propolis extracts

	α – glucosidase inhibition %					**IC_{50} (µg/mL)**
Extracts concentration	**15.625 µg/mL**	**31.25 µg/mL**	**62.5 µg/mL**	**125 µg/mL**	**250 µg/mL**	
MEPC	15.77±0.21^{c}	44.48±0.52^{c}	92.58±0.03^{a}	92.99±0.24^{a}	93.34±0.19^{a}	34.92±0.37^{c}
MEPH	6.68±1.36^{d}	39.46±0.19^{d}	71.69±1.54^{c}	73.90±4.58^{d}	76.07±0.51^{c}	41.66±0.32^{d}
MEPG	9.01±2.56^{d}	40.78±1.03^{d}	64.12±0.87^{d}	70.42±0.35^{d}	71.83±0.97^{d}	43.58±0.89^{e}
MEPM	64.65±2.94^{a}	71.32±3.15^{a}	78.08±0.80^{b}	81.19±0.19^{b}	85.65±0.66^{b}	11.40±0.58^{a}
MEPO	60.55±1.22^{b}	63.26±1.04^{b}	73.99±3.83^{c}	78.35±0.58^{c}	83.15±1.31^{b}	13.99±0.17^{b}
Standard concentration	**78.125 µg/mL**	**156.25 µg/mL**	**312.5 µg/mL**	**625 µg/mL**	**1250 µg/mL**	
Acarbose	27.43±2.18	38.91±3.20	54.86±1.79	67.29±2.63	80.19±1.66	275.43±1.59^{f}

Note: Data are expressed as inhibition (%) and IC_{50} mean ± SD (n= 3). The values with different superscripts (a, b, c, d, e or f) in the same columns are significantly different ($p < 0.05$).
Abbreviations: MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi.

The IC_{50} values varied significantly ($p<0.05$) between most extracts and were found to be within the range 11.40±0.58 – 43.58±0.89 µg/mL (Table 9). Based on the IC_{50} values, the samples can be classified in their effectiveness against α-glucosidase as follows: MEPM > MEPO > MEPC > MEPH > MEPG > Acarbose. These findings indicated the strong ability of propolis extracts on inhibiting α-glucosidase better than acarbose. Similarly, Ibrahim *et al.* (2016) reported that Malaysian propolis exhibited a potent antidiabetic activity (IC_{50} of 2.5 µg/mL and 30 µg/mL)

than acarbose (IC_{50} = 190 µg/mL). Such result could be due to the high content of propolis on phenolic and flavonoid compounds. However, weak correlation between the α-glucosidase inhibition and TPC (r = 0.4417) and TFC (r = -0.08036) were observed in this study. This result indicates that there are other compounds in the extracts responsible for α-glucosidase inhibition. Similarly, Mccue *et al.* (2005) stated that a high phenolic content does not always confer a high inhibition of α-glucosidase activity, which may in fact be due to the nonphenolic compounds in the samples. However, the biological effects of propolis are mainly related to the synergistic effects of its chemical composition (Abdullah *et al*., 2019).

To the best of our knowledge, there is no scientific report on the antidiabetic effect of Algerian propolis. Only few works have been carried out on some propolis form other countries. Laaroussi *et al.* (2021) found that Moroccan propolis exhibited variable inhibitory activities against α-glucosidase with IC_{50} values ranged between 90.99–876.24 µg/mL. Such results were higher than those obtained in the current study. Taleb *et al.* (2020) studied the Turkish propolis and found IC_{50} of 40.40 ± 0.09 µg/mL against α-glucosidase, which was closer to our results. However, our results were not in accordance with those of Abd El-Hady *et al.* (2016) who reported that Sudanese propolis was ineffective in inhibiting α-glucosidase.

II.4.3. Antimicrobial Activity

II.4.3.1. Disc diffusion assay

The screening of antimicrobial activity of propolis extracts and essential oils was firstly performed by disc diffusion method against eight human pathogen microorganisms. The diameter of inhibition zone values are presented in Table 11. Overall, Gram-positive bacteria and yeast were mainly more susceptible to the action of propolis extracts and EOs than Gram-negative bacteria. This is consistent with previous works on Algerian propolis that have shown a high antimicrobial activity against Gram-positive bacteria and limited activity against Gram-negative bacteria (Nedji & Loucif-Ayad, 2014; Boufadi *et al*., 2016). Such results could be explained by variable cell wall and membrane structure of the tested microorganisms (Al-Ani *et al*., 2018). It is believed that the low sensitivity of Gram-negative bacteria is due to their outer membrane (phospholipids, proteins, and lipopolysaccharides structure) that inhibits and/or retards the penetration of propolis (Benhanifia *et al*., 2014).

Table 11. Antimicrobial activity of propolis extracts, EOs and antibiotics estimated by diameter of inhibition zone in mm

	Diameter of inhibition zone (mm)												
Sample / Strains	**MEPC**	**MEPH**	**MEPT**	**MEPG**	**MEPM**	**MEPO**	**MEPB**	**EOPO**	**EOPB**	**Ampicillin**	**Kanamycin**	**Erythromycin**	**Fluconazole**
Gram-Positive Bacteria													
B. subtilis RSKK 244	13.12±0.08^{f}	11.22±2.06^{h}	17.70±0.70^{c}	12.60±0.30^{g}	14.20±0.13^{e}	15.27±0.39^{d}	13.15±0.59^{c}	11.40±0.34^{h}	12.90±0.70^{f,g}	36.81±0.33^{a}	17.76±0.49^{c}	20.21±0.4^{b}	NA
B. cereus RSKK 863	13.09±0.36^{g}	14.25±0.15^{f}	18.84±0.56^{d}	12.37±0.57^{h}	14.11±0.22^{f}	16.28±0.13^{e}	14.28±0.13^{f}	11.15±0.33^{i}	14.20±0.50^{f}	34.95±0.26^{a}	24.53±0.12^{b}	21.43±0.32^{c}	NA
S. aureus ATCC *25923*	13.24±0.58^{d}	18.32±2.17^{c}	13.53±0.68^{d}	16.56±0.50^{c}	13.40±0.14^{d}	13.76±0.17^{d}	16.79±0.27^{c}	9.68±0.18^{e}	8.87±0.32^{f}	32.48±0.25^{a}	17.50±0.21^{c}	26.44±0.37^{b}	NA
Gram-Negative Bacteria													
S. enteritidis ATCC 13076	11.29±0.63^{c,d}	10.33±1.23^{d}	10.76±1.22^{c,d}	11.88±0.52^{c}	10.37±0.87^{d}	10.88±0.73^{c,d}	11.53±0.97^{c}	9.50±0.73^{e}	10.57±0.30^{d}	26.46±0.23^{a}	17.84±0.26^{b}	12.58±0.31^{c}	NA
E. coli ATCC 11229	11.26±0.23^{e,f}	10.34±0.79^{f}	12.29±0.49^{d}	10.40±0.52^{f}	11.06±0.37^{e}	11.00±0.90^{e}	10.31±0.26^{f}	10.13±0.54^{f}	10.62±0.41^{f}	24.59±0.38^{b}	18.58±0.21^{c}	29.10±0.36^{a}	NA
E. coli O157:H7	9.04±0.36^{e,f}	9.58±0.23^{e}	8.84±0.08^{f}	9.21±0.23^{e}	9.90±0.20^{d,e}	8.95±0.15^{f}	9.34±0.27^{e}	9.49±0.59^{e}	10.62±0.41^{d}	25.95±0.26^{a}	19.89±0.89^{b}	18.83±0.11^{c}	NA
P. aeruginosa ATCC 27853	9.32±1.94^{d}	11.11±0.72^{b}	10.47±0.62^{c}	9.67±0.54^{c,d}	10.16±0.08^{c}	10.19±0.39^{c}	10.29±0.06^{c}	8.09±0.29^{e}	11.51±0.85^{b}	-	14.51±0.18^{a}	11.77±0.58^{b}	NA
Yeast													
C. albicans ATCC 10231	13.42±0.28^{e}	14.00±0.51^{d}	16.55±0.32^{b}	13.91±0.36^{d,e}	13.24±0.78^{e}	15.24±0.13^{c}	15.53±0.33^{c}	9.93±0.28^{f}	9.37±0.41^{f}	NA	NA	NA	17.08±0.09^{a}

Note: Data are presented as mean±SD (n=3). The values with different superscripts (a, b, c, d, e, f, g, h or i) in the same columns are significantly different ($p < 0.05$).

Abbreviations: NA: not applicable. (-): No activity. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from Batna.

The diameter of inhibition zone varied from 11.15±0.33 to 18.84±0.56 mm for *B. cereus* RSKK 863 followed by *S. aureus* ATCC 25923 with a diameter ranged from 8.87±0.32 to 18.32±2.17 mm, *C. albicans* ATCC 10231 (9.37±0.41– 16.55±0.32 mm), *B. subtilis* RSKK 244 (11.22±2.06 – 15.27±0.39 mm), *E. coli* ATCC 11229 (10.13±0.54 – 12.29±0.49 mm), *S. enteritidis* ATCC 13076 (9.50±0.73 – 11.88±0.52 mm), *P. aeruginosa* ATCC 27853 (8.09±0.29 –11.51±0.85 mm) and *E. coli* O157:H7 (8.84±0.08 – 10.62±0.41mm). Such findings are consistent with those of Benhanifia *et al*. (2014) who studied Algerian propolis and found diameter of inhibition zone varied from 8.05±0.07 to 20.15±0.21 mm for *S. aureus*, from 10.05±0.05 to 17.5±0.70 mm for *B. subtilis* and from 9.2±0.28 to 18.55±0.63 mm for *B. cereus.*

In the present study, the highest antibacterial activity was exhibited by MEPT against *B. cereus* RSKK 863 (18.84±0.56 mm) and MEPH against *S. aureus* ATCC 25923 (18.32±2.17 mm). MEPH and EOPB, however, showed a marked activity against *P. aeruginosa* ATCC 27853 with inhibition zones 11.11±0.72 mm and 11.51±0.85 comparable to that of Erythromycin (11.77±0.58 mm) while Ampicillin was ineffective against this strain. The highest antifungal activity against *C. albicans* ATCC 10231 was exerted by MEPT with inhibition zone diameter of 16.55±0.32 mm closer to that of Fluconazole (17.08±0.09 mm). The high antimicrobial potential of MEPT and MEPH could be linked to their phenolic and flavonoid content. However, the Pearson's correlation revealed a weak relationship between TPC and TFC and antimicrobial activity against most tested strains except for *S. enteritidis* ATCC 13076 and *C. albicans* ATCC 10231, in which a strong negative correlation was observed between TPC and TFC and antimicrobial activity, and a strong positive correlation between TPC and antibacterial activity against *E. coli* O157:H7 ($r = 0.5048$). These results indicate that the antimicrobial potential of propolis extracts is related to the synergistic effect of its components (Hasan *et al*., 2011).

II.4.3.1.Microdilution assay

To better understand the mode of action of propolis extracts and EOs against the tested microorganisms, MIC, MBC and MFC were determined using microdilution method and then MBC/MIC and MFC/MIC ratios were calculated. The results are indicated in Tables 12, 13 and 14. Overall, the MIC values of propolis extracts and EOs generally varied within the range 0.0156 – 2 µg/µL.

Table 12. MIC values of propolis extracts and EOs

Sample / Strains	MIC (µg/µL)								
	MEPC	**MEPH**	**MEPT**	**MEPG**	**MEPM**	**MEPO**	**MEPB**	**EOPO**	**EOPB**
Gram-Positive Bacteria									
B. subtilis RSKK 244	0.25	0.125	0.25	0.125	0.125	0.25	0.125	0.25	2
B. cereus RSKK 863	0.0156	0.0156	0.125	0.0156	0.125	0.5	0.25	0.5	1
S. aureus ATCC *25923*	0.25	0.25	0.125	0.25	0.25	0.125	0.25	1	1
Gram-Negative Bacteria									
S. enteritidis ATCC 13076	0.25	0.125	0.25	0.5	0.25	0.5	0.125	0.5	2
E. coli ATCC 11229	0.25	0.25	0.5	0.125	0.125	0.5	0.25	1	0.5
E. coli O157:H7	0.5	1	1	1	1	1	1	0.5	2
P. aeruginosa ATCC 27853	1	0.5	1	1	1	1	1	1	0.5
Yeast									
C. albicans ATCC 10231	0.125	0.03125	0.0625	0.0625	0.125	0.03125	0.0625	0.25	2

Abbreviations: MIC: minimum inhibitory concentration. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from Batna.

The lowest MIC value of 0.0156 µg/µL was found in MEPC, MEPH and MEPG against *B. cereus* RSKK 863. The MBC values ranged from 0.03125 to 8 µg/µL. The lowest MBC value (0.03125 µg/µL) was exerted by MEPC, MEPH and MEPG against *B. cereus* RSKK 863, whilst the lowest MFC value of 1 µg/µL against *C. albicans* ATCC 10231 was recorded by MEPT and MEPO. The MBC and MFC values of propolis extracts and EOs were found to be similar or even higher than their MIC values. This could be attributed to variation in the rate of the extracts or EOs penetration through the cell wall and cell membrane structures (Jiang *et al*., 2011). However, The MBC/MIC and MFC/MIC values in the present study were found to be higher than their MIC values. This phenomenon may be explained by the impure form of the bioactive compound(s) (Mewari & Kumar, 2011).

Compared to literature, the MIC values obtained in the current study were lower than those reported by Boufadi *et al.* (2016) who found MIC value range between 0.3 – 9 mg/mL of Algerian propolis and closer to those obtained by Moroccan propolis (MIC range 0.002 –1.12 mg/mL) (El Menyiy *et al*., 2021). However, our results of MBC were higher than those obtained by Moroccan propolis (MBC range (MIC range 0.002 –1.12 mg/mL) (El Menyiy *et al*., 2021).

According to the MBC/MIC ratio (Krishnan *et al*., 2010) and MFC/MIC ratio (Hazen, 1998), the inhibition of bacterial growth by propolis extracts and EOPO, in the present study, was through two mechanisms which are bactericidal (MBC/MIC ≤ 4) or bacteriostatic (MBC/MIC > 4), whereas, the inhibition of yeast growth was through fungistatic mode (MFC/MIC > 4). However, EOPB was bactericidal for all tested pathogenic bacteria and fungicidal for *C. albicans* ATCC 10231. The cidal effect of EOPB could be correlated to its monoterpenic content, especially, to its high amount of α-pinene (56.1%), which has been reported to possess cidal effect (Jiang *et al*., 2011). α-pinene is used as antibacterial due to its toxic effects on membranes (Salehi *et al*., 2019). It has been reported in the literature that the bactericidal and bacteriostatic activity of propolis is attributed to the inhibition of protein synthesis and prevention of cell division, whilst the fungicidal and the fungistatic action could be due to the induced expression of apoptotic and necrotic factors alongside the formation of reactive oxygen species (Chamandi *et al*., 2015; Torres *et al*., 2018).

Table 13. MBC and MFC values of propolis extracts and EOs

Sample / Strains	MBC or MFC (µg/µL)								
	MEPC	**MEPH**	**MEPT**	**MEPG**	**MEPM**	**MEPO**	**MEPB**	**EOPO**	**EOPB**
Gram-Positive Bacteria									
B. subtilis RSKK 244	1	2	0.25	1	2	1	0.5	1	4
B. cereus RSKK 863	0.03125	0.03125	4	0.03125	0.25	1	0.5	1	2
S. aureus ATCC *25923*	2	1	1	2	1	1	1	2	2
Gram-Negative Bacteria									
S. enteritidis ATCC 13076	2	1	2	2	1	2	4	2	4
E. coli ATCC 11229	0.5	2	0.5	0.125	0.5	2	2	2	2
E. coli O157:H7	4	4	2	4	4	2	4	4	4
P. aeruginosa ATCC 27853	4	4	4	4	4	4	8	8	2
Yeast									
C. albicans ATCC 10231	4	2	1	2	2	1	2	2	2

Abbreviations: MBC: minimum bactericidal concentration, MFC: minimum fungicidal concentration. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from Batna.

Table 14. MBC/MIC and MFC/MIC ratios of propolis extracts and EOs

	MBC/MIC or MFC/MIC								
Sample / Strains	**MEPC**	**MEPH**	**MEPT**	**MEPG**	**MEPM**	**MEPO**	**MEPB**	**EOPO**	**EOPB**
Gram-Positive Bacteria									
B. subtilis RSKK 244	4	16	1	8	16	4	4	4	2
B. cereus RSKK 863	2	2	32	2	2	2	2	2	2
S. aureus ATCC *25923*	8	4	8	8	4	8	4	2	2
Gram-Negative Bacteria									
S. enteritidis ATCC 13076	8	8	8	4	4	4	32	4	2
E. coli ATCC 11229	2	8	1	1	4	4	8	2	4
E. coli O157:H7	8	4	2	4	4	2	4	8	2
P. aeruginosa ATCC 27853	4	8	4	4	4	4	8	8	4
Yeast									
C. albicans ATCC 10231	32	64	16	32	16	32	32	8	1

Abbreviations: MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration, MFC: minimum fungicidal concentration. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPT: Methanolic extract of propolis from El-Taref. MEPG: Methanolic extract of propolis from Grarem. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna. EOPO: Essential oil of propolis from Oum el Bouaghi. EOPB: Essential oil of propolis from Batna.

II.4.4. Toxic effect

As a preliminary toxicity assessment tool, brine shrimp lethality bioassay was used. This test is also used to identify samples with a potential of having anticancer activity. It is, therefore, possible that the samples exhibited toxicity to brine shrimps may also be toxic to cancer cells (Ngassapa *et al.*, 2022).

In the present study, the lethality of brine shrimp larvae was found to be directly proportional to the concentration of propolis extracts (Table 15). Total mortality of *Artemia salina* nauplii was observed at 400 µg/mL concentration of MEPC and MEPM, and at 200 µg/mL of MEPM. All extracts, however, caused mortality below 50% at concentration of 50 and 100 µg/mL and showed no toxicity at 25 µg/mL concentration and below. All the tested extracts were less toxic than the standard potassium dichromate (LC_{50} = 21.11±3.47 µg/mL). Based on the LC_{50} values, the toxic potential of the extracts was in the following order: Potassium dichromate > MEPM > MEPG > MEPC > MEPO > MEPH.

Table 15. Toxic effect of propolis extracts against brine shrimp larvae

	Mortality % at different concentrations							LC_{50} (µg/mL)
	6.25 µg/mL	**12.5 µg/mL**	**25 µg/mL**	**50 µg/mL**	**100 µg/mL**	**200 µg/mL**	**400 µg/mL**	
MEPC	0±0.00	0±0.00	0±0.00	0±0.00	13.33±5.77	66.67±5.77^{a}	100±0.00	201.61±7.27^{d}
MEPH	0±0.00	0±0.00	0±0.00	10±0.00	16.67±5.77	30±0.00^{b}	40±0.00	>400
MEPT	ND	ND	ND	ND	ND	ND	ND	ND
MEPG	0±0.00	0±0.00	0±0.00	13.33±5.77	26.67±5.77	50±17.32	96.67±5.77	186.08±15.08^{c}
MEPM	0±0.00	0±0.00	0±0.00	6.67±5.77	26.67±5.77	100±0.00	100±0.00	131.55±5.15^{b}
MEPO	0±0.00	0±0.00	0±0.00	16.67±5.77	30±0.00	33.33±5.77	80±17.32	263.49±5.50^{e}
MEPB	ND	ND	ND	ND	ND	ND	ND	ND
	10 µg/mL	**20 µg/mL**	**40 µg/mL**	**80 µg/mL**	-	-	-	
Potassium dichromate	0±0.00	50±10.00	80±0.00	100±0.00	-	-	-	21.11±3.47^{a}

Note: Data are presented as LC_{50} mean±SD (n=3).The values with different superscripts (a, b, c, d or e) in the same column are significantly different ($p < 0.05$).

Abbreviations: ND: Not determined. MEPC: Methanolic extract of propolis from Collo, MEPH: Methanolic extract of propolis from El-Harrouch. MEPG: Methanolic extract of propolis from Grarem. MEPT: Methanolic extract of propolis from El-Taref. MEPM: Methanolic extract of propolis from El-Menia, MEPO: Methanolic extract of propolis from Oum el Bouaghi. MEPB: Methanolic extract of propolis from Batna.

According to Meyer's toxicity index, extracts with LC_{50} < 1000 µg/mL are considered as toxic, while extracts with LC_{50} > 1000 µg/mL are considered as non-toxic (Meyer *et al*., 1982). Clarkson's toxicity criterion classifies extracts into three sub-categories: extracts with LC_{50} above 1000 µg/mL are non-toxic, LC_{50} of 500 - 1000 µg/mL are low toxic, extracts with LC_{50} of 100 - 500 µg/mL are medium toxic, while extracts with LC_{50} of 0 - 100 µg/mL are highly toxic (Clarkson *et al*., 2004). The LC_{50} values found in the present study were within the range 100 - 500 µg/mL, indicating moderate toxic properties of propolis extracts, which may be due to the presence of toxic compounds that possess larvicidal properties (Obayed Ullah *et al*., 2013). In this study, there was found a weak negative correlation of toxicity with TPC and toxicity (r = -0.1486) and moderate positive correlation with TFC (r = 0.4986), indicating that the toxicity of propolis may be due to the interaction between its constituents (Asong *et al*., 2019).

Compared to literature, our results were higher than the value recorded by propolis from Bangladesh (LC_{50} = 57.99 µg/mL) and lower than that of Malaysian propolis (LC_{50} from 501.2 - 670.8 µg/mL) (Tanvir *et al*., 2018; Yusop *et al*., 2019). In addition, Ngassapa *et al*. (2022) studied 28 Tanzanian propolis extracts and found variable levels of toxicity to brine shrimp larvae, with LC_{50} values ranging from 7.75 to 1244.64 µg/mL, in which 14 (50%) out of 28 propolis extracts were found very toxic. The difference between our results and those of literature could be explained by the variation in the chemical composition of propolis samples.

II.4.5. Anticancer Activity

II.4.5.1. Cytotoxicity

The results of cytotoxicity assay revealed a clear dose-dependent cytotoxicity response against HepG2 cells 72-hour posttreatment with MEPC, MEPG, MEPM and MEPO (Figure 11). The maximum inhibition percentages 83.22±1.01, 81.98±0.15%, 81.83±0.22% and 81.70±1.27% were reached at the final assay concentration (200 µg/mL) of MEPM, MEPO, MEPC and MEPG, respectively. These values, however, did not show any significant difference ($p>0.05$). The IC_{50} values varied significantly ($p<0.05$) between the extracts and were found to be 12.22±0.05 µg/mL for MEPC, 18.68±0.33 µg/mL for MEPO, 32.78±0.34 µg/mL for MEPM and 60.39±1.82 µg/mL for MEPG, indicating a stronger cytotoxic effect of MEPC on HepG2 cells compared with the other extracts.

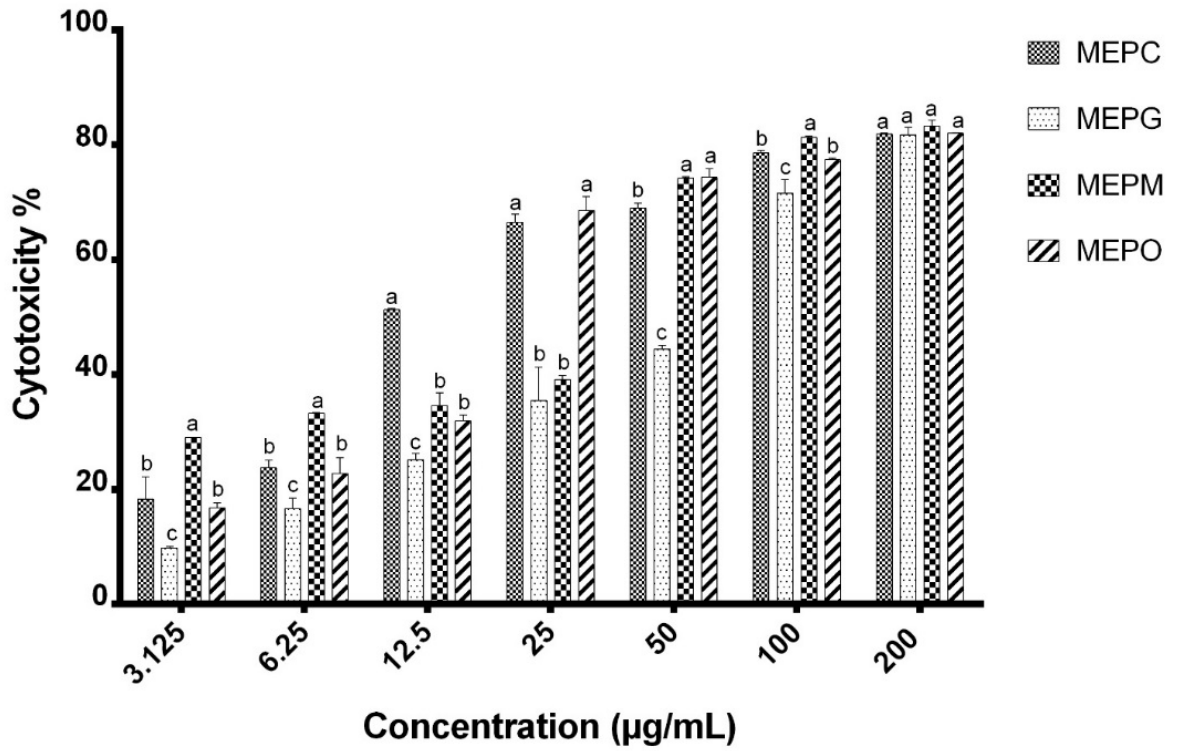


Figure 11. Dose-dependent cytotoxicity response against HepG2 cells 72-hour posttreatment with propolis extracts. Data are expressed as cytotoxicity (%) mean ± SD (n= 3). Columns with different letters indicate statistically significant differences ($p<0.05$). Vertical bars represent the standard deviation.

Previous studies on propolis from different geographical origins have stated its anticancer potential on human hepatocellular carcinoma cells and have described a variety of ranges of IC_{50} values. Turan *et al.* (2015) and Gokduman (2019) investigated the cytotoxic effect of Turkish propolis and reported IC_{50} values of 27.0±0.8 µg/mL and 25.62±1.50 µg/mL, which were closer to the value exhibited by MEPM in the current study. Abu Shady *et al.* (2016) and Abd El-Hady *et al.* (2016) indicated that Egyptian and Sudanese propolis exhibited anticancer potentials against HepG2 with IC_{50} values within the range of 62.5-70.9 µg/mL and 57-60 µg/mL, respectively. Such results were closer to that of MEPG. In another study by Sadeghi-Aliabadi *et al.* (2015), Iranian propolis was found to exhibit a potent cytotoxicity with an IC_{50} value of 15 µg/mL, which is closer to the results obtained by MEPC and MEPO.

II.4.5.2. Cell morphology analysis

The cytotoxic effects of MEPC, MEPG, MEPM and MEPO, were further studied by morphological cellular imaging (Figures 12, 13, 14 and 15).

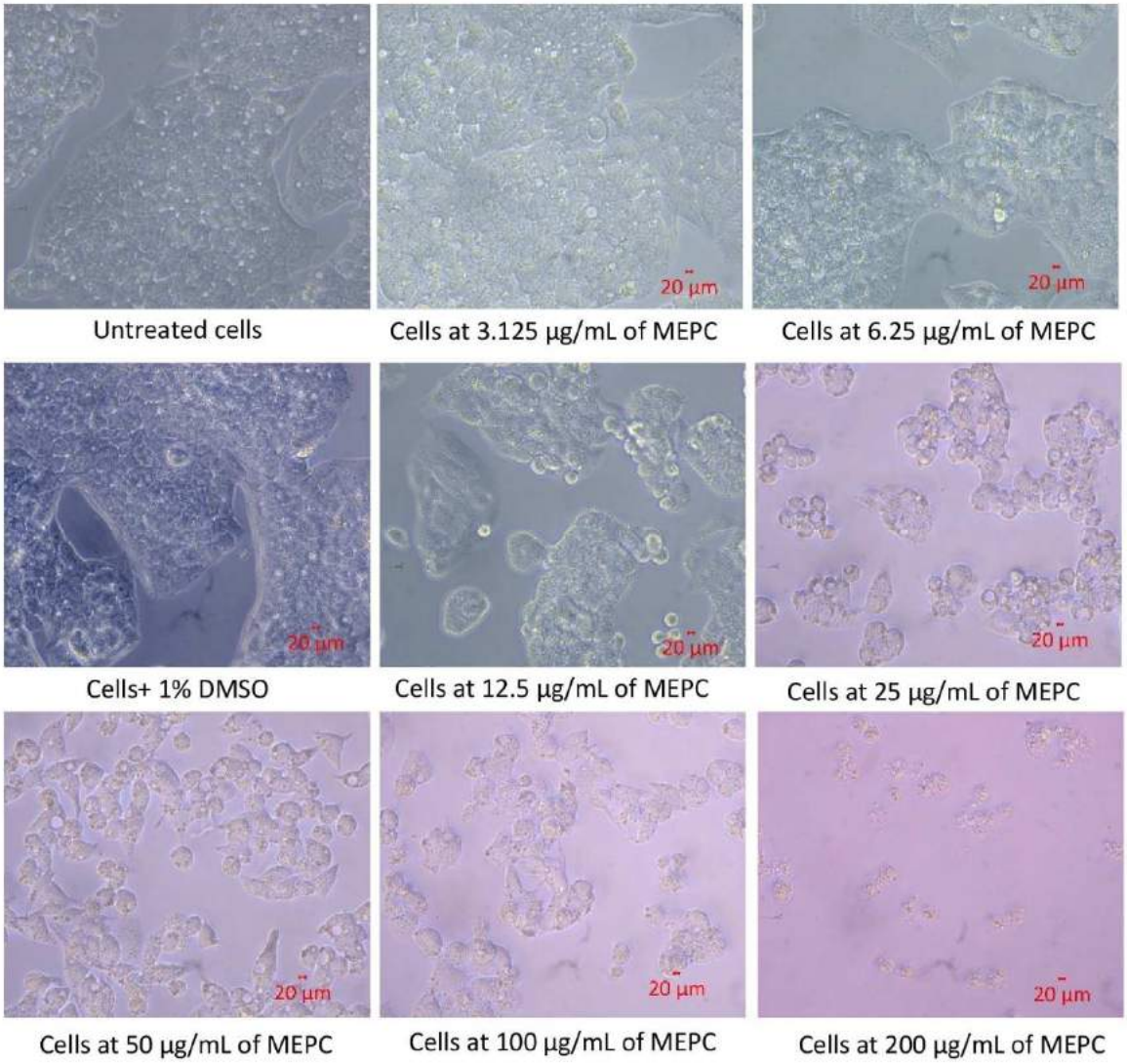


Figure 12. Morphological effects of MEPC on HepG2 cells observed using inverted microscope (40X magnification)

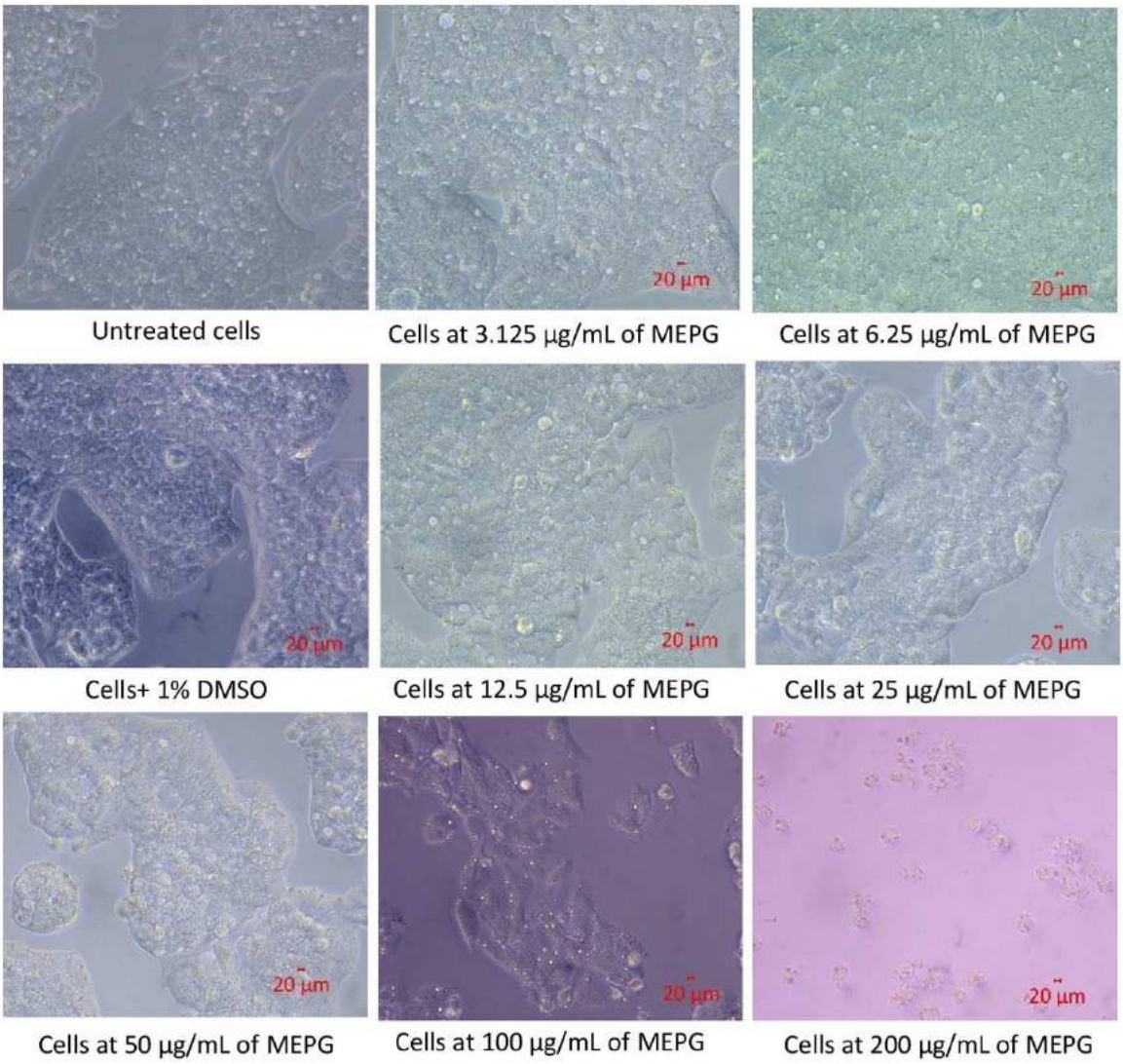


Figure 13. Morphological effects of MEPG on HepG2 cells observed using inverted microscope (40X magnification)

It was observed that untreated and 1%DMSO‑treated cells maintained a normal morphology. However, HepG2 cells treated with the extracts within 72-h period lost the typical morphology in a concentration dependent manner. At lower concentrations (12.5 µg/mL and less of MEPC and MEPO, 25 µg/mL and less of MEPM, 50 µg/mL and less of MEPG), the changes were less significant. However, at higher concentrations (25 µg/mL and higher of MEPC and MEPO, 50 µg/mL and higher of MEPM, 100 µg/mL and higher of MEPG), the changes were much

more severe including loss of normal morphology and cellular junctions, reduction in cell volume and formation of apoptotic bodies. Most cells at higher concentrations lost contact with adjacent cells and acquired a spherical shape compared to untreated cells. These morphological alterations indicated that propolis extracts mediated cytotoxic effect against HepG2 cells possibly via induction of apoptosis, which is in agreement with previous works that demonstrated the apoptotic effect of extracts from Algerian propolis in cancer cells (Kebsa *et al*., 2018; Rouibah *et al*., 2018).

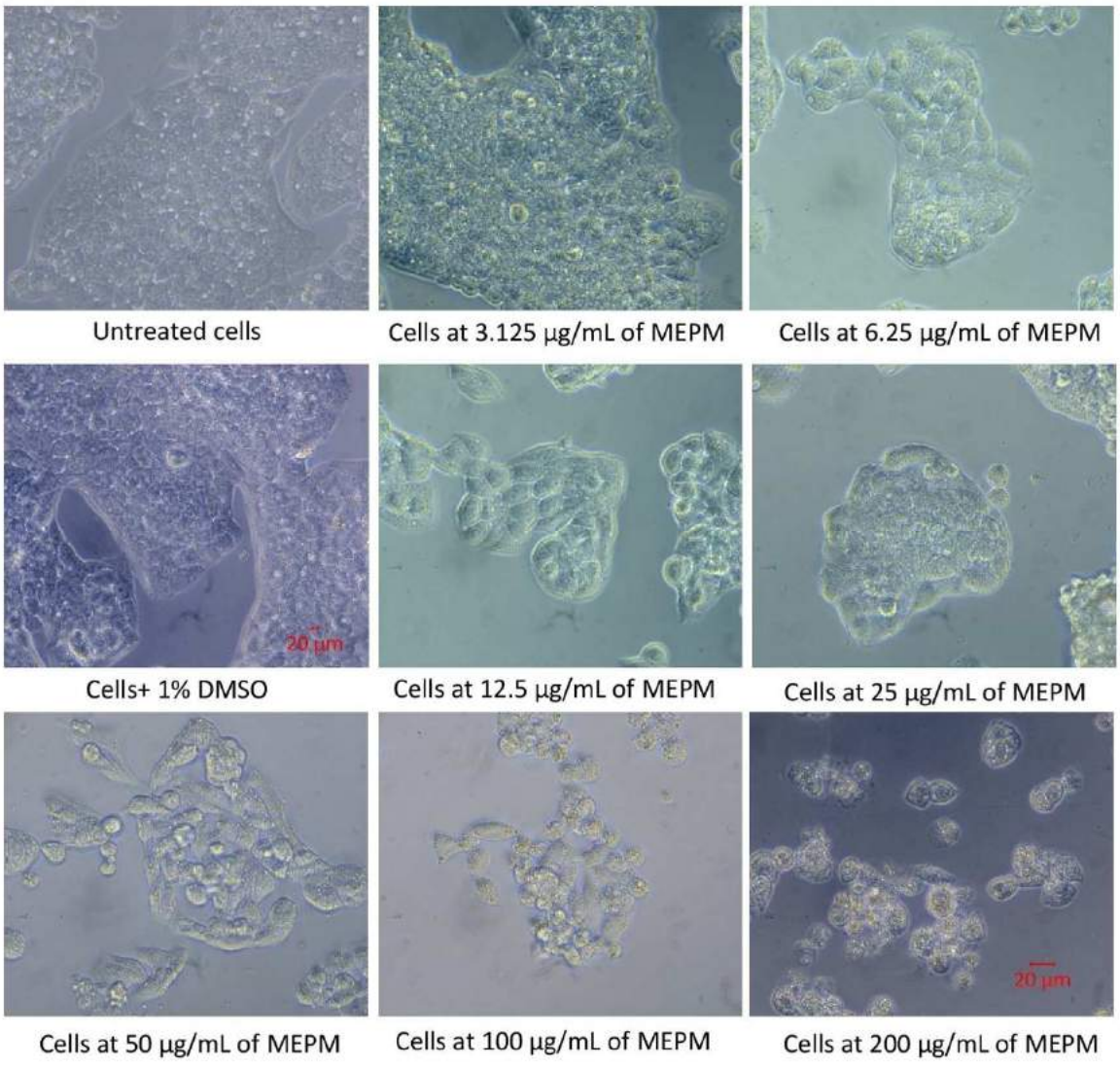


Figure 14. Morphological effects of MEPM on HepG2 cells observed using inverted microscope (40X magnification)

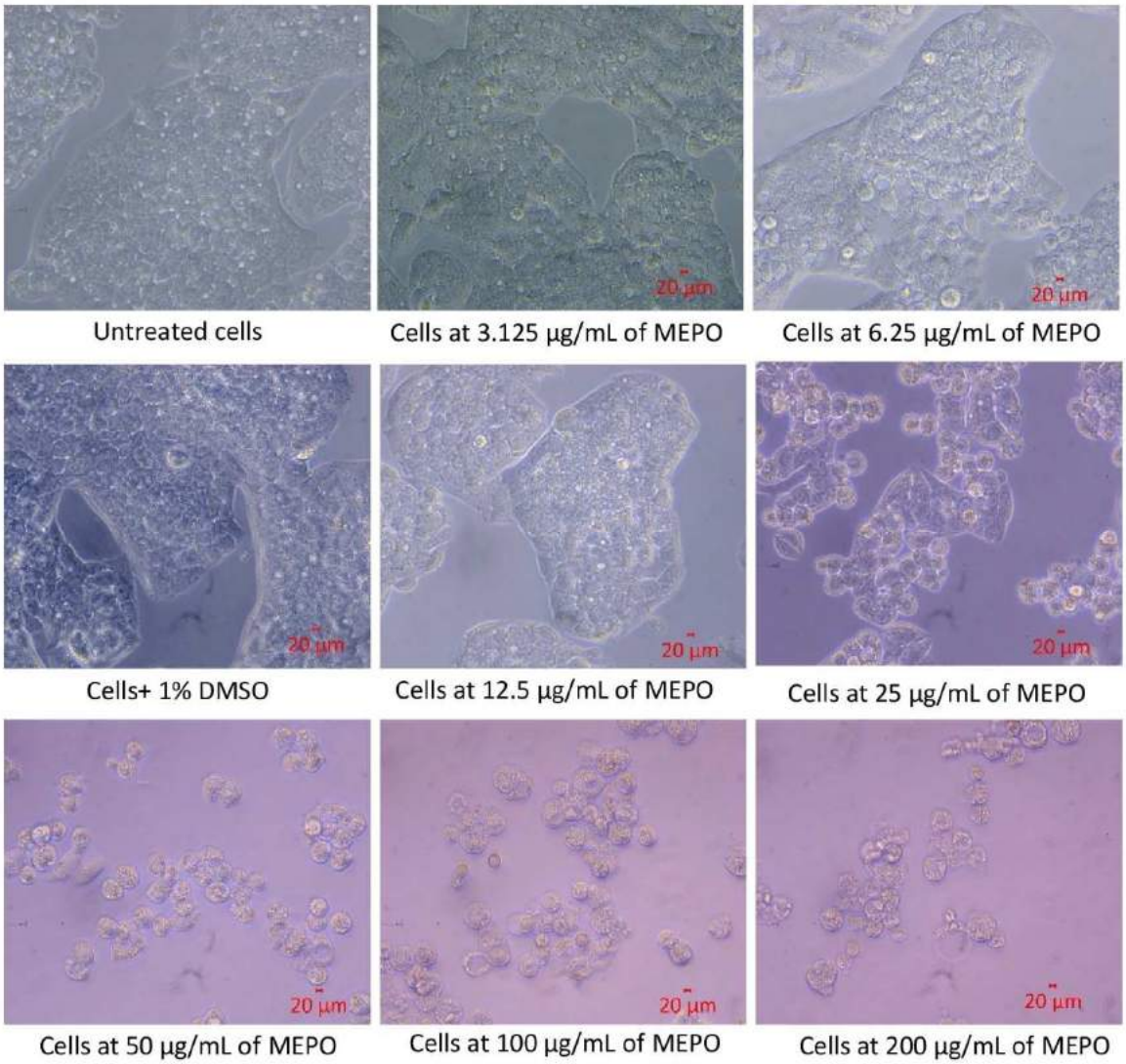


Figure 15. Morphological effects of MEPO on HepG2 cells observed using inverted microscope (40X magnification)

II.5. LC–MS/MS analysis of the potent extracts

The phenolic profiles of the extracts that showed potent anticancer effect were analyzed by LC-MS/MS triple quadrupole. Table 16 shows the content of each propolis extract. The results revealed some qualitative and quantitative differences between the three extracts, which could be explained by the difference in geographical origin of propolis, season and collection time (Sorucu & Oruç, 2019; Soltani *et al*., 2020; Kasote *et al*., 2022). Twenty-one phenolic compounds were detected in MEPM, twenty-two compounds were detected in MEPC, whereas twenty-three

compounds were identified in MEPO. Twenty-one phenolics were common between the three propolis but with different amounts. One compound was detected only in MEPO, which is 3,4-dihydroxyphenylacetic acid.

Table 16. Phenolic compounds of propolis extracts determined by LC-MS/MS

		MEPC	MEPM	MEPO
Compound	**RT (min)**	**Quantification (ng/mg E)**	**Quantification (ng/mg E)**	**Quantification (ng/mg E)**
Phenolic acids				
Gallic acid	8.808	1434.39±12.22	358.63±3.41	153.30±5.96
Protocatechuic acid	10.59	691.61±15.57	228.87±3.87	541.04±6.01
3,4-Dihydroxyphenylacetic acid	10.905	ND	ND	6.07±0.11
Chlorogenic acid	11.786	443.33±30.40	88.34±3.54	415.96±3.51
3-Hydroxybenzoic acid	12.854	ND	ND	ND
4-Hydroxybenzoic acid	12.114	367.71±0.86	143.07±5.55	319.38±19.10
2,5-Dihydroxybenzoic acid	11.988	ND	ND	ND
homovanillic acid	12.642	180.32±16.03	114.50±20.78	215.19±3.28
Caffeic acid	12.651	5236.90±83.84	3633.65±16.81	5745.61±262.38
Syringic acid	12.782	77.40±5.96	9.84±1.06	31.19±0.11
Verbascoside	13.468	488.04±9.47	236.59±27.03	2726.14±64.31
p-Coumaric acid	13.802	1376.51±7.97	1308.51±11.43	1329.28±28.35
Sinapic acid	13.874	ND	ND	ND
Ferulic acid	13.934	7103.17±55.45	1694.90±42.65	3126.90±74.21
Rosmarinic acid	14.508	ND	ND	ND
2-Hydroxycinnamic acid	14.846	ND	ND	ND
Total phenolic acids		17,399.38±49.43	7816.89±2.32	14,610.05±467.32
Flavonoids				
(+)-Catechin	11.37	ND	ND	ND
(-)-Epicatechin	12.379	ND	ND	ND
Taxifolin (dihydroquercetin)	13.713	10.59±3.17	45.98±3.43	88.12±1.50
Luteolin 7-glucoside	14.273	ND	ND	ND
Hesperidin	14.303	252.77±3.52	351.56±6.90	1116.47±71.19
Hyperoside (quercetin-3-O-galactoside)	14.489	135.60±0.72	55.99±0.59	326.81±9.89
Apigenin 7-glucoside	14.74	9.91±0.46	ND	12.73±0.68
Eriodictyol	15.072	78.87±5.12	111.67±0.11	153.37±8.01
Quercetin	15.571	1130.32±49.96	2141.92±1.09	2572.27±96.47
Luteolin	15.81	201.17±6.80	276.73±5.55	382.97±10.46
Kaempferol	16.106	1457.16±0.88	2369.63±9.08	2109.85±54.77
Apigenin	16.245	2053.56±37.81	2320.70±11.35	2028.32±19.76
Total flavonoids		5329.97±86.04	7674.18±20.71	8790.92±140.90
Lignans				
Pinoresinol	14.944	138.05±4.52	140.68±2.14	284.03±53.47
Total lignans		138.05±4.52	140.68±2.14	284.03±53.47
Other polyphenols				
3-hydroxytyrosol	10.268	ND	ND	ND
Pyrocatechol	10.891	119.62±0.35	124.63±20.51	418.62±11.23
Vanillin	13.071	437.13±17.13	130.24±16.75	171.63±15.55
Oleuropein	14.607	ND	ND	ND
Total of other polyphenols		278.37±17.48	254.87±37.26	590.26±4.32
Total identified phenolic compounds		23,424.134±23.65	15,886.61±62.42	24,275.26±550.43

Note: Data are presented as mean±SD of two measurements.

Abbreviations: RT: retention time. MEPC: Methanolic extract of propolis from Collo. MEPM: Methanolic extract of propolis from El-Menia. MEPO: Methanolic extract of propolis from Oum El Bouaghi. ND: Not detected.

The molecules identified in the extracts belong to four phenolic sub-classes including phenolic acids, flavonoids, lignans and other polyphenols. Phenolic acids were the major phenolic sub-class found in the three extracts followed by flavonoids. The predominant individual phenolic compounds in MEPM were caffeic acid followed by kaempferol, apigenin and quercetin. The major components of MEPC were ferulic and caffeic acids followed by apigenin and kaempferol. Gallic acid, *p*-coumaric acid and quercetin were also found at high amount. The most abundant components in MEPO, however, were ferulic and caffeic acids followed by verbascoside and quercetin. Kaempferol and apigenin were also determined in high quantity.

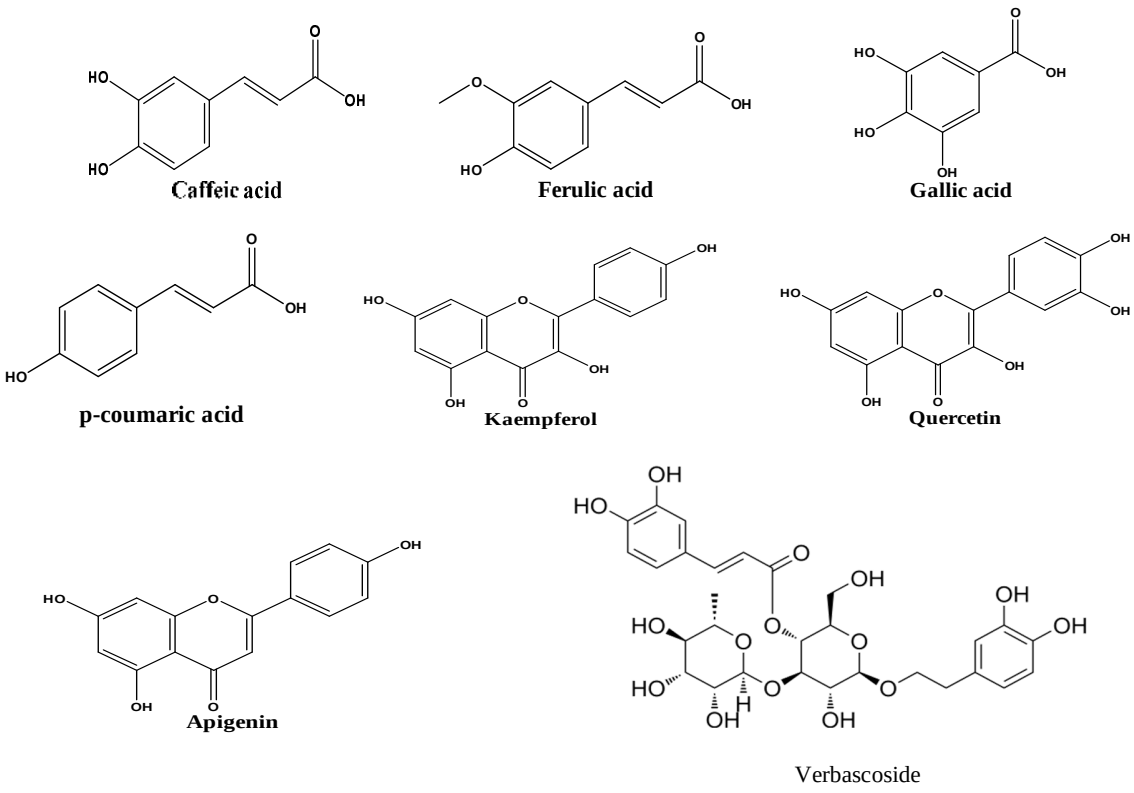


Figure 16. Abundant phenolic components in MEPC, MEPM and MEPO

Similar to other Algerian propolis, gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid, quercetin, apigenin, kaempferol have also been identified in MEPC, MEPM and

MEPO (Hegazi *et al*., 2012; Segueni *et al*., 2017; Chaa *et al*., 2019; Daikh *et al*., 2019), which could be used for quality determination and standardization of Algerian propolis. However, the other compounds reported in the present study have never been identified in Algerian propolis but have been detected in propolis from other countries. Apigenin 7-glucoside, hesperidin, hyperoside and verbascoside have been detected in Cypriot propolis (Nalbantsoy *et al*., 2022). Protocatechic acid, *p*-hydroxybenzoic acid, syringic acid, eriodictiol, luteolin and vanillin have been found in Turkish propolis (Ahu Kahraman *et al.*, 2022). Homovanillic acid and pinorisenol have been characterized in Brazilian propolis (Righi *et al*., 2013; Ribeiro *et al.*, 2021). Taxifolin has been found in Moroccan propolis (Belmehdi *et al*., 2021). Pyrocatechol has been identified in *Trigona laeviceps* stingless bee propolis from Indonesia (Wibowo *et al*., 2021). 3,4-Dihydroxyphenylacetic acid, however, has not been reported in propolis samples to the best of our knowledge.

The anticancer activity of MEPC and MEPO in this study was very interesting because of the possible cytotoxic effects of their phytoconstituents. Their chemical profiles revealed the presence of several phenolic compounds that have been reported to exhibit anticancer activity cells such as ferulic acid, cafeic acid, apigenin and quercetin (Ou *et al*., 2004; Prasad *et al*., 2011; Madunic *et al*., 2018; Azeem *et al*., 2022). In addition, the Pearson's correlation analysis revealed a strong positive correlation between cytotoxicity of propolis and their contents in TPC (r = 0.6796) and TFC (r = 0.8584).

Conclusion

The present study provides first data about Algerian propolis from seven Northeastern regions namely: Collo (Skikda), El Harrouch (Skikda), Bouteldja (El-Taref), Grarem (Mila), El-Menia (Constantine), Oum El Bouaghi (Oum El Bouaghi) and Mestaoua & Chelala mountains (Batna).

The methanolic extracts from these propolis showed variable total phenolic and flavonoid contents, which were correlated to the difference in geographic origin of propolis samples.

The phenolic profile identification of methanolic extracts of propolis from Collo, El-Menia and Oum El Bouaghi, performed through LC-MS/MS allowed the identification of twenty-three phenolic compounds known for their pharmacological activities. The most abundant compounds were those commonly observed in Algerian propolis, which could be used for quality determination and standardization of Algerian propolis. However, there was a new phenolic compound identified in propolis from Oum El Bouaghi which is 3,4-Dihydroxyphenylacetic acid.

The GC-MS analysis of propolis essential oils from Grarem, Oum El Bouaghi and Batna allowed the identification of 112 compounds, in which α-pinene, limonene, *trans*-pinocarveol, α-terpinenyl acetate and δ-Cadinene were common between the three oils.

The antioxidant tests revealed strong antioxidant properties of propolis extracts, expressed by the capacity to scavenge radicals, reduce ions and inhibit lipid peroxidation. Algerian propolis, therefore, could be a promising remedy for radical-mediated diseases.

The anti-enzymatic assays demonstrated the strong cholinesterase and α-glucosidase inhibitory potentials of propolis extracts and revealed their potency to be used as a strong source of future therapeutic agents in Alzheimer and diabetes.

The antimicrobial assays revealed the broad-spectrum antimicrobial activity of propolis extracts and essential oils, suggesting their use in managing microbial resistance and in treating the pathological damage caused microbial infections.

The Brine shrimp lethality test showed that propolis extracts from Collo, El Harrouch, Grarem, El-Menia, Oum El Bouaghi are medium toxic and exhibit no toxicity at 25 µg/mL concentration and below.

The anticancer assay against human hepatocellular carcinoma (HepG2) cell line revealed the strong cytotoxic activity of propolis extracts especially propolis from Collo and Oum El Bouaghi, which support the potential health benefits of propolis as a potential source of bioactive principles for therapeutic application in liver cancer treatment.

Further studies, however, are needed to be carried out in order to isolate the active chemical constituents responsible for the observed biological activities and to determine in depth their functional properties and their mechanisms of action.

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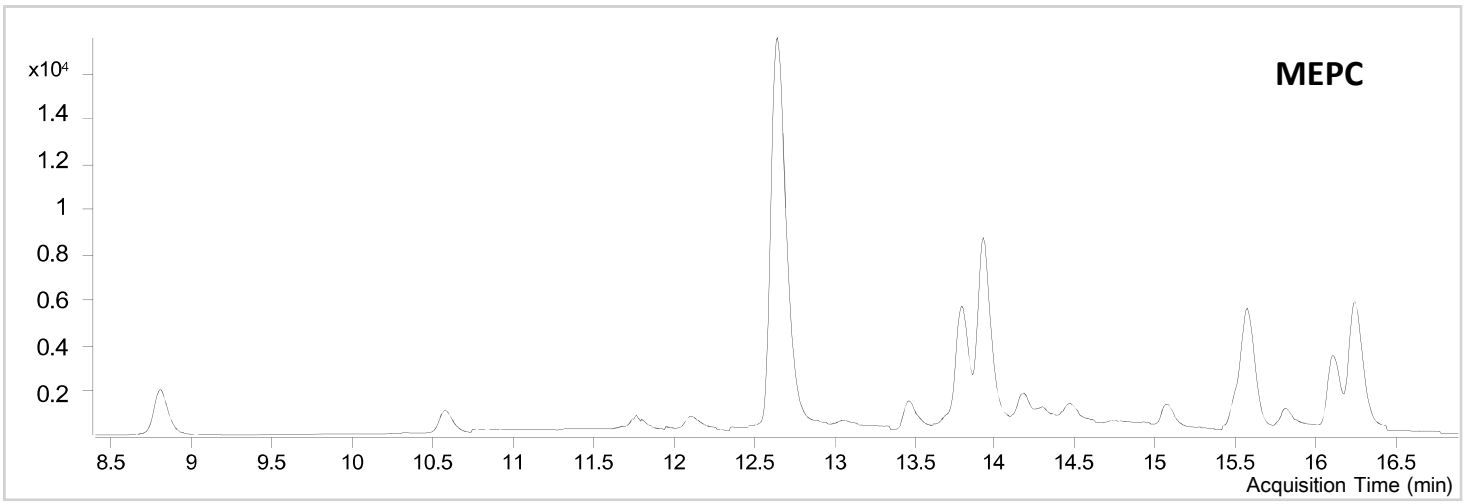
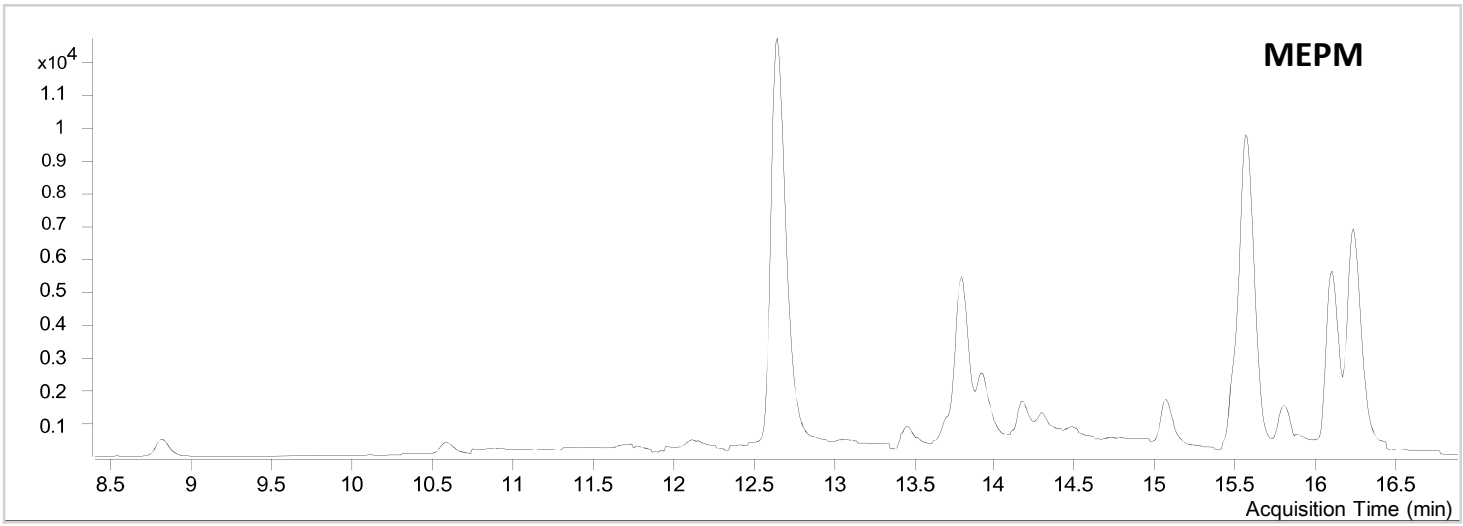
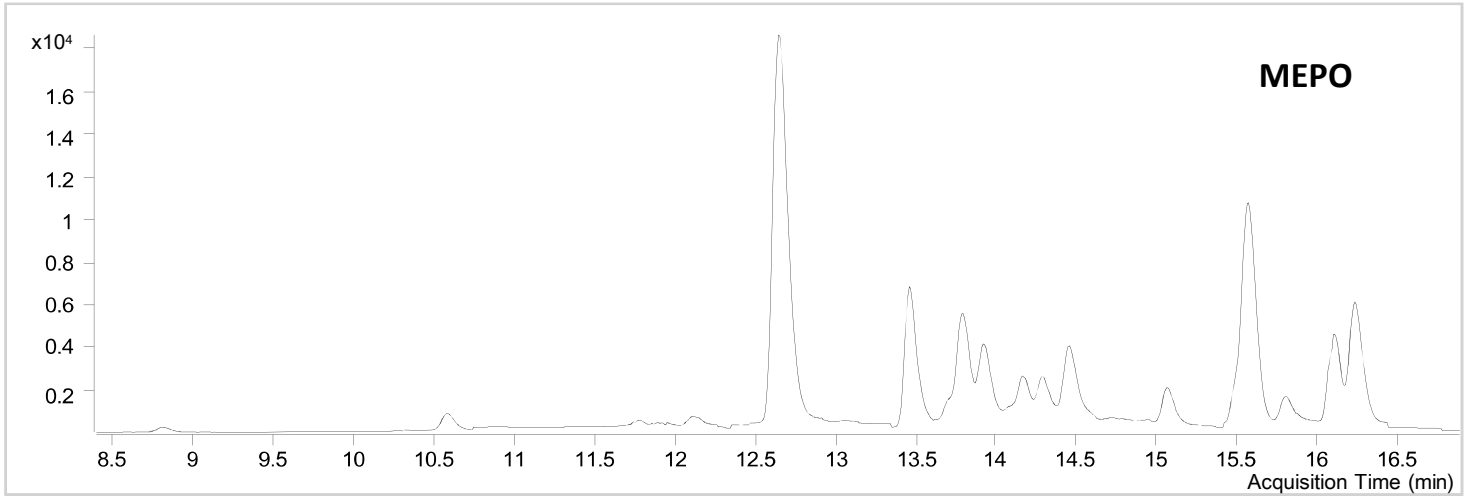
Annexes

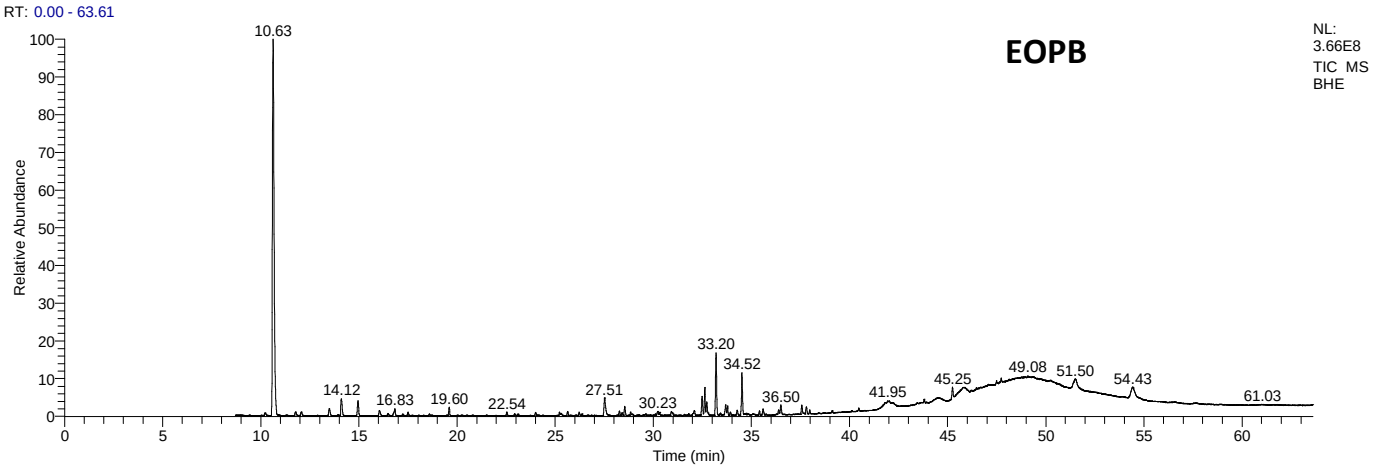
Annexe 1. LC-MS/MS chromatograms

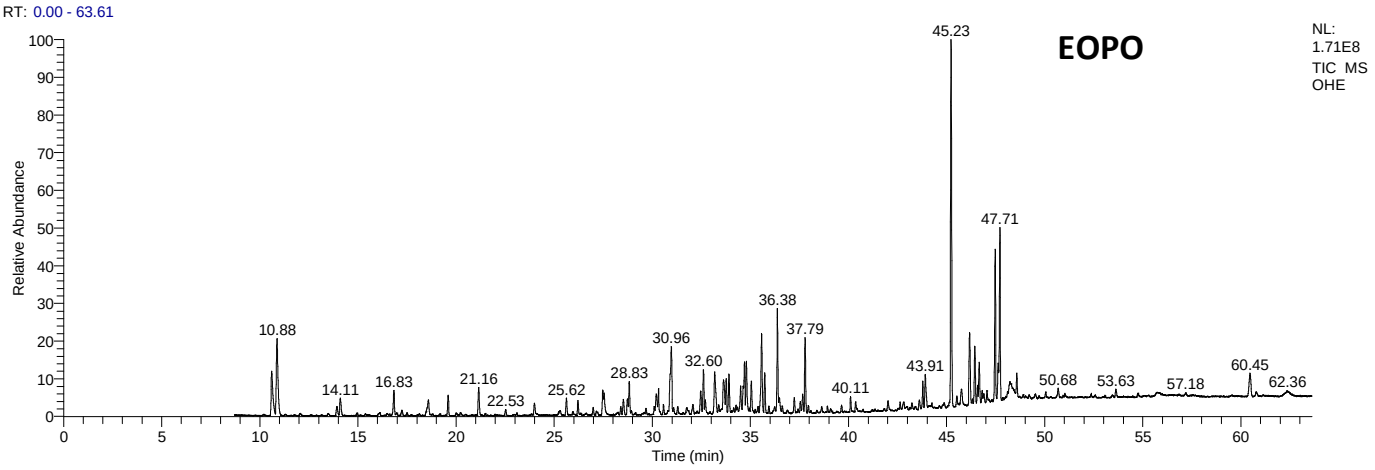






Annexe 2. GC-MS chromatograms

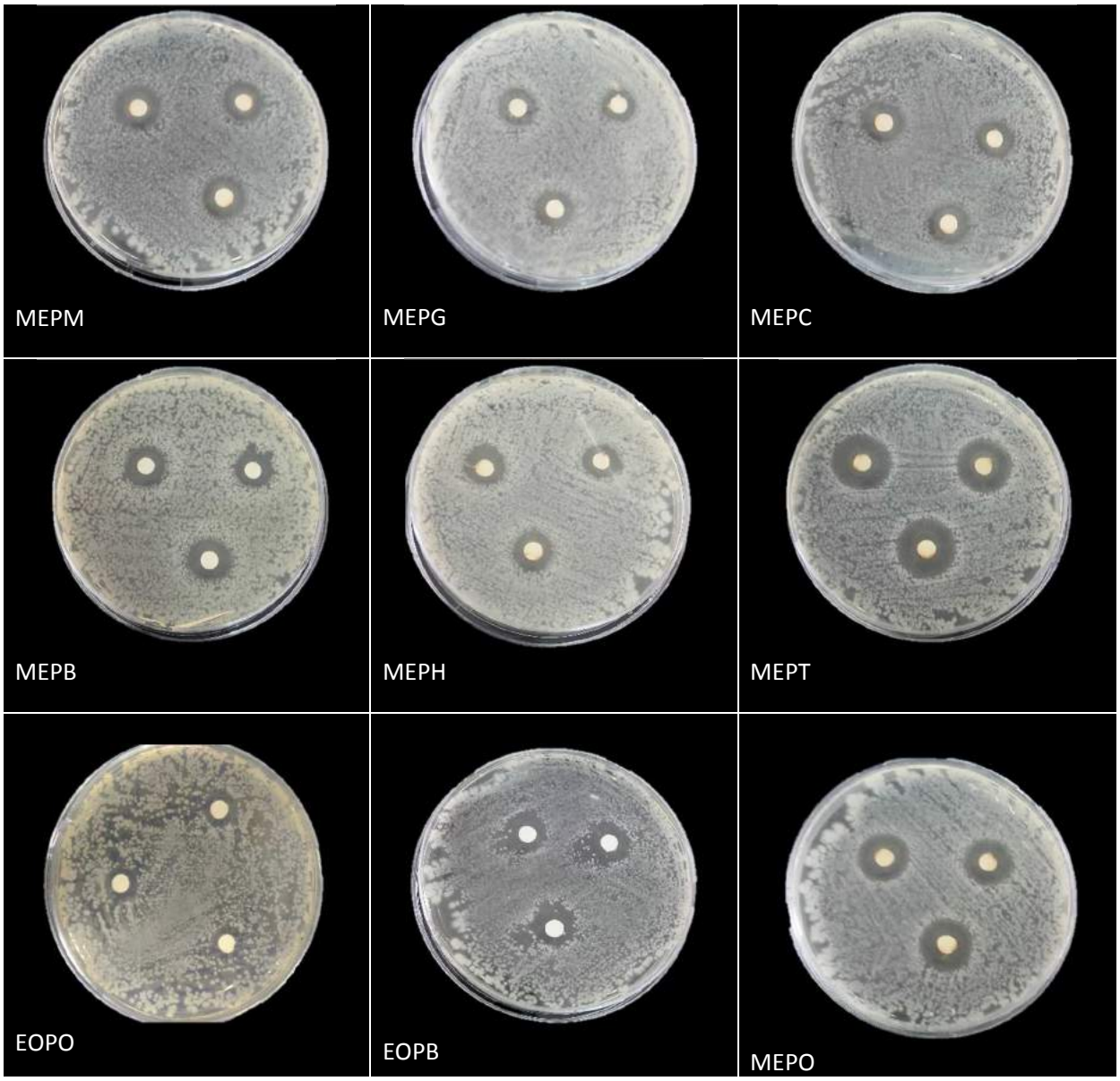




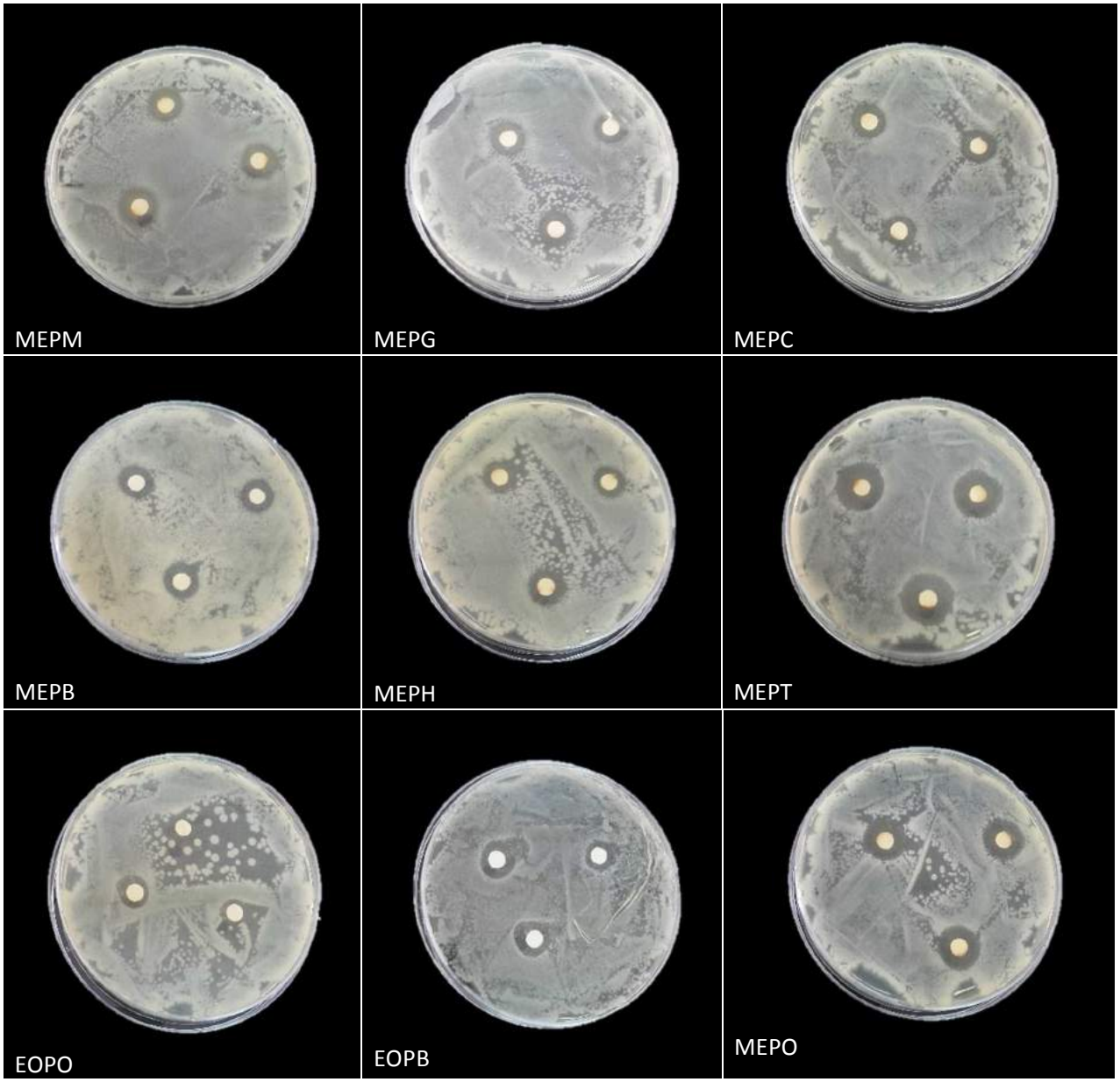
Annexe 3. Pearson's correlation coefficient (*r*) between TPC, TFC and biological activities

Biological activity	**TPC**	**TFC**
DPPH scavenging	0.7241	0.9004
ABTS scavenging	0.7328	0.8964
Superoxide scavenging	0.8340	0.1213
CUPRAC	0.7750	0.6126
Ferric reducing	-0.1907	0.08391
Lipid peroxidation	-0.04886	0.2658
Alpha glucosidase inhibition	0.4417	-0.08036
AChE inhibition	0.2421	0.7552
BChE inhibition	0.3786	0.6401
Toxicity	-0.1486	0.4986
Cytotoxicity against HepG2	0.6796	0.8584
***B. subtilis* RSKK 244**	-0.3507	0.08589
***B. cereus* RSKK 863**	-0.3472	-0.09683
***S. aureus* ATCC 25923**	-0.1564	-0.3889
***S. enteritidis* ATCC 13076**	-0.7907	-0.7719
***E. coli* ATCC 11229**	0.003993	0.2188
***E. coli* O157:H7**	0.5048	0.2689
***P. aeruginosa* ATCC 27853**	-0.02601	-0.1584
***C. albicans* ATCC 10231**	-0.7994	-0.5406

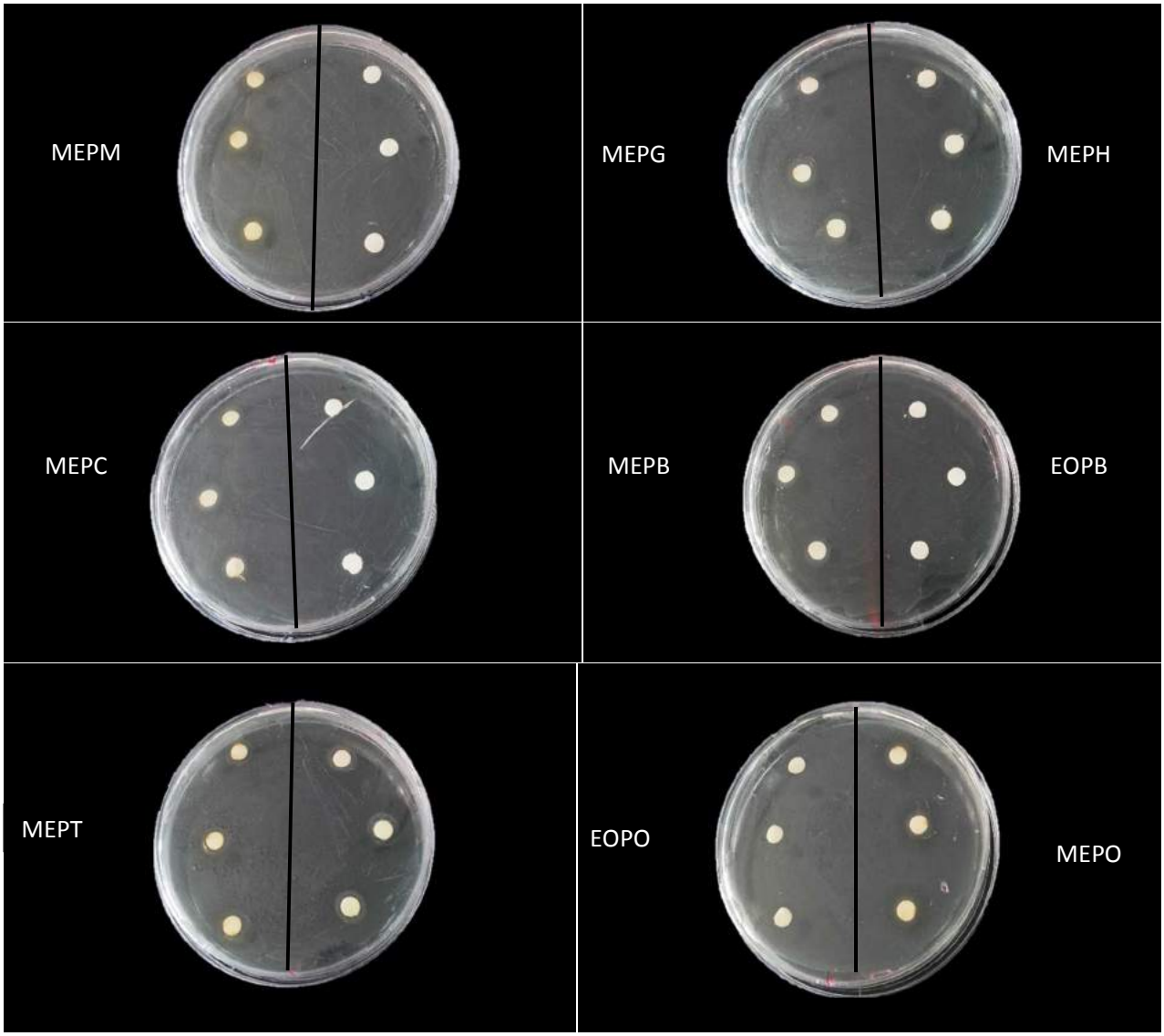
Annexe 4: Antibacterial activity images of propolis methanolic extracts and essential oils against *Bacillus cereus* RSKK 863



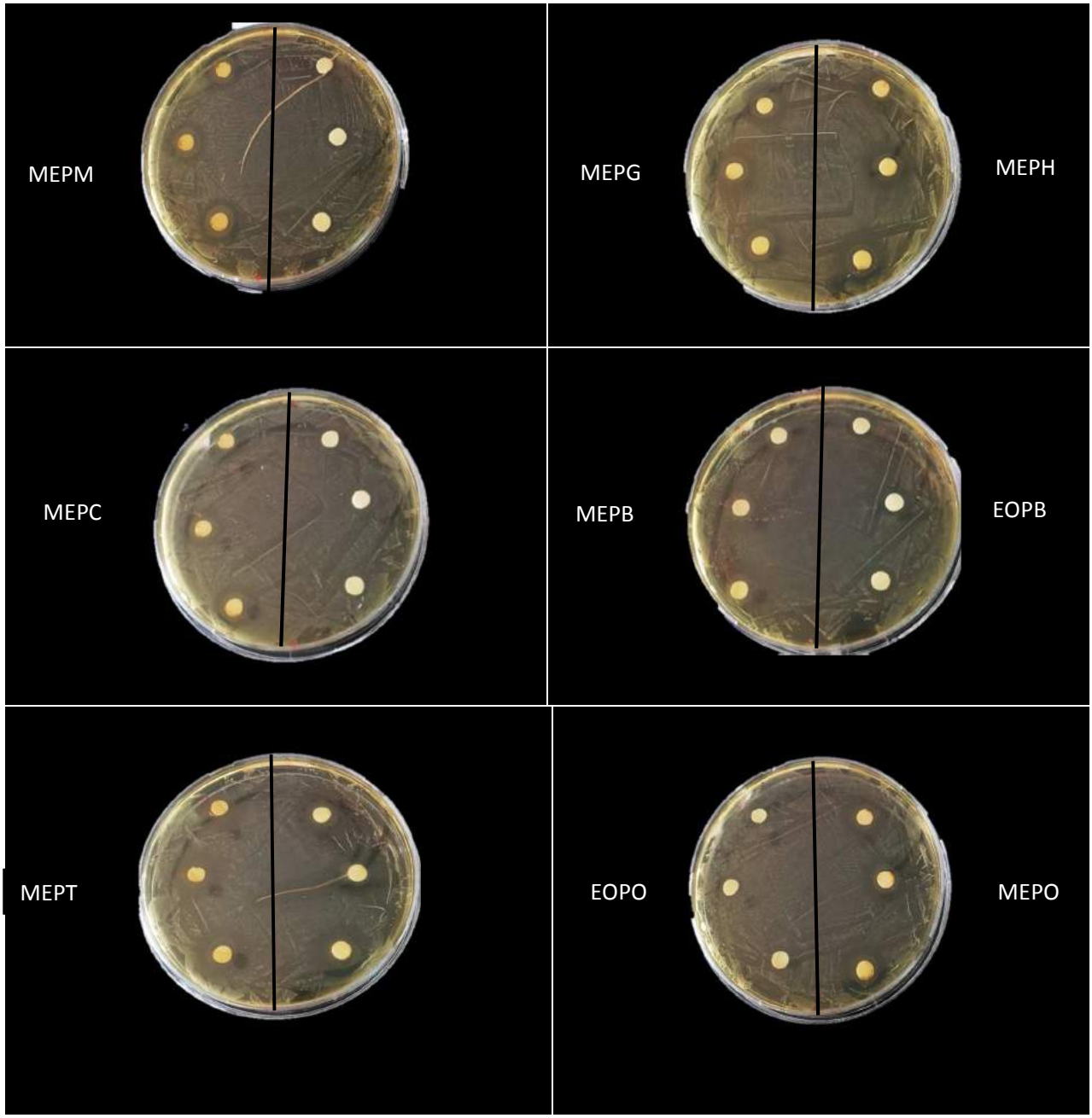
Annexe 5: Antibacterial activity images of propolis methanolic extracts and essential oils against *Bacillus subtilis* RSKK 224



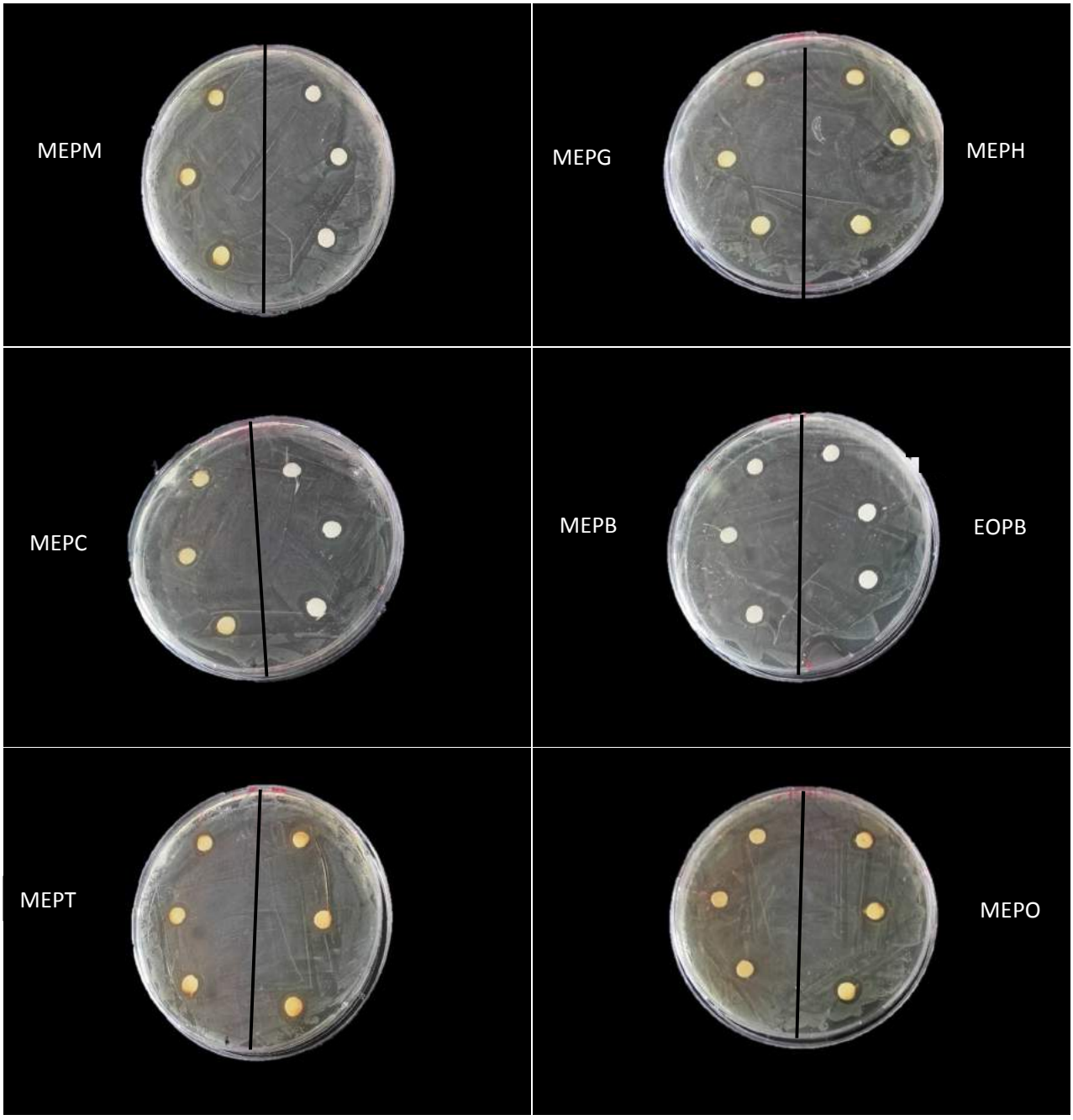
Annexe 6: Antibacterial activity images of propolis methanolic extracts and essential oils against *Staphylococcus aureus* ATCC 25923



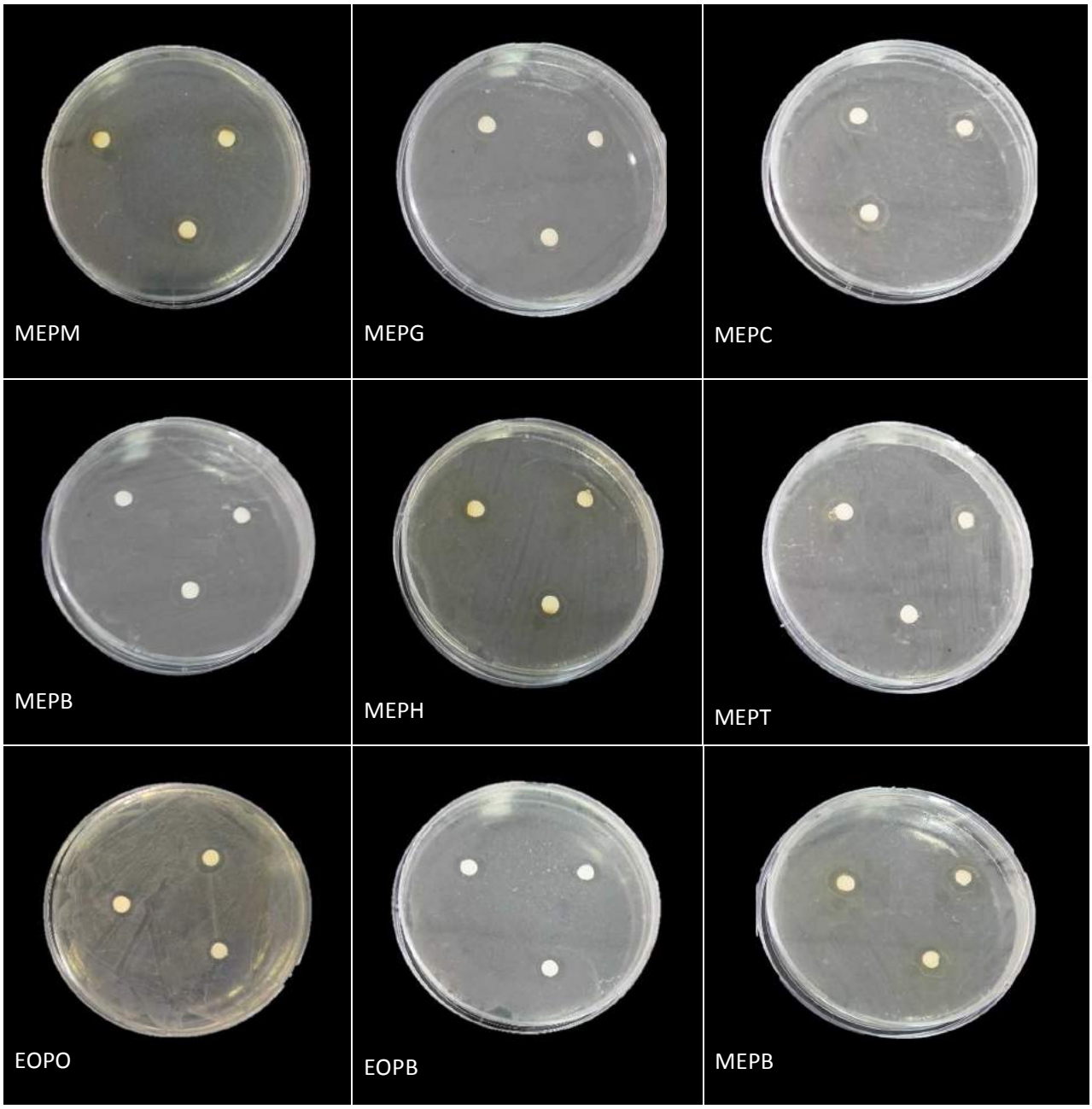
Annexe 7: Antifungal activity images of propolis methanolic extracts and essential oils against *Candida Albicans* ATTCC 10231



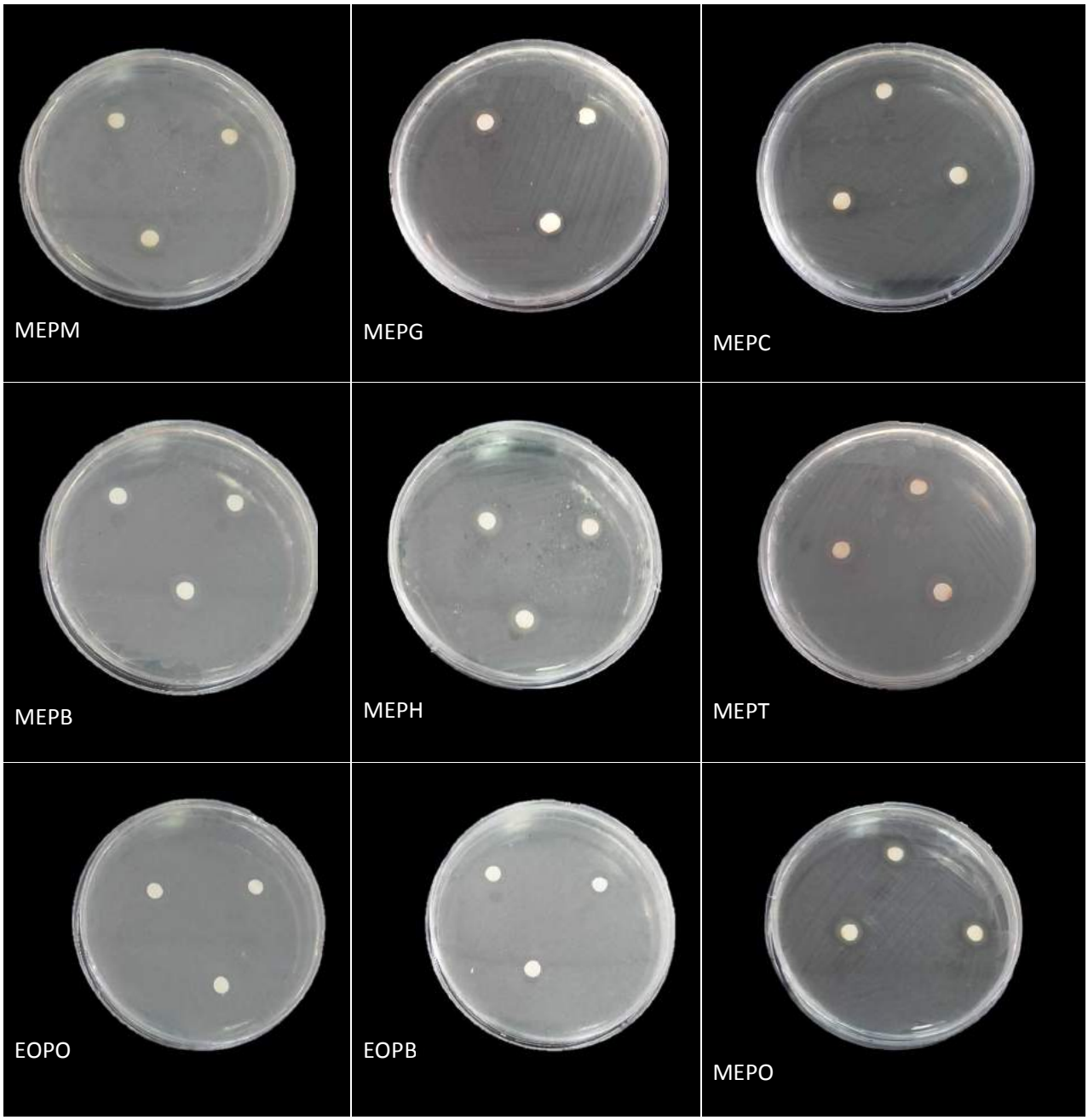
Annexe 8: Antibacterial activity images of propolis methanolic extracts and essential oils against *Escherichia coli O157:H7*



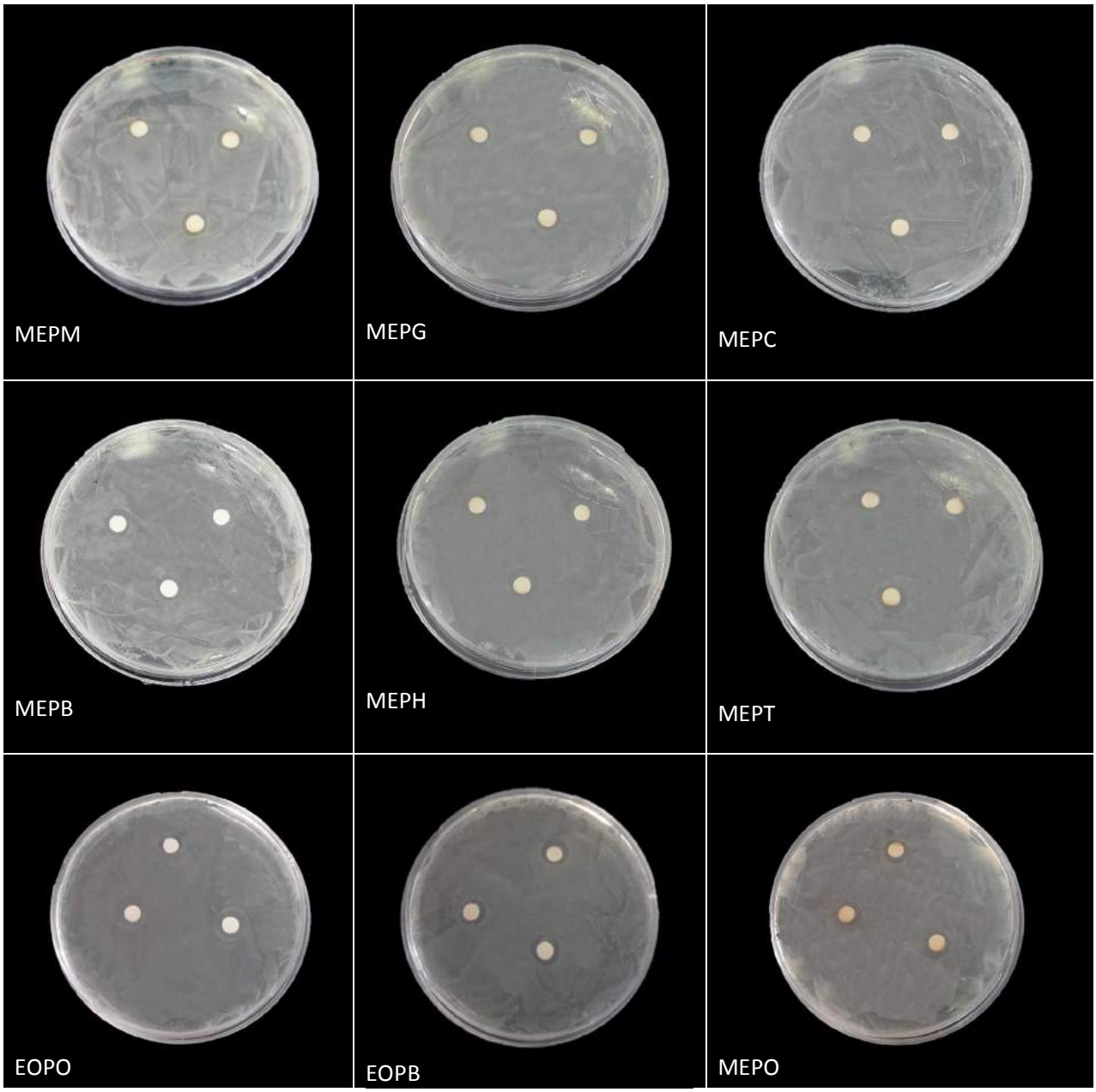
Annexe 9: Antibacterial activity images of propolis methanolic extracts and essential oils against *Salmonella enteritidis* ATCC 13076



Annexe 10: Antibacterial activity images of propolis methanolic extracts and essential oils against *Escherichia coli* ATCC 11229



Annexe 11: Antibacterial activity images of propolis methanolic extracts and essential oils against *Pseudomonas aeruginosa* ATCC 27853



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Investigation of Antioxidant and Anticholinesterase Potential of Essential Oil and Methanolic Extract of Propolis from Mila Region

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Abstract: The essential oil (EO) obtained by hydrodistillation of the propolis of Grarem located in Mila region (East of Algeria) have been studied by GC/MS. Seventy eight compounds were identified representing 93.52 % of the essential oils. The main constituents were: *p*-Mentha-1,5-dien-8-ol (23.69 %), α-Pinene (9.50 %), Bornyl acetate (9.13 %), β-Pinene (4.02 %) and Spathulenol (3.47 %). The total phenolic and flavonoid contents of propolis methanolic extract were evaluated according to the Folin-Ciocalteu procedure, and a colorimetric method. The total phenolic amount was 279.72 ± 2.07 µg GAE/mg E, whereas the flavonoids were 60.43 ± 0.65 µg QE/mg E. The antioxidant activity of propolis essential oil and methanolic extract were determined using ABTS radical scavenging and β-carotene-linoleic acid assays. Anticholinesterase activity was screened against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) which are the chief enzymes of Alzheimer's disease. Both of EO and MeOH extract had capability to inhibit BChE more than AChE enzyme, whereas propolis methanolic extract exhibited highest inhibitory activity (IC_{50} value: 20.30 ± 0.52 µg/mL) against BChE, even higher than galantamine (IC_{50} value: 34.75 ± 1.99 µg/mL).

Key words: Propolis; essential oil; GC-MS; methanolic extract; antioxidant, anticholinesterase.

Introduction

Alzheimer's disease (AD) is a severe disease of the central nervous system involving the loss of cholinergic neurons [1], an imbalance of the AChE and BChE activity and the deposition of intra- and extracellular β-amyloid in the brain tissue [2]. It is a progressive neurodegenerative disorder clinically characterized by loss of memory and cognition. Cholinergic deficit and oxidative stress have been implicated in the pathogenesis of AD. Therefore, inhibition of AChE and oxidation are the two promising strategies in the de-

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velopment of drug for AD [3]. Oxidative stress is directly related to neurodegenerative diseases; therefore, the antioxidant potentials of various natural products extracts can be helpful to provide neuroprotection [4]. According to Rahman & Choudhary compound having acted as antioxidants may be used in the treatment of neuronal diseases [5].

Propolis is a complex resinous material produced by bees from several plant exudates. *Apis mellifera* species obtain their propolis by addition of waxes, salivary secretions, or gummy and balsamic material collected from various plant species [6]. It contains a large number of biologically active components including different flavonoids, polyphenolic esters, terpenoids, steroids, amino acids, caffeic acids and their esters [7]. The flavonoids and polyphenolic compounds are the major constituents of propolis making 45-55 % in most samples from different countries [8]. Volatile compounds are known to be present in low concentration in propolis but their aroma and variety of biological activities make them valuable [9, 10]. Propolis has plenty of biological and pharmacological properties such as anti-inflammatory [11-14] antimicrobial, antioxidant, antitumor [15] and neuroprotective activity [16, 17].

In continuation of our research on propolis of Algeria [18-20] and considering the above-mentioned facts, this study was conducted to investigate the chemical composition and antioxidant and anticholinesterase activities of essential oil and methanolic extract of propolis collected in April 2015 from Grarem located in Mila region (East of Algeria).

Experimental

Material

Extraction of methanolic extract

The methanolic extract was obtained by the methodology described by Park & Ikegaki [21]. Air-dried powdered material (20 g) of propolis was extracted three times with 200 mL hydroalcoholic solution (80 % MeOH, 20 % Distillated water) for 72 h. After filtration, the filtrate was evaporated, concentrated and was stored under dry conditions at 4°C until analyzed. The yield was 9.5 %.

Determination of total phenolic content

Total phenols were assayed according to Singleton & Rossi [22]. Briefly, a 200 µL of diluted extract (0.5 mg/mL) was added to 1 mL of Folin-Ciocalteu reagent. After incubation in the dark for 4 min, 800 µL of 7.5 % Na_2CO_3 was added. After incubation in the dark for 2 h, absorbance at 765 nm was read versus a prepared blank. The total phenol content of propolis extract was expressed as micrograms of Gallic acid equivalents per milligram of extract (µg GAE/mg E) from a calibration curve with Gallic acid.

Determination of flavonoids content

The Aluminum chloride colorimetric method was used to measure the flavonoids content of all plant extracts [23]. 1 mL of Extract solution (0.5 mg/mL) of propolis was added to 1 mL of 2 % Aluminium chloride. After incubation in the dark for 10 min, the absorbance of the reaction mixture was measured at 430 nm with a UV/VIS spectrophotometer immediately. Quercetin was used as the standard for the calibration curve. Flavonoids content were expressed as µg of Quercetin equivalent (QE)/mg of extract.

Extraction of essential oil

The essential oil of Mila propolis (100 g) was obtained via hydrodistillation by using a Clevenger type apparatus for 3 h. The oil was dried over anhydrous sodium sulphate and stored under nitrogen until required. The yield of essential oil was 0.09 % (volume/dry-weight).

Gas chromatography analysis

GC analysis of the oil were performed using a Shimadzu GC-17 AAF, V3, 230V LV Series (Kyoto, Japan) gas chromatography, equipped with a FID and a DB-1 fused silica column [30 m x 0.25 mm (i.d.), film thickness 0.25 µm]; the oven temperature was held at 60°C for 5 min, then programmed to 240°C at 4°C/min and held isothermal for 10 min; injector and detector temperatures were 250°C and 270°C respectively; carrier gas was He at a flow rate of 1.3 mL/min; Sample size, 1.0 µL; split ratio, 50:1. The percentage composition of the essential oil was determined with a Class-GC 10 computer program.

Gas chromatography-mass spectrometry (GC-MS)

The analysis of the essential oil was performed using a Varian Saturn 2100 (Old York Rd., Ringoes, NJ, USA), ion trap machine, equipped with a DB-1 MS fused silica non-polar capillary column [30 m x 0.25 mm (i.d.),film thickness 0.25 µm]. Carrier gas was helium at a flow rate of 1.4 mL/min. The oven temperature was held at 60°C for 5 min, then increased up to 240°C with 4°C/min increments and held at this temperature for 10 min. Injector and transfer line temperatures were set at 250°C and 180°C, respectively. Ion trap temperature was 200°C. The injection volume was 0.2 µL and split ratio was 1:30. EI-MS measurements were taken at 70 eV ionization energy. Mass range was from *m/z* 28 to 650 amu. Scan time was 0.5 s with 0.1 s inter scan delays. Identification of components of the essential oils was based on GC retention indices and computer matching with the Wiley, NIST-2005 and TRLIB Library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and, whenever possible, by co-injection with authentic compounds. GC and GC-MS spectra were performed at the Department of Chemistry, Faculty of Sciences, Mugla Sitki Koçman University [24].

Biological activities

Antioxidant activity

Two methods were used to evaluate the antioxidant activity of methanolic extract and essential oil obtained from propolis: ABTS and β-Carotene-linoleic acid assay.

ABTS cation radical scavenging assay

The ABTS scavenging activity was done by the method of Re *et al.* [25], with slight modifications. The ABTS was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days with storage in the dark at room temperature. Before usage, the ABTS solution was diluted with ethanol to get an absorbance of 0.700±0.020 at 734 nm. Then, 160 µL of ABTS solution were added to 40 µL of sample solution in methanol at different concentrations. After 10 min, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance (methanol). The scavenging capability of ABTS was calculated using the following equation:

$ABTS^{\bullet+}$ scavenging activity (%) = [(A Control - A Sample) /A Control]×100

β-Carotene/linoleic acid bleaching assay

The slightly modified β-Carotene-linoleic acid test system was used to assay lipid-peroxidation inhibitory activity [26]. β-Carotene (0.5 mg) in 1 mL of chloroform and 25 µL of linoleic acid were dissolved in 200 µL of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 50 mL of distilled water saturated with oxygen, were added by vigorous shaking. The assay mixture, containing 160 µL β-carotene emulsion and 40 µL methanolic extract or EO, was incubated at 45°C. The decrease in the absorbance of β-carotene was measured at 470 nm for 120 min at 30-min intervals. The antioxidant activity was expressed as percent inhibition relative to the control after a 120 min incubation using the equation:

$$AA\ (\%) = [1- (A_{H0} - A_{Ht}) / (A_{C0} - A_{Ct})] \times 100$$

Where AA is the antioxidant activity, A_{H0} is the initial absorbance at time 0 in the presence of the sample, A_{Ht} is the absorbance at time 120 min in the presence of the sample, A_{C0} is the initial absorbance at time 0 in the presence of the control and A_{Ct} is the absorbance at time 120 in the presence of the control.

Anticholinesterase activity

The inhibition activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured by spectrophotometric method developed by Ellman & al., in 1961 [27], with slight modification [28]. AChE from electric eel and BChE from horse serum were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. Ellman's Reagent 5,5'-dithio-bis-[2-nitrobenzoic acid]

(DTNB) was used for the measurement of the activity. Galantamine was used as a positive reference compound. The results were given as IC_{50} value (mg/mL) corresponding to the concentration shows 50 % inhibition.

Statistical analysis

The antioxidant and the anticholinesterase activity assays were performed in triplicate analyses. The data were recorded as means ± standard error meaning. Student's t-test were used to determine the significant differences between means; $p < 0.05$ were regarded as significant. Microsoft Office Excel 2010® Program was employed for statistical analysis.

Results and discussion

Total phenolic and flavonoid contents

The total phenolic contents of MeOH extract of propolis of Grarem was performed spectrophotometrically using Gallic acid as standard compound. The phenolic content was 279.72 ± 2.07 µg GAE /mg E. Compared to the study carried out by Belfar *et al.* [29] on methaolic extracts of Algerian propolis, our result is in accordance with the polyphenol contents of propolis from Boumerdes (262.338 ± 0.810 µg GAE /mg E), and higher than those from other regions: Ghardaia (185.074 ± 1.336 µg GAE /mg E) and Bejaia (81.141 ± 0.538 µg GAE /mg E). A study carried out by Nedji and Loucif-Ayad [30] on ethanolic extracts of propolis from four regions of Annaba, North easten Algeria: Seraidi, Chetaibi, Berrehal and El-Bouni, the total polyphenol content ranged between 100.90-257.40 µg GAE /mg E.

In the present study the total flavonoids content of MeOH extract of propolis was 60.43 ± 0.65 µg Q/mg E. A larger variability in flavonoid contents was shown in propolis collected in different regions of Algeria (Bejaia: 19.626 ± 0.301 µg Q/mg E Ghardaia: 74.827 ± 0.995 µg Q/mg E and Boumerdes: 210.884 ± 0.754 µg Q/mg E). In addition, Propolis ethanolic extracts from Annaba contained flavonoids at levels of 58.99-91.44 µg Q/mg E. However, Propolis contains a wide variety of phenolic compounds, mainly flavonoids. Variation in the flavonoid content of propolis is mainly attributable to the difference in the preferred regional plants collected by honeybees [31].

Chemical composition of propolis essential oil

The essential oil obtained by hydrodistillation of propolis of Mila region (East of Algeria) have been studied by GC/MS. Seventy eight compounds were identified representing 93.52 of the essential oil. The composition of volatile oil is given in table 1.

The main constituents were: *p*-mentha-1,5-dien-8-ol (23.69 %), α-Pinene (9.50 %), bornyl acetate (9.13 %), β-pinene (4.02 %) and spathulenol (3.47 %).

As shown in the table 2, oxygenated monoterpenes are the main constituents (43.07 %) of the monoterpene group followed by monoterpenic hydrocarbons fraction (23.80 %). While Sesquiterpenic hydrocarbons (15.75 %) are more abundant than oxygenated sesquiterpenes (8.58 %). It is observed that diterpenes were less representative (0.23 %).

When our findings were compared with the literature, the results showed some qualitative and quantitative differences between the compounds of the essential oils of propolis from other localities of Algeria. The volatile fraction of propolis from El-malha (Mila) was dominated by 2-hexenal, myristic acid, linoleic acid and spathulenol, whereas in propolis from Benibelaîd (Jijel) isooctane, linoleic acid, undecane, myristic acid, hexadecane, *p*-cymene, palmitic acid and 4-terpineol dominated; and the major constituents of the essential oil of propolis of Kaous (Jijel) were 2-hexenal, myristic acid, linoleic acid, carvacrol, α-cedrol and *p*-cymene [18]. It is interesting to note that chemical composition depends on many factors (type of bee, flora accessed, environment, management, season, vegetation and geographical area of collection [6, 32].

Biological activities

Antioxidant activities

Many studies demonstrate that antioxidants protect against the chronic disease and aging by inhibiting or reducing the oxidation processes that produce free radicals [33]. In order to determine the antioxidant activity of the essential oil and the

Table 1. Constituents of the essential oil of propolis of Mila region

Peaks	Compounds	%	KI
1	Santolina triene	0.33	908
2	α-Thujene	0.27	930
3	α-Pinene	9.50	939
4	Camphene	1.70	954
5	Thuja-2,4(10)-diene	0.32	960
6	Sabinene	0.94	975
7	β-Pinene	4.02	979
8	α-Phellandrene	0.94	1002
9	α-Terpinene	0.62	1017
10	o-Cymene	0.55	1026
11	Limonene	2.78	1029
12	γ-Terpinene	1.51	1059
13	Cymenene <meta->	0.17	1085
14	Terpinolene	0.43	1088
15	Heptenol acetate <(3Z)->	0.64	1099
16	α-Campholenal	0.80	1126
17	*trans*-Pinocarveol	0.66	1139
18	*trans*-Verbenol	0.66	1144
19	Pinocarvone	0.62	1164
20	*p*-Mentha-1,5-dien-8-ol	23.69	1181
21	Menthol <iso->	1.22	1182
22	Thuj-3-en-10-al	0.66	1184
23	α-Terpineol	0.27	1188
24	Myrtenol	0.25	1195
25	Safranal	0.30	1196
26	Dihydro carvone <trans->	1.45	1200
27	*cis*-Carveol	0.19	1229
28	Ascaridole	0.12	1237
29	Carvone	0.12	1243
30	Phenylethyl acetate <2->	0.01	1256
31	Thujanol acetat <iso-3->	0.23	1270
32	Bornyl acetate	9.13	1288
33	α-Terpinyl acetate	2.69	1349
34	α-Cubebene	0.65	1352
35	Isoledene	0.45	1374
36	α-Copaene	0.64	1376
37	β-Bourbonene	0.67	1385
38	β-Cubebene	0.16	1390
39	Sibirene	0.19	1399
40	α-Gurjunene	0.54	1409
41	β-Copaene	0.55	1432
42	Aromadendrene	0.35	1441
43	α-Himachalene	0.60	1451
44	Alloaromadendrene	0.45	1460
45	Cadina-1(6),4-diene<cis->	0.37	1463
46	γ-Muurolene	0.22	1479

table 1. (continued).

Peaks	Compounds	%	KI
47	Germacrene D	1.52	1481
48	α-Amorphene	1.03	1484
49	β-Selinene	2.04	1490
50	Muurola-4(14),diene <trans->	0.22	1493
51	γ-Amorphene	0.69	1495
52	α-Muurolene	0.55	1496
53	δ-Amorphene	0.15	1510
54	γ-Cadinene	0.69	1513
55	Calamenene	0.25	1520
56	δ-Cadinene	2.02	1523
57	α-Calacorene	0.21	1545
58	β-Calacorene	0.54	1565
59	Spathulenol	3.47	1578
60	Caryophylene oxide	0.89	1582
61	Cedrol	0.39	1599
62	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	0.39	1609
63	Cubenol<1,10-di-epi->	0.23	1622
64	γ-Eudesmol	0.19	1632
65	Hexenyl phenyl acetate <(3Z)->	0.10	1634
66	tau.-Cadinol	0.09	1640
67	Cubenol	0.08	1646
68	β-Eudesmol	0.51	1649
69	Eudesmol <7-epi-α->	0.95	1663
70	Eudesma-4(15)7,dien-1β-ol	1.12	1688
71	*n*-Nonadecane	0.12	1900
72	Manoyl oxide	0.06	1987
73	Abietatriene	0.03	2056
74	Heneicosane	0.22	2100
75	Totarol	0.14	2314
76	Octadecoxyethanol	0.48	2328
77	*n*-Tetracosane	0.19	2400
78	*n*-Pentacosane	0.33	2500
		93.52	

Table 2. Composition of particular classes of compounds in Propolis essential oil

Grouped components	%
Monoterpenic hydrocarbons	23.80
Oxygenated monoterpenes	43.07
Sesquiterpenic hydrocarbons	15.75
Oxygenated sesquiterpene	8.58
Oxygenated ditertpène	0.20
Ditertpenichydrocarbons	0.03
Hydrocarbures	0.86
Others	1.23

methanolic extract of propolis, ABTS cation radical scavenging and lipid peroxidation inhibition by β-carotene-linoleic acid assays were used.

As shown in Table 3, MeOH extract was more active than EO in both assays (ABTS and β-carotene-linoleic acid). However, none of the extracts exhibited higher activity than those of antioxidant standards (BHT and BHA). The IC_{50} value of MeOH extract and EO were found to be 10.08 ± 0.11 µg/mL and 516.05 ± 11.66 µg/mL against ABTS respectively, 43.46 ± 0.03 µg/mL and 198.01 ± 6.71 µg/mL against lipid peroxidation inhibition by β-carotene-linoleic acid respectively. These results indicate that propolis MeOH extract has strong scavenging power for ABTS radicals. It may be related to its contents from total polyphenol and flavonoid. Relevantly, Propolis contains a wide variety of phenolic compounds, mainly phenolic acids and flavonoids. Indeed, flavonoids and various phenolic compounds have already been studied as antioxidants and demonstrated to be very active [34, 35]. However, the best antioxidant activity of propolis essential oil was observed with the β-carotene-linoleic acid assay. Previous studies have shown that propolis essential oil inhibits lipid peroxidation [36, 37]. Relevantly, it has been reported that oxygenated monoterpenes and monoterpene hydrocarbons are mainly responsible for the antioxidant potential of essential oil [38]. However, we can not attribute the antioxidant effect of a total essential oil only to the major compounds, minor molecules may make significant contributions to the oil activity [39].

Anticholinesterase activity

The anticholinesterase activity of the EO and MeOH extract of propolis against AChE and BChE enzymes was given in Table 4. Galantamine was the standard drug used for comparison. The preliminary screening of different concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200 µg/mL) of MeOH and EO showed inhibitory activity in a dose dependent manner. However, at high concentration of 200 µg/mL, MeOH and EO gave 66.56 ± 0.10 % and 43.96 ± 1.28 % inhibition of AChE, respectively and 77.07 ± 0.89 % and 72.36 % inhibition of BChE. The EO (IC_{50} value: > 200 µg/mL) and MeOH (IC_{50} value: 124.50 ± 2.46 µg/mL) showed less inhibitory activity against AChE compared with that of galantamine (IC_{50} value: 6.27 ± 1.15 µg/mL). The MeOH extract (IC_{50} value: 20.30 ± 0.52 µg/mL) exhibited highest inhibitory activity against BChE, even higher than galantamine (IC_{50} value: 34.75 ± 1.99 µg/mL). However, the EO had a some BChE inhibitory activity (IC_{50} value: 115.70 ± 6.59 µg/mL). Compared to previous reports, the IC_{50} value of propolis ethanolic extract from Marocco and Egypt ranged between 43±0.006 - 743±0.006 µg/mL and 360 - 600 µg/mL against AChE enzyme respectively [40, 41].

It is observed that Both of EO and methanolic extract of propolis had capability to inhibit BChE more than AChE enzyme. These results can be explained by the chemical constituents.In the present study we found that EO contains (-)-Carvone, Dihydrocarvone, β-Pinene, Menthol, α-

Table 3. **Antioxidant activities by β-carotene linoleic acid and ABTS of propolis methanolic extract and essential oil**

Samples	ABTS cation radical scavenging assay		β-Carotene/linoleic acid bleaching assay	
	Scavenging Activity (%)	IC_{50} Value (µg/mL)	Activity (%)	IC_{50} Value (µg/mL)
MeOH	92.03 ± 0.10	10.08 ± 0.11	91.98 ± 0.44	43.46 ± 0.03
EO	27.51 ± 0.26	516.05 ±11.66	48.51 ± 6.40	198.01± 6.71
BHT	96.68 ± 0.39	1.29 ± 0.30	95.28 ± 3.25	1.05 ± 0.01
BHA	95.39 ± 2.62	1.81 ± 0.10	99.76 ± 0.14	0.90 ± 0.02

All data are expressed as mean ± SD (n = 3)
Scavenging activity (%) was determined at 200 µg/mL

Table 4: **Anticholinesterase activity of methanolic extract (MeOH) and essential oil (EO) obtained from propoli**

Samples	Concentrations (µg/mL)	AChE inhibition (%)	IC_{50} (µg/mL)	BChE inhibition (%)	IC_{50} (µg/mL)
EO	3.125	-	> 200	-	115.7 ± 6.59
	6.25	-		-	
	12.5	-		-	
	25	-		6.70 ± 1.99	
	50	-		28.73 ± 1.02	
	100	21.29 ± 0.88		46.63 ± 1.11	
	200	43.96 ± 1.28		72.36 ± 0.82	
MeOH	3.125	11.80 ± 3.04	124.5 ± 2.46	10.11 ± 2.95	20.30 ± 0.52
	6.25	20.67 ± 2.96		13.70 ± 4.85	
	12.5	22.64 ± 2.87		37.61 ± 0.50	
	25	28.33 ± 2.64		57.53 ± 1.31	
	50	43.09 ± 3.69		66.57 ± 0.34	
	100	44.61 ± 0.72		73.08 ± 1.18	
	200	66.56 ± 0.10		77.07 ± 0.89	
Galanthamine [b]	3.125	35.93 ± 2.28	6.27 ± 1.15	3.26 ± 0.62	34.75 ± 1.99
	6.25	43.77 ± 0.00		6.93 ± 0.62	
	12.5	68.50 ± 0.31		24.03 ± 2.94	
	25	80.69 ± 0.41		45.13 ± 2.60	
	50	85.78 ± 1.63		63.87 ± 2.85	
	100	91.80 ± 0.20		73.57 ± 0.77	
	200	35.93 ± 2.28		78.95 ± 0.58	

[a] Values expressed as means ± S.D of three parallel measurements

[b] Reference compounds

pinene, γ-Terpinene, Camphene and Bornyl acetate. In the study carried out by Orhan *et al.* [42] , (-)-Carvone, Dihydrocarvone and β-Pinene showed a notable inhibitory activity against BChE more than AChE. Besides, Menthol exhibited significant inhibition on BChE (70.0 ± 0.71 %) and no effect against AChE. On the contrary, α-pinene showed a potent inhibitory activity against AChE (76.3 ± 1.27 %) more than BChE (23.5 ± 1.08 %). While, γ-Terpinene, Camphene and Bornyl acetate were not active against both enzymes [42]. However, the phytochemical study for the chemical constituents of Algerian propolis proved the presence of pectolinarigenin, pilosin, ladanein, Chrysin, apigenin, caffeic acid, ferulic acid, pinobanksin, caffeic acid phenethyl ester, chrysin, pinocembrin, galangin, phenethyl caffeate, cinnamyl caffeate, and tectochrysin [19, 43, 44]. Relevantly, it has been reported that ferulic acid, anthocyanins pelargonidin, delphinidin, cyanidin, flavones apigenin, luteolin, flavonols quercetin, kaempferol, myricetin, dihydrochalcone phloridzin and prenylated chalcone xanthohumol are the most efficient *in vitro* inhibitors of AChE and/or BChE [45, 46].

On the other hand, many studies showed that phenolic efficient cholinesterase inhibitors singly in the solution, but combined phenolic acids, as well as phenolic acids coupled with flavonoids, were less efficient inhibitors than could have been expected (calculated) from the sum of activities exerted by both compounds [47]. However, drug synergism and antagonism; can explain why a subfraction could have biological activity and some of its isolated compounds have no activity and vice versa [48].

Conclusion

It could be concluded that propolis is rich in bioactive substances with high antioxidant capacity. Also, our study revealed the ability of propolis essential oil and methanolic extract to inhibit AChE and BChE. Finally, the investigation of chemical composition and biological activities of propolis from unexplored regions of Algeria is considered a promising way to find and discover new bioactive agents.

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Chemical composition, antioxidant, and antimicrobial activities of two essential oils from Algerian propolis

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Abstract: This study aims to investigate the chemical composition, antioxidant, and antimicrobial activity of two essential oils (EOs) from Algerian propolis. The volatile constituents were analyzed by gas chromatography-mass spectrometry. Fifty components were identified from the oils. The major components were found to be: cedrol (17.0%), β-eudesmol (7.7%), and α-eudesmol (6.7%) in EO of propolis from Oum El Bouaghi (EOPO) whilst α-pinene (56.1%), *cis*-verbenol (6.0%), and cyclohexene,3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl (4.4%) in EO of propolis from Batna (EOPB). The antioxidant properties of EOPO and EOPB were determined using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS•+) and cupric reducing antioxidant capacity (CUPRAC assays), respectively. Both EOs had more cupric ion reducing ability than scavenging ABTS•+ radicals. The antimicrobial potential of the two EOs against eight pathogens was assayed by the agar diffusion method and the mode of action was determined by microdilution assay. The results revealed that EOPB was bactericidal for all tested pathogenic bacteria and fungicidal for *Candida albicans* ATCC 10231, whereas, EOPO showed bacteriostatic effect against *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* ATCC27853 and fungistatic effect against *C. albicans* ATCC 10231. Thus, the obtained results suggest the important use of propolis EOs as preservative agents.

Keywords: antimicrobial; antioxidant; essential oil; GC-MS; propolis.

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1 Introduction

The use of essential oils (EOs) as antioxidants, antimicrobials, and food preservative agents is of concern because of several reported side effects of synthetic oils, which have raised attention on various natural antioxidants and antimicrobials [1, 2]. Propolis is a natural product made by bee-released and plant-derived compounds [3]. Propolis is mainly composed of around 50% resins, 30% waxes, 10% EOs, 5% pollen, and 5% of various organic compounds [4–6]. Volatile compounds are present in low concentrations of propolis but their aroma and variety of biological activities make them valuable [7, 8]. It has been reported in the literature that propolis EOs have plenty of biological and pharmacological activities such as antibacterial, antifungal, antiparasitic, antioxidant, neuroprotective, and immunostimulatory effects [9–21]. The chemical profile of propolis volatiles has been little studied, especially Algerian propolis. Up to now, only a few data are available on the chemical composition of propolis EOs from humid and subhumid zones. It has been reported that monoterpenes were the most abundant constituents in propolis EOs from subhumid regions, while propolis volatiles from the humid regions were found to be rich in acids, hydrocarbons, alcohols, aldehydes, and ketones [22, 23]. No information, however, exists on propolis volatiles from semi-arid regions of Algeria. Since the biological properties of propolis are mainly attributed to the presence of active compounds, which are strongly dependent on the type of vegetation, the climatic conditions, and geographical origin [24], the

present study aimed to determine the chemical composition, the antioxidant, and the antimicrobial activity of two propolis volatiles from Oum El Bouaghi and Batna (semi-arid regions).

2 Materials and methods

2.1 Collection of propolis and extraction of essential oils (EOs)

Two propolis samples were collected, during September 2018, from *Apis mellifera* hives located at Northeastern semi-arid regions of Algeria. The collection sites are abbreviated as follows: P Propolis, O refers to P collected from the Oum El Bouaghi region, while B refers to P collected from the Batna region. After separation of impurities, crude propolis samples (100 g) were subjected to hydrodistillation using a Clevenger type apparatus for 3 h. The obtained oils EOPO (EO of propolis from Oum el Bouaghi) and EOPB (EO of propolis from Batna) were dried over anhydrous sodium sulfate and stored at 4 °C.

2.2 Gas chromatography-mass spectrometry (GC-MS) analysis

EOs were chemically characterized using a Thermo Scientific TRACE 1310 Gas Chromatography equipped with TriPlus RSH Autosampler (Thermo Scientific) and attached with ISQ LT single quadrupole Mass Spectrometer (Thermo Scientific). A thermo TG-WAXMS capillary column (60 m × 0.25 mm I.D., film thickness 0.25 µm), with the stationary phase of acid optimized Polyethylene Glycol, was used for the separation. The conditions of analysis were programmed as follows: a volume of 1 µL of the diluted sample (1/20 in chloroform, v/v) was injected at a split ratio of 1:12, helium was used as a carrier gas at 1.2 mL/min constant flow mode, injector temperature 230 °C, the oven temperature was programmed from 60 to 230 °C at 4 °C/min. Ion source and transfer line temperatures were maintained at 250 °C. Mass spectra were recorded in electronic impact mode at 70 eV ionization energy, scanning the range 50–500 *m*/*z*. The volatile compounds were identified by comparing their retention time (RT) or mass spectra with those of databases (Main library, Wiley 9, and NIST). Data analyses were performed using the Thermo Xcalibur software. The constituents were expressed as percentages from peak area normalization, assuming that the total injection was 100% of EO. The retention index was calculated from RTs relative to that of the *n*-alkane series and compared with those reported in the literature [25–28].

2.3 Antioxidant properties

Antioxidant activities of EOs were analyzed by using ABTS radical scavenging and CUPRAC assays.

2.3.1 ABTS•+ cation radical scavenging assay: The ABTS•+ radical scavenging activity was done by the method of Re et al. [29] with slight modifications. The ABTS•+ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. Before usage, the ABTS•+ solution was diluted with ethanol to get an absorbance of 0.70 ± 0.02 at 734 nm. Then, 160 µL of ABTS•+ solution was added to 40 µL of the sample solution in methanol at different concentrations. After 10 min, the percentage inhibition at 734 nm was calculated. The scavenging activity of ABTS•+ was calculated using the following equation:

$$I\% = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

I%: inhibition percentage, Abs: absorbance.
The results are given as IC_{50} value.

2.3.2 Cupric ion reducing antioxidant capacity (CUPRAC) assay: CUPRAC was determined according to the method developed by Apak et al. [30]. The method comprises mixing of 40 µL of sample solution with 60 µL of ammonium acetate aqueous buffer (pH 7), 50 µL of neocuproine alcoholic solution, and 50 µL of a copper (II) chloride solution. After 60 min, the absorbance was read at 450 nm. The results were given as $A_{0.50}$, which corresponds to the concentration producing 0.50 absorbance.

2.4 Antimicrobial activity

2.4.1 Test microorganisms: *In vitro* antimicrobial activity of propolis EOs was tested against eight pathogens. These included three Gram-positive bacteria (*Bacillus cereus* RSKK 863, *Bacillus subtilis* RSKK 244, and *Staphylococcus aureus* ATCC 25923), four Gram-negative bacteria (*Escherichia coli* ATCC 11229, *E. coli* O157:H7, *Salmonella enteritidis* ATCC 13076, and *Pseudomonas aeruginosa* ATCC 27853), and one yeast (*Candida albicans* ATCC 10231). Bacterial strains were cultured overnight at 37 °C in the nutrient broth while yeast was cultured for 48 h at 30 °C in Yeast Peptone Dextrose broth medium.

2.4.2 Preparation of propolis essential oil solutions: Ten milligrams of each propolis EO were dissolved in 1 mL of dimethyl sulphoxide to obtain a final concentration of 10 mg/mL. Then, the obtained solutions were sterilized by 0.45 µm Millipore filters.

2.4.3 Disc diffusion assay: The disc diffusion method was used to determine the antimicrobial potential of the investigated oils [31]. The culture suspensions were adjusted by comparing with 0.5 McFarland. Then, a volume of 100 µL of suspension was spread on agar plates. Thereafter, sterile 6-mm-diameter filter discs (Whatman paper n° 3) were placed on the inoculated plates and impregnated with 15 µL (150 µg/disc) of each EO solution. The treated Petri dishes were kept at 4 °C for 1 h to enable prediffusion of each EO into the agar. Finally, the inoculated plates were incubated at 37 °C for 24 h for bacterial strains and 30 °C for 48 h for yeast. Ampicillin (AM, 10 µg/disc), Kanamycin (K, 30 µg/disc) and Erythromycin (E, 15 µg/disc) were chosen as standard antibacterial while Fluconazole (FCA, 25 µg/disc) was chosen as a standard antifungal. The results were obtained by measuring the diameter of the growth inhibition zone surrounding the discs and expressed in mm.

2.4.4 Determination of minimum inhibitory (MIC), minimum bactericidal (MBC), and minimum fungicidal (MFC): The minimum inhibitory (MIC), minimum bactericidal (MBC), and minimum fungicidal

(MFC) concentrations of propolis oils were determined using two-fold microdilution method according to the protocol described by Koneman et al. [32] with slight modification. The EOs were added to each growth medium to obtain a final concentration of 8 µg/µl and diluted to 4, 2, 1, 0.5, and 0.25 µg/µL in tubes. The total volume was 100 µL in each tube. 1.25 µL of each tested bacteria or yeast (adjusted to 0.5 McFarland) were inoculated into each tube. The content of the tubes was mixed and they were incubated at appropriate temperatures for 24 and 48 h. The MIC value was defined as the lowest concentration of EOs, which inhibited bacterial or fungal growth. MBC and MFC were determined by sub-culturing 5 µL of the test dilutions from each clear tube on solid growth medium and incubating for 24 and 48 h at appropriate temperatures. The lowest concentration that did not show bacterial growth was defined as the MBC value whereas the MFC value was determined as the lowest concentration with no fungal growth. The results are expressed as µg/µL.

2.5 Statistical analysis

Results are reported as mean value ± SD of three measurements; the IC_{50} and $A_{0.50}$ values were calculated by linear regression analysis. The student's *t*-test was applied using Microsoft Excel to determine standard deviation and *p*-value. *p*-values > 0.05 indicated no significant differences while *p*-values < 0.05 were regarded as significant.

3 Results and discussion

3.1 Chemical composition of propolis essential oil

The hydrodistillation of propolis samples produced yellow EOs with a yield (% w/w) of 0.61% for EOPO and 0.27% for EOPB. The GC-MS analysis of the two volatile oils allowed the identification of a total of 50 compounds: 33 for EOPO (99.7% of the total oil) and 25 for EOPB (99.8% of the oil). The constituents of volatile oils are given in Table 1. Eight components were common between the oils of the samples, namely *α*-pinene, limonene, verbenol, *trans*-pinocarveol, *cis*-verbenol, *α*-terpinenyl acetate, cyclohexene,3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl, and *δ*-cadinene; but with different percentage values. The major constituents found in EOPO were cedrol (17.0%), *β*-eudesmol (7.7%), and *α*-eudesmol (6.7%), whereas *α*-pinene (56.1%), *cis*-verbenol (6.0%), and cyclohexene,3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl (4.4%) were mainly detected in EOPB.

Oxygenated sesquiterpenes (42.4%) and sesquiterpenic hydrocarbons (30.9%) were the main constituents of EOPO, whereas monoterpenic hydrocarbons (59.9%) and oxygenated monoterpenes (21.7%) were more abundant in EOPB. Compared to previous studies on EOs of Algerian propolis from other localities, there were some qualitative and quantitative differences. The chemical analysis of EOs of propolis collected from two sites in Mila (a sub-humid region) showed that the main constituents of EO of propolis from Grarem were: *p*-mentha-1,5-dien-8-ol, *α*-pinene, bornyl acetate, *β*-pinene, and spathulenol, whereas the volatile fraction of propolis from El-malha was dominated by 2-hexenal, myristic acid, linoleic acid, and spathulenol [22, 23]. Moreover, the chemical profile of EOs of propolis from two localities in Jijel (a humid region) revealed that isooctane, linoleic acid, undecane, myristic acid, hexadecane, *p*-cymene, palmitic acid, and 4-terpineol were predominant in propolis from Benibelaîd, whereas 2-hexenal, myristic acid, linoleic acid, carvacrol, *α*-cedrol, and *p*-cymene were more abundant in EO of Kaous [23]. These variations in the chemical composition, between the oils of the current study and those from the literature, depend on multiple factors such as the type of vegetation, climatic conditions, and geographical location, among others [24].

3.2 Antioxidant properties

The antioxidant capacity of the EOs from propolis was tested by using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and cupric reducing antioxidant capacity CUPRAC assays. The results revealed that the EOPO rich in sesquiterpenes had better values for the antioxidant activities than the EOPB rich in monoterpenes, which could explain the differences in the antioxidant activity between them (Table 2). Both oils showed more cupric ion reducing ability than scavenging ABTS•+ radicals. However, they were less active than the antioxidant standard (BHT).

Many reports have stated the strong antioxidant properties of propolis EOs [11, 14, 16–19]. In contrast, EOs in this study did not show strong antioxidant properties and this can be explained by their major constituents, the ones that have already had weak activity reported by the literature, such as cedrol and *α*-pinene [33], where in the current study cedrol is found to be the major constituent of EOPO and *α*-pinene was the main component of EOPB.

Nevertheless, the overall antioxidant activity of EOs is usually the result of interaction between all components [34]. This interaction may produce a synergistic effect, when the interaction enhances the effect of the oil, or antagonistic, when the interaction negatively affects the antioxidant potential of the oil in the study, which makes it very important to investigate the antioxidant properties of EOs without considering only its major constituents [35].

Table 1: Volatile components identified in EOPO and EOPB.

N°	Compounds	RI[a]	RI[b]	RT	EOPO (%)	EOPB (%)
1	Dimethylvinylcarbinol	601	621	10.61	5.3	–
2	2-Buten-1-ol, 3-methyl	762	773	10.76	1.4	–
3	*α*-Pinene	931	942	10.88	3.5	**56.1**
4	2- *α*-Pinene	948	953	13.49	–	1.0
5	*Delta*-3-Carene	1005	1021	14.12	–	1.8
6	Limonene	1020	1032	18.83	1.4	1.0
7	*o* -Cymene	1042	1051	19.60	–	0.9
8	Linalool	1082	1095	25.83	1.4	–
9	Verbenone	1119	1128	26.95	–	2.2
10	Camphor	1121	1132	28.55	–	0.9
11	*p*-Mentha-1,5-dien-8-ol	1125	1136	32.73	–	1.3
12	*trans*-Pinocarveol	1131	1142	33.60	2.0	2.7
13	Verbenol	1136	1151	34.47	1.1	1.8
14	*α*-Fenchyl alcohol	1138	1153	35.69	–	1.1
15	Borneol	1180	1187	35.91	1.7	–
16	*cis*-Verbenol	1188	1196	36.18	2.7	**6.0**
17	Myrtenol	1191	1198	36.50	–	1.0
18	1-Carveol	1206	1211	37.57	–	1.0
19	*α*-Copaene	1221	1226	38.47	1.1	–
20	Bornyl acetate	1277	1281	39.49	1.2	–
21	*α*-Terpinenyl acetate	1327	1336	40.76	1.4	0.8
22	Aromadendrene	1386	1391	41.63	2.2	–
23	*β*-Eudesmene	1432	1439	43.69	2.1	–
24	*γ*-Cadinene	1440	1448	45.73	1.6	–
25	Benzene,1-(1-formylethyl)-4-(1-buten-3-yl)-	1454	1456	46.81	–	0.7
26	*α*-Guaiene	1458	1461	47.66	1.8	–
27	Cyclohexene, 3-acetoxy-4-(1-hydroxy-1-methylethyl)-1-methyl	1471	1476	48.50	1.4	**4.4**
28	Eremophilene	1474	1479	49.80	2.3	–
29	*γ* -Gurjunene	1479	1482	51.38	4.9	–
30	*trans*-Caryophyllene	1494	1498	52.96	4.9	–
31	*α*-Muurolene	1496	1502	53.63	0.8	–
32	*α*-Campholene aldehyde	1500	1506	54.51	–	3.0
33	Cyclohexanemethanol,4-ethenyl-*α*,*α*,4-trimethyl-3-(1-methylethenyl)-,[1R-(1α,3α,4α)]-	1522	1532	55.91	1.9	–
34	*cis*-calamenene	1537	1542	57.79	3.4	–
35	Cedrol	1543	1548	58.23	**17.0**	–
36	*δ*-Cadinene	1556	1559	59.57	4.3	0.7
37	Cubenol	1580	1586	60.79	1.3	–
38	*β*-Eudesmol	1593	1597	61.71	**7.7**	–
39	Guaiol	1614	1621	62.17	3.8	–
40	Valencene	1713	1715	64.05	1.4	–
41	*α*-D-Mannofuranoside, farnesyl-	1870	1876	66.95	–	0.9
42	*τ*-Muurolol	2178	2181	68.62	1.5	–
43	Bulnesol	2202	2210	69.44	2.5	–
44	*α*-Eudesmol	2237	2241	71.47	**6.7**	–
45	Glycerol 1-palmitate	2482	2486	72.50	–	1.7
46	6,9,12,15-Docosatetraenoic acid,methylester	2507	2510	74.25	–	1.1
47	Octaethylene glycol monododecyl ether	2628	2631	75.53	–	1.5
48	Finasteride	2689	2692	77.78	–	2.7
49	15,15′-*Bi*-1,4,7,10,13-pentaoxacyclohexadecane	3629	3633	78.43	–	3.5
50	1-Heptatriacotanol	3942	3949	79.45	1.9	–
	Total identified compounds (%)				**99.7**	**99.8**
	Monoterpenic hydrocarbons				4.9	59.9
	Oxygenated monoterpenes				12.9	21.7
	Sesquiterpenic hydrocarbons				30.9	0.7
	Oxygenated sesquiterpenes				42.4	–
	Others				8.6	17.5

RI[a]: Retention Index from literature; RI[b]: Retention Index calculated from retention times relative to that of *n*-alkane series; RT, Retention time (min). Values in bold correspond to the major components of the EOs.

Table 2: Antioxidant activities of propolis essential oils by ABTS and CUPRAC assays.

Samples	ABTS assay IC_{50} µg/mL	CUPRAC assay $A_{0.50}$ µg/mL
EOPB	>800	651.11 ± 15.40^{c}
EOPO	505.28 ± 19.02^{b}	351.52 ± 14.71^{b}
BHT	1.29 ± 0.30^{a}	8.97 ± 3.94^{a}

BHT: butylated hydroxytoluene. Data are expressed as Mean ± SD of three parallel measurements. The values with different superscripts (a, b, or c) in the same columns are significantly different ($p < 0.05$).

3.3 Antimicrobial activity

Antimicrobial efficiency is one of the most important properties of propolis that is considered a potent chemical weapon against bacteria, viruses, and other pathogenic microorganisms that may invade the bee colony [36]. In the current study, the antimicrobial activity of EOPB and EOPO, against *B. cereus* RSKK 863, *B. subtilis* RSKK 244, *S. aureus* ATCC 25923, *E. coli* ATCC 11229, *E. coli* O157:H7, *S. enteritidis* ATCC 13076 *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231, was qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters, MIC, MBC, and MFC values. The antimicrobial properties were initially estimated by the disc diffusion method for primary screening (Table 3).

The results indicated that both EOPB and EOPO inhibited the growth of all tested microorganisms with inhibition zones ranged between 8.09 ± 0.29–14.20 ± 0.50 mm). This is in agreement with previous studies that have proven the potential antimicrobial activity of propolis EOs against the above-mentioned pathogens [9–13]. The highest antibacterial activity was exhibited by EOPB against *B. cereus* RSKK 863 with an inhibition zone diameter of 14.20 ± 0.50 mm followed by 12.90 ± 0.70 mm against *B. subtilis* RSKK 244 and a marked activity (11.51 ± 0.85 mm) against *P. aeruginosa* ATCC 27853 compared with Erythromycin (11.77 ± 0.58 mm) while Ampicillin failed to inhibit *P. aeruginosa*.

However, the highest inhibition activity against *S. aureus* ATCC 25923 was shown by EOPO with a diameter of 9.68 ± 0.18 mm, but still lower than the result of Kujumgiev et al. who reported inhibition zone diameters against *S. aureus* ranged from 11.29 ± 0.3–23.09 ± 1.3 mm [9]. EOPO and EOPB exhibited antifungal activity against *C. albicans* ATCC 10231 with inhibition zone diameters of 9.93 ± 0.28 and 9.37 ± 0.41 mm, respectively. Both EOs were less active than FCA (25 µg/disc). However, appropriate concentrations of both EOs may be used as natural antifungal source alternatives to synthetic drugs.

The MIC, MBC, MFC, MBC/MIC, and MFC/MIC ratios were estimated using the broth microdilution method. The results are given in Table 4. Both propolis EOs showed antimicrobial effect against all tested microorganisms with MIC values of EOPO and EOPB ranging from 0.25–1 and 0.5–2 µg/µL, respectively. The lowest MIC value of 0.25 µg/µL was found in EOPO against *B. subtilis* RSKK 244 and *C. albicans* ATCC 10231, whereas the lowest MIC of EOPB (0.5 µg/µL) was recorded against *E. coli* ATCC 11229 and *P. aeruginosa* ATCC 27853. Our findings were not in accordance with those of Melliou et al. [10] who studied EOs of Greek propolis and reported MIC values ranged from 4.1 to 6.7 mg/mL against *S. aureus*, 3.4–4.9 mg/mL against *E. coli*, 5.2–7.1 mg/mL for *P. aeruginosa* and 5.2–5.9 mg/mL for *C. albicans*, which were higher than the MIC values obtained in the present study. This could be due to the difference in the chemical composition of the oils. Hames-Kocabas et al. showed that MIC values of Turkish propolis volatiles were 0.25–1 mg/mL

Table 3: Antimicrobial activity of propolis essential oils and antibiotics estimated by diameter of inhibition zone in mm.

Strains	Propolis essential oils		Antibiotics			
	EOPO	EOPB	Ampicillin	Kanamycin	Erythromycin	Fluconazole
Gram-positive bacteria						
B. subtilis RSKK 244	11.40 ± 0.34^{e}	12.90 ± 0.70^{d}	36.81 ± 0.33^{a}	17.76 ± 0.49^{c}	20.21 ± 0.4^{b}	NA
B. cereus RSKK 863	11.15 ± 0.33^{e}	14.20 ± 0.50^{d}	34.95 ± 0.26^{a}	24.53 ± 0.12^{b}	21.43 ± 0.32^{c}	NA
S. aureus ATCC *25923*	9.68 ± 0.18^{d}	8.87 ± 0.32^{e}	32.48 ± 0.25^{a}	17.50 ± 0.21^{c}	26.44 ± 0.37^{b}	NA
Gram-negative bacteria						
S. enteritidis ATCC 13076	9.50 ± 0.73^{d}	10.57 ± 0.30^{d}	26.46 ± 0.23^{a}	17.84 ± 0.26^{b}	12.58 ± 0.31^{c}	NA
E. coli ATCC 11229	10.13 ± 0.54^{d}	10.62 ± 0.41^{d}	24.59 ± 0.38^{b}	18.58 ± 0.21^{c}	29.10 ± 0.36^{a}	NA
E. coli O157:H7	9.49 ± 0.59^{e}	10.62 ± 0.41^{d}	25.95 ± 0.26^{a}	19.89 ± 0.89^{b}	18.83 ± 0.11^{c}	NA
P. aeruginosa ATCC 27853	8.09 ± 0.29^{c}	11.51 ± 0.85^{b}	–	14.51 ± 0.18^{a}	11.77 ± 0.58^{b}	NA
Yeast						
C. albicans ATCC 10231	9.93 ± 0.28^{b}	9.37 ± 0.41^{b}	NA	NA	NA	17.08 ± 0.09^{a}

NA: not applicable. (–): No activity. Data are expressed as Mean ± SD of three parallel measurements. The values with different superscripts (a, b, c, d, or e) in the same lines are significantly different ($p < 0.05$).

Table 4: MIC, MBC, MFC, MBC/MIC, and MFC/MIC ratios values of EOPO and EOPB.

Strains	EOPO			EOPB		
	MIC (µg/µL)	MBC or MFC (µg/µL)	MBC/MIC or MFC/MIC	MIC (µg/µL)	MBC or MFC (µg/µL)	MBC/MIC or MFC/MIC
Gram-positive bacteria						
B. subtilis RSKK 244	0.25	1	4	2	4	2
B. cereus RSKK 863	0.5	1	2	1	2	2
S. aureus ATCC *25923*	1	2	2	1	2	2
Gram-negative bacteria						
S. enteritidis ATCC 13076	0.5	2	4	2	4	2
E. coli ATCC 11229	1	2	2	0.5	2	4
E. coli O157:H7	0.5	4	8	2	4	2
P. aeruginosa ATCC 27853	1	8	8	0.5	2	4
Yeast						
C. albicans ATCC 10231	0.25	2	8	2	2	1

against *S. aureus,* >1 mg/mL against *E.coli* O157:H7 >1 mg/mL against *P. aeruginosa* and 0.25–1 mg/mL against *C. albicans* [11]. These results were close to our findings, which could be explained by the similarity in the chemical composition. Indeed, more than 10 volatile compounds were common between Turkish propolis [11] and propolis used in the present study.

The results of MBC revealed that EOPO was more effective than EOPB against Gram-positive bacteria. Regarding Gram-negative bacteria, the lowest MBC value of 2 µg/µL was found in EOPO against *S. enteritidis* ATCC 13076 whilst EOPB displayed the lowest MBC against *P. aeruginosa* ATCC 27853. Both EOs showed the same MBC and MFC values against *E. coli* strains and *C. albicans,* respectively. However, the MBC and MFC values of EOPB and EOPO were similar or even higher than their MIC values. This could be attributed to variation in the rate of EO penetration through the cell wall and cell membrane structures [37]. According to Krishnan et al., antibacterial agents are categorized into two classes: bacteriostatic when MBC/MIC ratio > 4 and bactericidal when MBC/MIC ratio ≤ 4 [38]. Similarly, an agent is considered fungistatic when the ratio MFC/MIC > 4 and fungicidal when MFC/MIC ≤ 4 [39]. Following these classifications, EOPB was bactericidal for all tested pathogenic bacteria and fungicidal for *C. albicans* ATCC 10231, whereas, EOPO was bacteriostatic for *E. coli* O157:H7 and *P. aeruginosa* ATCC 27853 and had a fungistatic effect against *C. albicans* ATCC 10231.

The cidal effect of POPB against *E. coli*, *P. aeruginosa*, and *C. albicans* strains could be correlated to its monoterpenic content, especially, to its high amount of *α*-pinene (56.1%), which has been reported to possess a cidal effect against these strains [37]. *α*-pinene is used as an antibacterial due to its toxic effects on membranes [40]. It is noteworthy, however, that the antibacterial activity exhibited by propolis EOs may be explained by synergic or even antagonistic effects among their different compounds. EOs are lipophilic and hence easily permeable through the cell membrane. The interactions of EOs and their components with polysaccharides, fatty acids, and phospholipids make the bacterial membranes more permeable, which cause the loss of membrane integrity, leakage of cellular contents, and unbalance in intracellular pH and, consequently, lead to cell death [17, 41]. Similarly, the antifungal activity of EOs against *C. albicans* may be exhibited through the inhibition of membrane ergosterol and signaling pathways, leakage of cytoplasmic contents, and cell cycle inhibition [41].

4 Conclusion

The present paper provides the first data about Algerian propolis volatiles from Northeastern semi-arid regions (Oum el Bouaghi and Batna). The chemical analysis showed that the volatile compounds of the two propolis were variable. EO from propolis of Oum el bouaghi was dominated by sesquiterpenes while that of Batna was dominated by monoterpenes, which were observed as the result of their botanic and geographic origin. Our study has also demonstrated the antioxidant and antimicrobial properties of propolis oils and revealed that propolis oil possesses antimicrobial property, which may have interesting applications in food and pharmaceutical industries. Further investigations are needed to determine in depth their functional properties.

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ORIGINAL PAPER

Anticholinesterase, anti-α-glucosidase, antioxidant and antimicrobial effects of four Algerian propolis

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Abstract
This study aimed to investigate the functional properties of four Algerian propolis collected from El-Menia, Oum el Bouaghi, El Harrouch and Collo regions. The total bioactive content, antioxidant, anti-enzymatic and antimicrobial effects of the four propolis methanolic extracts were evaluated using in vitro assays. The highest amount of total phenolic (561.99 ± 3.50 µg GAE/mg E) and flavonoid content (76.98 ± 0.26 µg QE/mg E) was found in propolis from El-Menia. Antioxidant tests (DPPH, ABTS, CUPRAC and ferric reducing power) revealed the strong scavenging and reducing abilities of the extracts. Anti-enzymatic assays against acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and α-glucosidase showed that all extracts possessed a potent inhibitory effect on α-glucosidase better than acarbose and revealed the ability of extracts to inhibit BChE more effectively than AChE. However, the extract of propolis from El Menia was the best inhibitor of the three key enzymes with IC_{50} values of 11.40 ± 0.58 µg/mL 16.06 ± 0.85 µg/mL and 71.29 ± 2.73 µg/mL against α-glucosidase, BChE and AChE, respectively. The antimicrobial assay indicated that all extracts were mainly active against Gram-positive bacteria and yeast and had bactericidal action in certain bacteria and bacteriostatic action in other ones. However, they had all fungistatic effect on *C. albicans* ATCC 10231. According to these results, Algerian propolis can be considered as a source of natural bioactive principles for dietary, pharmacological and medicinal applications.

Keywords Propolis · Cholinesterase · Antioxidant · α-Glucosidase · Antimicrobial

Introduction

Nowadays, the emerging evidences revealed the ever-increasing demand of propolis as nutraceuticals, functional food and food supplements, which is attributed to its imperative health-promoting bioactive constituents and functional properties such as antimicrobial, antioxidant, anticancer, anti-infammatory, antidiabetic and neuroprotective effects [1, 2]. This natural bee product mainly consists of resins (50%), waxes (30%), essential oils (10%), pollen (5%) and other organic substances (5%) [3]. It contains a large number of biologically active components including different flavonoids, polyphenolic esters, terpenoids, steroids, amino acids, caffeic acids and their esters, which are responsible for the broad spectrum of its biological activities [4]. The constituents as well as various properties of propolis are significantly influenced by geographical location, climatic zones, flora, strength of bee colony and production season, which gives diversity and uniqueness to propolis of each country, state and zone [2]. Propolis from Algeria has recently begun to be studied and gain interest. Studies indicate that it contains many flavonoids and phenolic acids such as chrysin, apigenin, pectolinarigenin, pilosin, ladanein, galangin, Pinocembrin and caffeic acid derivatives [5–7]. Only a few amount of literature has been published on the biological activities of Algerian propolis, most of them have focused

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on the antioxidant and antimicrobial activities. Many authors have validated the antioxidant capacities of propolis extracts using in vitro assays and revealed a correlation between the phenolic content and the antioxidant activity [7–9]. By in vivo studies, Brihoum et al. [10] showed that propolis from Jijel region has a potential to reduce oxidative stress caused by benzo(a)pyrene and prevent damage by increasing enzymatic and non-enzymatic antioxidants, as well as decreasing lipid peroxidation. Similarly, Boutabet et al. [11] reported that propolis protects rat kidney against acute oxidative stress induced by doxorubicin. Algerian propolis was also reported to possess antimicrobial potentials. Nedji & Loucif-Ayad [12] have proved the effectiveness of propolis from Annaba against foodborne pathogens and attributed the strong antimicrobial activity to high total phenolic and flavonoid contents. Some other biological and pharmacological properties of Algerian propolis have been noted such as antitumor, anti-inflammatory, wound healing, immunomodulatory and neuroprotective [7, 10, 13–16]. Only two research exist on the anti-Alzheimer effect of Algerian propolis from Grarem and Djebel-El-ouahch regions [7, 16], which have distinct geographical and botanical origins from the samples used in the current study. There is no scientific report, however, on the antidiabetic effect of Algerian propolis. This paper, therefore, aimed to provide scientific information on the phenolic and flavonoid contents, anti-Alzheimer, antidiabetic, antioxidant and antimicrobial properties of propolis from four regions El-Menia, Oum el Bouaghi, El Harrouch and Collo (Northeastern of Algeria). This is the first study in the literature on the biological activities of propolis from these regions.

Materials and methods

Collection of propolis

Four propolis samples were collected from *Apis mellifera* hives located at Northesastern regions of Algeria. The collection sites are abbreviated as follows: P Propolis, M refers to P collected from El-Menia (Constantine city), O refers to P collected from Oum El Bouaghi region, C refers to P collected from Collo (Skikda city) and H refers to P collected from EL-Harrouch region (Skikda city). After removing impurities such as parts of plants and insects using pince, crude propolis samples were kept in freezer and then the frozen propolis was powdered using a blender and stored at 4 °C.

Preparation of methanolic extracts (ME)

The methanolic extract (ME) was obtained by the methodology described by Park and Ikegaki [17]. Air-dried powdered material (20 g) of propolis was extracted three times with 200 mL hydroalcoholic solution (80% MeOH, 20% Distillated water) for 72 h. After filtration, the filtrate was evaporated by rotary evaporator (under 50 °C temperature) to obtain dry extract and stored under dry conditions at 4 °C until analysed.

Total phenolic content (TPC)

Total phenols were assayed according to Singleton & Rossi [18]. Briefly, a 200 µL of diluted extract (0.5 mg/mL) was added to 1 mL of Folin–Ciocalteu reagent. After incubation in the dark for 4 min, 800 µL of 7.5% Na_2CO_3 was added. After incubation in the dark for 2 h, absorbance at 765 nm was read versus a prepared blank. The total phenol content of propolis extracts was expressed as micrograms of Gallic acid equivalents per milligram of extract (µg GAE/mg E) from a calibration curve with Gallic acid.

Total flavonoid content (TFC)

Total flavonoid content (TFC) was determined using aluminium chloride assay [19]. 1 mL of extract solution (0.5 mg/mL) was added to 1 mL of 2% aluminium chloride. After incubation in the dark for 10 min, the absorbance of the reaction mixture was measured at 430 nm with a UV/VIS spectrophotometer immediately. Quercetin was used as the standard for the calibration curve. Flavonoid content was expressed as µg of Quercetin equivalent (QE)/mg of extract.

Antioxidant activities

DPPH radical scavenging assay

One of the most popular techniques to evaluate the antioxidant capacity is the method employing 2,2-diphenyl-1-picrylhydrazyl (DPPH) [20]. The DPPH radical scavenging assay was conducted using the method of Blois [21]. Briefly, 40 µL of sample solution was mixed with 160 µL of DPPH solution. The reaction mixture was incubated for 30 min at 25 °C, and the absorbance was measured at 517 nm. The radical scavenging activity was calculated using formula as follows:

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

The results are expressed as IC_{50} value (µg/mL).

ABTS•+ cation radical scavenging assay

The $ABTS^{\bullet+}$ scavenging activity was done by the method of Re et al. [22] with slight modifications. The $ABTS^{\bullet+}$ was produced by the reaction between 7 mM ABTS in H_2O and

2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. The oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days with storage in the dark at room temperature. Before usage, the $ABTS^{\bullet+}$ solution was diluted with ethanol to get an absorbance of 0.70 ± 0.02 at 734 nm. Then, 160 µL of $ABTS^{\bullet+}$ solution were added to 40 µL of sample solution in methanol at different concentrations. After 10 min, the percentage inhibition at 734 nm was calculated. The scavenging capability of $ABTS^{\bullet+}$ was calculated using the following equation and the results were given as IC_{50} value.

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

.Cupric ion reducing antioxidant capacity (CUPRAC) assay

CUPRAC was determined according to the method developed by Apak et al. [23]. The method comprises mixing of 40 µL of sample solution with 60 µL of ammonium acetate aqueous buffer (pH 7), 50 µL of neocuproine alcoholic solution and 50 µL of a copper(II) chloride solution. After 60 min, the absorbance was read at 450 nm. The results were given as $A_{0.50}$, which corresponds to the concentration producing 0.50 absorbance.

Ferric reducing ability assay

The ferric reducing power was determined by the method of Oyaizu [24] with slight modifications. Sample solution (10 µL) were mixed with 40 µL sodium phosphate buffer (pH 6.6) and 50 µL of 1% potassium ferricyanide. The mixture was intensively shaken, then incubated at 50 °C for 20 min. Thereafter, 50 µL of 10% trichloroacetic acid (w/v) was added and the resulted mixture was mixed with 40 µL distilled water and 10 µL of 0.1% ferric chloride. The absorbance was spectrophotometrically measured at 700 nm. Butylatedhydroxytoluene (BHT) was used as a positive reference compound. The results were given as $A_{0.50}$, which corresponds to the concentration producing 0.50 absorbance.

Enzyme inhibitory properties

Cholinesterase inhibitory assay

The inhibition activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured by spectrophotometric method developed by Elman et al. [25] with slight modification [26]. AChE from electric eel and BChE from horse serum were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. 5,5′-Dithiobis(2-nitrobenzoic) (DTNB) acid was used for the measurement of the activity. Galantamine was used as a positive reference compound. The results were given as IC_{50} value (µg/mL) corresponding to the concentration shows 50% inhibition.

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

α-Glucosidase inhibitory assay

α-Glucosidase inhibitory activity was conducted according to Lordan et al. [27] with some modifications. A volume of 50 µL of sample solution and 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution prepared in phosphate buffer (pH 6.9) was incubated at 37 °C for 10 min.Then, 100 µL of α-glucosidase solution (0.1 U/mL) prepared in phosphate buffer (pH 6.9) was added. The absorbance was mesured at 405 nm for 30 min at 10-min intervals. The α-glucosidase inhibitory activity was calculated using the following equation and the results were given as IC_{50} value.

$$I\% = \frac{Abs\ Control - Abs\ Sample}{Abs\ Control} x100$$

I%: inhibition percentage, Abs: absorbance.

Antimicrobial activity

Test microorganisms

In vitro antimicrobial activity of propolis extracts was tested against eight human pathogens including three Gram-positive bacteria (*Bacillus cereus* RSKK 863, *Bacillus subtilis* RSKK 244 and *Staphylococcus aureus* ATCC 25923), four Gram-negative bacteria (*Escherichia coli* ATCC 11229, *Escherichia coli* O157:H7, *Salmonella enteritidis* ATCC 13076 and *Pseudomonas aeruginosa* ATCC 27853) and one yeast (*Candida albicans* ATCC 10231). Bacterial strains were cultured overnight at 37 °C in nutrient broth while yeast was cultured for 48 h at 30 °C in YPD (Yeast Peptone Dextrose) broth medium.

Preparation of propolis solutions

10 mg of propolis methanolic extracts were dissolved in 1 mL of Dimethyl sulphoxide (DMSO) to obtain a final concentration of 10 mg/mL. Then, the obtained solutions were sterilized by 0.45 µm Millipore filter.

Disc diffusion assay

The disc diffusion method was used to determine the antimicrobial potential of the investigated extracts [28].The culture suspensions were adjusted by comparing with 0.5 McFarland. Then, a volume of 100 µL of suspension was spread on agar plates. Thereafter, sterile 6-mm-diameter filter discs (Whatman paper n° 3) were placed on the inoculated plates and impregnated with 15 µL (150 µg/disc) of propolis extracts solutions. The treated petri dishes were kept at 4 °C for 1 h to enable prediffusion of the extracts into the agar. Finally, the inoculated plates were incubated at 37 °C for 24 h for bacterial strains and 30 °C for 48 h for yeast. Ampicillin (AM, 10 µg/disc), Kanamycin (K, 30 µg/disc) and Erythromycin (E, 15 µg/disc) were chosen as standard antibacterial while Fluconazole (FCA, 25 µg/disc) was chosen as standard antifungal. The results were obtained by measuring the diameter of growth inhibition zone surrounding the discs and expressed in mm.

Microdilution assay

Microdilution assay is the most used method for evaluating the minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal (MFC) concentrations [29]. It was performed in this study according to the protocol described by Koneman et al. [30] with slight modification. The propolis extracts were added to each growth medium to obtain a final concentration of 4 µg/µL and diluted to 2, 1, 0.5 and 0.031 µg/µL in tubes. The total volume was 100 µL in each tube. 1.25 µL of each tested bacteria or yeast (adjusted to 0.5 McFarland) were inoculated into each tube. The content of the tubes was mixed and they were incubated at appropriate temperatures for 24 h and 48 h. The MIC value was defined as the lowest concentration of the extract, which inhibited bacterial or fungal growth. MBC and MFC were determined by subculturing 5 µL of the test dilutions from each clear tube on solid growth medium and incubating for 24 h and 48 h at appropriate temperature. The lowest concentration that did not show bacterial growth was defined as the MBC value whereas the MFC value was determined as the lowest concentration with no fungal growth. The results are expressed as µg/µL.

Statistical analysis

Results are reported as mean value ± SD of three measurements; the IC_{50} and $A_{0.50}$ values were calculated by linear regression analysis. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism software (version 9.0.1). Results were considered statistically significant at $p < 0.05$.

Results

Total bioactive content (TPC and TFC)

In the present study, the yield of extraction, TPC and TFC were influenced by the geographic origin of propolis samples (Table 1). The yield of extraction varied between 20.5 and 39%. The highest yield was obtained by MEPM. The quantitative estimation of total bioactive content showed significant difference ($p < 0.05$) between the extracts with regard to TPC and TFC, except MEPC and MEPH which showed no significant difference between them regarding TFC. The highest amounts of TPC (561.99 ± 3.50 µg GAE/mg E) and TFC (76.98 ± 0.26 µg QE/mg E) were recorded with MEPM.

Antioxidant activities

In the current study, the antioxidant activity of MEPM, MEPO, MEPC and MEPH was evaluated in vitro using four different methods. DPPH and ABTS assays were used to assess the radical scavenging ability while CUPRAC and ferric reducing assays were used to assess the ability of the extracts to reduce copper and ferric ions, respectively.

All extracts exhibited a strong scavenging activity for DPPH and ABTS radicals (Table 2). However, among the extracts, MEPH had the highest capacity to trap DPPH with IC_{50} value (22.24 ± 0.43 µg/mL) which was similar ($p > 0.05$) to that of BHT (22.32 ± 1.19 µg/mL), whereas MEPM exerted the most potent scavenging activity for ABTS radicals with IC_{50} values of 5.81 ± 0.48 µg/mL.

Table 1 Extraction yield, TPC and TFC of propolis methanolic extracts

Sample	Extraction yield (%)	TPC (µg GAE/mg E)	TFC (µg QE/mg E)
MEPM	39	561.99 ± 3.50^{d}	76.98 ± 0.26^{c}
MEPO	38	270.62 ± 1.91^{a}	54.35 ± 0.20^{b}
MEPC	20.5	504.21 ± 2.23^{b}	46.66 ± 0.98^{a}
MEPH	36	524.95 ± 2.54^{c}	47.31 ± 2.54^{a}

Data are expressed as Mean ± SD of three parallel measurements ($p < 0.05$). The values with different superscripts (a, b, c or d) in the same columns are significantly different ($p < 0.05$)

TPC total phenolic content is expressed as µg Gallic acid equivalent/mg of extract, *TFC* total flavonoid content is expressed as µg Quercetin equivalent/ mg of extract, *MEPM* methanolic extract of propolis from El-Menia, *MEPO* methanolic extract of propolis from Oum el Bouaghi, *MEPC* methanolic extract of propolis from Collo, *MEPH* Methanolic extract of propolis from El-Harrouch

Regarding the reducing ability, all extracts had more ability to reduce copper ions than ferric ions. However, MEPM and MEPO showed ferric reducing power activity higher than that of BHT with $A_{0.50}$ values of 31.46 ± 1.08 µg/mL and 40.14 ± 0.42 µg/mL, better than that of BHT (41.67 ± 2.61 µg/mL).

Enzyme inhibitory properties

The anticholinesterase activity of propolis extracts was evaluated by using a combination of two complementary methods: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity assays. The results showed that all propolis methanolic extracts and galantamine (the standard drug) inhibited AChE activity in a concentration dependent manner (Fig. 1). Galantamine showed the best percent inhibition at all concentrations. The maximum inhibition was observed at the final assay concentration of 200 µg/mL and the decreasing order of AChE inhibition percent was: galantamine (94.77 ± 0.34%) > MEPM (82.98 ± 2.10%) > MEPO (62.84 ± 1.80%) > MEPH (53.09 ± 0.74%) > MEPC (22.86 ± 2.85%).

The BChE inhibitory activity of propolis methanolic extracts and galantamine was also increased with increasing concentration (Fig. 2). MEPM showed the best percentage inhibition at all concentrations. All extracts reached their maximum inhibition at the final assay concentration of 200 µg/mL where the highest inhibition activity was observed with MEPM (99.48 ± 2.71%) followed by MEPC (82.95 ± 0.42%), galantamine (78.95 ± 0.58%) and MEPO (76.82 ± 3.19%), whereas MEPH (58.19 ± 2.97%) had the lowest activity.

In contrast to galantamine, all propolis methanolic extracts were more selective inhibitors of BChE than AChE enzyme (Table 3). Among propolis extracts, MEPM was the most active againt BChE and AChE with IC_{50} of 16.06 ± 0.85 µg/mL and 71.29 ± 2.73 µg/mL, respectively.

Regarding the anti-diabetic activity, all tested extracts was able to inhibit α-glucosidase in a dose-dependent manner (Fig. 3). The maximum inhibition percentages (93.34 ± 0.19%, 85.65 ± 0.66%, 83.15 ± 1.31%, 76.07 ± 0.51%) were reached at the final assay concentration (250 µg/mL) of MEPC, MEPM, MEPO, and MEPH, respectively. Acarbose, however, reached 80.19 ± 1.66% at a concentration of 1250 µg/mL (Fig. 3). Based on the IC_{50} values (Table 3), the samples can be classified in their effectiveness against α-glucosidase as follows: MPEM > MEPO > MEPC > MEPH > Acarbose.

Antimicrobial activity

The screening of antimicrobial activity of MEPM, MEPO, MEPC and MEPH was firstly performed by disc diffusion

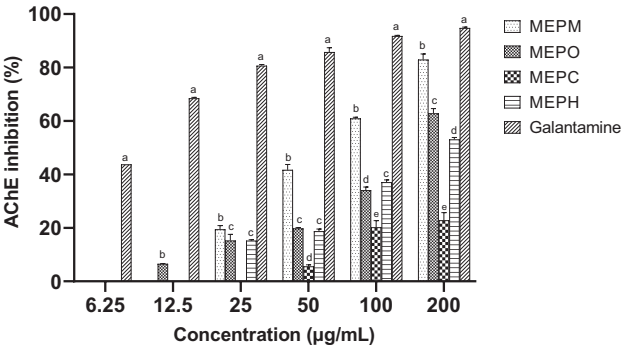

Fig. 1 Dose-dependent inhibition of acetylcholinesterase by propolis methanolic extracts and galantamine. Data are expressed as inhibition (%) mean ± SD (n = 3). Columns with different letters indicate statistically significant differences ($p < 0.05$). Vertical bars represent the standard deviation

Table 2 Antioxidant activities of propolis methanolic extracts

Sample	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	CUPRAC $A_{0.50}$ (µg/mL)	Ferric reducing $A_{0.50}$ (µg/mL)
MEPM	29.06 ± 0.20^{b}	5.81 ± 0.48^{b}	18.01 ± 2.15^{b}	31.46 ± 1.08^{a}
MEPO	42.02 ± 1.15^{c}	6.99 ± 0.16^{c}	15.98 ± 1.10^{b}	40.14 ± 0.42^{b}
MEPC	41.33 ± 0.61^{c}	8.73 ± 0.32^{d}	18.25 ± 2.34^{b}	47.32 ± 0.36^{b}
MEPH	22.24 ± 0.43^{a}	7.60 ± 0.32^{c}	11.83 ± 0.12^{a}	69.53 ± 2.93^{c}
BHT	22.32 ± 1.19^{a}	1.29 ± 0.30^{a}	9.62 ± 0.87^{a}	41.67 ± 2.61^{b}

Data are presented as IC_{50} mean ± SD (n = 3) and $A_{0.50}$ mean ± SD (n = 3).The values with different superscripts (a, b, c or d) in the same columns are significantly different ($p < 0.05$)

BHT butylatedhydroxytoluene, *MEPM* methanolic extract of propolis from El-Menia, *MEPO* methanolic extract of propolis from Oum el Bouaghi, *MEPC* methanolic extract of propolis from Collo, *MEPH* methanolic extract of propolis from El-Harrouch

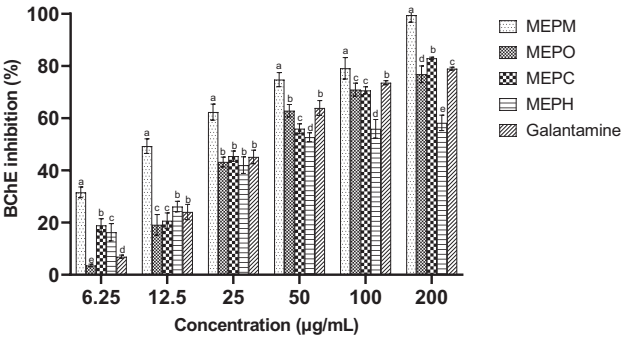

Fig. 2 Dose-dependent inhibition of butyrylcholinesterase by propolis methanolic extracts and galantamine. Data are expressed as inhibition (%) mean ± SD (n = 3). Columns with different letters indicate statistically significant differences ($p < 0.05$). Vertical bars represent the standard deviation

Table 3 Enzyme inhibitory activities of propolis methanolic extracts

Extract	AChE inhibitory assay IC_{50} µg/mL	BChE inhibitory assay IC_{50} µg/mL	α- glucosidase inhibitory assay IC_{50} µg/mL
MEPM	71.29 ± 2.73^{b}	16.06 ± 0.85^{a}	11.40 ± 0.58^{a}
MEPO	155.48 ± 1.67^{c}	33.57 ± 0.68^{b}	13.99 ± 0.17^{b}
MEPC	>200	35.70 ± 1.06^{b}	34.92 ± 0.37^{c}
MEPH	180.80 ± 3.56^{d}	44.04 ± 2.52^{c}	41.66 ± 0.32^{d}
Galantamine[RC]	6.27 ± 1.15^{a}	34.75 ± 1.99^{b}	NA
Acarbose[RC]	NA	NA	275.43 ± 1.59^{e}

Data are presented as IC_{50} mean ± SD (n = 3). The values with different superscripts (a, b, c or d) in the same columns are significantly different ($p < 0.05$)

RC reference compound, *NA* not applicable, *MEPM* methanolic extract of propolis from El-Menia, *MEPO* methanolic extract of propolis from Oum el Bouaghi, *MEPC* methanolic extract of propolis from Collo, *MEPH* methanolic extract of propolis from El-Harrouch

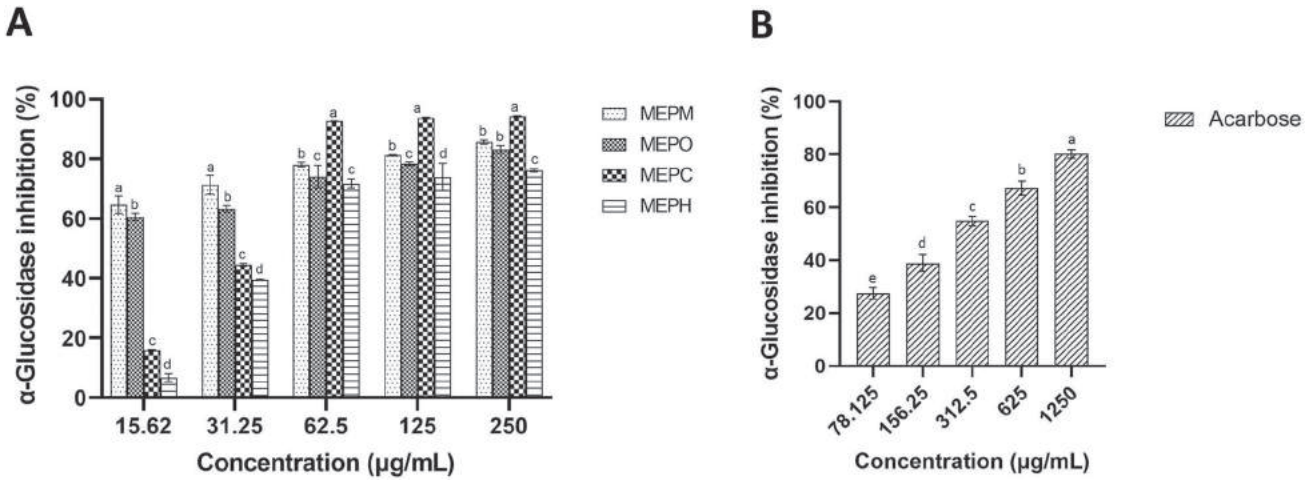

Fig. 3 **A** Dose-dependent inhibition of α-glucosidase by propolis methanolic extracts. **B** Dose-dependent inhibition of α-glucosidase by Acarbose. Data are expressed as inhibition (%) mean ± SD (n = 3). Columns with different letters indicate statistically significant differences ($p < 0.05$). Vertical bars represent the standard deviation

method against eight human pathogens. The diameter of inhibition zones values are presented in Table 4. All extracts inhibited the growth of all tested microorganisms and were found mainly active against Gram-positive bacteria (11.22 ± 2.06 mm – 18.32 ± 2.17 mm) and yeast (13.24 ± 0.7–15.24 ± 0.13 mm). The highest antimicrobial activity was exhibited by MEPH against *S. aureus* ATCC 25923 with inhibition zone diameter of 18.32 ± 2.17 mm which was better than that of Kanamycin the antibiotic standard (17.50 ± 0.21 mm), followed by MEPO against *B. cereus* RSKK 863 (16.28 ± 0.13 mm) and *C. albicans* ATCC 10231 (15.24 ± 0.13 mm) with inhibition zone closer to that of Fluconazole (17.08 ± 0.09 mm), a standard antifungal. Gram-negative bacteria, however, were less sensitive to the effect of propolis extracts (The inhibition zones ranged from 8.95 ± 0.15 mm to 11.29 ± 0.63 mm).

MEPH showed a marked activity against *P. aeruginosa* ATCC 27853 with an inhibition zone (11.11 ± 0.72 mm) comparable to that of Erythromycin (11.77 ± 0.58 mm) while Ampicillin was ineffective against this strain.

To better understand the mode of action of the extracts against the tested microorganisms, MIC, MBC, MFC, MBC/MIC and MFC/MIC ratios were determined using microdilution method; the results are indicated in Table 5 and 6. The MIC values of propolis extracts ranged from 0.0156 to 1 µg/µL. The lowest MIC value of 0.0156 µg/µL was found in MEPH against *B. cereus* RSKK 863. The MBC values of the extracts were higher than their MIC values; the lowest MBC value (0.031 µg/µL) was exerted by MEPC and MEPH against *B. cereus* RSKK 863, whilst the lowest MFC value of 1 µg/µL against *C. albicans* ATCC 10,231 was recorded by MEPO.

Table 4 Antimicrobial activity of MEPM, MEPO, MEPC, MEPH and antibiotics estimated by diameter of inhibition zone in mm

	Sample							
	Diameter of zone of inhibition (mm)							
Strains	MEPM	MEPO	MEPC	MEPH	Ampicillin	Kanamycin	Erythromycin	Fluconazole
Gram-positive bacteria								
B. subtilis RSKK 244	14.20 ± 0.13^{d}	15.27 ± 0.39^{d}	$13.12 \pm 0.08^{d,e}$	11.22 ± 2.06^{e}	36.81 ± 0.33^{a}	17.76 ± 0.49^{c}	20.21 ± 0.4^{b}	NA
B. cereus RSKK 863	14.11 ± 0.22^{e}	16.28 ± 0.13^{d}	13.09 ± 0.36^{f}	14.25 ± 0.15^{e}	34.95 ± 0.26^{a}	24.53 ± 0.12^{b}	21.43 ± 0.32^{c}	NA
S. aureus ATCC *25923*	13.40 ± 0.14^{d}	13.76 ± 0.17^{d}	13.24 ± 0.58^{d}	18.32 ± 2.17^{c}	32.48 ± 0.25^{a}	17.50 ± 0.21^{c}	26.44 ± 0.37^{b}	NA
Gram-negative bacteria								
S. enteritidis ATCC 13076	10.37 ± 0.87^{d}	$10.88 \pm 0.73^{c,d}$	$11.29 \pm 0.63^{c,d}$	10.33 ± 1.23^{d}	26.46 ± 0.23^{a}	17.84 ± 0.26^{b}	12.58 ± 0.31^{c}	NA
E. coli ATCC 11229	11.06 ± 0.37^{d}	11.00 ± 0.90^{d}	11.26 ± 0.23^{d}	10.34 ± 0.79^{d}	24.59 ± 0.38^{b}	18.58 ± 0.21^{c}	29.10 ± 0.36^{a}	NA
E. coli O157:H7	9.90 ± 0.20^{d}	8.95 ± 0.15^{e}	9.04 ± 0.36^{e}	9.58 ± 0.23^{d}	25.95 ± 0.26^{a}	19.89 ± 0.89^{b}	18.83 ± 0.11^{c}	NA
P. aeruginosa ATCC 27853	10.16 ± 0.08^{b}	10.19 ± 0.39^{b}	9.32 ± 1.94^{c}	11.11 ± 0.72^{b}	–	14.51 ± 0.18^{a}	11.77 ± 0.58^{b}	NA
Yeast								
C. albicans ATCC 10231	13.24 ± 0.78^{c}	15.24 ± 0.13^{b}	13.42 ± 0.28^{c}	14.00 ± 0.51^{c}	NA	NA	NA	17.08 ± 0.09^{a}

Data are presented as mean ± SD (n = 3). The values with different superscripts (a, b, c or d) in the same columns are significantly different ($p < 0.05$)

NA not applicable, *(–)* no activity, *MEPM* methanolic extract of propolis from El-Menia, *MEPO* methanolic extract of propolis from Oum el Bouaghi, *MEPC* methanolic extract of propolis from Collo, *MEPH* methanolic extract of propolis from El-Harrouch

Table 5 MIC, MBC and MFC of propolis extracts

	Extracts							
	MIC (µg/µL)				MBC or MFC (µg/µL)			
Strains	MEPM	MEPO	MEPC	MEPH	MEPM	MEPO	MEPC	MEPH
Gram-positive bacteria								
B. subtilis RSKK 244	0.125	0.25	0.25	0.125	2	1	1	2
B. cereus RSKK 863	0.125	0.5	0.0156	0.0156	0.25	1	0.031	0.031
S. aureus ATCC *25923*	0.25	0.125	0.25	0.25	1	1	2	1
Gram-negative bacteria								
S. enteritidis ATCC 13076	0.25	0.5	0.25	0.125	1	2	2	1
E. coli ATCC 11229	0.125	0.5	0.25	0.25	0.5	2	0.5	2
E. coli O157:H7	1	1	0.5	1	4	2	4	4
P. aeruginosa ATCC 27853	1	1	1	0.5	4	4	4	4
Yeast								
C. albicans ATCC 10231	0.125	0.031	0.125	0.031	2	1	4	2

MIC minimum inhibitory concentration, *MBC* minimum bactericidal concentration, *MFC* minimum fungicidal concentration, *MEPM* methanolic extract of propolis from El-Menia, *MEPO* methanolic extract of propolis from Oum el Bouaghi, *MEPC* methanolic extract of propolis from Collo, *MEPH* methanolic extract of propolis from El-Harrouch

Based on MBC/MIC ratios [31], the bactericidal effect was confirmed for MEPM and MEPO for most strains tested (ratios ≤ 4) except for *B. subtilis* RSKK 244 for MEPM and *S. aureus* ATCC 25923 for MEPO (Table 6). MEPC, however, was bactericidal against four stains and bacteriostatic against three other ones, whilst MEPH showed bacteriostatic effect for the majority of tested bacteria. According to the MFC/MIC ratio, it is possible to identify an extract's

Table 6 MBC/MIC and MFC/MIC ratios of propolis extracts

Strains	Extracts			
	MBC/MIC or MFC/MIC			
	MEPM	MEPO	MEPC	MEPH
Gram-positive bacteria				
B. subtilis RSKK 244	16	4	4	16
B. cereus RSKK 863	2	2	2	2
S. aureus ATCC *25923*	4	8	8	4
Gram-negative bacteria				
S. enteritidis ATCC 13076	4	4	8	8
E. coli ATCC 11229	4	4	2	8
E. coli O157:H7	4	2	8	4
P. aeruginosa ATCC 27853	4	4	4	8
Yeast				
C. albicans ATCC 10231	16	32	32	64

MIC minimum inhibitory concentration, *MBC* minimum bactericidal concentration, *MFC* minimum fungicidal concentration, *MEPM* methanolic extract of propolis from El-Menia, *MEPO* methanolic extract of propolis from Oum el Bouaghi, *MEPC* methanolic extract of propolis from Collo, *MEPH* methanolic extract of propolis from El-Harrouch

antifungal profile (fungistatic and/or fungicidal). A ratio of MFC/MIC > 4 indicates fungistatic activity, whereas a ratio of MFC/MIC ≤ 4 defines a fungicidal effect [32]. Hence, all extracts of the current study had a fungistatic action against *C. albicans* ATCC 10231 (Table 6).

Discussion

Phenolic and flavonoids are the bioactive compounds having important roles for maintenance of overall human health [33]. In the current study, the phenolic levels of propolis extracts ranged from 270.62 ± 1.91 to 561.99 ± 3.50 µg GAE/mg E. Such results were higher than those obtained by Nedji & Loucif-Ayad [12] who studied propolis from other localities in Algeria and found phenolic levels ranged between 100.90 and 257.40 mg GAE/g E. Literature references, however, describe a variety of ranges for total phenolic content of propolis from different geographical origins.

Lagouri et al. [34] reported phenolic levels from 21.8 ± 0.62 to 179.99 ± 3.43 mg GAE/g of Greek propolis while Misir et al. reported phenolic content of 114.7 ± 0.02 mg GAE/g of Turkish propolis [35]. Ethiopian propolis was reported to contain phenolic amounts from 63.09 ± 3.55 to 82.07 ± 3.72 mg GAE/g [36]. In regards to the flavonoid content, our findings were quite similar to those obtained by Nedji & Loucif-Ayad [12] and the Greek propolis (5.96 ± 0.85–88.26 ± 1.58 µg QE/mg E) [34]. However, they were higher than the Turkish (36.02 ± 0.08 mg QE/g E) and the Ethiopian propolis (17.26 ± 0.35–24.42 ± 0.53 mg QE/g E) [35, 36]. This variation in phenolic and flavonoid contents is mainly attributable to the difference in the preferred regional plants visited by honeybees, geographical location, altitudes, seasons, solvent and extraction method [3, 37–39].

Antioxidant capacity is one of the most important properties of propolis [40]. Previous studies have investigated and confirmed the antioxidant potential of Algerian propolis [41–43]. In this study, our propolis extracts exhibited potent antioxidant activities, which can be attributed to their high content in total phenolic and flavonoid compounds. Relevantly, Moreno et al. [44], Hamasaka et al. [45] and Segueni et al. [9] investigated the antioxidant activity of Argentinian, Japanese and Algerian propolis, respectively, and reported that the correlation between polyphenols, flavonoid contents and antioxidant activity is significant.

Inhibition of cholinesterase has become a widely used clinical approach to treating the AD symptoms [46]. Previous studies on propolis have demonstrated its inhibition potential of cholinesterase enzymes and thus could be beneficial in the treatment of Alzheimer's disease [7, 16]. In this study, the anticholinesterase activity of propolis methanolic extracts (MEPM, MEPO, MEPC and MEPH) against AChE and BChE was evaluated. All extracts inhibited BChE more effectively than AChE, which can be explained by the fact that BChE enzyme can accept a wide range of substrates over AChE, because of its low substrate specificity [47]. This is in accordance with our previous study on propolis from Mila region, which exhibited anticholinesterase activity with IC_{50} values of 20.30 ± 0.52 µg/mL against BChE and 124.50 ± 2.46 µg/mL against AChE [16]. In another study on Algerian propolis, Bouaroura et al. [7] reported that among four propolis extracts (petroleum ether, Chloroform, ethyl acetate and methanolic extracts), chloroform extract demonstrated the highest inhibitory effect against both enzymes with IC_{50} values of 55.70 ± 2.12 µg/mL for BChE and 81.21 ± 6.06 µg/mL for AChE. However, our result was in disagreement with the results of Bouaroura et al. [7] who found that no cholinesterase inhibitory effect exerted by methanolic extract of propolis from Djebel El-ouahch (Constantine city), while in the current study we found that the methanolic extract of propolis from El-Menia (Constantine city) was the most potent extract. This difference could be due to the variation in propolis composition, which is extremely variable and depends on the plant resin sources that grow around the apiary [48]. Referring to the literature, studies on the anticholinesterase activity of propolis from different geographical origins showed variable cholinesterase inhibitory effects. According to El-Guendouz et al. [49], Moroccan propolis samples exhibited antiacetylcholinesterase effect with IC_{50} values ranging from 0.002 ± 0.051 to 3.555 ± 0.051 mg/mL. Another study by Baltas et al. [50]

indicated that ethanolic extracts from Turkish propolis exerted acetylcholinesterase inhibitory ability with values ranging from 0.081 to 1.353 mg/mL. Abd El-Hady et al. [51] found that Sudanese propolis possessed variable inhibitory activities against AChE with values ranged between 25.5– 91.7%, where the high activity of propolis was linked to its high content of several classes of compounds that is known to possess high activity against the enzyme such as flavonoids, phenolic acids and their esters. It is interesting to note that the biological properties of propolis are mainly attributed to the presence of active compounds in propolis extracts, which are strongly depended on the geographical origin, solvent, extraction method, operating conditions of propolis as well as the different botanical species that honeybees use as resin sources [9, 48].

The α-glucosidase inhibitors are currently used as therapeutic agents for diabetes. Acarbose is a commercially available enzyme inhibitor for type II diabetes [52, 53]. However, it is reported to cause adverse effects such as abdominal distention, flatulence and diarrhea. As a result, searching for safe and effective inhibitors from natural materials is of emerging interest. [52, 53]. Propolis is a natural product that has been reported to exert an antidiabetic effect [49, 54]. No information, however, concerning Algerian propolis efficacy on α-glucosidase inhibition. In this study, therefore, we investigated the effect of MEPM, MEPO, MEPC and MEPH on α-glucosidase enzyme. The findings revealed the strong ability of our extracts on inhibiting α-glucosidase with values better than that of acarbose. Similarly, Ibrahim et al. [55] reported that Malaysian propolis exhibited a potent antidiabetic activity than acarbose. In the present study, among the extracts, MEPM exhibited the best activity against α-glucosidase, which could be due to its high content on phenolic and flavonoid compounds. MEPO, however, showed a good inhibition against α-glucosidase regardless its low total phenolic content. This could be due to its high amount of total flavonoids. Importantly, Popova et al. [56] demonstrated that the capacity of inhibiting α-glucosidase was better in propolis samples in which phenolics and particularly flavonoids predominated. Indeed, phenolic and flavonoid compounds have been known to possess high inhibitory potential towards α-glucosidase enzyme [57, 58].

Natural products are promising natural antimicrobial agents with potential applications in pharmaceutical or food industries [12]. In this study, therefore, we investigated the antimicrobial effects of four propolis methanolic extracts against a range of bacteria and yeast. All extracts were active against the tested microorganisms with high antimicrobial activity against Gram-positive bacteria and yeast. This is in agreement with previous studies on Algerian propolis that have shown a high antimicrobial activity against Gram-positive bacteria and limited activity against Gram-negative bacteria [12, 59]. The MBC/MIC and MFC/MIC values in the present study were found to be higher than their MIC values. This phenomenon may be explained by the impure form of the bioactive compound(s) [60]. All extracts had bactericidal action in certain bacteria and bacteriostatic action in other ones. However, they had all fungistatic effect on *C. albicans* ATCC 10231. The bactericidal and bacteriostatic effects of propolis can be associated with their combined action manifested by inhibition of protein synthesis and bacterial growth by preventing cell division. However, the fungistatic effect could be due to the induced expression of apoptotic and necrotic factors by propolis alongside the formation of reactive oxygen species [61]. Additionally, the antimicrobial potential of propolis can be attributed to the synergistic effects of phenolic compounds such as cinnamic acid and ester derivatives, including caffeic acid and CAPE, as well as flavonoids [62].

Conclusion

The present study highlights that Algerian propolis especially propolis from El-Menia region possess strong cholinesterase and α-glucosidase inhibitory potentials and reveals their potency to be used as a strong source of future therapeutic agents in Alzheimer and diabetes. Our study also indicates that Algerian propolis may be beneficial for treating the pathological damage caused by radicals' activities and bacterial infections with the prospect to be used in many industries, such as food, pharmaceuticals and cosmetics. Further work needs to be carried out in order to isolate the active chemical constituents, which might be helpful in studying the precise mechanisms of cholinesterase and α-glucosidase inhibitory, antioxidant and antimicrobial potentials.

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Declarations

Conflict of interest Authors have no conflicts of interest.

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ORIGINAL PAPER

LC–MS/MS analysis, antioxidant and anticancer effects of phenolic-rich extracts from Algerian propolis: a comparative study

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Abstract
The present study was aimed to compare the phenolic composition and the functional properties (antioxidant and anticancer) of phenolic-rich extracts of two different propolis collected from Collo (PREPC) and Oum El Bouaghi (PREPO) regions. The phenolic composition was determined using LC/MS–MS. The antioxidant activity of the extracts was evaluated using alkaline DMSO and β-carotene-linoleic acid tests. Anticancer effect on HepG2 human hepatocellular carcinoma cells was determined using CCK-8 assay. Twenty-two phenolic compounds in PREPC and twenty-three in PREPO were detected and quantified by LC–MS/MS. Ferulic and caffeic acids were found to be the predominant compounds. Both extracts were able to inhibit lipid peroxidation and demonstrated their ability to scavenge superoxide radicals more effectively than the standards. The highest lipid peroxidation inhibition ($IC_{50} = 17.58 \pm 1.98$ µg/mL) and superoxide radical scavenging effects ($IC_{50} = 6.19 \pm 0.24$ µg/mL) were exhibited by PREPO. However, PREPC showed stronger cytotoxic activity against HepG2 ($IC_{50} = 12.22 \pm 0.05$ µg/mL) than PREPO ($IC_{50} = 18.68 \pm 0.33$ µg/mL). These results demonstrate the potential of extracts from Algerian propolis to be used in functional formulations.

Keywords Propolis · Antioxidant · LC–MS/MS · Anticancer · HepG2

Introduction

Liver cancer is the 5th most common cancer type and was reported as the 3rd common deadly cancer worldwide [1]. It is occurred by the presence of hepatocellular damage through reactive oxygen species and the generation of chronic inflammation related to hepatocarcinogenesis [2]. Emerging evidence states that diet is recognized as a potential lifestyle-related risk factor for the development of liver cancer. Hence, a healthy diet may play a preventative role in the development of such a life-threatening disease [3]. Bioactive foods with anticancer potential not only provide nutritional benefits, but also inhibit cancer progression within the human body [4]. Propolis, a natural bee product,

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is extensively used as an ingredient in functional foods [5]. It is known to have many diverse biological properties such as antimicrobial, anti-inflammatory, anticancer, and antioxidant activities [6]. Its therapeutic properties are due to its chemical composition and are mainly associated with the presence of biologically active components including different flavonoids, polyphenolic esters, caffeic acids and their esters [7]. The chemical composition of propolis is affected by botanical and geographical factors leading to variations in propolis bioactivities [8]. Hence, propolis from different regions may contain different bioactive compounds and could exhibit different biological activities.

Research on the chemical composition and functional potential of Algerian propolis are still very scarce. At present, there are no published studies on the cytotoxic effect of Algerian propolis against human liver cancer cells. The goal of this investigation, therefore, was to analyze and compare the phenolic composition of two phenolic-rich extracts obtained from two different propolis. Their antioxidant and anticancer effects against HepG2 human hepatocellular carcinoma cells were also evaluated.

Materials and methods

Reagents and chemicals

Standard compounds (purity ≥ 99%) used for LC–MS/MS analysis were purchased from Sigma-Aldrich Chemical Co., Ltd (St. Louis, Missouri, USA). Standards and reagents used in antioxidant activity were purchased from Sigma Chemical Co. (Sigma-Aldrich GmbH, Stern-heim, Germany). Solvents used for extraction and analysis were of analytical and HPLC-MS grades, respectively. Human hepatocellular carcinoma (HepG2) cell line was obtained from the American Type Culture Collection (USA). Cell Counting Kit-8 (CCK-8) was purchased from Abcam (UK). Fetal bovine serum (FBS), Penicillin and Streptomycin were purchased from PAN-Biotech (GmbH, Germany).

Propolis collection and phenolic-rich extracts preparation

Two propolis samples produced by the honey bee *Apis mellifera*, were collected by beekeepers from beehives. Propolis (PO) was collected from hives located in Oum El Bouaghi (35° 52′ 39″ N, 7° 06′ 49″ E), which is a semi-arid region and propolis (PC) was collected from Collo (37° 00′ 23″ N, 6° 33′ 39″ E), which is a humid region. The samples were collected by scraping frames, walls and the entrance of the beehive. After removing impurities such as parts of plants and insects, the crude propolis sample was kept in freezer and then the frozen propolis was powdered.

The preparation of PREPO (phenolic-rich extract from PO) and PREPC (phenolic-rich extract from PC) was carried out according to Park and Ikegaki [9]. Briefly, 20 g of propolis was extracted three times with 200 mL hydroalcoholic solution (80% Methanol, 20% Distillated water) for 72 h. After filtration, the filtrate was evaporated by rotary evaporator (under 50 °C temperature) to obtain dry extract and stored under dry conditions at 4 °C until analyzed.

For chemical and antioxidant studies, 1 mg of each extract was dissolved in 1 mL of methanol. However, for anticancer assay, 2 mg of each extract was dissolved in 1% DMSO (50 µL DMSO, 4950 µL growth medium) and diluted with growth medium to the desired concentration prior to exposure.

Chemical composition analysis by LC–MS/MS

The phenolic component of the extracts was analyzed by using an LC (Agilent 1260 Infinity) system coupled to a triple quadrupole mass spectrometer (Agilent 6420 Triple Quadrupole LC–MS). The chromatographic separation of the phenolic compounds was carried out on a C18 reversed-phase ODS column (25 × 4.6 mm × 5 µm). The injection volume of the standards and the samples was 2 µL. The mobile phase consisted of water/0.1% formic acid (eluent A), methyl alcohol (eluent B) at a flow rate of 0.4 mL/min. The elution conditions were as follows: 2% B for 3 min, 25% B for 6 min, 50% B for 10 min, 95% B for 14 min, 2% B for 17.5 min. MS analysis was performed in both positive and negative ionization modes. The multiple reaction monitoring (MRM) mode was used to quantify the analytes. The LC–MS/MS data were collected and processed by Mass Hunter software (version B.07.01). The phenolic compounds of samples were identified by comparing their retention time, UV profile and mass spectra with those of authentic standards. All the phenolics detected were quantified using the calibration curves of corresponding standard solutions and the results were expressed as nanograms per milligram of dried propolis extract.

Antioxidant activities

Superoxide radical scavenging activity

The scavenging activity of extracts towards the superoxide radical ($O_2^{\cdot -}$) was measured in terms of inhibition of generation of $O_2^{\cdot -}$. The method was performed by using alkaline DMSO method as reported in Bensouici et al. [10]. Superoxide radical (O2· −) is generated by the addition of sodium hydroxide (NaOH) to air saturated DMSO. The generated superoxide remains stable in solution and reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature, which can be measured at 560 nm [11]. Briefly, to the

reaction mixture containing 40 µL of extract (or standard compound) at various concentrations and 130 µL of alkaline DMSO (100 mL DMSO containing, 20 mg NaOH in 1 mL distilled water), 30 µL NBT (1 mg/mL solution in distilled water) was added and absorbance was noted at 560 nm against blank samples. The decrease in the absorbance of the reaction sample indicated the increase in superoxide anion scavenging activity. The percent inhibition of superoxide anion generation was calculated using the following formula:

$$I\% = \frac{Ac - As}{Ac} \times 100$$

I%: inhibition percentage, Ac: absorbance in the presence of the control. As: Absorbance in the presence of the sample.

The results are expressed as IC_{50} value (µg/mL).

Lipid-peroxidation inhibitory activity

The lipid peroxidation inhibitory potential of the extracts was determined by the β-carotene-linoleic acid test system as mentioned in Bensouici et al. [10]. In this model, β-carotene undergoes rapid discoloration in the absence of an antioxidant because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical (formed upon the withdrawal of a hydrogen atom from one of its diallylic methylene groups) attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized and partly broken down; subsequently the system loses its chromophore [12]. The addition of an antioxidant inhibits lipid peroxidation and thus delays β-carotene bleaching. Briefly, β-carotene (0.5 mg) in 1 mL of chloroform and 25 µL of linoleic acid were dissolved in 200 µL of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 50 mL of distilled water saturated with oxygen, were added by vigorous shaking. The assay mixture, containing 160 µL β-carotene emulsion and 40 µL of extract solution, was incubated at 45 °C. After 120 min, the decrease in the absorbance of β-carotene was measured at 470. The antioxidant activity was expressed as percent inhibition relative to the control using the following equation:

$$I\% = \left[1 - \frac{As0 - Ast}{Ac0 - Act}\right] \times 100$$

where I% is the inhibition percentage, A_{S0} is the initial absorbance at time 0 in the presence of the sample, A_{St} is the absorbance at time 120 min in the presence of the sample, A_{C0} is the initial absorbance at time 0 in the presence of the control and A_{Ct} is the absorbance at time 120 min in the presence of the control.

The results are expressed as IC_{50} value (µg/mL).

Anticancer study

Cell culture

The human hepatocellular carcinoma (HepG2) cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified incubator with 5%CO_2. Cells were checked under Zeiss PrimoVert inverted microscope, and subculturing was performed when cells reached 80% confluency.

Cytotoxicity assay

The cytotoxicity of the extracts on HepG2 cells was determined by using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. Briefly, the cells were counted using a trypan blue solution. Next, 100 µL of cell suspension (1×10^5 cells per well) was plated into 96-well plate and incubated at 37 °C in a CO_2 incubator (5%) for 24 h. Then, cells were treated with serial concentrations (3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) of each extract (100 µL) and incubated for 72 h. Thereafter, cells were washed and 100 µL of fresh medium was added. Then, 10 µL of CCK-8 solution was added to each well and incubated at 37 °C for 3 h. Absorbance at 450 nm was determined using a microplate reader. The cytotoxic activity was measured using the following equation and the results were given as IC_{50} value.

$$Cytotoxicity\ \% = 100\% - \left[\frac{Abs\ treated\ cells}{Abs\ untreated\ cells} \times 100\right]$$

Cell morphology analysis

The morphological changes in HepG2 cells exposed to increasing concentrations (3.125–200 µg/mL) of PREPC and PREPO were investigated using an inverted phase microscope (PrimoVert, Zeiss) at 40 X magnification and compared with control cells.

Statistical analysis

Except LC–MS/MS, the results were illustrated as means ± standard deviation of three measurements. The IC_{50} values were calculated by linear regression analysis. Data were analyzed by Student t-test using GraphPad Prism software (version 6.0.1). Results were considered statistically significant at $p < 0.05$.

Results

Identification and quantification of phenolic compounds by LC–MS/MS analysis

The phenolic profiles of the extracts were analyzed by LC–MS/MS triple quadrupole. Figure 1 shows the chromatograms while Table 1 shows the content of each propolis extract. Twenty-two phenolic compounds were detected in PREPC, whereas twenty-three compounds were identified in PREPO. Twenty-two phenolics were common between the two propolis but with different amounts. One compound was detected only in PREPO, which is 3,4-dihydroxyphenylacetic acid. The molecules identified in the extracts belong to four phenolic sub-classes including phenolic acids, flavonoids, lignans and other polyphenols. Phenolic acids were the major phenolic sub-class found in both extracts followed by flavonoids. The predominant individual phenolic compounds in PREPC were ferulic and caffeic acids followed by apigenin and kaempferol. Gallic acid, *p*-coumaric acid and quercetin were also found in high amount. The most abundant components in PREPO, however, were ferulic and caffeic acids followed by verbascoside and quercetin. Kaempferol and apigenin were also determined in high quantity.

Antioxidant activities

Superoxide radical scavenging activity

The scavenging of superoxide radical by PREPO and PREPC was evaluated by Alkaline DMSO method, in which $O2^{\cdot-}$ was produced chemically without the presence of enzymes. In non-enzymatic system, compounds can exert an antioxidant activity by reducing the production of $O2^{\cdot-}$, or by a stabilizing action of the radical when donating or receiving electrons to the $O2^{\cdot-}$ radical [13]. As shown in Fig. 2, both extracts showed a concentration-dependent increase in inhibition of superoxide generation. Maximum $O2^{\cdot-}$ scavenging activity was observed at a final concentration of 200 µg/mL. Both extracts, however, exhibited a higher radical scavenging activity than the positive standards

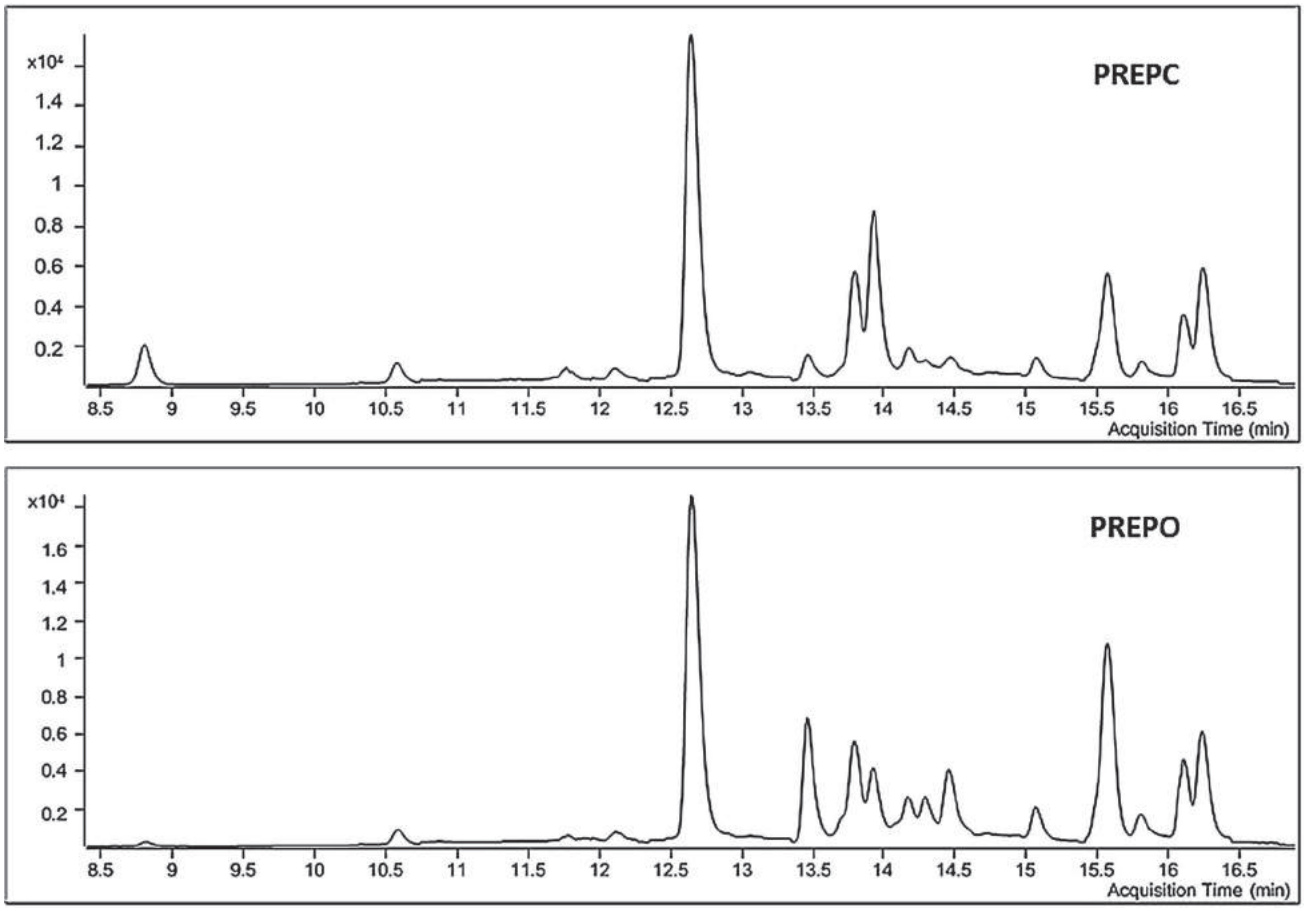

Fig. 1 LC–MS/MS chromatograms of PREPC and PREPO

Table 1 Phenolic compounds of propolis extracts determined by LC–MS/MS

Compound	MRM transition	RT (min)	PREPC Composition (ng/mg E)	PREPO Composition (ng/mg E)
Phenolic acids				
Gallic acid	168.9–> 125.0	8.808	1434.39 ± 12.22	153.30 ± 5.96
Protocatechuic acid	152.9–> 108.9	10.59	691.61 ± 15.57	541.04 ± 6.01
3,4-Dihydroxyphenylacetic acid	167.0–> 123.0	10.905	ND	6.07 ± 0.11
Chlorogenic acid	355.0–> 163.0	11.786	443.33 ± 30.40	415.96 ± 3.51
3-Hydroxybenzoic acid	137.0–> 93.0	12.854	ND	ND
4-Hydroxybenzoic acid	136.9–> 93.1	12.114	367.71 ± 0.86	319.38 ± 19.10
2,5-Dihydroxybenzoic acid	152.9–> 109.0	11.988	ND	ND
Homovanillic acid	181.0–> 137.1	12.642	180.32 ± 16.03	215.19 ± 3.28
Caffeic acid	179.0–> 135.0	12.651	5236.90 ± 83.84	5745.61 ± 262.38
Syringic acid	196.9–> 181.9	12.782	77.40 ± 5.96	31.19 ± 0.11
Verbascoside	623.0–> 160.8	13.468	488.04 ± 9.47	2726.14 ± 64.31
p-Coumaric acid	162.9–> 119.0	13.802	1376.51 ± 7.97	1329.28 ± 28.35
Sinapic acid	222.9–> 207.9	13.874	ND	ND
Ferulic acid	193.0–> 134.0	13.934	7103.17 ± 55.45	3126.90 ± 74.21
Rosmarinic acid	359.0–> 160.9	14.508	ND	ND
2-Hydroxycinnamic acid	162.9–> 119.1	14.846	ND	ND
Total phenolic acids			17,399.38 ± 49.43	14,610.05 ± 467.32
Flavonoids				
(+)-Catechin	289.0–> 245.0	11.37	ND	ND
(−)-Epicatechin	291.0–> 139.1	12.379	ND	ND
Taxifolin (dihydroquercetin)	303.0–> 285.1	13.713	10.59 ± 3.17	88.12 ± 1.50
Luteolin 7-glucoside	447.1–> 285.0	14.273	ND	ND
Hesperidin	611.1–> 303.0	14.303	252.77 ± 3.52	1116.47 ± 71.19
Hyperoside (quercetin-3-O-galactoside)	465.1–> 303.1	14.489	135.60 ± 0.72	326.81 ± 9.89
Apigenin 7-glucoside	433.1–> 271.0	14.74	9.91 ± 0.46	12.73 ± 0.68
Eriodictyol	287.0–> 151.0	15.072	78.87 ± 5.12	153.37 ± 8.01
Quercetin	301.0–> 151.0	15.571	1130.32 ± 49.96	2572.27 ± 96.47
Luteolin	287.0–> 153.1	15.81	201.17 ± 6.80	382.97 ± 10.46
Kaempferol	285.0–> 229.1	16.106	1457.16 ± 0.88	2109.85 ± 54.77
Apigenin	271.0–> 153.0	16.245	2053.56 ± 37.81	2028.32 ± 19.76
Total flavonoids			5329.97 ± 86.04	8790.92 ± 140.90
Lignans				
Pinoresinol	357.0–> 151.0	14.944	138.05 ± 4.52	284.03 ± 53.47
Total lignans			138.05 ± 4.52	284.03 ± 53.47
Other polyphenols				
3-Hydroxytyrosol	153.0–> 123.0	10.268	ND	ND
Pyrocatechol	109.0–> 52.9	10.891	119.62 ± 0.35	418.62 ± 11.23
Vanillin	151.0–> 136.0	13.071	437.13 ± 17.13	171.63 ± 15.55
Oleuropein	539.2–> 275.1	14.607	ND	ND
Total of other polyphenols			278.37 ± 17.48	590.26 ± 4.32
Total identified phenolic compounds			23,424.134 ± 23.65	24,275.26 ± 550.43

Data are presented as mean ± SD of two measurements

PREPO Phenolic-rich extract of propolis from Oum El Bouaghi, *PREPC* Phenolic-rich extract of propolis from Collo, *ND* Not detected

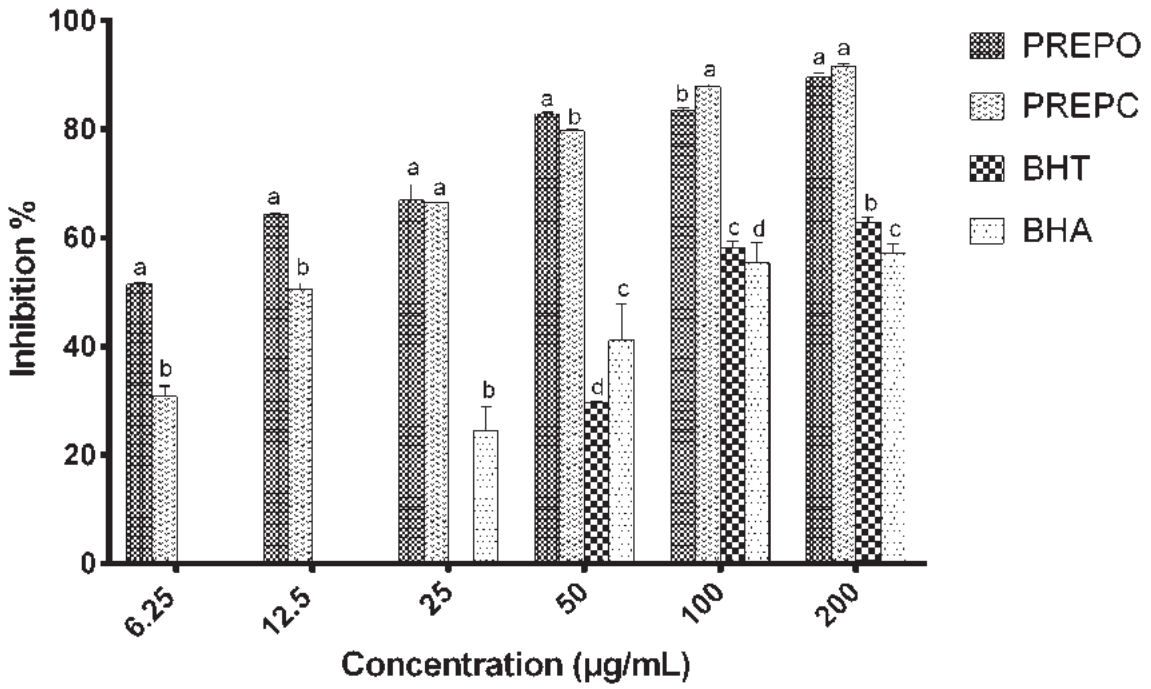

Fig. 2 Dose-dependent inhibition of superoxide radical generation by propolis extracts and standards. Data are expressed as inhibition (%) mean ± SD (n = 3). Columns with different letters indicate statistically significant differences ($p < 0.05$). Vertical bars represent the standard deviation

Table 2 Antioxidant activities of phenolic-rich extracts from propolis

	PREPO	PREPC	BHT	BHA
$O2^{\cdot-}$ scavenging IC_{50} (µg/mL)	6.19 ± 0.24^{a}	14.86 ± 0.15^{b}	85.30 ± 2.08^{c}	86.33 ± 3.53^{c}
Lipid peroxidation Inhibition IC_{50} (µg/mL)	17.58 ± 1.98^{b}	30.59 ± 0.01^{c}	1.05 ± 0.01^{a}	0.90 ± 0.02^{a}

Data are presented as IC_{50} mean ± SD (n = 3). The values with different superscripts (a, b or c) in the same line are significantly different ($p < 0.05$)

BHT butylatedhydroxytoluene, *BHA* butylatedhydroxyanisole, *PREPO* Phenolic-rich extract of propolis from Oum El Bouaghi. *PREPC* Phenolic-rich extract of propolis from Collo

BHT and BHA (Table 2). Based on the IC_{50} values, the scavenging potential of the extracts was in the following order: PREPO > PREPC > BHT > BHA.

Lipid-peroxidation inhibitory activity

The effect of extracts on lipid peroxidation inhibition was determined by β-carotene/linoleic acid system. Both extracts showed good ability in this respect and inhibited the bleaching of β-carotene in a concentration-dependent manner (Fig. 3). The maximum inhibition percentages (99.76 ± 0.14%, 95.45 ± 1.70%, 95.28 ± 3.25% and 94.06 ± 1.22%) were reached at the final assay concentration (200 µg/mL) of BHA, PREPC, BHT and PREPO, respectively. Based on the IC_{50} values (Table 2), the samples can be classified in their effectiveness against β-carotene bleaching as follows: BHA > BHT > PREPO > PREPC.

Anticancer study

Cytotoxicity

The results of cytotoxicity assay revealed a clear dose-dependent cytotoxicity response against HepG2 cells 72-h posttreatment with PREPC and PREPO (Fig. 4). The maximum inhibition percentages 81.98 ± 0.15% and 81.83 ± 0.22% were reached at the final assay concentration (200 µg/mL) of PREPO and PREPC, respectively. These

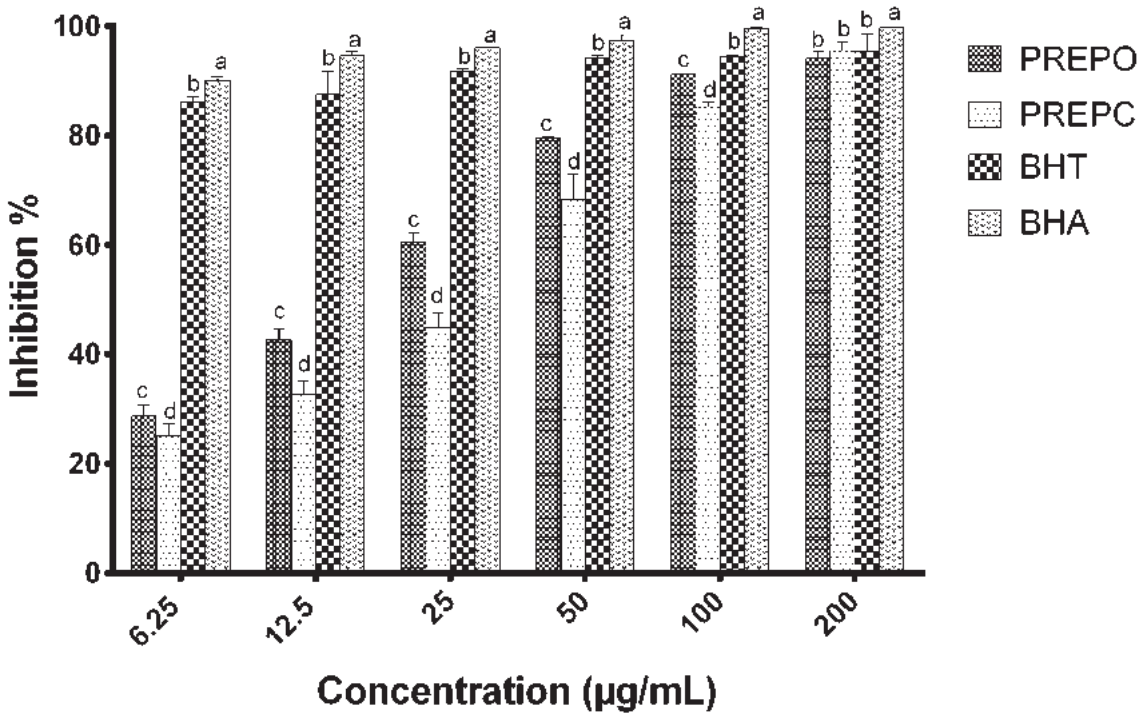

Fig. 3 Dose-dependent inhibition of lipid peroxidation by propolis extracts and standards. Data are expressed as inhibition (%) mean ± SD (n = 3). Columns with different letters indicate statistically significant differences ($p < 0.05$). Vertical bars represent the standard deviation

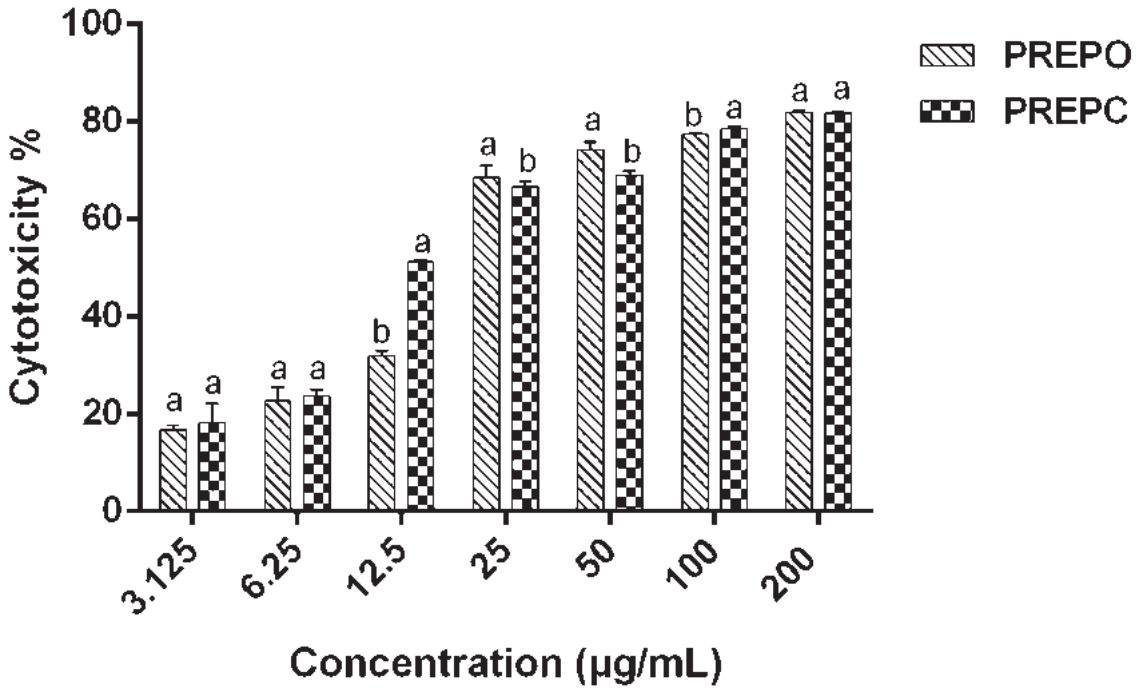

Fig. 4 Dose-dependent cytotoxicity response against HepG2 cells 72-h posttreatment with PREPC and PREPO. Data are expressed as cytotoxicity (%) mean ± SD (n = 3). Columns with different letters indicate statistically significant differences ($p < 0.05$). Vertical bars represent the standard deviation

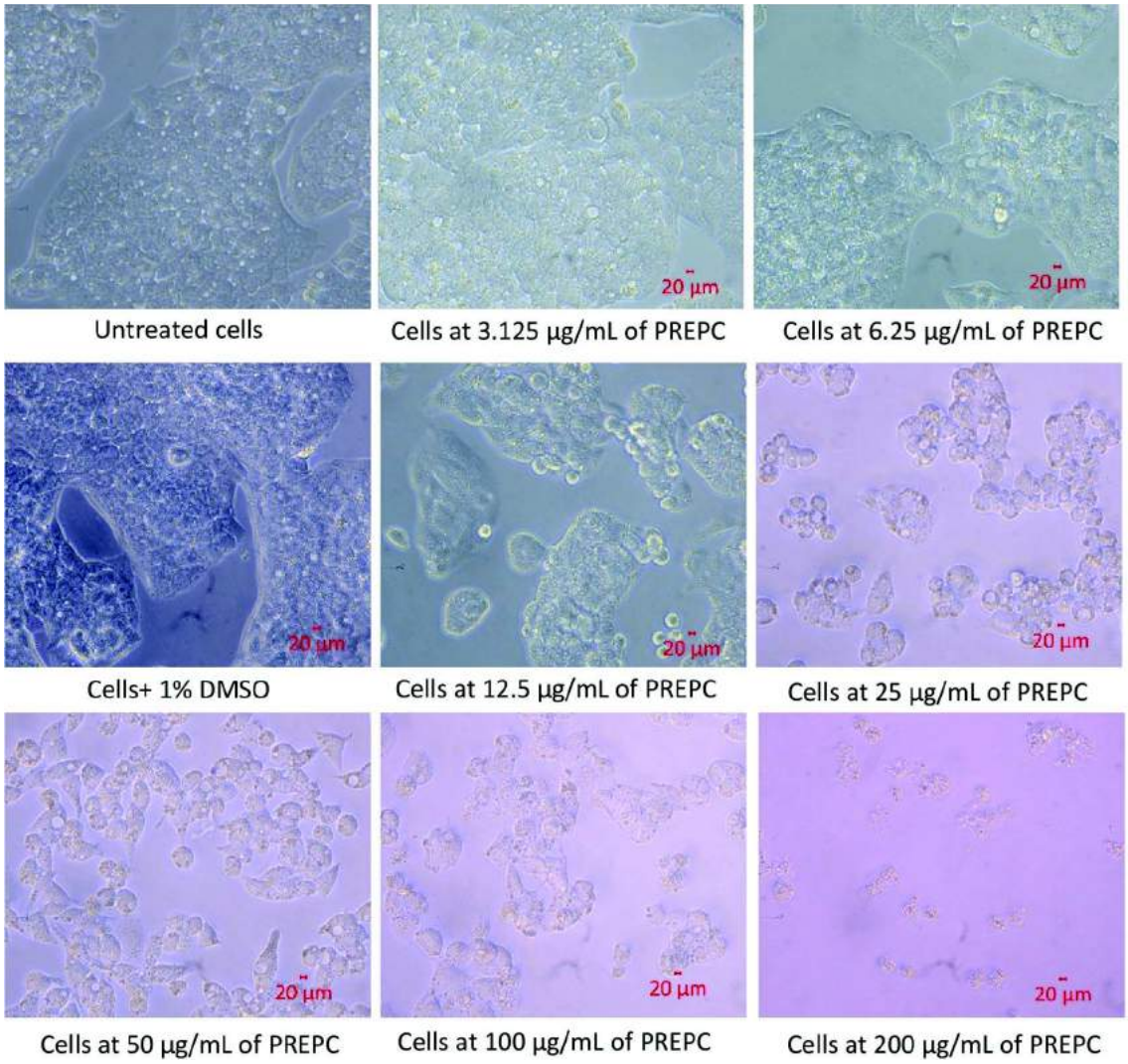

Fig. 5 Morphological effects of PREPC on HepG2 cells observed using inverted microscope (40 × magnification)

values, however, did not show any significant difference ($p > 0.05$). The IC_{50} values were found to be 12.22 ± 0.05 µg/mL for PREPC and 18.68 ± 0.33 µg/mL for PREPO, indicating a stronger cytotoxic effect of PREPC on HepG2 cells compared with that of PREPO.

Cell morphology analysis

The cytotoxic effects of PREPC and PREPO were further studied by morphological cellular imaging (Figs. 5 and 6). It was observed that untreated and 1%DMSO-treated cells maintained a normal morphology. However, HepG2 cells treated with PREPC and PREPO within 72-h period lost the typical morphology in a concentration dependent manner. At lower concentrations (12.5 µg/mL and less) of PREPC and PREPO, the changes were less significant while at 25 µg/mL and higher concentrations, the changes were much more severe including loss of normal morphology and cellular junctions, reduction in cell volume and formation of apoptotic bodies. Most cells at higher concentrations lost contact with adjacent cells and acquired a spherical shape compared to untreated cells. These morphological alterations induced by PREPC and PREPO in HepG2 cells could be attributed to an apoptotic mechanism.

Discussion

The phenolic profiles of PREPC and PREPO were analyzed by LC–MS/MS and have shown the presence of several components (Table 1). There were some qualitative and quantitative differences between the two extracts, which could be explained by the difference in geographical origin of propolis. In line with the current results, Soltani et al. [14]

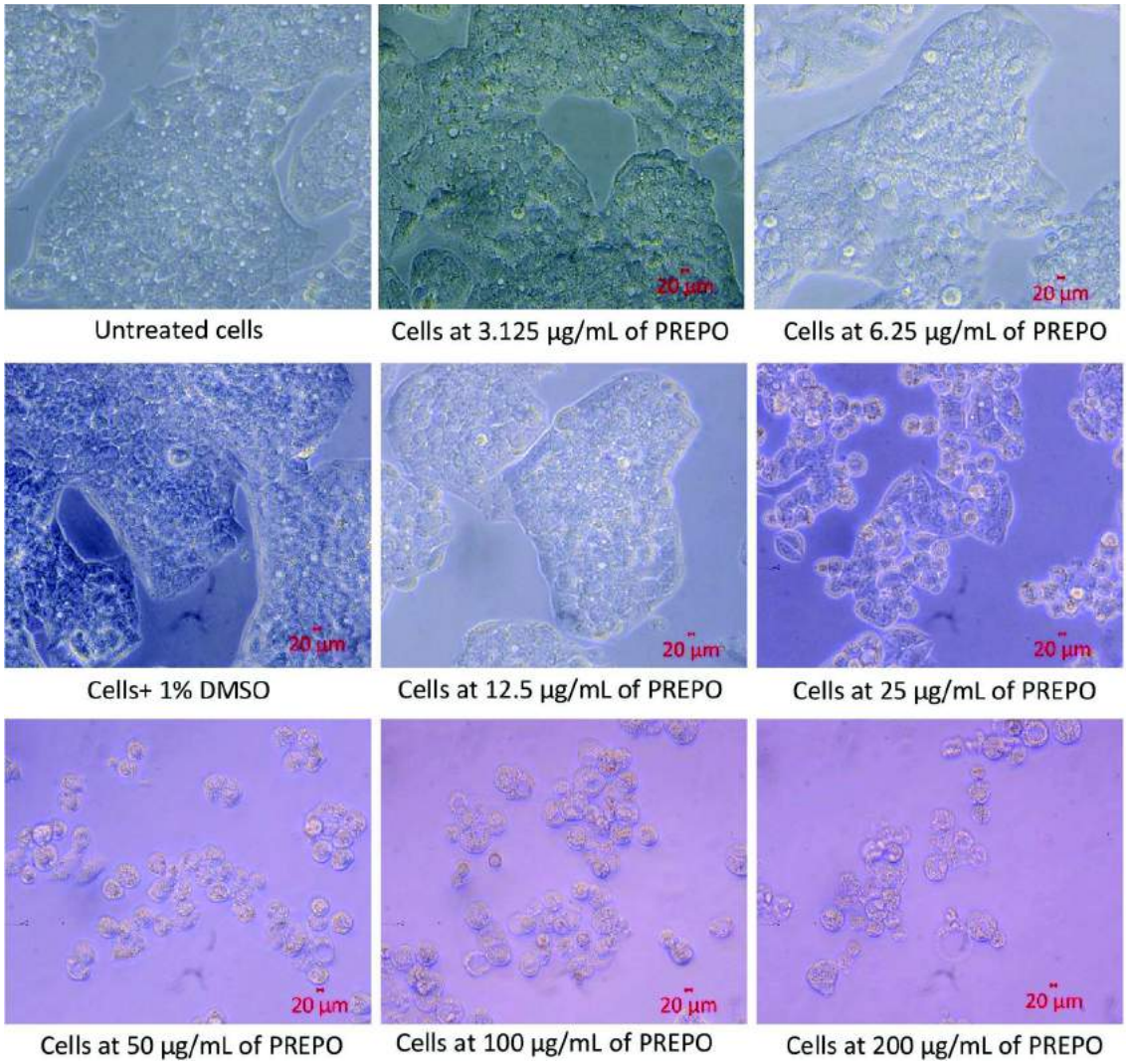

Fig. 6 Morphological effects of PREPO on HepG2 cells observed using inverted microscope (40 × magnification)

also studied extracts from propolis samples collected from different locations in Algeria and confirmed the influence of geographical origin on the variation of the chemical profile of this material. Similar to other Algerian propolis, gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid, quercetin, apigenin, kaempferol have also been identified in PREPC and PREPO [15–18], which could be used for quality determination and standardization of Algerian propolis. However, the other compounds reported in the current study have never been identified in Algerian propolis but have been detected in propolis from other countries. Apigenin 7-glucoside, hesperidin, hyperoside and verbascoside have been detected in Cypriot propolis [19]. Protocatechic acid, *p*-hydroxybenzoic acid, syringic acid, eriodictiol, luteolin and vanillin have been found in Turkish propolis [20]. Homovanillic acid and pinorisenol have been characterized in Brazilian propolis [21, 22]. Taxifolin has been found in Moroccan propolis [23]. Pyrocatechol has been identified in *Trigona laeviceps* stingless bee propolis from Indonesia [24]. 3,4-Dihydroxyphenylacetic acid, however, has not been reported in propolis samples to the best of our knowledge.

The antioxidative potential of PREPC and PREPO was determined by their ability to scavenge superoxide anion radicals and to inhibit lipid peroxidation. Both extracts were found to be more effective in scavenging superoxide anion radical than the standard antioxidants, which could be related to their contents of phenolic compounds that have been recognized as powerful antioxidant agents, mainly due to their hydroxyl groups [25]. Phenolic compounds can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [26]. In this study, PREPO showed higher superoxide radical scavenging and lipid peroxidation inhibition potentials than PREPC. This could be explained by the

chemical analysis that revealed that PREPO is qualitatively and quantitatively richer in phenolic content especially flavonoids than PREPC. These findings are in good accordance with previous studies that have correlated the phenolic and flavonoid composition of propolis extracts with its antioxidant properties [16, 27]. However, studies on superoxide radical scavenging and lipid peroxidation inhibition properties of propolis methanolic extracts from different geographical origins showed variable effects. Miguel et al. [28] stated that Portuguese propolis exhibited superoxide radical scavenging with IC_{50} values ranged from 0.001 ± 0.003 to 0.053 ± 0.003 mg/mL, while Ichikawa et al. [29] indicated an IC_{50} value of 6.2 mg/mL of Brazilian propolis. Bouaroura et al. [30] who investigated the capacity of some Algerian propolis methanolic extracts to inhibit lipid peroxidation in β-carotene-linoleic acid system indicated IC_{50} values ranged from 11.34 ± 0.17 to 40.38 ± 0.39 µg/mL. In another study, propolis from Mila has been reported to inhibit lipid peroxidation with an IC_{50} value of 43.46 ± 0.03 µg/mL [31]. These differences in the effects of propolis from different collection sites could be due to the variation in propolis composition [32].

Propolis is a widely used bee product with broad biological activities including antitumor properties [33]. Many reports have demonstrated the cytotoxic effects of Algerian propolis in several human cancer cell lines including breast adenocarcinoma MCF-7, mammary gland adenocarcinoma MDA-MB-231, epithelial adenocarcinoma HeLa, prostate cancer PC3, myelogenous leukemia K562, pancreatic PANC-1 cancer and lung adenocarcinoma A549 cell lines [34–37]. However, no scientific study using liver cancer cell lines has ever been carried out to confirm its potential in the management of liver cancer. This study, therefore, was performed to evaluate the cytotoxic effect of PREPC and PREPO against human liver cancer HepG2 cell line. The obtained results revealed that PREPC and PREPO possess significant potential to inhibit the HepG2 cancer cells. Previous studies on propolis extracts from various regions of the world have stated its anticancer potential on human hepatocellular carcinoma cells. Turan et al. [38] and Gokduman [39] investigated the cytotoxic effect of Turkish propolis and reported IC_{50} values of 27.0 ± 0.8 µg/mL and 25.62 ± 1.50 µg/mL, however, these values are higher than those obtained in the current study. Abu Shady et al. [40] and Abd El-Hady et al. [41] indicated that Egyptian and Sudanese propolis exhibited anticancer potentials against HepG2 with IC_{50} values within the range of 62.5–70.9 µg/mL and 57–60 µg/mL, respectively. Such results were also higher than our findings. In another study by Sadeghi-Aliabadi et al. [42], Iranian propolis was found to exhibit a potent cytotoxicity with an IC_{50} value of 15 µg/mL, which is closer to our results. The anticancer activity of PREPC and PREPO in this study was very interesting because of the possible cytotoxic effects of their phytoconstituents. Their chemical profiles revealed the presence of several bioactive compounds that have been reported to exhibit anticancer activity such as ferulic acid, cafeic acid, apigenin and quercetin [43–46].

Induction of cancer cell apoptosis is a beneficial mechanism for cancer treatment [47]. The morphological cellular imaging in this study revealed that PREPC and PREPO mediated cytotoxic effect against HepG2 cells possibly via induction of apoptosis, which is in agreement with previous works that demonstrated the apoptotic effect of extracts from Algerian propolis in cancer cells [34, 35]. However, the precise mechanisms of action remain to be elucidated.

Conclusion

This work is the first report about the chemical profile and anticancer activity of phenolic-rich extracts from propolis obtained from Collo and Oum El Bouaghi regions (Algeria). The extracts were found to be rich in phenolic compounds especially PREPO, in which a new phenolic compound 3,4-Dihydroxyphenylacetic acid was identified for the first time in propolis. The results showed also the antioxidant and anticancer importance of Algerian propolis, which support the potential health benefits of propolis as a potential candidate for developing functional food products. Further studies, however, are needed to determine their mechanisms of action and their safety.

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Author contributions SB: Data curation, Formal analysis, Investigation, Visualization, Writing-original draft, Writing-review and editing. ZA: Data curation, Formal analysis, Investigation, Methodology. HDT: Data curation, Formal analysis, Investigation, Methodology. AZ: Project administration, Supervision, Validation, Visualization, Writing-review and editing. RGA: Supervision, Methodology, Resources, Validation, Writing-review and editing. CB: Supervision, Methodology, Resources, Data curation, Validation. Writing-review and editing. FD: Formal analysis, Data curation. LK: Resources, Data curation. GD: Resources, Data curation. ML: Writing-review and editing.

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Data availability The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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