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DEDICATION

I dedicate this PhD thesis to

My mother and brothers, who believed in my abilities and supported me, their encouragement played an integral role in my accomplishments.

My friends' widad, ines, yasmine, safia, dicra, khaouther, Sonia, Fatima and nadia and all colleagues who helped and encouraged me

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Abstract

This study sought to investigate the phenolic and flavonoid content, antioxidant properties, antibacterial efficacy and antibiofilm activity of propolis extracts sourced from Algeria against *Enterococcus faecalis*, a prominent oral pathogen. Further investigations deleved into assessing their potential inhibitory effects quorum-sensing using *Chromobacterium violaceum 026* and *Chromobacterium violaceum 12472*. Moreover, the toxicity of the propolis extracts was evaluated on brine shrimp larvae.

Phenolic and flavonoid amounts in propolis extracts ranged from 752.82 ±13.25 to 303.41 ± 1.01 µg GAE/mg extract and from 271.46 ± 11.49 to 28.47 ± 0 µg QE/mg extract, respectively.

HPLC-DAD analysis revealed that one of the major phenolic acids present in all extracts was caffeic acid, while Chrysin was one of the most abundant flavones identified only in Chekfa-El milia and Kaous samples, Hesperetin was a flavone found exclusively in Kissir ouana, Taxanna, Kherrata and Tamalous samples, while naringenin was highly abundant in Tamalous sample. Cynarin was a new compound identified for the first time in Algerian propolis.

The results of antioxidant activity showed that propolis extracts possess good scavenging and reducing abilities. In addition, antibacterial and antibiofilm results indicated that propolis extracts have an effective antibacterial and antibiofilm effect against *E. faecalis*.

Taxanna extract had the greatest ability to inhibit quorum sensing in *CV026*, with an inhibition diameter of 13 mm. Propolis extracts effectively suppressed violacein secretion on *CV12472* at MIC with a value of 100 %, while only Bouteldja and Taxanna samples showed high inhibition potency (100 %) at both MIC and sub-MIC concentrations. A toxicity study on brine shrimp larvae suggests that propolis extracts have moderate toxicity.

These findings propose that propolis could be used in dentistry as a new source of drugs to inhibit and eliminate *E. faecalis* and its virulence factors from the oral cavity, particularly from the dental root canal.

Keywords: Propolis, HPLC-DAD, Antioxidant, *Enterococcus faecalis*, Antibiofilm, Quorum-sensing inhibition

Résumé

Le but de cette recherche était d'étudier la teneur en composés phénoliques, la teneur en flavonoïdes, les propriétés antioxydantes, l'activité antibactérienne et anti-biofilm des extraits de propolis collectés dans différentes régions d'Algérie contre *Enterococcus faecalis*, responsable de maladies bucco-dentaires, et de révéler sa capacité à inhiber le quorum sensing chez *Chromobacterium violaceum 026* et *Chromobacterium violaceum 12472*. En outre, la toxicité des extraits de propolis a été évaluée sur des larves de crevette *Artemia salina*.

Les teneurs en composés phénoliques et en flavonoïdes des extraits de propolis variaient respectivement de ± 13.25 à 303.41 ± 1.01 µg GAE/mg d'extrait et de 271.46 ± 11.49 à 28.47 ± 0 µg QE/mg d'extrait

L'analyse HPLC-DAD a révélé que l'un des principaux acides phénoliques dans tous les extraits était l'acide caféique, tandis que la chrysine, l'une des flavones les plus abondantes, n'a été identifiée que dans Chekfa-El milia et Kaous samples, l'hespérétine est une flavone trouvée exclusivement dans Kissir ouana, Taxanna., Kherrata et Tamalous samples, tandis que la naringénine était présente en grande abondance dans Tamalous sample; la cynarine est un nouveau composé identifié pour la première fois dans les extraits de propolis algérienne.

Des expériences antioxydants ont montré que les extraits de propolis ont une grande capacité à piéger et à réduire différentes radicaux et ions. En outre, des études antibactériennes et d'antibiofilm ont révélé que les extraits de propolis ont un effet antibactérien et antibiofilm efficace contre *E. faecalis*.

Taxanna sample ayant la plus grande capacité à inhiber le quorum sensing chez *CV026*, avec un diamètre d'inhibition de 13 mm.

Les extraits de propolis ont effectivement inhibé la production de violacéine chez *CV12472* à la CMI avec une valeur de 100 %, tandis que seuls Bouteldja et Taxanna samples ont montré une forte capacité d'inhibition (100 %) à la fois à la CMI et à la concentration inférieure à la CMI. Les études de toxicité sur les larves de crevette *Artemia Salina* indiquent que les extraits de propolis ont des propriétés toxiques modérées.

Ces résultats suggèrent que la propolis pourrait être utilisée en dentisterie comme nouvelle source de médicaments pour inhiber et éliminer *E. faecalis* et ses facteurs de virulence de la cavité buccale, en particulier du canal radiculaire.

Mots-clés : Propolis, HPLC-DAD, Antioxydant, *E. faecalis*, Antibiofilm, Anti-quorum-sensing

الملخص:

يهدف هذا البحث إلى دراسة المحتوى الفينولي والفلافونويد، الخصائص المضادة للأكسدة، الفعالية المضادة للبكتيريا والمضادة للأغشية الحيوية لمستخلصات العكبر التي تم جمعها من مناطق مختلفة من الجزائر ضد *Enterococcus faecalis* المسببة لأمراض الفم; إضافة إلى الكشف على قدرتها على تعطيل استشعار النصاب عند *Chromobacterium violaceum 026* و *Chromobacterium violaceum 12472*. علاوة على ذلك، تم تقييم سمية مستخلصات العكبر على يرقات الروبيان *Artemia salina*.

تراوحت المحتويات الفينولية والفلافونويدية في مستخلصات البروبوليس من 13.25± 752.82 إلى 1.01 ± 303.41 ميكروغرام مكافئ الغاليك/ملغ من المستخلص, ومن 0 ± 28.47 إلى 11.49 ± 271.46 ميكروغرام مكافئ الكرستين/ملغ من المستخلص على التوالي

كشف تحليل الكروماتوغرافي باستخدام كروماتوغرافيا السائلة ذات المردود العالي أن احد أهم الأحماض الفينولية الموجودة في جميع المستخلصات هو حمض الكافيك, في حين تم التعرف على مركب Chrysin وهو أحد أكثر flavones وفرة فقط في عينات الشقفة بالميلية و قاوس، hesperetin هو مركب تم الكشف عنه فقط في مستخلصات منطقة كيسير العوانة و تاكسانة (جيجل) و خراطة و تمالوس(سكسكدة) ، بينما يوجد naringenin بوفرة كبيرة في تمالوس. cynarin هو مركب جديد تم الكشف عنه لأول مرة في مستخلصات العكبر الجزائرية.

أظهرت التجارب المضادة للأكسدة أن مستخلصات العكبر لديها قدرة كبيرة على الكسح الجدري وإرجاع الايونات. بالإضافة إلى ذلك، كشفت الدراسات المضادة للبكتيريا ومضادات الاغشية الحيوية أن مستخلصات البروبوليس لها تأثير فعال مضاد للبكتيريا ومضاد للبيوفلم لعزلات *E. faecalis*.

اظهرت عينة تاكسانة قدرة على تثبيط استشعار النصاب لدى *CV026*، بقطر منطقة تثبيط يبلغ 13 ملم. أدت مستخلصات العكبر بشكل فعال إلى تثبيط إنتاج الفيولاسين لدى *CV12472* عند MIC بقيمة 100٪، بينما أظهر عينات بوتلجة و تاكسانة قدرة تثبيط قوية (100 ٪) فقط عند كل من MIC وأقل من تركيزات MIC. تشير دراسة السمية التي أجريت على على يرقات الروبيان *Artemia Salina* إلى أن مستخلصات العكبر لها قدرة سمية معتدلة.

تشير نتائج هدا البحث أنه يمكن استخدام البروبوليس في طب الأسنان كمصدر جديد للأدوية لتثبيط وإزالة *E.faecalis* وعواملها الممرضة من تجويف الفم، وخاصة من قناة جذرالاسنان.

الكلمات المفتاحية: العكبر ,HPLC , مضادات الأكسدة, *Enterococcus faecalis* ,النشاط المضاد للبيوفلم, الاستشعار المضاد لنصاب.

LIST OF ABBREVIATIONS

ABTS: Acide 2,2'-azino-bis (3-ethylbenzothiazolin-6-sulfonic)

$AlCl_3$: Aluminum chloride

$A_{0.5}$: Concentration in which the absorbance is 0.50

AHL : acylhomoserine lactone

ATB: Antibiotic

ANOVA: Analysis of Variance

BHA: Butylated hydroxyanisole

BHT: Butylated Hydroxytoluene

CUPRAC: Cupric ion reducing antioxidant capacity

$CH3COONH_4$: Ammonium Acetate

***CV12472*:** *Chromobacterium violaceum* (*CV12472*)

CV026: *Chromobacterium violaceum* (*CV026)*

CFU: Colony-Forming Unit

C6HSL: N-hexanoyl-L-Homoserine lactone

DMSO: Dimethyl sulfoxyde

DPPH: 2,2'-diphenyl-1-picrylhydrazyl

GOR: Galvinoxyl radical scavenging

GAE: Equivalent of Gallic acid

HPLC-DAD: high-performance liquid chromatography- Diode Array Detector

IC_{50}: The half-maximal inhibitory concentration

$K_2S_2O_8$: potassium persulfate

LB: Luria Bertani

MHA: Mueller-Hinton Agar

MeOH: Methanol

MIC: minimum inhibitory concentration

MBC: minimum bactericidal concentration

OD: Optical density

PBS: phosphate-buffered saline

BEA: Bile-Esculine-Azide

QE: Equivalent of quercetin

RT: Retention time

TFC: Total flavonoid content

TPC: Total phenolic content of retention

Tr: Trace

PJOK: Propolis collected from Ouana region (city Jijel)

PJCH: Propolis collected from Chekfa-El milia region (city Jijel)

PJT: Propolis collected from Taxanna region (city Jijel)

PJ5: Propolis collected from Kissir ouana region (city Jijel)

PJKT: Propolis collected from Kaous region (city Jijel)

PKH: Propolis collected from Kherrata region (city Bejaïa)

PTH: Propolis collected from Bouteldja region (city El-Taref)

PPS: Propolis collected from Tamalous region (city Skikda)

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INTRODUCTION

Introduction

The human mouth is home to over 700 species of bacteria, thriving in its various anatomical habitats such as teeth, gums, and tongue. This microbial diversity ranks second only to the intestines. Although these microbes generally coexist peacefully with their host, disruptions in their living conditions can lead to aggressive behavior, infections, and pathogen spread. Key to maintaining harmony, the oral microbiota achieves balance through homeostasis. However, factors like poor oral hygiene, dietary choices, and health conditions can disrupt this balance, allowing harmful microorganisms to dominate and induce oral diseases **(Persoon *et al*., 2017; Gao *et al*., 2018; Deo *et al*., 2019; Sterzenbach *et al*., 2020)**.

Oral biofilm is particularly significant in the development of conditions like periodontitis and dental caries. Predominantly, tooth infections stem from the oral cavity's natural flora, with a mix of aerobic and anaerobic bacteria responsible for these polymicrobial diseases. *Enterococcus faecalis*, notable for its prevalence in various oral infections, exemplifies the challenges in treating these conditions due to its resistance capabilities, including biofilm formation and evasion of immune defenses **(Stuart *et al*., 2006; Klein, 2011; Kouidhi *et al*., 2011; Suliman Al-Badah *et al*., 2015; Kanwar *et al*., 2017; Grenier *et al*., 2020)**.

During the infection process, the activation of phagocytes surface receptor results in a phagocytosis stimulation, a synthesis of ROS (reactive oxygen species), an activation of humoral and cellular responses in addition to a production of inflammatory mediators. As result, the normal redox of cells is disturbed by the accumulation of ROS which causes oxidative stress **(Hernández-Ríos *et al*., 2017)**.

Addressing these challenges, research has turned towards natural antimicrobial solutions, including apitherapy. This ancient method utilizes bee products like propolis, known for its broad medicinal properties, including antimicrobial and anti-inflammatory effects. Propolis, a resinous mixture produced by bees, has gained attention for its potential in treating resistant bacterial infections, including those caused by *Enterococcus faecalis*. Its effectiveness is attributed to its rich polyphenolic content, highlighting the importance of exploring natural remedies in combating oral pathogens and enhancing oral health **(Castaldo *et al*., 2002; Parolia *et al*., 2010;**

Gupta *et al*., 2014; Piccinelli *et al*., 2011; Campoccia *et al*., 2021; Zulhendri *et al*., 2021; Boulechfar *et al*., 2022)

The proposed thesis aims to explore the potential of propolis in treating resistant oral infections, focusing on its antimicrobial and antibiofilm activities. The research plan includes various methodologies, from the extraction and characterization of propolis components to evaluating their therapeutic effectiveness against resistant bacterial strains, underscoring the promise of natural substances in addressing oral health challenges.

Bibliographical STUDY

Chapter I

Apitherapy: propolis

1 Natural product and apitherapy

Since ancient times, natural products have been considered as a significant source of therapeutic agents. It has been estimated that 25–30% of medications are derived from plants, animals and microorganisms **(Soltani, 2017)**. According to the OMS, traditional medicine is getting a lot of attention around the world "In China, traditional medicine accounts for about 40 % of all health care services; in Chile and Colombia, 71 % and 40 % of the populations, respectively use traditional medicine; in India, 65 % of rural residents use Ayurveda and herbal medicine as primary health care services. In developed countries, traditional and complementary medicine is gaining popularity. For example, the percentage of the population that has used it at least once is 48 % in Australia, 31 % in Belgium, 70 % in Canada, 49 % in France and 42 % in the United States" **(OMS, 2013)**.

Apitherapy is a type of traditional medicine that uses propolis, royal jelly, pollen, venom and honey to prevent and treat various diseases **(Gupta *et al.*, 2014)**; Its usage in medicine dates back over 6,000 years to ancient Egypt; bee products were also utilized medicinally by the Greeks and Romans **(Kareem, 2022)**.

Many manuscripts describe the therapeutic properties of bee products against dental diseases, oral ulcers, cardiovascular diseases, Alzheimer's disease, diabetes, cancer, as well as their anti-inflammatory, antihypertensive, anti-estrogenic, anti-hypercholesterolemic and antioxidant properties **(Ahuja *et al.*, 2011; Andreea *et al.*, 2020).**

Propolis is the most interesting product of the hive, known for centuries for its biological and pharmacological properties, including antibacterial, antiseptic, anti-inflammatory and anesthetic properties **(Almuhayawi, 2020)**.

2 Propolis

Propolis is a resinous substance that bees (*Apis mellifera L.*) harvest and prepare from resins, waxes, and gums found on the buds and bark of various botanical species (**figure 1, 2**) **(Marco *et al.*, 2017)**. The term "**Propolis**" is derived from the Greek words "**Pro**" which means forward and "**Polis**" which is the city or the entrance to the hive **(figure 3, 4) (Kasiotis *et al.*, 2017)**.

Figure 1. Plant Gums (http://www.differencebetween.net/science/difference-between-gum-and-mucilage/)

Figure 2. Resin of a bud (https://coloradorealsoap.com/blog/2017/3/29/natural-pain-relief-healing-with-cottonwood-buds)

The bees transport this resinous substance to the hive (**figure 3, 4**), where they modify it before depositing it in the hive by adding some of their own secretions and then use it to seal and protect the hive **(Fokt *et al.*, 2010)**. Propolis is used by bees to defend themselves against insects and microorganisms. It is used as a cement to seal cracks or open spaces in the hive, to sterilize the queen bee site and to mummify invading insects or against weather threats like wind and rain **(Ramos *et al.*, 2007 ;Wagh, 2013) (figure 5)**.

Figure 3. The mastication of the hive
(https://bestbees.com/2023/05/26/bee-propolis/)

Figure 4. Aspect of propolis (https://www.compagnie-des-/sens.fr/propolis-guide-complet)

Figure 5. mummifies intruders
https://www.honeyrunfarm.com/honeyrunfarm/2020/8/1/propolis-and-more)

3 Origin of propolis

Propolis is obtained from different sources:

- Botanical origin in which worker bees collect resins from tree buds, plant exudates, or resins found in the stem, branches, or leaves of different plants, and triturate them with their mandibles before mixing them with other salivary substances to make propolis **(Dezmirean *et al*., 2021)**.
- Incidental materials introduced during propolis production (pollen, nectar or honey) **(Mărghitaş *et al*., 2013)**.

The different types of propolis, as well as their geographical and botanical origins, are presented in **Table (1)**.

Table 1. Types of propolis and their geographical and botanical origins **(Cardinault *et al.*, 2012)**

Type of propolis	Geographical origin	Botanical origin
Poplar amber to brown	Europe, North America, non-tropical regions Asia, New Zealand	*Populus spp.* and especially *P. nigra L.*
Brazilian Green	Tropical zone of Brazil	*Baccharis spp.* especially *B. dracunculifolia DC*
Birch	North of Russia	*Betula verrucosa*
Red propolis	Cuba, Brazil, Mexico	*Dalbergia ecastophyllum*
Red propolis	Cuba, Venezuela	*Clusia rosea*
Mediterranean	Sicily, Greece, Malta, Crete Turkey	Family of Cupressacea
Pacific	Pacific zone (Taiwan, Okinawa, Indonesia)	*Macaranga tanarius*

4 The characteristics of propolis

The different characteristics of propolis such as odor, color, constitution are really varied depending on:

- Botanical source **(Bankova, 2005)**.
- geographical region **(Chan *et al*., 2013)**.
- Climate, season and environmental conditions **(Barlak *et al*., 2011)**.

5 Propolis collected

The harvest of propolis is an operation carried out by the worker bees and the beekeeper:

5.1 By worker bees foraging

The worker bees in the hive play several important roles, they collect various resins found on some parts of plants and mix them by their own secretion (wax, saliva) to make propolis, the bees used it to protect their hive and their colonies against aggressors from sealing cracks and holes, mummify the dead intruder animals to avoid their decomposition inside the hive and also against moisture and drafts unfavorable to the hive **(Wagh, 2013)**. Propolis is harvested by bees during the spring season and in the fall at the end of the honey flow when the harvesting temperature is above 20 °C **(figure 6) (Melin, 2011)**.

The yield of harvested propolis depends on the race; it is the Caucasian bees that produce an abundant quantity of propolis than the other races and according to the type of flora within the other races and also according to the location and geographical conditions (Forest area) of the hive (**Philippe, 1994**).

Figure 6. Bees bridging the gaps of the hive using propolis (https://www.apistory.fr/PAGES/lapropolis.html, https://thebeestore.com.au/blogs/bee-blog/what-is-propolis-its-uses-and-how-to-collect-it)

5.2 By beekeeper

Propolis is harvested by beekeepers using two main methods:

- The scraping of the frames and the different parts of the hive **(Bruneau, 2000)**.
- By placing grids (plastic, metal) on top of the frame where the bees fill the holes in the grid with propolis **(Cuvillier, 2015)**.
- The grids are then put in the freezer for a while, after this time the propolis becomes crumbly and detaches easily from the grids, then the raw propolis is recovered by the beekeepers **(figure 7, 8)** (**Philippe, 1994**).

Figure 7. Recovery of propolis by scraping the frames of the hive (https://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=5190)

Figure 8. Propolis harvesting from a grid
(https://www.etsy.com/fr/listing/1518726727/propolis-brute-en-vrac)

6 Factors influencing the harvest of propolis

The harvest of propolis will be determined by a number of factors, including **(Mountford-McAuley *et al.*, 2021)**:

- Vegetable sources.
- Environmental conditions such as plant diversity, climate and seasonality
- Hive structure; wooden hives give a very high production compared to polystyrene, wooden and plastic hives; hives with spaced top bars
- Honey bee races: Honey bees (*A. mellifera*) have adapted to the ecological conditions within different countries, resulting in races of local ecotypes such as Caucasian bees (*A. mellifera caucasica*), Carniolan bees (*A. mellifera carnica*), Italian bees (*A. mellifera ligustica*), Anatolian bees (*A. mellifera anatoliaca*), German black bees (*A. mellifera mellifera*) and Africanised honey bees (*A. mellifera scutellata* and its hybrids).

7 Physicochemical characteristics of propolis

The consistency of propolis varies depending on temperature. It is soft, pliable and very sticky at high temperatures; on the other hand, when it is cooled, especially when it is frozen or almost frozen, it becomes hard and brittle **(Martinotti *et al.*, 2015)**. Propolis will become liquid at 60 °C to 70 °C, but the melting point of some samples

may be as high at 100 °C **(Wagh, 2013)**. The color of propolis varies considerably, from dark brown to yellow, through green or red **(Chasset *et al.*, 2016).**

Propolis smells different depending on its botanical origin and aromatic resins, but it has a pleasant and sweet aroma that is mixed with honey, wax, and other products (cinnamon, vanilla ...); Its flavor often pungent and sometimes bitter **(Benaskeur *et al.*, 2004)**.

Various solvents including water, methanol, ethanol, chloroform, dichloromethane, ether and acetone were used to remove inert material and extract the desired propolis compounds **(Wagh, 2013)**.

8 Chemical composition of propolis

Chemical profiling of propolis is accomplished using a variety of techniques, including HPLC-DAD, LC-MS, LC-MS-MS, GC-MS; because of the relatively polar nature of propolis constituents (in general, they have several OH groups in their molecules), and the introduction of soft ionization techniques compatible with liquid chromatography in the 1990s, HPLC-DAD and HPLC-MS became the preferred methods for analyzing propolis constituents **(Sforcin *et al.*, 2011)**. Between 2000 and 2012, 241 compounds were identified in propolis for the first time through a systematic database search, and they belong to chemical classes as diverse as flavonoids, phenylpropanoids, terpenenes, stilbenes, lignans, coumarins, and their prenylated derivatives, demonstrating a pattern consistent with around 300 previously reported compounds **(Huang *et al.*, 2014)**.

Propolis usually contains resins (50%), composed of flavonoids and phenolic acids, waxes (up to 30%), essential oils (10%), pollen (5%) and various organic compounds (5%) such as Fe, Zn, vitamins (B1, B2, B3 and B6), benzoic acid, ketones, lactones, steroids and sugar, as shown in figure (9) **(Vladimirov *et al.*, 1966; Hossain *et al.*, 2022)**. The proportion of these compounds varies depending on the type of bee, the climatic zone, the local trees and plants and even the time of day it is harvested **(Ahuja *et al.*, 2011).**

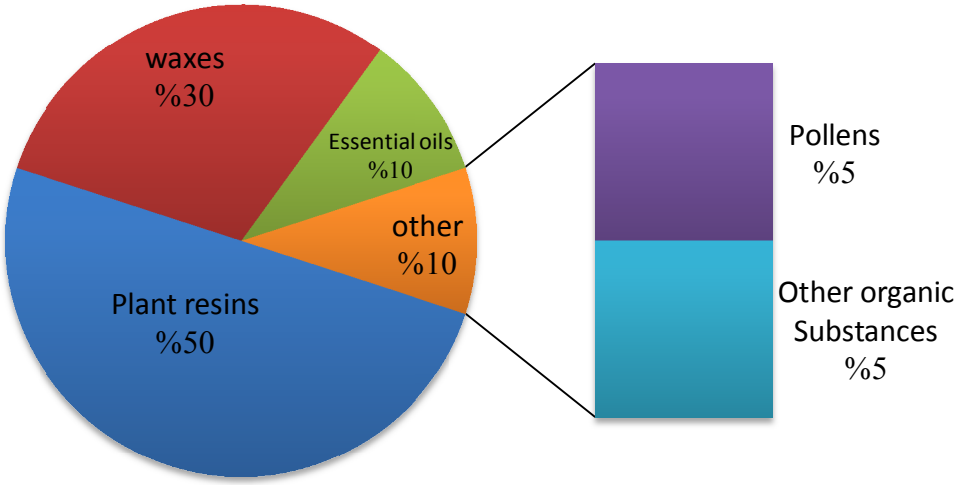


Figure 9. Chemical composition of propolis (%)

The composition of propolis is directly related to the origin of the resin collected by the bees from different botanical species such as populous (*Populus spp.*), beech (*Fagus sylvatica*), horsechestnut (*Aesculus hippocastanum*), birch (*Betula alba*), alder (*Alnus glutinosa*), various conifer trees, *Eucalyptus* species and *Baccharis*, *Populus trichocarpa, Populus tremuloides, Dalbergia ecastophyllum* (L) Taub. and *Symphonia globulifera* L.f. **figure (10) (Christov *et al.*, 2006; Saeed *et al.*, 2016 ; Freitas *et al.*, 2022)**.

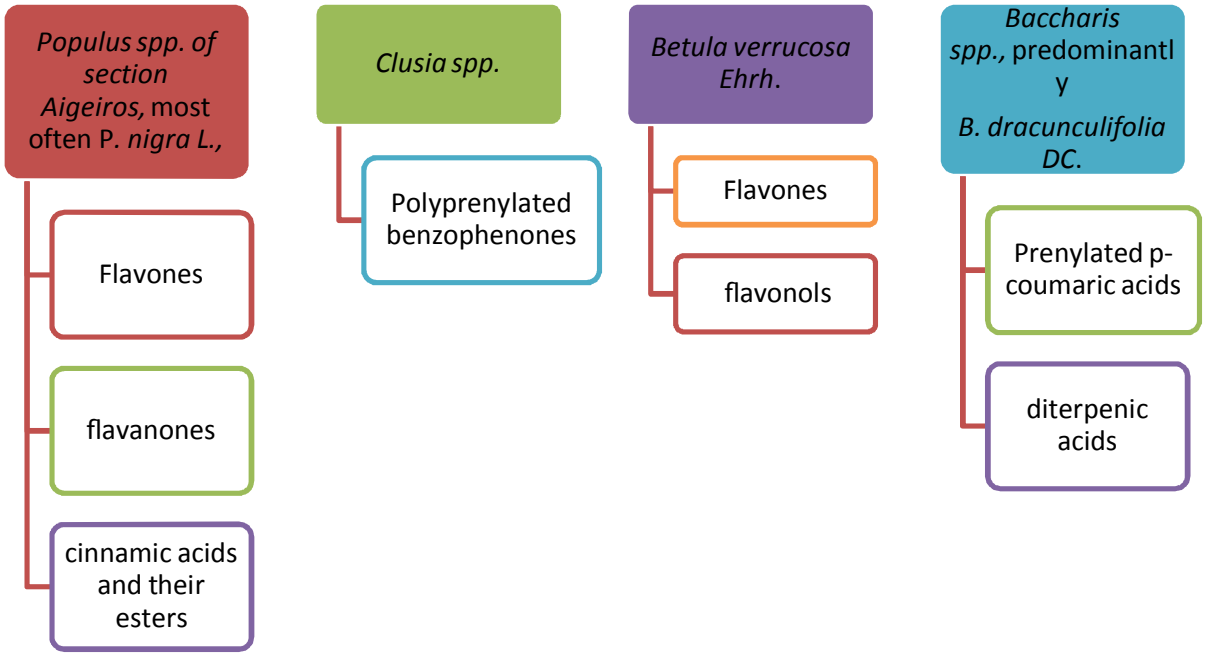


Figure 10. Substances and the plants that produce them in relation with propolis composition **(Bankova, 2005)**

8.1 Phenolic compounds

Propolis is the richest sources of phenolic molecules, mainly flavonoids, phenolic acids and their esters **(Asem *et al.*, 2020)**. Flavonoids, phenolic acids, tannins, lignans and coumarins are a class of metabolites derived from plant secondary pathways that are naturally found in fruits, vegetables, cereals, roots, and leaves, among other plant products. They are secondary metabolites produced by the shikimic acid and phenylpropanoid pathways **(Luna-Guevara *et al.*, 2018)**.

The resinous fraction of propolis contains a high concentration of phenolic compounds (flavonoids and phenolic acid derivatives), which are major active constituents of propolis resinous fraction, which is still widely used as a natural remedy for its antibacterial, antifungal and anticancer properties; many studies reported that propolis contains phenolic and flavonoid compounds such as chrysin, pinocembrin, galangin, pinobanksin, caffeic acid phenethyl ester, gallic acid,

caffeic acid, p-coumaric acid, ferulic acid, chlorogenic acid, quercetin, apigenin, kaempferol **(table 2) (Castaldo *et al.*, 2002; Medana *et al.*, 2008; Cho *et al.*, 2020; Boulechfar *et al.*, 2022).**

Table 2. The principal phenolic acids and flavonoids components in propolis

Groups of components	Subgroups	Constituents	References
Phenolic acids	Hydroxybenzoic acids	Gallic acid, benzoic acid, vanillic acid	**(Pellati *et al.*, 2013)**
	Hydroxycinnamic acid	Caffeic acid, ferulic acid, *p*-coumaric acid, cinnamic acid	**(Christov *et al.*, 2006; Barbarić *et al.*, 2011)**
Flavonoids	Flavones	Chrysin, tectochrysin, apigenin, rutin, luteolin	**(Barbarić *et al.*, 2011; Vică *et al.*, 2022; Zullkiflee *et al.*, 2022)**
	Flavonols	Galangin, Kaempferol, quercetin	**(Borrelli *et al.*, 2002; Coneac *et al.*, 2008)**
	Flavanones	Naringenin, hesperidin, pinocembrin	**(Quiroga *et al.*, 2006;) Laaroussi *et al.*, 2020**

8.2 Essential oils

Essential oils derived from propolis residues could be a natural source of different bioactive components such as alpha-pinene, beta-pinene, camphene, hexanal, p-cymene, myrcene, acetophenone, sesquiterpenes-caryophyllene, (E)-nerolidol and selina-3,7, diene, which responsible for its antioxidant, anti-Alzheimer, antibacterial and antifungal properties **(Melliou *et al.*, 2007; Albuquerque *et al.*, 2008; Ikeda *et al.*, 2021; Boulechfar, 2023)**.

9 Therapeutic properties of propolis

Propolis is known for its biological characteristics, which are due to its flavonoid, phenolic and other aromatic compounds. these properties have promising future applications in medicine and dentistry figure (11) **(Shruthi *et al.*, 2012)**.

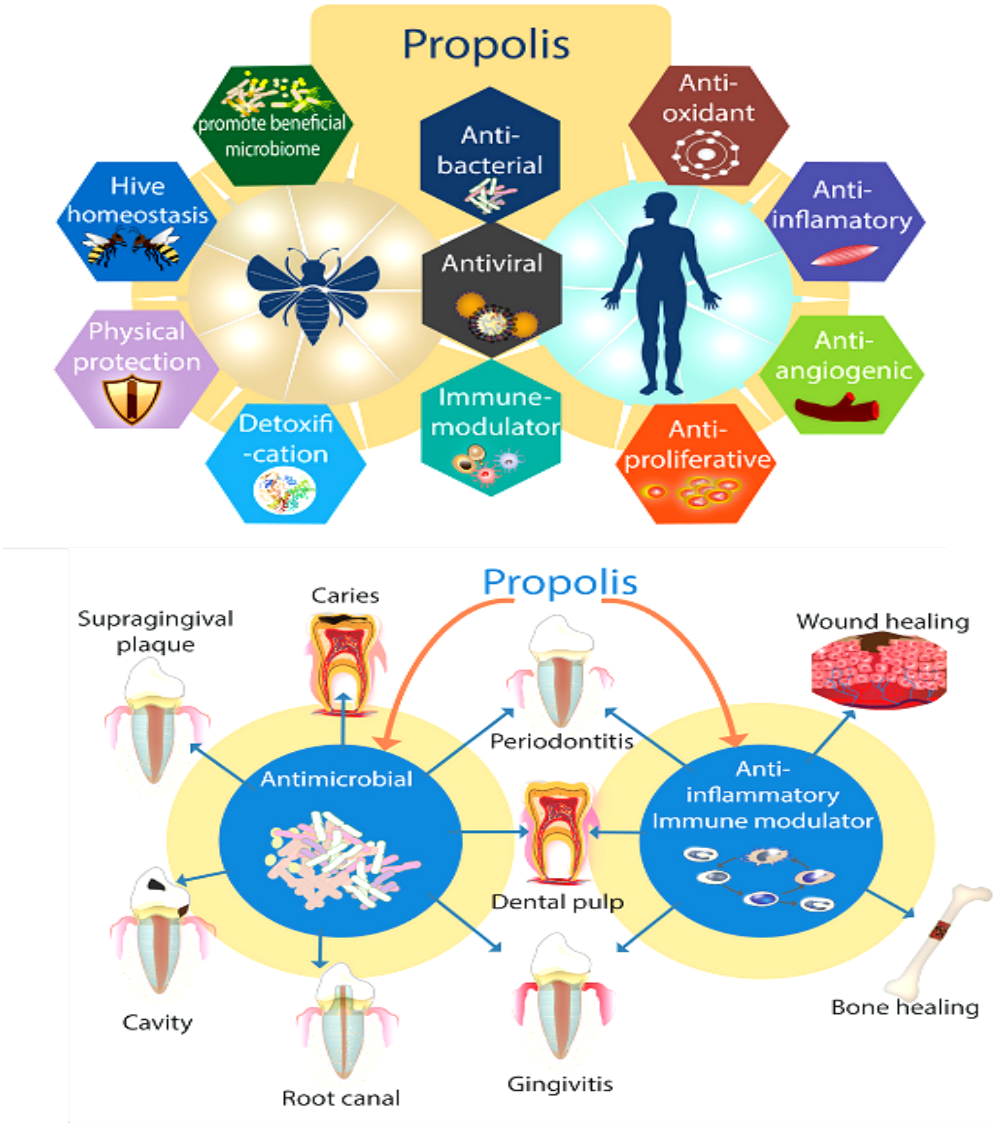


Figure 11. The main therapeutic properties associated with propolis **(Zulhendri *et al.*, 2021)**

9.1 Antibacterial, antibiofilm and antiquorum-sensing Effects

Propolis is a natural antibiotic that has been recognized as a powerful antibacterial agent in dental medicine. Their activity has been demonstrated particularly against Gram-positive cariogenic bacteria involved in the process of dental diseases, including *Streptococcus mutans, Streptococcus sobrinus, Lactobacillus acidophilus, Prevotella intermedia, Lactobacillus salivarius* subsp. *Salivarius* **(Akca *et al.*, 2016)**. It was found to be very effective against periodontitis bacteria (*Peptostreptococcus anaerobius, Porphyromonas gingivalis* and *Prevotella intermedia*); and effective activity against bacteria that cause endodontic infection *Enterococcus faecalis* **(Awawdeh *et al.*, 2009)**.

Propolis has a broad-spectrum inhibitory effect against a wide range of Gram-positive and Gram-negative bacteria such as *S. mutans*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Staphylococcus aureus, Staphylococcus epidermidis* **(Ugur *et al*., 2004; Graikou *et al*., 2016)**.

The bactericidal effects of propolis appear to be due to multiple mechanisms, manifested by alteration of bacterial membrane and cell wall structures, destroy DNA-dependant RNA polymerase, stop and inhibit bacterial cell growth, division and protein production **(Parolia *et al*., 2021; Vadillo-Rodríguez *et al*., 2021)**.

Propolis acts on bacteria through its secondary metabolites, including p-coumaric acid, apigenin, quercetin, caffeic acid phenethyl ester, galangin, pinocembrin and caffeic acid **(Kosalec *et al*., 2003; Salomão *et al*., 2008; Veloz *et al*., 2019)**.

Propolis can be used as an alternative therapeutic agent to combat multidrug-resistant bacteria in both forms planktonic and biofilm such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* **(Santos *et al*., 2020)**.

Propolis reduces biofilm formation in a variety of microorganisms, including clinical *Staphylococcus aureus* strains, methicillin-resistant *S. aureus, Streptococcus mutans, Candida glabrata, Pseudomonas aeruginosa*, which acts on biofilm by causing damage to the extracellular polymer matrix (**Veloz *et al*., 2015; Bryan *et al*., 2016; Doganli, 2016; Marco *et al*., 2017; Daikh *et al*., 2020; Fernández-Calderón *et al*., 2021)**.

Propolis contains tt-farnesol, apegenin, pinocembrin, caffeic and ferulic acids, all of which have mechanisms for inhibiting bacterial biofilm growth and development **(Wahjuningrum *et al*., 2014; Veloz *et al*., 2019)**.

Propolis has the ability to inhibit virulence mechanisms in bacteria such as the quorum-sensing, making it an effective way to combat infections and biofilm formation **(Bulman *et al*., 2011; Alıç *et al*., 2020; Sorucu *et al*., 2021)**. There are several compounds in propolis that inhibit quorum-sensing responses such as isoprenyl caffeate, pinocembrin **(Gemiarto *et al*., 2015; Savka *et al*., 2015)**.

9.2 Antifungal activity

Propolis possesses antifungal and antibiofilm properties that have been observed and demonstrated against three fungi responsible for onychomycoses, including *Fusarium solani, Fusarium oxysporumand, Fusarium subglutinans* **(Galletti *et al*., 2017)**.

Propolis extracts are considered a promising agent for the treatment of onychomycosis due to their activity against planktonic cells and the biofilm formed by *Trichophyton spp* **(Veiga *et al*., 2018)**. Propolis could be used in the future as an alternative product for the treatment of infectious diseases caused by *Candida albicans, Candida glabrata* and *Candida tropicalis* **(Siqueira *et al*., 2015)**.

9.3 Anti-inflammatory properties

Propolis acts as an anti-inflammatory agent, reducing IL-1ß production by inhibiting the inflammasome, inhibition of prostaglandin synthesis, inhibition of the proinflammatory cytokines TNF-α, IL-6, IL-8 and increase in the anti-inflammatory cytokines TGF-β and IL-10 **(MacHado *et al.*, 2012; Hori *et al*., 2013; Boufadi *et al*., 2021)**. Polyphenols play a very important role in immune system regulation through gene expression, regulation of immune cell, synthesis proinflammatory cytokines **(Yahfoufi *et al*., 2018)**.

9.4 Antioxidant activity

Oxidation is a necessary process of cellular metabolism involving the production of oxygen, whose uncontrolled metabolic production leads to the formation of free radicals (superoxide $O2^{\bullet-}$, hydroxyl $HO^{\bullet}$, alkoxyl $RO^{\bullet}$ et peroxyl $RO2^{\bullet}$) which can damage lipids, DNA, carbohydrates and proteins and lead to the development and progression of various pathologies **(Codoñer-Franch *et al*., 2011; Rashid *et al*., 2013; Sarr *et al*., 2015)**.

Oxidative stress causes the emergence and progression of severe diseases; to address this issue, several research teams are focusing on the search of new antioxidants to combat oxidative stress and related pathologies **(Oliveira *et al*., 2015; Surai, 2015)**.

Propolis is a natural product distinguished by its ability to scavenge and reduce radicals, which is directly linked to its abundance of polyphenols such as Kaempferol, caffeic acid phenethyl ester, and quercetin, Artepillin C, and galangin **(Kumazawa *et al*., 2004; Ahn *et al*., 2009; Narimane *et al*., 2017)**.

The process by which polyphenols exert their antioxidant action is as follows **(Kurek-Górecka *et al*., 2014)**:

- Inhibits the production of radicals by inhibiting the activity of enzymes involved in their creation
- Chelating ions of metals used to generate free radicals
- Scavenging reactive forms of oxygen (ROS), thus interrupting the cascade of reactions leading to the peroxidation of lipids
- Synergistic effect with other compounds having an antioxidant effect

9.5 Antiviral Activity

Propolis has a significant antiviral activity on the influenza virus and the COVID-19, a herpetic infection caused by the type 2 herpes simplex virus **(Shimizu *et al*., 2008; Nolkemper *et al*., 2010; Refaat *et al*., 2021)**.

9.6 Antiparasitic activity

Numerous research works have shown the effect of propolis on the trypanosoma protozoa (*Trypanosoma brucei*) and S*chistosoma mansoni* infection **(Alanazi *et al*., 2021; Silva *et al*., 2021)**.

10 Other propolis properties

Propolis is a bioactive substance with the following properties:

- Anti-tumor agents that influence cancer cell proliferation and apoptosis via the bioactive substance caffeic acid phenethyl ester (CAPE) **(Wu *et al*., 2011)**.
- Propolis has the ability to combat *Helicobacter pylori* the agent responsible of gastric inflammation, peptic ulcer, gastric cancer, and lymphomas of mucosa associated lymphoid tissues **(Shapla *et al*., 2018)**.
- effective in the treatment of gynecological problems and neurodegenerative diseases **(Imhof *et al*., 2005; Hussein *et al*., 2017)**.

11 The interest of propolis in oral health

Numerous scientific studies have demonstrated the benefits of propolis for oral health, including its ability to combat the bacteria that causes tooth decay, *Streptococcus mutans* **(Ophori *et al*., 2010; Zulhendri *et al*., 2021)**. The flavones and flavonols found in propolis inhibit polysaccharide synthesis by inhibiting the activity of virulence factors involved in the pathogenesis of tooth decay glucosyltransferases **(Koo *et al*., 2002)**.

Propolis is also considered an alternative product that can be used to disinfect the dental canal during endodontic therapy, it has potent effect on periodontopathogenic bacteria including *Tannerella forsythensis, Porphyromonas gingivalis, Prevotella intermedia and Treponema denticola*, as well as a powerful effect on candidiasis and aphthous stomatitis **(Samet *et al*., 2007; Madhubala *et al*., 2011; Sayyadi *et al*., 2020; Lisbona-González *et al*., 2021)**.

Chapter 2: Oral diseases

1 Oral cavity ecosystem

Oral cavity is a complex ecosystem composed of teeth, gingival sillon, hard, soft palate, amygdales and among others, which colonized by different types of microorganisms that estimated by over 600 prevalent taxa, with distinct subsets predominating at different habitats **figure (12) (Samaranayake *et al.*, 2017; Dewhirst *et al.*, 2010)**. A variety of factors, including temperature, pH, redox potential, nutrient, water, anatomy, salivary flow affect the growth of microorganisms and maintain the balance between bacterial communities; So environmental characteristics determine which microbes occupy a site, and the metabolic activities of these microbial populations modify these characteristics **(Marcotte *et al.*, 1998; Takahashi, 2005)**.

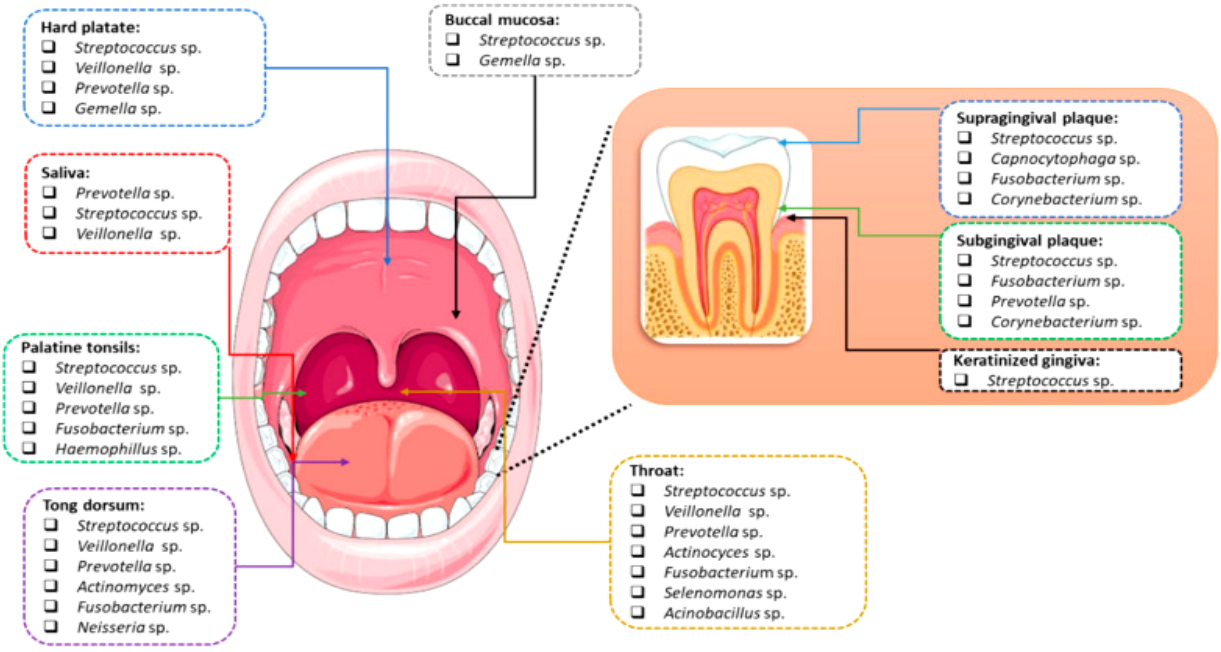


Figure 12. Oral Ecosystem **(Ptasiewicz *et al.*, 2022)**

2 Teeth

Teeth are hard and calcified organs found in the oral cavity that are attached to the upper jaw (maxilla) and lower jaw (mandible) and perform the following functions **(Ahmed, 2010; Morris *et al.*, 2021)**:

- cutting and crushing of food during chewing
- participate in articulated language
- participate in the development and protection of the tissues that fix them inside the dental alveoli

Teeth are classified in four groups according to their shape: incisors, canines, small molars, large molars **(Tafti *et al.*, 2021) figure (13)**

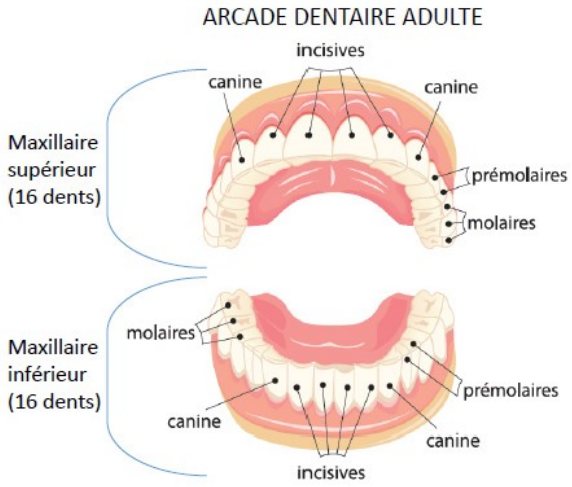


Figure 13. Different tooth classes (https://www.santepourtous.nc/les-thematiques/mes-dents-ma-sante/generalites/anatomie-de-la-bouche/171-les-dents)

Tooth is anatomically represented by two parts the crown and the root, each tooth is composed of four calcified tissues, it includes enamel, dentin, cementum and a specialized soft connective tissue, the pulp **(Lautrou, 1997) figure (14)**.

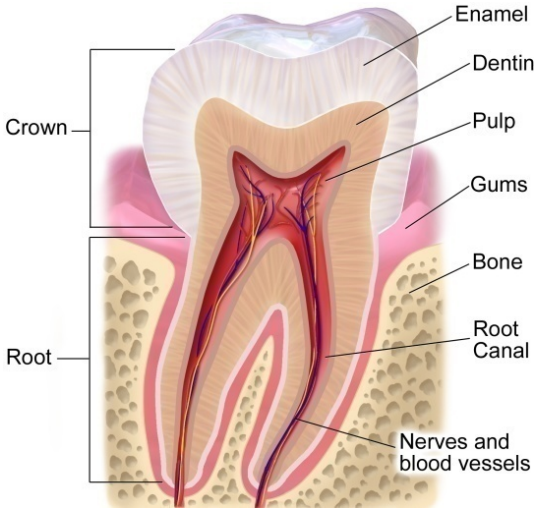


Figure 14. Anatomy of tooth **(Lacruz *et al.*, 2017)**

2.1 Enamel

Enamel is the most difficult material to create through biological processes. It originates from the epithelium and serves as the anatomical crown of the tooth. It is composed of approximately 96 % inorganic apatite crystals and 4% organic matter and water **(Lynnerup *et al.*, 2019)**. Enamel is formed by specific matrix proteins (amelogenin, ameloblastin, and enamelin) that are deposited in a highly organized manner and then gradually degraded **(Duverger, 2015)**. Teeth enamel protects the dentin and eventually the pulp from decay and infection **(Lacruz *et al.*, 2017)**.

2.2 Dentin

Dentin is the calcified tissue that makes up the majority of the structure of a tooth. It is composed of about 70 % calcium hydroxyapatite, 18 % organic matter (collagen fibers), 12 % water and making it harder than cementum but softer and less brittle than enamel **(Scheid *et al.*, 2012; Giudice *et al.*, 2015)**. Dentin contains a large number of small parallel tubules that are embedded in a highly mineralized collagen matrix. This structure is stronger than enamel in compressive, tensile and flexural strength **(Varley *et al.*, 2019)**. Dentine is produced by odontoblasts, which are cells that line the pulpal cavity. The odontoblaste serves as a reservoir for dormant cells. When there is a trauma or a dental caries, the odontoblasts become activated and contribute to the rapid formation of reparative dentine **(Huang *et al.*, 2009)**.

Dentin protects the pulp tissue from microbes and other harmful stimuli. It also provides essential support to the enamel and allows the enamel, which is highly mineralized and therefore fragile, to withstand occlusal and masticatory forces without breaking. In addition, it is the first vital tissue to be exposed to external irritation and rather than being a passive mechanical barrier, dentin may play a role in the defensive reactions of the dento-pulp complex **(Tjäderhane *et al.*, 2009)**.

Dentin, for example, contains several growth factors that can be released during wear or decay and help regulate the defense response at the dentin-pulp boundary or in the pulp itself **(Tjäderhane, 2019)**.

2.3 Root canal system

The root canal system consists of two parts: the pulp chamber, which is located in the anatomic crown of the tooth and the pulp or root canal (or canals), which is located in the anatomic root of the tooth **figure (15) (Gutmann, 2021)**. The dental pulp is contained within a rigid chamber of dentin, enamel and cement, which provides mechanical support and protection from the microbe-rich oral environment **(Yu *et al.*, 2007)**.

The dental pulp is a vital and vascularized tissue that ensures the formation, nutrition, protection and restoration characteristics of the teeth **(Bindal *et al.*, 2017)**. Dental pulp, the only soft tissue in teeth, composed of fibroblasts, odontoblasts, immune cells, nerves, blood vessels, extracellular matrix, interstitial fluid and other cellular components that nourish teeth, form dentin, transmit sensory information and provide immunoprotection **(Li *et al.*, 2021)**. Teeth with pulp are much more resistant to bacterial invasion in the tubules dentinaires than teeth with canal obturation; in these cases, bacteria can penetrate the teeth and reach the canal system in a relatively short period of time **(Nagaoka *et al.*, 1995).**

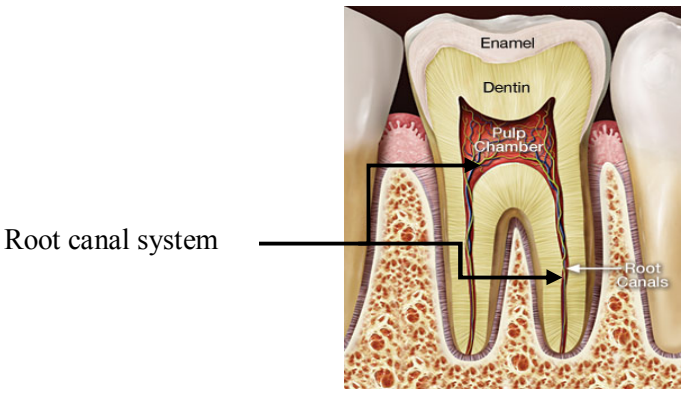


Figure15. Components of the root canal system (https://www.thorpdentalcenter.com/endodontics)

2.4 Periodontium

The periodontium is a connective tissue organ, protected by epithelium that attaches the teeth to the bone of the jaws and provides a continually adapting apparatus for their support during function. consisting of four components: the gingiva, the periodontal ligament, the cementum and the alveolar bone **(Melcher, 1976)**.

2.5 Cementum

Cementum is a thin layer of hard dental tissue that covers the anatomical roots of the teeth; it is formed by cementoblasts cells, which develop from undifferentiated mesenchymal cells in the dental follicle connective tissue. Cementum is slightly softer than dentin and composed of 45-50 % inorganic material (hydroxyapatite) and 50-55% organic material (collagen, protein polysaccharides and water) **(Boushell *et al.*, 2018)**. The cement serves two important functions: on the one hand, it ensures the attachment of the dent to the alveolar os through the insertion of parodontal ligament fibers and on the other hand, it prevents racine resorption during parodonte remodeling **(Hughes, 2015)**.

2.6 Periodontal ligament

Periodontal ligament is a highly specialized connective tissue that exists between the tooth and the alveolar bone. Its primary function is to connect the tooth to the jaw, which it must do in such a way that the tooth can withstand the significant forces of mastication **(Melcher, 1976)**. Periodontal ligament is composed of various types of cells, fibers and cellular components including osteoblasts, osteoclasts, fibroblasts,

malassez epithelial remnants, odontoblasts, cementoblasts, macrophages, and undifferentiated mesenchymal cells **(Miguez *et al*., 2018)**.

2.7 Alveolar bone

The alveolar bone is that part of the mandibular and maxillary bone which surrounds the teeth and forms the tooth sockets **(Hughes, 2015)**.

2.8 Gingival tissue

Gum tissue (commonly known as gums) is the soft tissue that covers the alveolar bone of the jaws and the teeth up to the exposed crown of the teeth **(Zwetchkenbaum *et al*., 2008)**. The gingiva is composed of two distinct stratified epithelia (the junctional epithelium and the oral epithelium) as well as a densely collagenous lamina propria containing the supra-alveolar fibrous apparatus, blood and lymphatic vessels, and nerves **(Schroeder *et al*., 1997)**. The gingiva is a masticatory mucosa component that acts as an internal defense against pathogens and mechanical stress **(Yildirim *et al*., 2017)**.

3 Oral diseases

Oral diseases are among the most common diseases worldwide, with serious health and economic consequences that significantly reduce the quality of life for those affected **(Peres *et al*., 2019)**. After the gut, the oral cavity has the second largest and most diverse microbiota, containing over 700 species of bacteria. It supports a wide range of microorganisms, including bacteria, fungi, viruses and protozoa; mouth with its various niches an unusually complex environment in which microbes colonize the surfaces of the teeth and the mucus tissues **(Deo *et al*., 2019)**.

Throughout a person's life, a homeostatic balance is maintained between the host and the oral microbial community via a variety of bidirectional communication and regulatory mechanisms **(Li *et al*., 2022)**; when this balance is broken and affected by changes in the condition (poor oral hygiene, diet rich in carbohydrates, inflammatory, autoimmune diseases and immunodeficiency disorders), some microbes can dominate and cause diseases **(Persoon *et al*., 2017; Sterzenbach *et al*., 2020)**. Oral microorganisms have been associated with a multitude of diseases of the oral cavity, including dental caries, periodontal disease, endodontic infections and even oral cancer **(Sampaio-Maia *et al*., 2016)**.

Enterococcus faecalis is one of microorganism that plays a significant role in human oral cavity infections including periodontitis, dental carie and especially in endodontic treatment failure **(Kouidhi *et al.*, 2011; Rams *et al.*, 2013; Najafi *et al.*, 2020)**.

3.1 Dental carie

Tooth decay is a chronic infectious disease caused by cariogenic bacteria adhering to teeth, which metabolize sugars to produce acid, demineralizing the tooth structure over time **(Yu *et al.*, 2007)**. *Streptococcus mutans* is regarded as one of the primary agents responsible for dental caries. One important virulence property of the bacteria its ability to form a biofilm on the surfaces of the teeth. This organism also produces glucosyltransferases, multiple glucan-binding proteins, protein antigen c and collagen-binding protein, as well as surface proteins that coordinate that combine to form the dental plaque, which results in dental caries **(Matsumoto-Nakano, 2018; Chen *et al.*, 2021)**.

3.2 Periodontal diseases

Periodontal diseases are a group of inflammatory diseases that affect the structures that support the teeth (gingiva, bone and periodontal ligament), causing tooth loss and contributing to systemic inflammation **(Lisbona-González *et al.*, 2021)**. They are caused by certain bacteria found in the bacterial plaque *A. actinomycetemcomitans, P. gingivalis, P. intermedia, B. forsythus, C. rectus, E. nodatum, P. micros, S. intermedius and Treponema sp, Enterococcus faecalis* **(Lovegrove, 2004; Sun *et al.*, 2012)**.

3.3 Oral candidiasis

Oral candidiasis is a common opportunistic infection of the oral cavity caused by a *Candida* species overgrowth, the most common of which is *Candida albicans* **(Tanda, 2020)**. *Candida albicans*, is a highly versatile commensal organism that is well adapted to its human host; however, changes in the host microenvironment can promote the transition from one of commensalism to pathogen **(Vila *et al.*, 2020)**.

Saliva plays an important role in maintaining microbial homeostasis in the oral cavity, while salivary gland hypofunction predisposes the oral mucosa to pathologic alteration and increases the risk for oral candidiasis **(Mahajan *et al.*, 2015)**. It causes a variety of diseases in the oral cavity, including pseudomembranous candidiasis (oral

thrush), erythematous candidiasis, denture stomatitis and angular cheilitis **(Wilson and Wilson, 2021)** .

3.4 Endodontic infections

The root canal system is in its healthy and intact state free from infection. Unlike the oral cavity, the root canal system has no commensal microbiota and any microorganism detected here can be regarded as a potential pathogen **(Persoon *et al*., 2017)**. Endodontic diseases are caused by the loss of integrity of the outer surface of the tooth as a result of caries, traumatic dental injuries, tooth wear and/or dental surgical procedures. These allow the root canal space to be exposed to the microbiota normally present in the oral cavity **(Thebault *et al*., 1995)**.

Root canal infections are caused by microorganisms that have colonized the root canal system after entering the dental pulp. They cause inflammation of the pulp tissue, which leads to pulp death and spread of the inflammation/infection to the periradicular tissues **(Wong *et al*., 2021)**

Endodontic disease is defined as inflammation (pulpitis) or necrosis (partial or total) of pulp tissue. Pulpitis can be reversible or irreversible, depending on the severity of the attack. Reversible pulpitis is usually caused by a minor injury that allows the tooth to survive. Irreversible pulpitis is caused by severe pulp inflammation and results in the death of the tooth **(Niemiec, 2005)**. Root canal infections are primarily characterized by microbial biofilm that adhere to the root canal dentin and spread to the apical foramina and in some cases beyond **(Gulabivala *et al*., 2015)**.

Endodontic infections are multimicrobial in nature; in primary infections, anaerobic bacteria dominate the microbiota and many microorganisms associated with chronic infections have been linked to intra- and extra-radicular infections **(Abusrewil *et al*., 2020)**.

Endodontic disease has been found to contain a variety of bacteria, including Gram-negative anaerobic rods; periodontal pathogens such as *Prevotella intermedia, Prevotella denticola, Prevotella sp, Porphyromonas endodontalis, Porphyromonas gingivalis, Porphyromonas gingivalis*, *Tannerella forsythia* is an obligate anaerobic asaccharolytic bacterium, coccobacilli that are Gram-negative bacteria with periplasmic flagella include *Dialister sp*, *Fusobacterium nucleatum, Fusobacterium periodonticum, Denticola Treponema,* Gram-positive anaerobic cocci, *Treponema*

sacranskii, Treponema sp, Streptococcus spp, Peptostreptococcus micros and Enterococcus faecalis **(Narayanan *et al*., 2010)**.

Endodontic infections are classified based on their anatomic location (intraradicular or extraradicular infection) and the time at which the microorganisms involved gained access to the radicular canal (primaire, secondaire or persistante) **(Siqueira *et al*., 2005)**.

Primary endodontic infections are caused by microorganisms involved in the initial invasion of the pulp and subsequent colonization of necrotic tissue. Secondary infections are caused by microorganisms introduced into the root canal as a result of professional intervention. Microorganisms that have survived in the rare environment of treated root canals and have proven resistant to chemical debridement procedures cause chronic endodontic infections **(Siqueira, 2002; Wong *et al*., 2021)**.

3.4.1 Etiology and pathogenicity of endodontic diseases

The presence of certain bacteria inside the root canal system such as *Enterococcus faecalis* is the primary cause of endodontic failure. These bacteria are more resistant to disinfectants, resulting in a chronic intraradicular or extraradicular infection **(Alghamdi *et al*., 2020)**.

Enterococcus faecalis is a Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacterium that inhabits the gastrointestinal tract, oral cavity and vagina as normal commensal in humans. It can withstand a variety of stresses and hostile environments, including extreme temperatures (5-65°C), pH (4.5-10.0), and high NaCl concentration, allowing it to colonize a diverse range of niches **(Jett *et al*., 1994 ; Fisher *et al*., 2009)**.

The microorganism *Enterococcus faecalis* was found in 24 % to 77 % of asymptomatic and persistent endodontic infections. This finding could be attributed to a number of survival and virulence factors such as its ability to compete with other microorganisms, invade dentinal tubules and resist nutritional deprivation **(Stuart *et al*., 2006)**.

Enterococcus faecalis are opportunistic pathogens that can form biofilm during endodontic infections. They have many virulence factors, including extracellular surface protein, gelatinase, aggregation substance, *E. faecalis* collagen adhesion, serine protease, lipoteichoic acid and antigen A are implicated in *E. faecalis* adhesion,

colonization and resistance during infections **(Najafi *et al.*, 2020)**. While some bacterial products may be directly linked to periradicular tissue damage, the host response to the bacterium and its products is most likely to blame for a large portion of the tissue damage **(Kayaoglu *et al.*, 2004)**.

- Gene ace is responsible for the production of the collagen-binding adhesin protein, which is also responsible for biofilm adhesion to root canal walls.
- Gene esp produces a surface protein that aids biofilm adhesion to root canal walls **(Francisco *et al.*, 2021)**
- Through lipoteichoc acids (LTA), *E. faecalis* can bind to a variety of eukaryotic cells, including platelets, erythrocytes, leukocytes, lymphocytes, and epithelial cells; induce apoptosis of certain cell lines, including macrophages osteoblasts, osteoclasts, periodontal ligament fibroblasts, macrophages and neutrophils; related to the resistance of *E. faecalis* to adverse conditions and may also be involved in resistance against root canal medicaments applied during endodontic treatment **(Kayaoglu *et al.*, 2004)**.
- Gelatinase (GelE) is an extracellular zinc metalloprotease that hydrolyzes gelatin, collagen and casein involved in biofilm formation **(Mohamed *et al.*, 2007)**.
- Aggregation substance (Asa) is a pheromone-inducible surface protein that promotes aggregation during bacterial conjugation, increase adherence and invasion of cells as well as promote biofilm formation **(Lins *et al.*, 2013; Anderson *et al.*, 2016)**.
- Serine protease (Spr), an endopeptidase enzyme aids in root canal adhesion **(Najafi *et al.*, 2020)**.
- *E.faecalis* antigen A (efA) is throught to contribute to the adhesion of *E.faecalis* to cardiac cells in endocarditis, as well as its role in biofilm formation and pathogenesis of *E.faecalis* in dental root canals **(Reynaud *et al.*, 2007; Beomidehagh *et al.*, 2018)**

4 Oral biofilm

Oral biofilm are functionally and structurally organized polymicrobial communities embedded in an extracellular matrix of exopolymers on mucus and tooth surfaces **(Rosier *et al.*, 2014 ; Kriebel *et al.*, 2018)**.

Quorum sensing is a method of bacterial cell communication in biofilm that involves the production and detection of diffusible signaling molecules that control a wide range of responses including bacterial surface adhesion, extracellular matrix production, spore formation, competence, bioluminescence and virulence factor expression **(Parashar *et al*., 2015)**.

Bacterial association within a biofilm results in synergistic interaction, including **(Agarwal *et al*., 2019)**:

- Metabolic cooperation between species facilitates the catabolism of complex macromolecules, increasing the overall efficiency of the community.
- Cell-to-cell communication; aids in the transport of information between cells of the same species, such as coordinating gene expression with the secretion of small peptides.
- Confer antibiotic resistance genes, as well as antagonism; the production of inhibitory molecules may result in a competitive advantage as well as the exclusion of undesirable microbes.

5 Stages of oral biofilm creation

The formation of the acquired pellicule is the first stage of bioadhesion on dental surfaces. It primarily consists of a cellulosic layer that forms instantly on all solid surfaces exposed to oral fluids. It is composed of proteins, glycoproteins and lipids which are then followed by cyclic processes that require **(figure 16) (Reich *et al*., 2013 ; Abebe, 2021)**:

- Reversible attachment of planktonic bacteria to conditioned solid surfaces such as teeth surfaces
- Synthesis of glue-like exopolysaccharide matrix
- Cells are firmly attached to the surface which is irreversible
- Production of matured biofilm structure
- Dispersion of an organized structure
- Seeking new habitats

There are numerous parameters that influence bacterial attachment including pH, temperature, substrate surface energy, nutritional availability, bacterial contact time, bacterial cell surface charge and surface hydrophobicity **(Narayanan *et al*., 2010)**.

6 Properties of biofilm

Microorganisms in a biofilm must meet four fundamental requirements **(Garg *et al.*, 2018)**:

- Autopoiesis : capacity to self-organize
- Homeostasis: resistance to environmental disturbances
- Synergy: more effective in collaboration than in isolation
- Communality: the ability to respond to environmental changes as a group rather than as individuals.

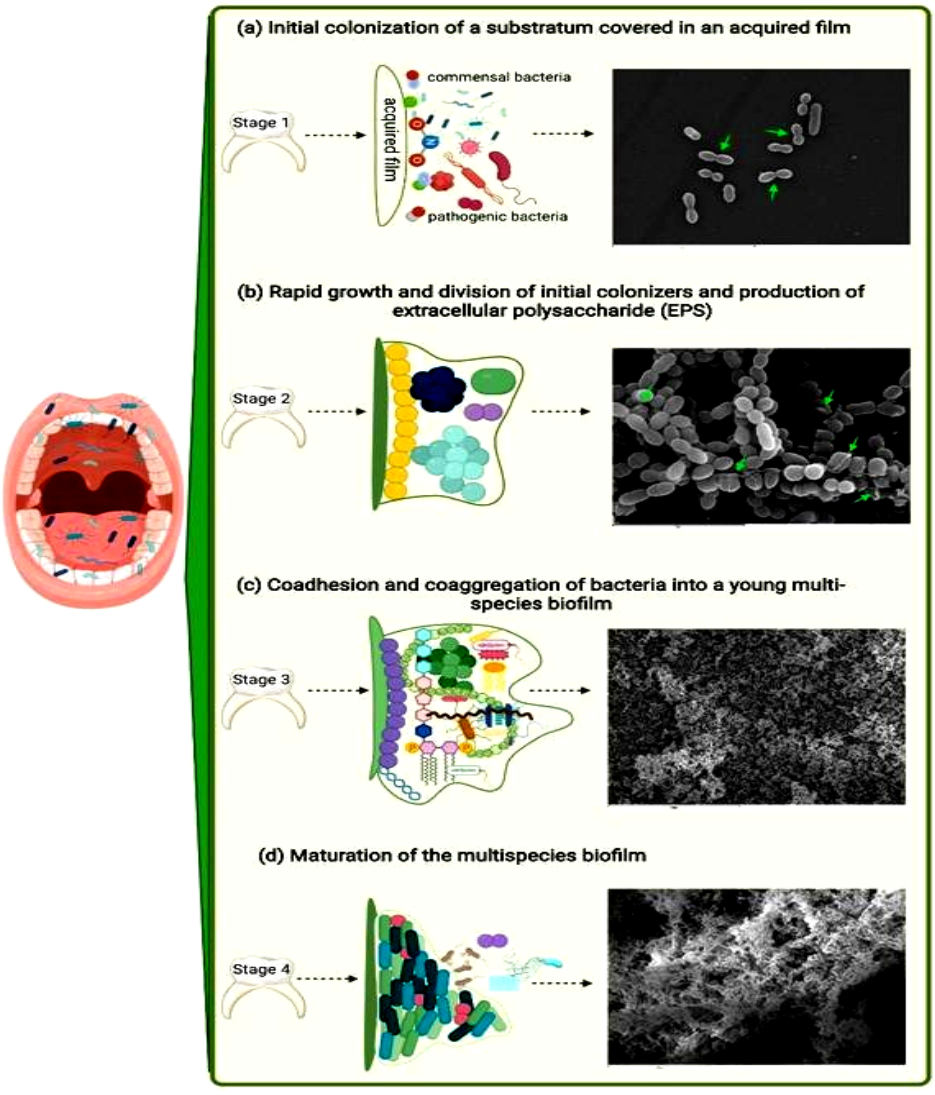


Figure 16. Stages of oral biofilm formation **(Hernández *et al.*, 2022)**

7 Classification of oral biofilm

Based on the infection and biofilm location, oral biofilm can be classified into several types **(Colombo *et al*., 2015; Kriebel *et al*., 2018)**:

- Supragingival biofilm formed above the gingival margin
- Subgingival biofilm formed below the gingival margin
- Cariogenic biofilm
- Periodontopathogenic biofilm
- Endodontic biofilm
- Fungal Biofilm

7.1 Endodontic biofilm

The root canal provides an exceptional microenvironment for several bacterial species to attach to the surface of the dentine and form dense biofilm, which are common on most humid surfaces and can cause environmental problems while also being a frequent source of persistent infections **(Estrela *et al*., 2009)**. Endodontic biofilm are multicellular microbial communities in which microbes are enmeshed in their own extracellular polymeric substance (EPS, typically a polysaccharide) and firmly attached to surfaces (root canal walls, inside isthmuses, ramifications, dentinal tubules, accessory and lateral canals, as well as on external root areas) **(Colombo *et al*., 2015; Yoo *et al*., 2019)**.

Endodontic bacterial biofilm are classified into four types, the most common of which is intracanal, which forms on the root canal dentin of an infected tooth **(Jhajharia *et al*., 2015)**, the bacteria in this biofilm are mostly collections of cocci, rods, filaments, spirochets and other bacterial wastes. The ability of *Enterococcus faecalis* to form a calcified biofilm on root canal dentin may be a major factor contributing to its persistence and resistance to treatment **(Garg and Garg, 2018)**. Root surface biofilm are formed on the root (cementum) surface adjacent to the root apex of endodontically infected teeth, periapical microbial biofilm are isolated biofilm found in the periapical region of endodontically infected teeth, biomaterial centered infection is caused when bacteria adhere to the surface of an artificial biomaterial and form biofilm structures **(Narayanan and Vaishnavi, 2010)**.

8 Quorum-sensing

Quorum sensing (QS) is a type of cell-to-cell communication in bacteria that couples gene expression via the accumulation of signaling molecules, resulting in the production of several virulence factors and the modulation of bacterial behaviors **(Wang *et al*., 2022)**. The quorum sensing mechanism is divided into three categories based on the signal molecules that aid in cell-to-cell communication. These compounds play a role in the synthesis of antibiotics, exoenzymes, virulence factors, and biofilm **(Martinelli *et al*., 2004; Ray, 2021)**:

- LuxI/LuxR-type quorum sensing, which is facilitated by signal molecules acyl-homoserine lactones (AHL) in Gram-negative bacteria.
- Oligopeptide-two-component-type quorum sensing is only for the Gram-positive bacteria where bacterial cells use small peptides as signal molecules.
- LuxS-encoded autoinducer 2 (AI-2) quorum sensing where signal molecules are found in both Gram-positive and Gram-negative bacteria in the gene regulatory mechanism

Chromobacterium violaceum is a gram-negative bacterium that has been used widely in microbiology labs involved in quorum sensing research **(Kothari *et al*., 2017)**. *Chromobacterium violaceum* is a pathogenic bacterium that communicates via quorum sensing (QS), via the C6-homoserine lactone signal (C6-HSL). It is well known that QS regulates the production of the pigment violacein in this microorganism; in fact, violacein production dependent on QS is widely used as a marker to assess the efficacy of potential anti-QS molecules **(Oca-Mejía *et al*., 2015)**.

9 Biofilm related to oral diseases

Bacteria in the mouth are responsible for dental health and disease, including caries, periodontal diseases, and endodontic infections. The development of bucco-dental diseases is inextricably linked to the ability of buccal bacteria to form and reside in a multi-species adhering consortium known as biofilm **(Kreth *et al*., 2015)**.

Oral diseases are caused by bacteria embedded in the dental biofilm that forms on the surface of the teeth. bacteria within a biofilm can communicate between themselves by producing, detecting, and responding to small diffusible signal molecules in a process known as quorum sensing, which benefits host colonization, biofilm

formation, competition defense, and adaptation to environmental changes **(Donlan *et al*., 2002; Li *et al*., 2012; Kriebel *et al*., 2018)**.

The biofilm is an effective survival organization that protects the resident microorganisms from exogenous, potentially harmful factors and permits cooperative interactions between cells of the same or different species, Moreover, biofilm allow bacteria to develop resistance to host immune responses, antibiotics **(Colombo *et al*., 2015)**. Cells embedded in a biofilm are up to a thousand times more resistant to antibiotics than planktonic cells **(Kouidhi *et al*., 2015)**. Microbes present at the supragingival level are mainly related to gingivitis and root caries, whereas subgingival species advance the destruction of teeth supporting tissues and thus cause periodontitis **(Aruni *et al*., 2015)**. Endodontic disease is an infection caused by a biofilm and the primary goal of endodontic treatment is to remove the bacterial biofilm from the canal system **(Jhajharia *et al*., 2015)**. The high prevalence of *E. faecalis* in endodontic infections could be caused by its ability to form biofilm and to coaggregate with other bacteria present in root canals **(Al-Ahmad *et al*., 2009)**. Biofilm formed by *Enterococcus faecalis* are able to resist destruction by enabling the bacteria to become 1000 times more resistant host immune and drugs **(Usha *et al*., 2010)**.

10 Resistance to antimicrobial drugs

Antimicrobial resistance has become a major public health concern in recent years, owing to the emergence of multi-drug resistant (MDR) pathogenic bacterial strains. Antibiotics have been widely used in medicine, veterinary medicine, agriculture, all of which have contributed significantly to the development of bacterial resistance. MDR was defined as acquired resistance to at least one antimicrobial agent from three or more antimicrobial categories **(Babchinskii, 1963; Naha *et al*., 2020; Rahman *et al*., 2020)**.

Enterococcus faecalis has a number of virulence factors, toxins such as cytolysin, haemolysin, gelatinase, aggregation substance, serine protease and cell wall polysaccharide have been shown to facilitate adherence, colonization, biofilm formation, resistance, pathogenicity and evasion of the host immune response **(Barbosa-Ribeiro *et al*., 2016; Colaco, 2018)**.

Bacterial drug resistance mechanisms typically involve one or more of the following **(Kitagawa *et al*., 2016)**:

- Enzymatic modification or destruction of the drug itself
- Alteration of the drug target in the bacterial cell
- Reduction of membrane drug permeability
- Limitation of drug accumulation as a result of active drug efflux in the cell membrane

Oral *Enterococcus faecalis* developed high levels of resistance to a variety of antimicrobial agents, including vancomycin, gentamicin, tetracycline, erythromycin, chloramphenicol, ciprofloxacin, and azithromycin **(Kouidhi *et al*, 2011; Lins *et al*, 2013** ; **Benbelaïd *et al*, 2014; Prado *et al*, 2017)**. Antibiotic resistance mechanisms in enterococci can be intrinsic to the species or acquired through mutation of intrinsic genes or horizontal genetic material exchange for resistance determinants **(Hollenbeck *et al*., 2012)**. However, *E. faecalis* has demonstrated resistance to intracanal medications such as calcium hydroxide. The following explanations have been proposed to explain why *E. faecalis* can survive intracanal calcium hydroxide treatment **(Stuart *et al*., 2006; Mohammadi *et al*., 2012):**

- pH homeostasis is passively maintained by *E. faecalis*. This is due to ions penetrating the cell membrane as well as the cytoplasm's buffering capacity.
- *E. faecalis* has a proton pump, which adds to its ability to maintain pH homeostasis. This is accomplished by "pumping" protons into the cell in order to reduce the internal pH.
- *E. faecalis* can feed on the fluid in the periodontal ligament, penetrate dentinal tubules, attach to collagen and form biofilm to protect itself from host resistance and disinfectants **(Alghamdi *et al*., 2020)**.

11 Role of biofilm in bacterial resistance

Bacteria that live in biofilm are more resistant to antibiotics than planktonic cells and can easily avoid phagocytosis **(Jung *et al*., 2019)**. The biofilm community protects bacteria from the host defense system, rendering them more resistant to the various disinfectants used for oral hygiene or the treatment of infections **(Jhajharia *et al*., 2015)**.

Resistance within biofilm can be explained by different mechanisms **(Kouidhi *et al*., 2015)**:

- restricted penetration of antimicrobial agents
- induction of the general stress response
- decreased growth rates and metabolism
- increasing expression of multiple drug resistance (MDR) pumps
- activating quorum sensing systems
- changing profiles of outer membrane proteins

The main cause of endodontic failure is the presence of certain bacteria in the root canal system, in particular *Enterococcus faecalis*, whose resistance to disinfectants leads to persistent intra- or extra-radicular infection **(Alghamdi *et al*., 2020)**.

E. faecalis has the ability to create a biofilm, invade dentinal tubules and resist in root canals where nutritional supply is limited. Because of these characteristics, *E. faecalis* can be resistant to conventional endodontic disinfection therapy **(Tinoco *et al*., 2016)**

The ability of *E. faecalis* to create a biofilm inside root canals gives it great potential to spread virulence and resistance genes by horizontal gene transfer **(Distel *et al*., 2002; Rosier *et al*., 2014)**. *E. faecalis* is one of the most common multidrug resistant hospital pathogens, capable of forming biofilm and acquiring resistance determinants via horizontal gene transfer. The genetic basis for intrinsic and acquired antibiotic resistance in *E. faecalis* is of great interest **(Dale *et al*., 2015; Yadav *et al*., 2022)**. *E. faecalis* in dentinal tubules can withstand intracanal calcium hydroxide dressings for more than ten days by forming a biofilm, which allows the bacteria to become hundreds of times more resistant to phagocytosis, antibodies and antimicrobials than non-biofilm producing organisms **(Jhajharia *et al*., 2015)**.

The virulence of microbial biofilm is normally restricted to the oral cavity. However, when these microbes or their components enter the connective tissues or circulatory system, they may increase the risk of certain systemic diseases such as cardiovascular disease, diabetes and immune-dysfunctional diseases **(Xu *et al*., 2018)**.

Because most commercial intracanal drugs have cytotoxic reactions and are unable to eliminate bacteria from dental tubules, the trend in recent medicine has been to use biological drugs extracted from natural plants in endodontics **(Sinha *et al*., 2014)**.

Materials and methods

1 Experimental part

1.1 Microbiology section

A comprehensive investigation was conducted to assess the general oral health status of patients in Oum el-Bouaghi region. The study aimed to identify prevalent dental diseases and associated bacteria to guide future research. The results of this investigation revealed that the most common dental diseases in the studied population were dental caries, endodontic infections, and dental abscesses. Analysis of various samples collected from patients' oral cavities unveiled the presence of different bacteria, among which *Staphylococcus saprophyticus, Streptococcus sp* and *Enterococcus faecalis* were predominant. Notably, *Enterococcus faecalis* exhibited a significant prevalence, indicating its potential role in oral health issues in these region, the research efforts have been directed towards understanding and addressing this specific bacterium.

1.1.1 Sample collection

Before taking samples, the mouth should be rinsed with water to remove any food debris present in the mouth. Microbiological samples were then obtained from different affected teeth (root canal, carious plaque, dental abscess) using a sterilized curette and swab from patients of different ages and in good general health. These samples were collected at the dental clinic in Oum El Bouaghi and then stored in phosphate-buffered saline (PBS) and transported to the laboratory for microbiological studies.

1.1.2 Isolation and identification of *Enterococcus faecalis*

Isolation allows us to obtain a pure bacterial culture, which is crucial step to begin microbiological studies, including the morphology, biochemical and physiological tests **(Ruangpan et al., 2004)**.

1.1.3 Morphologic Characteristics

Morphological characteristics (Gram reaction, motility, shape and arrangement) can be used to identify bacteria at genus level.

In this research, the isolation of *Enterococcus* is carried out on selective culture media BEA and incubated for 48 h at 37 °C. After incubation, each isolate is purified on BEA medium, then examined by Gram staining, motility, with

identification based on phenotypic, physiological (growth at 6.7 % NaCl, 45 °C, 50 °C, 10 °C and PH 9.6), biochemical characteristics (catalase, oxidase, Esculin hydrolysis, hemolytic activity on blood agar, capacity to reduce 0.04 % potassium tellurite) and its appearance in chromaagar orientation.

1.1.4 Antibiotic susceptibility and selection of resistant bacteria

The recommendations of the Institute of Clinical and Laboratory Standards 2020 were used to determine the effect (sensitivity, resistance and intermediate resistance) of various antibiotics including penicillin G (10 µg) , erythromycin (15 µg), tetracycline (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg) and amoxicillin (30 µg) on *Enterococcus faecalis* isolates **(CLSI, 2020)**.

2 Biochemical section

2.1 Propolis harvesting and extract preparation

Propolis samples were harvested in October 2018 at various locations in Algeria figure (17).

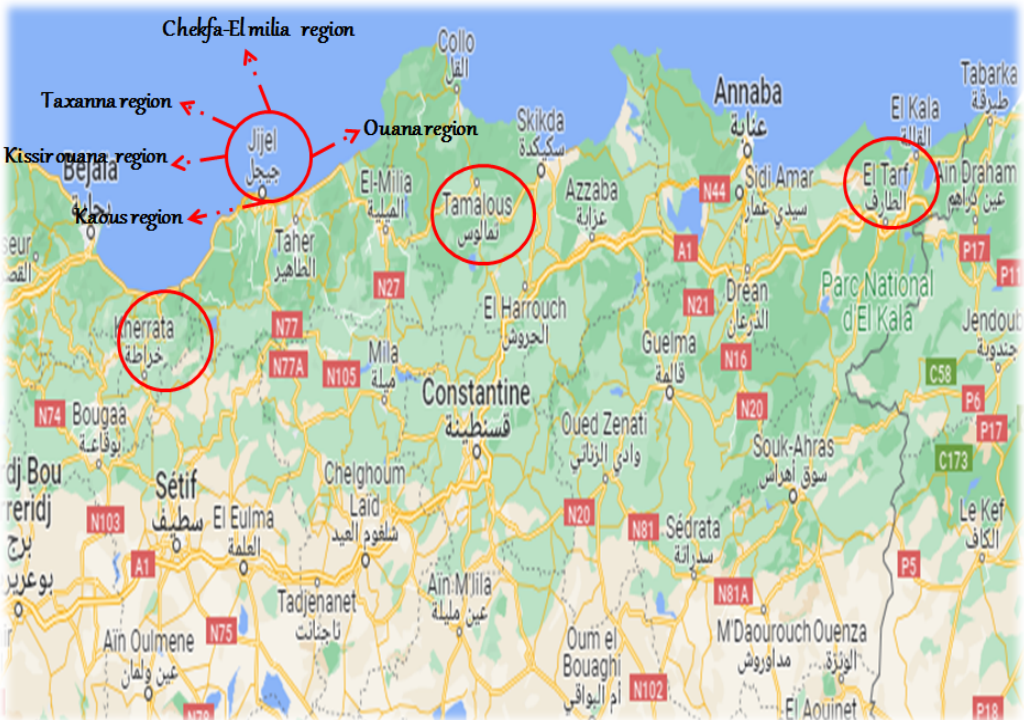


Figure 17. Geographical origins of propolis samples

An 80 % ethanolic solution was used to macerate 20 g of propolis samples. The mixture was incubated at room temperature for 5 days, this procedure was repeated two times for each sample, after filtering the mixture with Whatman N°1 paper, the filtrate was evaporated and concentrated at 45 °C, then stored under dry at 4 °C until analysis.

The following formula was used to calculate extraction yields:

$$\textbf{Yield}\ (\%) = \frac{\text{dry crude extract}}{\text{dry initial sample before extraction}} X100$$

2.2 Thin layer chromatography

Different phenolic and flavonoid compounds in propolis extracts were revealed by thin-layer chromatography.

Thin layer chromatography (TLC) is a method that separates the components of a mixture by entrainment with a mobile phase along a stationary phase.

The stationary phase consists of a thin layer of adsorbent material (silica gel: GF 254). The mobile phase is a mixture of organic solvents; the best solvent system was (n-hexane/ Diethyl-ether /Méthanol) (7 : 4 : 0.75), however other solvent systems were also tried, such as (Diethyl-ether/n-hexane) (3 : 1) and (n-hexane/ethyl acetate (6 : 1). It can be placed at the base of the plate so that it rises by capillarity. A solution with the highest affinity for the solvent and the lowest affinity for the support migrate the fastest. The affinity of a substance for a solvent is determined by the polarity of the molecules (due to the type of binding of the molecules, polar or apolar). Once the sample is applied and the solvent has passed through the TLC plate, the plate is then dried.

The chromatography plate is then placed under UV light at 254 nm. As a result, each substance has been identified by its UV fluorescence.

2.3 Phytochemical analysis

2.3.1 Estimation of phenolic contents

Folin-Ciocalteu method is the technique used to assess phenol content in extracts, the phenolic compounds in propolis extract react with the Folin-Ciocalteu reagent to form a blue complex that can be quantified by visible-light spectrophotometry at a wavelength of 765 nm using gallic acid as standard **(Lizcano *et al.*, 2019)**.

The mixture of 100 µL Folin Ciocalteu reagent, 20 µL extract and 75 µL Na_2CO_3 (7.5 %) was incubated for 2 hours in the dark, then their absorbance was determined spectrophotometrically at 765 nm. The results are indicated in µg GAE/mg Extract **(Singleton and Rossi, 1965)**.

2.3.2 Estimation of flavonoid contents

The basic principle of this technique is that the hydroxyl group of flavonoids reacts with aluminum (Al^{3+}) and generates the stable flavonoid-Al^{3+} complex, which appears in the reaction as an instant golden-yellow coloration with intensity proportional to the concentration of flavonoids in the extract; quercetin was used as a standard **(Ekpo *et al.*, 2020)**.

The content of flavonoids was determined by reacting 130 µL of methanol with 50 µL of diluted extract, 10 µL of potassium acetate (1M) and 10 µL of aluminum nitrate (10%). The reaction mixture was incubated for 40 min and then their absorbance was measured at 415 nm. The result was given in µg QE/mg Ext **(Topçu *et al.*, 2007)**.

2.3.3 Identification of polyphenols by HPLC-DAD

Different classes of polyphenols in propolis were identified and quantified using the HPLC method. In this research, Various standards, including fumaric acid, gallic acid, p-benzoquinone, protocatechuic acid, theobromine, theophylline, catechin, 4-hydroxybenzoic acid, 6,7-dihydroxycoumarin, methyl-1,4 benzoquinone, vanillic acid, caffeic acid, vanillin, chlorogenic acid, p-coumaric acid, ferrulic acid, cynarine, coumarin, prophylgallate, rutin, trans-cinnamic acid, ellagic acid, myricetin, fisetin, kersetin, trans-cinnamik acid, luteolin, rosmarinic acid, Kaempferol, apigenin, chrysin, pyrocatechol, 4-hydroxy benzaldehid, epicatechin, 2,4-dihydroxybenzaldehide, hesperedin, oleuropein, naringenin, hesperetin, genistein, curcumin were used to identify this molecules by comparing the retention time of each identified compound with the retention time of the various standards tested.

The system used to reveal and determine the various molecules present in propolis extracts consists of a Shimadzu (Shimadzu Cooperation, Japan) reverse-phase high-performance liquid chromatography system, comprising the Shimadzu SPD-M20A diode array detection system and the Shimadzu LC-20AT solvent delivery unit controlled by LC-solution software (Shimadzu CBM-20A System Controller). The mobile phase for the separation consists of a mixture of 0.1% aqueous acetic acid (A)

and methanol (B), the column temperature was regulated at 35 °C. Intertsil ODS-3 column (4 µm, 4.0 mm × 150 mm) and Inertsil ODS-3 guard column were used for separation. Detection of various components was performed using a diode array detector (DAD) at 254 nm after injection of 20 µL of sample solution (8 mg/mL) through an Agilent 0.45 filter µm. Finally, results are given in mg/g of raw propolis **(Boutellaa *et al.*, 2019)**.

2.3.4 Biological activities

2.3.4.1 Ability to scavenge and reduce free radicals

2.3.4.1.1 DPPH radical scavenging test

The 2,2-diphenyl-1-picrylhydrazyl radical is a stable, dark purple compound capable of reacting with antioxidant molecules, which can donate an electron or hydrogen atom that reduces to pale yellow 2, 2-diphenyl-1-hydrazine (DPPH-H) **(figure 18) (Mfotie Njoya, 2021)**.

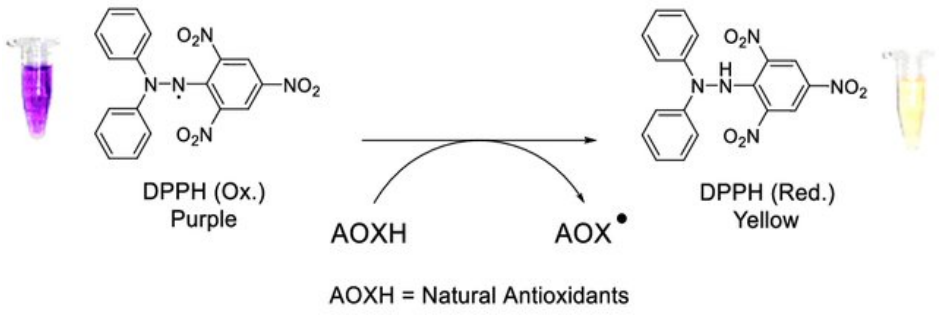


Figure 18. Reaction of DPPH with natural antioxidants **(Arce-Amezquita *et al.*, 2019)**

The procedure involves reacting 40 µL of diluted extract with 160 µl of DPPH solution in the dark for 30 minutes, then determining the absorbance of the mixture at 517 nm. BHA used as a standard.

The scavenging ability of the DPPH radical was estimated on the basis of the following formula:

$$\text{Inhibition percentage} = \frac{\text{A control} - \text{A sample}}{\text{A control}} X100$$

A: absorbance

2.3.4.1.2 $ABTS^{\bullet+}$ radical scavenging test

The principle is achieved when the dark blue radical cation 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) reacts with an antioxidant compound, it is reduced to colorless ABTS, that can be quantified spectrophotometrically (figure 19) **(Dasgupta *et al.*, 2014)**.

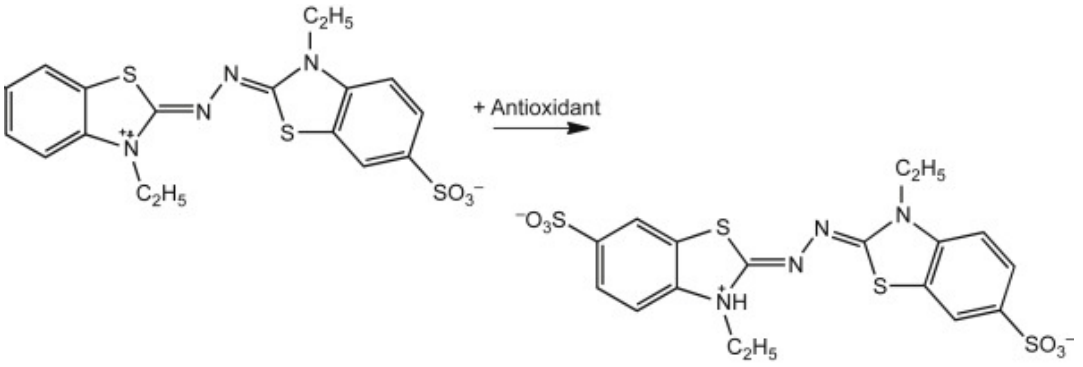


Figure.19 Reaction of ABTS with natural antioxidants **(Hernández-Rodríguez *et al.*, 2019)**

7mM ABTS and 2.45 mM potassium persulfate ($K_2S_2O_8$) were mixed and incubated for 12 h in the dark before being diluted with distilled water to an absorbance of 0.700 ± 0.020 at 734 nm; 40 µL of diluted extract was added to 160 µL of ABTS solution. The mixture was then incubated for 10 minutes before measuring their absorbance at 734 nm. BHA and BHT were used as standards **(Mebrek *et al.*, 2018)**. results are expressed as IC_{50} values

The percentage of inhibition was determined by the following formula:

$$\text{ABTS scavenging activity (\%)} = \frac{\text{A Control} - \text{A Sample}}{\text{A Control}} 100$$

A: absorbance

2.3.4.1.3 Cupric ion reducing ability

In the CUPRAC reaction, antioxidant molecules reduce Cu (II) to Cu (I) in the presence of the chromogenic redox reagent bis(neocuproine), which generates a Cu(I) chelate (figure 20) **(Suktham *et al.*, 2019)**.

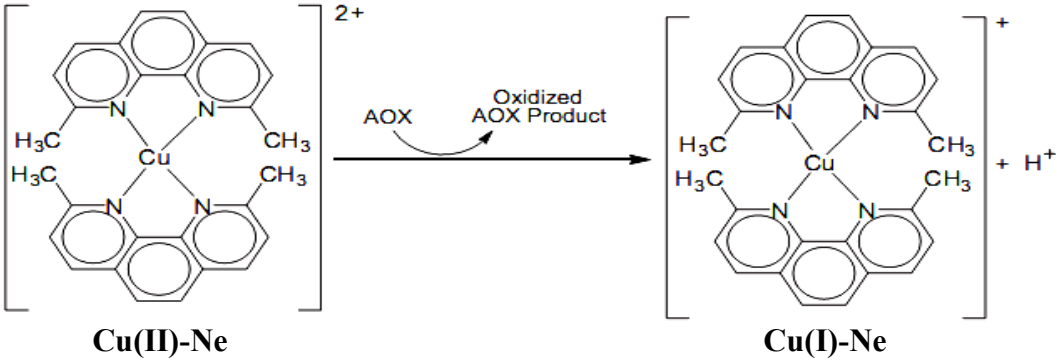


Figure 20. CUPRAC reaction **(Özyürek *et al.*, 2011)**

In the CUPRAC assay, 40 µL of extract at different concentrations were mixed with 50 µL of copper(II) chloride solution, 50 µL of ethanolic neocuproin solution (50 µL) and 60 µL of CH3COONH4 (1 M), then incubated for 1h. Absorbance was measured using a spectrophotometer at 450 nm. BHA and BHT were used as standards for comparison, and results were expressed as $A_{0.5}$ values (µg/mL) **(Lekouaghet *et al.*, 2020)**.

2.3.4.1.4 GOR free radical test

The basis of the technique is that the stable galvinoxyl radical (GoxN) reacts with antioxidants that can give it a hydrogen molecule, reducing it to GoxH **(figure 21) (Barzegar *et al.*, 2011)**.

The GOR scavenging method is based on reaction between 40 µL of diluted extract and 160 µL of galvinoxyl solution (0.1mM) in the dark at room temperature for 120 minutes. The reaction mixture is measured at 428 nm. The standard antioxidants used are BHT and BHA **(Barzegar *et al.*, 2011)**.

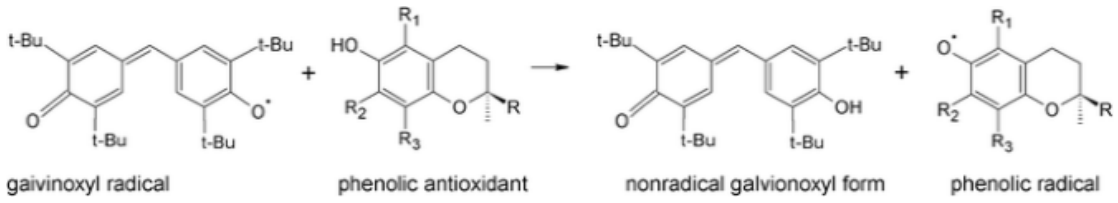


Figure 21. Reaction of GOR with natural antioxidants **(Jerzykiewicz *et al.*, 2013)**

2.3.4.1.5 Reducing power assay

The concept of this method is that compounds with a reducing potential generate potassium ferrocyanide (Fe^{2+}) after reacting with potassium ferricyanide (Fe^{3+}), which then reacts with ferric chloride to produce a ferric-ferrous complex that exhibits an absorption maximum at 700 nm **figure (22) (Bhalodia *et al.*, 2013)**.

The reducing capacity of propolis molecules was revealed by reacting 10 µL of sample solution with 40 µL of phosphate buffer (pH 6.6) and 50 µL of potassium ferricyanide (1%) (K3Fe (CN) 6) (1 g of K3Fe (CN) 6 in 100 ml H2O) for 20 min at 50 °C. After incubation, 50 µL of trichloroacetic acid (10%) (1 g TCA in 10 ml H2O), 40 µL of distilled water and 10 µL of ferric chloride solution (0.1%) (0.1 g FeCl3 in 100 ml H2O) were added. Absorbance was determined at 700 nm **(Elkolli *et al.*, 2022)**.

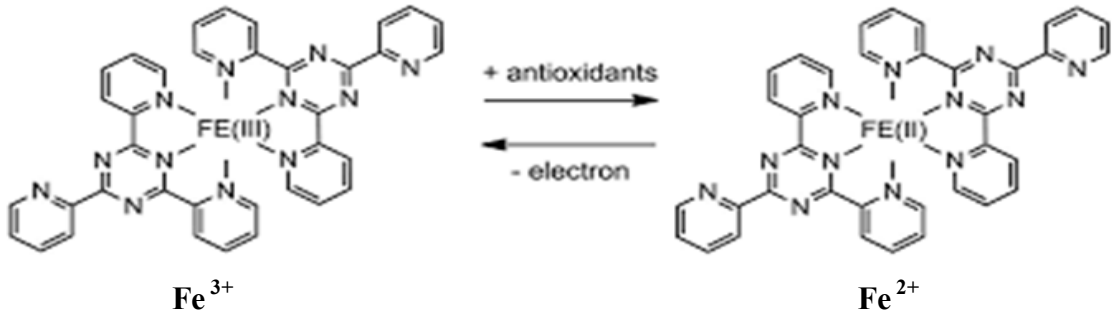


Figure 22. Reaction of ferric with natural antioxidants **(Shalaby *et al.*, 2013)**

2.3.4.1.6 Phenanthroline assay

In the phenanthroline method, antioxidant molecules reduce Fe^{3+} to Fe^{2+}ion and then react with ortho-phenanthroline to form a red-orange complex figure (23) **(Yefrida *et al.*, 2018)**.

The phenanthroline assay protocol is performed by mixing 50 µL ferric chloride (0.2%) + 30 µL phenanthroline (0.5%) + 110 µL MeOH with 10 µL extract at

different concentrations. Absorbance is read at 510 nm after incubation for 30°C/20 min. Results were expressed as $A_{0.5}$ value.

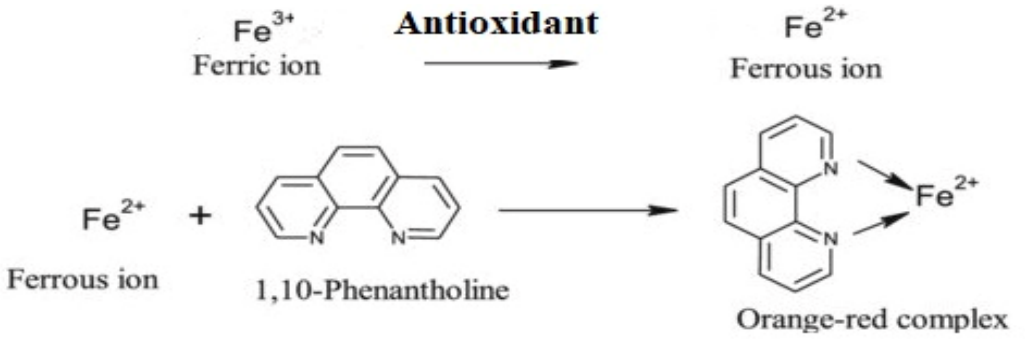


Figure 23. Reaction of phenanthroline with natural antioxidants **(Naraparaju *et al.*, 2020)**

2.3.5 Anti-bacterial properties

2.3.5.1 Culture conditions

Enterococcus faecalis bacteria were grown on nutrient agar supplemented with 0.5 % glucose (with slight modification), then incubated at 37 °C for 24 hours. The bacterial suspension was prepared in saline solution (0.9 %) and then its turbidity was adjusted to the 0.5 Mc Farland turbidity standard.

2.3.5.2 Disc diffusion assay

20 mg of propolis extracts were dissolved in 1 mL of Dimethyl sulphoxide (DMSO) to obtain a final concentration of 20 mg/mL, followed by a series of dilutions to 0.625 mg/mL.

The activity of propolis extracts against *Enterococcus faecalis* bacteria was assessed on Mueller-Hinton agar using the disk diffusion technique. Sterile filter paper discs (6 mm) impregnated with 20 µL of different propolis concentrations (20-0.625 mg/mL, respectively) were placed on the surface of agar medium spread with 100 µL of suspensions using a sterile cotton swab. Prediffusion of the extract into the medium was achieved by maintaining the petri dish at 4 °C for 1 hour in the refrigerator. The zones of inhibition around the discs were measured in millimeters using a ruler after incubation of the treated petri dish at 37 °C for 24 h. The negative control was dimethyl sulfoxide **(CLSI, 2020)**.

2.3.5.3 Broth microdilution technique

MIC and MBC values were tested in sterile 96-well plates by placing 10 µL of bacterial suspension diluted to 0.5 Mc-Farland in each well in the presence of 170 µL of Mueller-Hinton broth and propolis extract at different concentrations (0.625-20 mg/mL). The lowest concentration that inhibited bacterial growth was considered as MIC, while the MBC value was determined by subculturing 10 µL of liquid culture from each well onto Mueller-Hinton agar. Plates were then incubated at 37°C for 24 hours. MBC is the lowest concentration at which no growth is visible on Mueller-Hinton agar **(CLSI, 2020)**.

2.3.5.4 Antibiofilm test

To evaluate the effect of propolis extracts on the biofilm of *Enterococcus faecalis* bacteria, 20 µL of diluted extract at different concentration was combined in wells with 10 µL of bacterial suspension at the concentration $5x10^5$ CFU/mL and 170 µL of tryptose-Soy medium, the bacterial suspension was used as a control. The microplates were then kept at 37°C for 48 h. After incubation, the planktonic bacteria were cleared with distilled water from the wells, which were then stained with 0.1% crystal violet solution for 10 min at room temperature; the wells were then washed again to remove the crystal violet. The biofilm is removed from the walls with 200 µL glacial acetic acid (33%), the optical density was determined at 550 nm and biofilm inhibition was calculated according to the equation below **(Ceylan *et al*., 2015)**:

$$\text{Biofilm inhibition } (\%) = \frac{\text{A c} - \text{A s}}{\text{A c}} \text{X100}$$

Ac: Absorbance of control

As: Absorbance of sample

2.3.5.5 Antiquorum-sensing activity on *CV026*

The capacity of propolis to disrupt quorum-sensing was determined on *CV026* using the procedure of **Koh and Tham. (2011)**. Five milliliters of warm molten Soft Top Agar were seeded with warm molten Soft Top Agar and 20 µL of 100 µg/mL C6HSL (exogenous source of AHL), then mixed and immediately poured onto the Luria-Bertani Agar surface, 5 mm diameter wells filled with 50 µL of different sample

concentrations. Positive results for QSI were expressed by the appearance of a white or cream halo around this well against a purple lawn of activated *CV026* bacteria. The inhibition diameter was then measured in millimeters using a ruler after incubation at 30 °C for 72 h.

2.3.5.6 Inhibition of violacein production in *CV12472*

CV 12472 was used as a model strain to determine the capacity of propolis to inhibit violacein synthesis. In this assay, 20 µL of propolis extract at MIC and sub-MIC concentrations were mixed with 10 µL of overnight culture of *CV12472* (0.4 OD at 600 nm) in the presence of 170 µL of LB broth in a sterilized microplate. The *CV12472* suspension was used as a positive control. Plates were then maintained at 30 °C for 24 h, and absorbance was measured at 585 nm. Violacein pigment reduction was calculated according to the following equation **(Tamfu *et al.*, 2022)**:

$$\text{I}\,(\%) = \frac{\text{Ab 585 control} - \text{Ab 585 sample}}{\text{Ab 585 control}} \times 100$$

Ab: Absorbance

I: Inhibition percentage of Violacein

2.3.5.7 Cytotoxic activity

- **Lethality assay on brine shrimp**

The cytotoxicity test was performed on brine shrimp based on the method of **Meyer *et al.* (1982)**. Brine shrimp eggs were hatched into a 2000 mL beaker filled with sea water and exposed to light for 48 h at 30 °C under lighting to allow growing and producing shrimp larvae known as *Artemia nauplii*. Using a micropipette, *Artemia nauplii* were collected and placed in another glass petri dish with sea water after incubation. 100 µL of propolis extracts at various concentrations were combined with 4.9 mL of filtered seawater and ten brine shrimp larvae (nauplii) and then incubated for 24 hours. After incubation the survived shrimps were counted and the percentage of mortality at each dose was calculated.

2.3.5.8 Statistical study

Linear regression analysis was used to determine IC_{50} and $A_{0.5}$ values. Results are presented as the mean value ±SD of three parallel measurements. Data were statistically analyzed by Graph Pad Prism software (version 8.0.2) using one-way

ANOVA with Tukey's multiple comparisons test; when the P value < 0.05, the results are considered significant. Correlations between antioxidant activities and phenolic and flavonoid contents were determined by Pearson's correlation coefficient r.

RESULTS and DISCUSSION

1 Phytochemical analysis

Phenolic compounds are the most common secondary metabolites in plants, which have attracted growing interest in recent years and display a wide range of distinct biological activities. The general chemical structure of phenolic molecules comprises an aromatic ring with hydroxyl substituents, which can be classified into numerous categories, including flavonoids, phenolic acids, tannins, stilbenes and lignans. Spectrophotometry and HPLC are among the methods used to quantify phenolic compounds in plant **(Zhang *et al*., 2022)**.

1.1 Propolis extraction yield

Secondary metabolites from propolis samples are extracted by maceration in a hydroalcoholic solution (80/20: v/v). Yields of extracted propolis expressed as a percentage of the initial weight of the dry matter are represented in the **figure 24**.

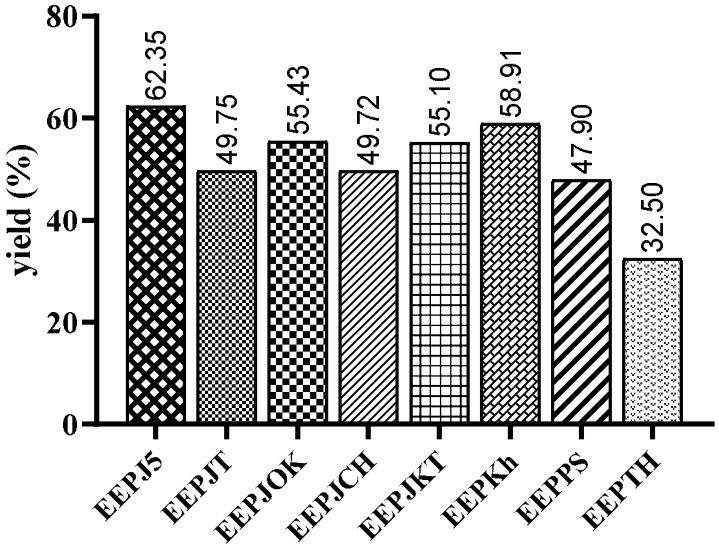


Figure 24. Extractions yield of propolis

The colors of the propolis filtrate obtained in eight different regions were light-yellow, brown, yellow-black and yellow-brown, dark brown. The results obtained show that among the eight samples studied, EEPJ5 (62.35 %) represents the highest yield followed by the EEPKH (58.91 %), while the lowest value is observed for EEPTH (32.50 %).

Extraction is an essential process, requiring a suitable procedure to obtain the desired components from propolis. Maceration is the most widely used and traditional technique for extracting the active components of propolis. Polyphenolic content increase with the concentration of ethanol in the solvent, mainly due to the fact that propolis polyphenolic substances are more soluble in ethanol than in water. The ideal concentration of ethanol in water is around 70-95% alcohol, with 70-80% being the most common **(Bankova *et al.*, 2021)**.

The results of this study indicate that the extraction yield of propolis extracts is significantly superior to the extraction yield of propolis extracts obtained in regions of El-Menia (39 %), Oum el bouaghi (38 %), Collo (20.5 %), El-Harrouch (36 %); while the percentage yield of EEPTH (32.50 %) is higher than those obtained in El Oued (11.3 %), Jijel (20.88 %), Tipaza (17.02 %) and Tlemcen (16.22 %) regions **(Rebiai *et al.*, 2021; Boulechfar *et al.*, 2022)**. Furthermore, a study by **Belfar *et al.* (2015)** showed that the yield of propolis extracts from Boumerdes (41.10 %), Ghardaia (15.57 %) and Mostaganem (30.01 %) regions were lower than that of the propolis extracts in our study.

Given the polarities that distinguish bioactive constituents, the most suitable solvent systems for extracting polyphenols are ethanol/water combinations, yield differences are due to differences in propolis origin and solvent polarity **(Dent *et al.*, 2013)**.

1.2 Phytochemical Screening by Thin Layer Chromatography

The results of qualitative phytochemical screening of propolis extracts by thin-layer chromatography (TLC) revealed various classes of chemical compounds after visualization under UV light at 365 nm. Thin-layer chromatographic profiling showing the presence of spots yellow-green, and purplish pink under UV light (figure 25) indicating the presence of flavonoids and phenolic acids **(Gwatidzo *et al.* 2018; Burman *et al.* 2019)**.

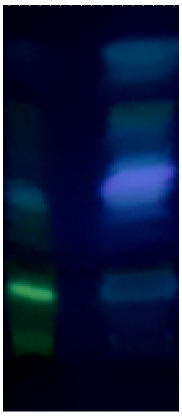


Figure 25. TLC of propolis extracts

1.3 Estimation of phenolic and flavonoid amounts

The richness of propolis extracts with polyphenolic compounds was revealed by the Folin-Ciocalteu colorimetric reaction and the aluminum chloride method. Gallic acid is the standard most often used in the Folin-Ciocalteu assay, and results are given in µg gallic acid equivalent/mg extract, whereas quercetin is the most commonly used standard in the aluminum chloride method and the amounts obtained are quantified in µg quercetin equivalent mg/of extract. The amount of phenols in propolis extracts was determined using the calibration curve created from gallic acid (y= 0.003x +0.104, R^2= 0.997) **(Figure 26)**, while flavonoid contents was measured using the calibration curve created from quercetin (y=0.004, R^2= 0.997) (**Figure 27**).

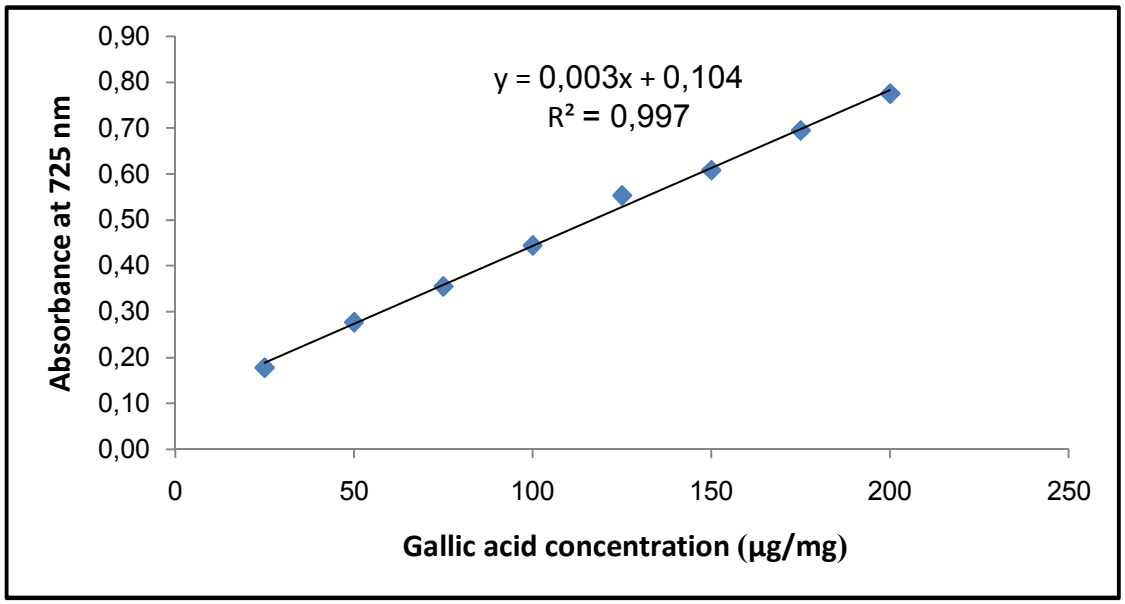


Figure 26. Calibration curve for gallic acid

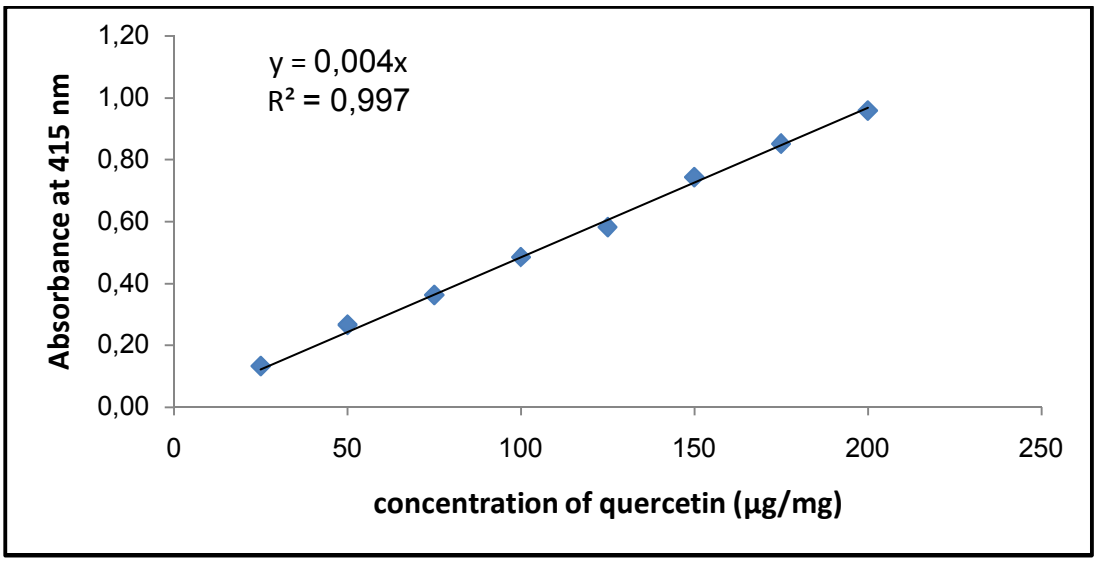


Figure 27. Calibration curve for quercetin

Table 3. Quantities of phenolic and flavonoid compounds in propolis extracts

Propolis extracts	TPC (µg GAE/mg extract)	TFC (µg QE/mg extract)
EEPJOK	303.41 ± 1.01^{e}	28.47 ± 0 ^{b}
EEPJT	630.37 ± 3.23^{f}	246.18 ± 0 ^{ac}
EEPJCH	752.82 ±13.25 ^{a}	271.46 ± 11.49 ^{a}
EEPJKT	742.53±9.17 ^{a}	248.33±2.36 ^{ac}
EEPJ5	526.55±9.34 ^{b}	268.19±5.45 ^{a}
EEPKh	734.39±11.54^{a}	224.30±0 ^{c}
EEPPS	553.22±7.30^{d}	260.14±3.09 ^{a}
EEPTH	677.98 ± 0.44^{c}	254.29 ±3.05 ^{a}

Results are expressed as means ± SD of three parallel measurements. Different letters in the columns represent statistically significant differences ($p<0.05$).

The results presented in the **table (3)** allow us to conclude that ethanolic extracts of propolis contain considerable quantity of phenolic and flavonoid components.

EEPJCH recorded the highest content of phenolic and flavonoid constituents (752.82 ±13.25 µg GAE/mg extract) with no significant difference ($p>0.05$) with EEPJKT (742.53 ± 9. 17 µg GAE/mg extract) and EEPKh (734.39 ± 11.54 µg GAE/mg extract), whereas the EEPJOK, EEPJT, EEPJ5, EEPPS, and EEPTH differed significantly ($p<0.05$).

The total flavonoid contents (TFC) ranged from 28.47 ± 0 µg QE/mg E to 271.46 ± 11.49 µg QE/mg E. The lowest amounts were found in EEPJOK (28.47 ± 0 µg QE/mg extract), which was significantly different ($p<0.05$) than the other extracts.

The findings of this work indicate that propolis extracts are particularly rich in TPC and TFC when compared to propolis extract from the Oum el Bouaghi region, which had a total phenolic and flavonoid values of 270.62 ± 1.91 µg GAE/mg E, 54.35 ± 0.20 µg QE/mg E, respectively **(Boulechfar *et al.*, 2022)**. **Segueni *et al.* (2020)** indicated that propolis extracts from Kheir Oued Adjoul (Jijel), El-malha (Constantine), Ouad athmania (Mila) and Djemaa beni habibi (Jijel) regions contain TPC and TFC contents of 19.51 ± 0.86 to 128.11 ± 1.55 mg GAE/g, respectively and 5.27± 0.07 to 74.57 ± 1.03 mg QE/g, respectively.

Nedji and Loucif-Ayad (2014) reported that the contents of phenolic and flavonoid compounds in propolis extracts varied from 100.90 ± 2.72 to 257.40 ± 3.01 mg GAE/g sample and from 58.99 ± 2.49 to 91.44 ± 4.42 mg/g extract, respectively within four regions of Annaba.

Studies on Algerian propolis have reported phenolic and flavonoid contents from Tipaza (TPC: 384.7± 18.0 mg/g, TFC: 37.27±1. 86 mg/g), Constantine (TPC: 105. 17±2.77 mg/g, TFC : 2.152±0.546 mg/g), Tebessa (TPC : 353.2±13.9 mg/g, TFC : 35.674±0.833 mg/g) and El-Oued (TPC : 42.12±1.42 mg/g, TFC : 29.42 ±3.57 mg/g) regions, respectively **(Kouadri *et al*., 2021)**

Our results confirm previous research indicating that hydroalcoholic solution is the best extraction solvent for obtaining the highest phenolic content from propolis **(Sun *et al*., 2015)**. In addition, various factors, including botanical source, harvesting period, geographical location, climatic changes and types of bees at the collection site, influenced the amount of phenolic and flavonoid compounds **(Hossain *et al*., 2022)**.

1.4 Chemical composition of propolis extracts

The results of HPLC-DAD analysis of the molecules present in propolis extracts have been expressed in mg/g extract in table (4).

The chromatograms and the chemical structure of the molecules detected in propolis extracts are shown in **Figure 28 and 29**. Caffeic acid was the richest phenolic acid in all extracts, with values of EEPJCH (28.50 mg/g), EEPJOK (30.03 mg/g), EEPJ5 (8.98 mg/g), EEPJT (28.84 mg/g), EEPKH (23.79 mg/g), EEPP5 (21.26 mg/g) and EEPJKT (12.88 mg/g), respectively, while it was found in trace concentration in EEPTH. One of the most abundant flavones is chrysin, found only in EEPJCH (29.01 mg/g) and EEPJKT (21.73 mg/g), respectively. Hesperetin is a flavonone that was found exclusively in EEPJ5 (5.06 mg/g) and EEPJT (11.40 mg/g), with high content in EEPKH (15.42 mg/g) and EEPPS (15.45 mg/g) while naringenin was present with high abundance in EEPPS (25.77 mg/g) and in variable amounts in the other extracts with the exception of EEPTH, which was completely absent. Ellagic acid was only detected in EEPJ5 (4.49 mg/g), EEPJT (4.09 mg/g) and EEPJKT (3.89 mg/g).

For the first time, we have recorded the presence of cynarin in Algerian propolis extracts. Kaempferol and apigenin were found in the composition of all extracts, with differences in concentration between regions. Other compounds such as

protocatechuic acid, 4-oh-benzoic acid, chlorogenic acid, ferulic acid, rutin, quercetin, luteolin, hesperidin and oleuropein were identified with the lowest values.

Caffeic and ferulic acids were the major compounds detected and identified in propolis extracts from different regions of eastern Algeria, ferulic and caffeic acids were more abundant, with a content of 100.62 ± 0.02 mg/g and 24.54 ± 0.02 mg/g, respectively for Wad sabt region, while their concentrations for Oum tboul region were 19.83 ± 0.02 mg/g and 4.427 ± 0.008 mg/g, respectively **(Daikh *et al*., 2020)**.

Boulechfar et *al*.(2022) studied propolis extracts from two Algerian regions, the predominant individual phenolic compounds found in Collo region were ferulic (7103.17± 55.45 ng/mg) and caffeic acids (5236.90 ± 83.84 ng/mg) followed by apigenin (2053.56 ± 37.81 ng/mg) and kaempferol (1457.16 ± 0.88 ng/mg), gallic acid (1434.39 ± 12.22 ng/mg), p-coumaric acid (1376.51 ± 7.97 ng/mg) and quercetin (1130.32 ± 49.96 ng/mg), while the most abundant components in the Oum El Bouaghi region were ferulic (3126.90 ± 74.21 ng/mg) and caffeic acid (5745.61 ± 262.38 ng/mg) followed by verbascoside (2726.14 ± 64.31 ng/mg), quercetin (2572.27 ± 96.47 ng/mg), Kaempferol (2109.85 ± 54.77 ng/mg) and apigenin (2028.32 ± 19.76 ng/mg).

On the other hand, various molecules were identified in Turkish propolis, including caffeic acid (630.67 µg/mL), chrysin (641.33 µg/mL), pinocembrin (572.67 µg/mL), galangin (534.11 µg/mL), naringenin (372.39 µg/mL), kaempferol, trans-cinnamic acid and quercetin **(Bozkuş *et al*., 2021)**. while naringin (290.19 ± 0.2 mg/kg), hesperidin (271.77 ± 0.0 mg/kg) and rosmarinic acid (222.02 ± 6.2 mg/kg) have been detected in Moroccan propolis **(Laaroussi *et al*., 2020)**.

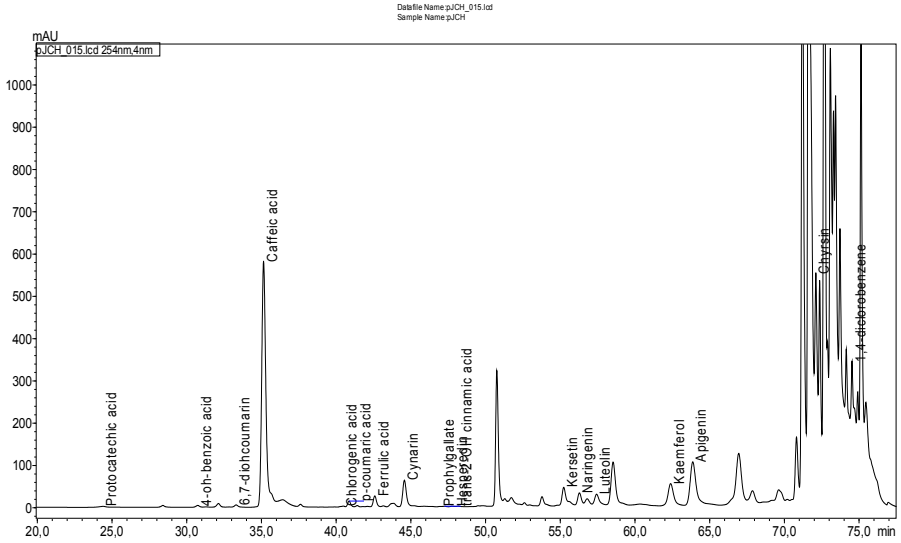
Rutin (0.45 ± 0.02 mg/g), quercetin (3.19 ± 0.03 mg/g), chlorogenic acid (0.73 ± 0, 01 mg/g), caffeic acid (1.32 ± 0.03 mg/g), ellagic acid (1.28 ± 0.01 mg/g), luteolin (7.51 ± 0.01 mg/g) and apigenin (2.97 ± 0.04 mg/g) the identified molecules of Brazilian propolis **(Regueira *et al*., 2017)**. Hesperetin (183.73 µg/mL), chlorogenic acid (96.92 µg/mL), caffeic acid (90.28 µg/mL) and apigenin (66.59 µg/mL) have been revealed in Egyptian propolis **(Saleh *et al*., 2023)**.

There are quantitative and qualitative variation in the chemical constituents of propolis due to various factors, including botanical source, climate, type of bee flora, collection seasons and the geographic region visited by the honeybees **(Barlak *et al*., 2011; Hossain *et al*., 2022)**. However, some molecules are sometimes identified just

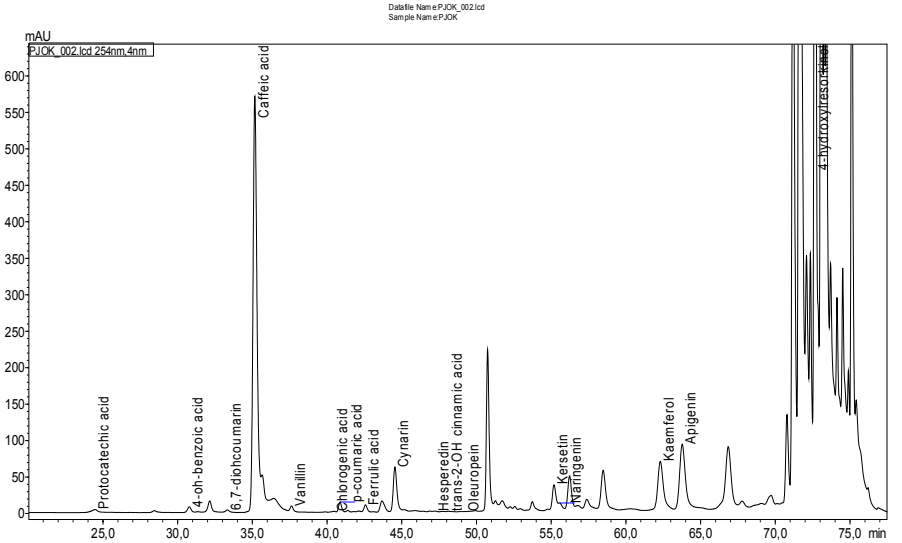
as trace amounts, meaning that the herb is rare around the hive or is not favorite by the bees **(Falcão *et al*., 2013)**.

Table 4. HPLC analysis of propolis ethanolic extracts

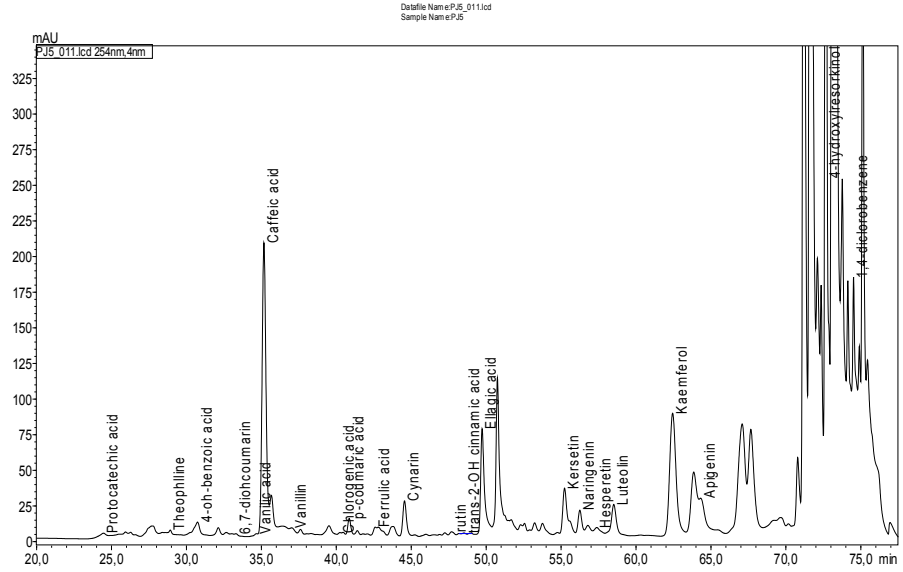
Compounds	RT (min)	Concentration (mg/g)							
		EEPJCH	EEPJOK	EEPJ5	EEPJT	EEPKH	EEPTH	EEPP5	EEPJKT
Protocatechuic acid	**22.39**	0.14	0.23	0.13	0.18	0.12	0.12	0.15	0.08
Theophlline	**29.44**	-	-	Tr	-	-	-	-	-
4-oh-benzoic acid	**31.69**	0.00	0.06	0.19	0.07	0.07	tr	0.07	0.03
6.7-dihydroxycoumarin	**33.43**	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
Vanilic acid	**34.68**	-	-	Tr	-	-	Tr	-	-
Caffeic acid	**35.19**	28.50	30.03	8.98	28.84	23.79	tr	21.26	12.88
Vanillin	**37.12**		Tr	Tr	Tr	Tr	Tr	Tr	Tr
Chlorogenic acid	**38.88**	0.21	0.17	0.21	0.16	-	-	-	-
p-coumaric acid	**40.81**	Tr	Tr	Tr	Tr	1.89	-	Tr	Tr
Ferulic acid	**42.92**	0.84	0.13	Tr	0.43	0.57	Tr	3.19	0.56
Cynarin	**43.85**	9.15	9.34	7.14	8.97	7.59	8.32	7.42	7.35
Propyl-gallate	**46.98**	0.11	-	-	-	-	-	-	-
Rutin	**47.52**	-	-	0.04	-	-	2.11	-	-
trans-2-hydroxycinnamic acid	**48.07**	Tr	Tr	Tr	Tr			Tr	Tr
Ellagic acid	**50.00**	-	-	4.49	4.09	-	-	-	3.89
Quercetin	**55.42**	1.62	1.03	1.25	1.17	1.15	0.57	1.49	0.93
Trans Cinnamic acid	**55.92**	-	-	-	-	-	0.82	-	-
Luteolin	**57.87**	1.16	-	0.86	2.21	2.25	0.48	2.06	0.78
Kaempferol	**62.48**	2.09	2.42	3.32	1.93	3.43	0.55	1.97	1.78
Apigenin	**64.07**	5.85	4.75	0.89	4.09	5.91	0.89	3.12	4.55
Chrysin	**72.77**	29.01	-	-	-	-	-	-	21.73
Hesperidin	**47.38**	0.61	0.27	-	0.52	0.49	-	0.64	-
Oleuropein	**49.54**	-	Tr	-	-	0.11	-	-	-
Naringenin	**55.51**	13.12	3.87	7.08	10.49	4.90	-	25.77	3.54
Hesperetin	**57.47**	-	-	5.06	11.40	15.42	-	15.45	-



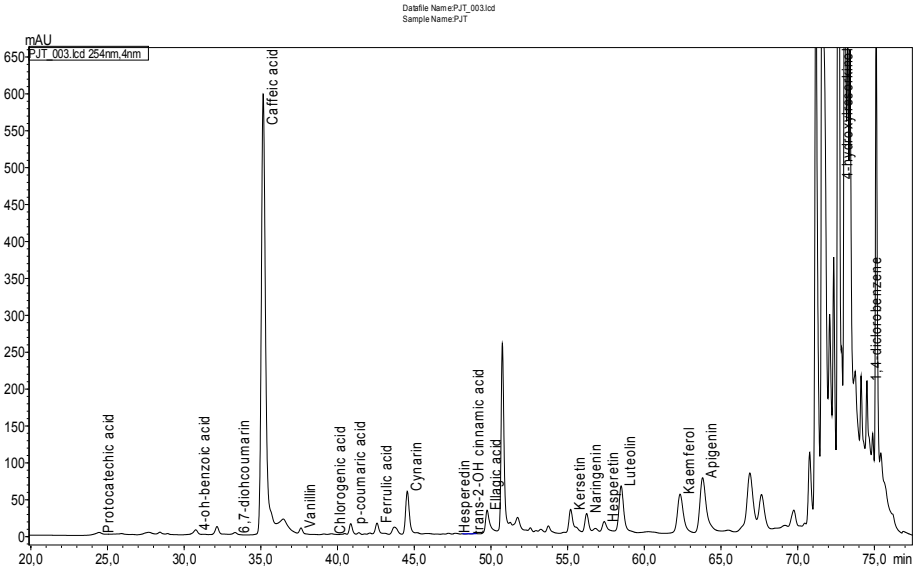
EEPJCH



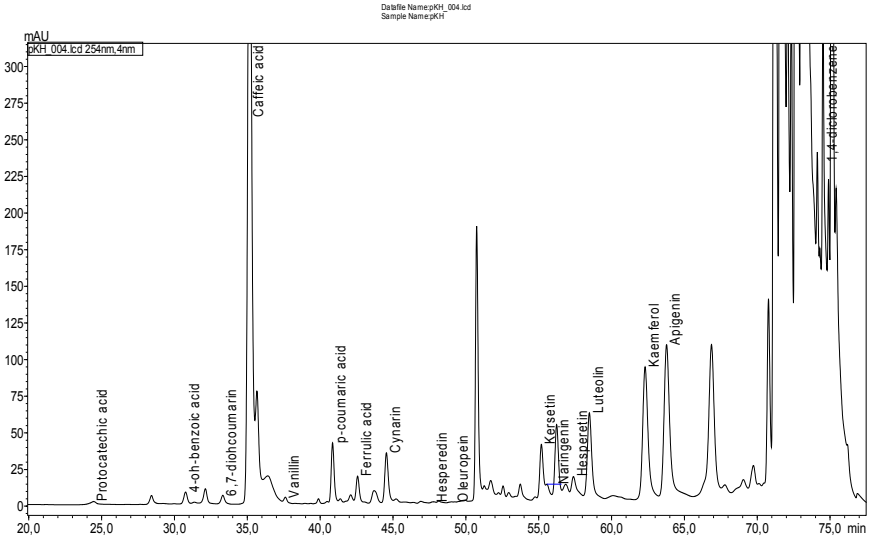
EEPJOK



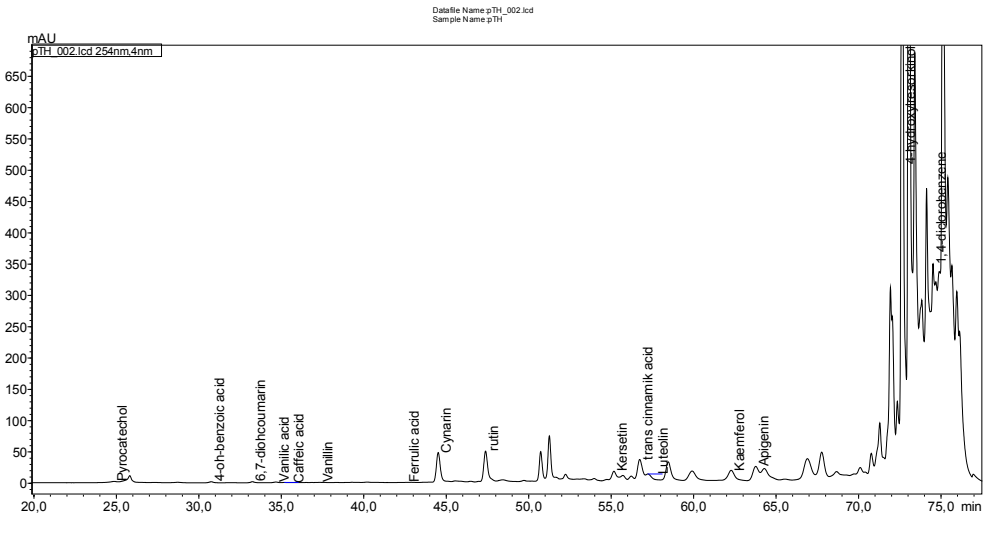
EEPJ5



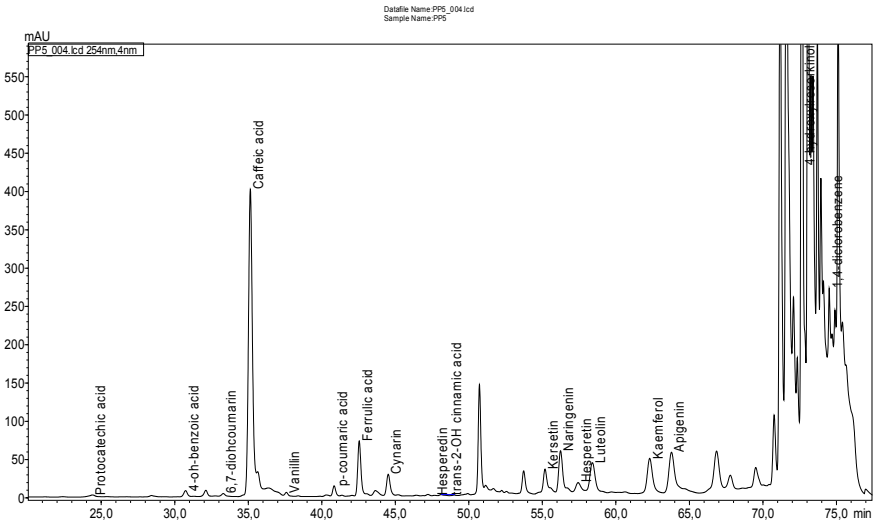
EEPJT



EEPKH

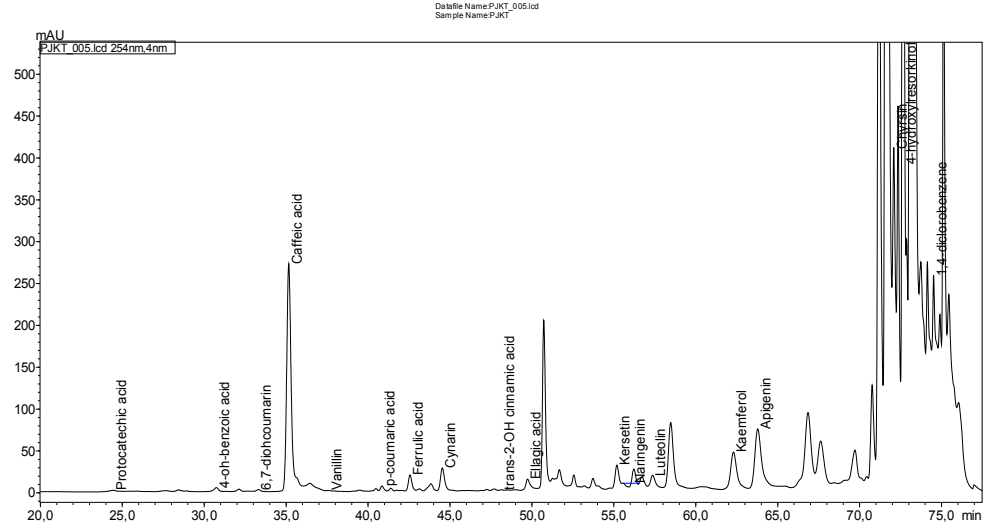


EEPTH

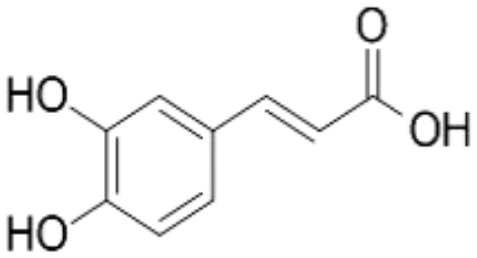


EEPPS

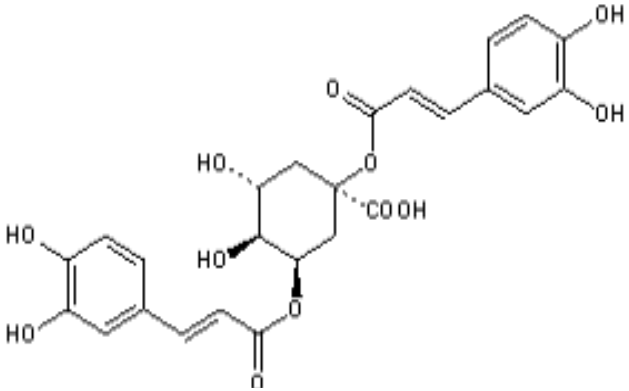
Figure 28. HPLC chromatograms of ethanolic extracts of propolis



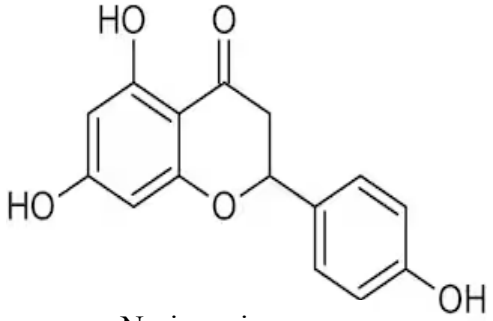
EEPJKT



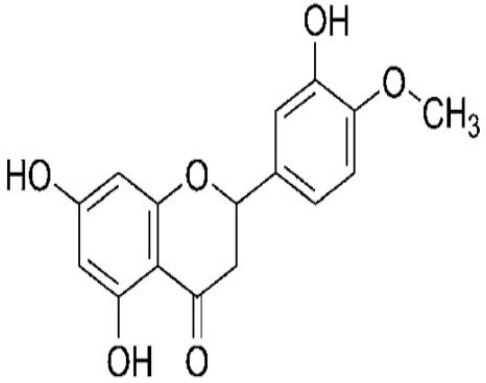
Caffeic acid



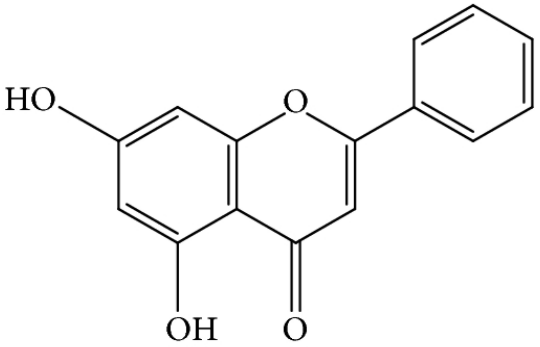
Cynarin



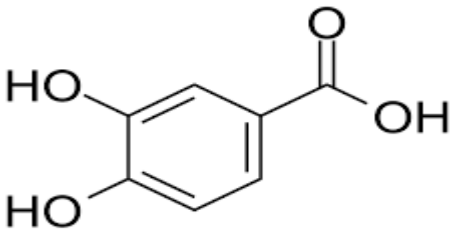
Naringenin



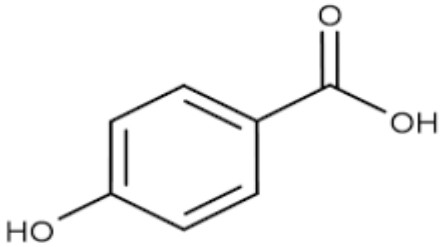
Hesperetin



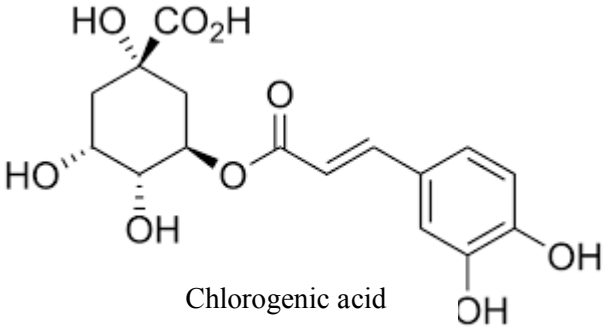
Chrysin



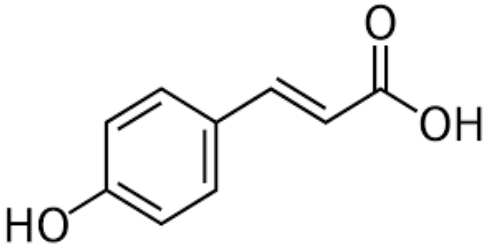
Protocatechuic acid



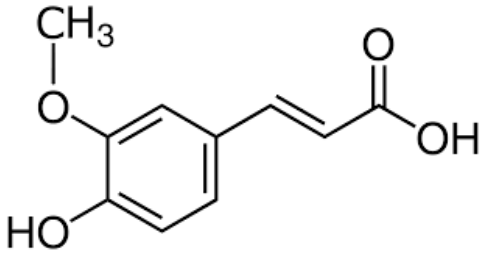
4-oh-benzoic acid



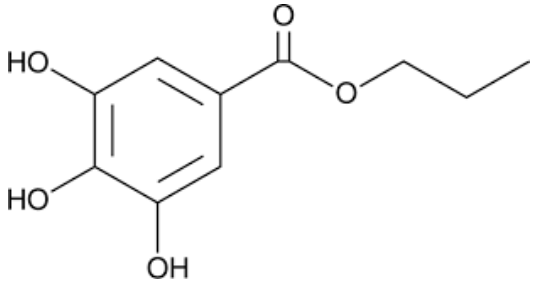
Chlorogenic acid



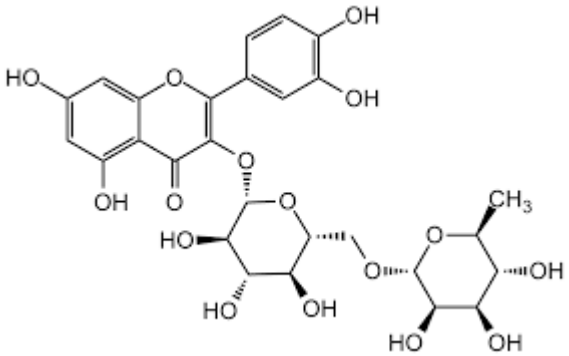
p-coumaric acid



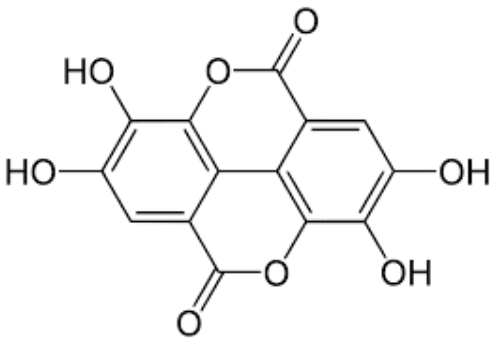
Ferulic acid



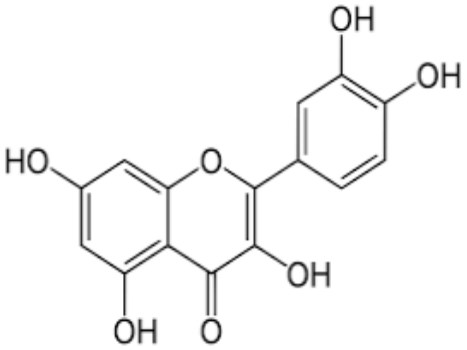
Propyl-gallate



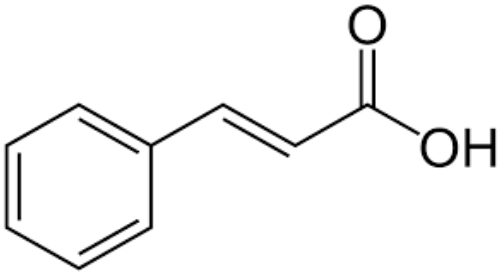
Rutin



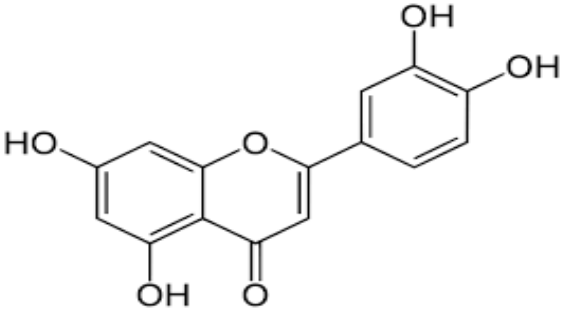
Ellagic acid



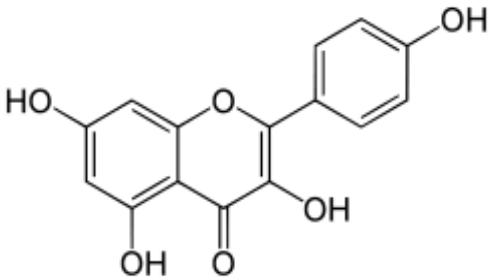
Quercetin



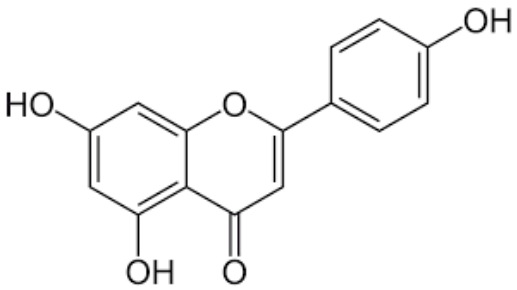
Trans Cinnamic acid



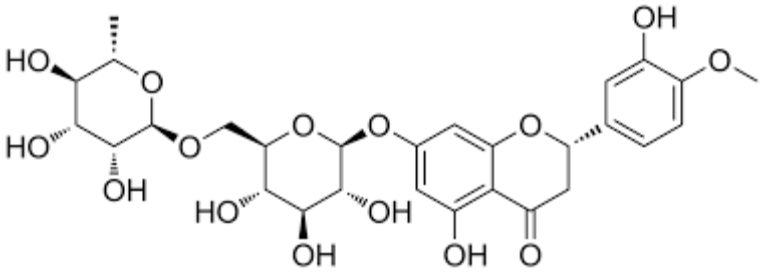
Luteolin



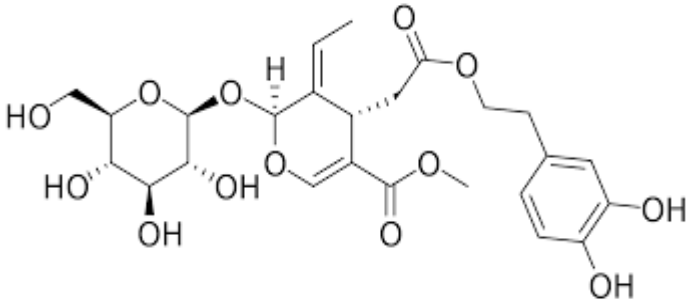
Kaempferol



Apigenin



Hesperidin



Oleuropein

Figure 29. Structures of compounds identified in propolis extracts

2 Biological activities

2.1 Antioxidant activity

Several techniques have been applied to determine the antioxidant capacity of propolis extracts; in this study, six techniques were used, including DPPH, ABTS, GOR, CUPRAC, reducing power and phenanthroline tests. Results are represented as percentage inhibition at different concentrations and expressed with IC_{50} (concentration capable of scavenging 50 % of radicals) for DPPH, ABTS, GOR and $A_{0.5}$ (concentration, indicating 0.50 absorbance) for CUPRAC, reducing power and phenanthroline assays compared with BHA, BHT and ascorbic acid as reference standards.

2.1.1 DPPH free radical assay

DPPH is a free radical that can transform into a stable substance when it accepts an electron or hydrogen. The color of the reaction changes from violet to diphenylpicrylhydrazine yellow with a decrease in absorbance at wavelength 517 nm, meaning that the reaction between DPPH radical and antioxidant molecules been achieved by hydrogen donation **(Gangwar *et al*., 2014; Fatiha *et al*., 2019)**.

The results presented in figure (30) show that propolis extracts and standard BHA have a dose-dependent scavenging capacity. The propolis extracts examined showed good DPPH radical scavenging activity, with a very close percentage of inhibition at concentration 25 µg/mL (PJCH: 86.88±0.90 %, PJOK: 87.43±0.29 %, PJT: 87.32±1.33 %, EEPKH: 86.88±0.82 %, EEPJ5: 86.85±0.64 %, EEPJKT: 87.67±0.34 %), 50 µg/mL (PJCH: 86.58±0.62 %, PJOK: 87.14±0,51 %, PJT: 87.37±1.11 %, PKH: 87.12±1.12 %, PJ5: 86.99±0.77 %, PJKT: 87.97±0.17 %,) and 100 µg/mL (PJCH: 86.63±0.37%, PJOK: 86.46±0.45%, PJT: 86.53±1.65 %, PKH: 86.29±1.05 %, PJ5: 86.46±1,22%, PJKT: 87.24±0.37%) compared with the standard antioxidant BHA (76.77±0.54 %, 78.67±1.31 %, 79.01±0.89 %, respectively).
A stationary phase was observed at these concentrations, justifying an almost total reduction of free radicals to non-radical form.

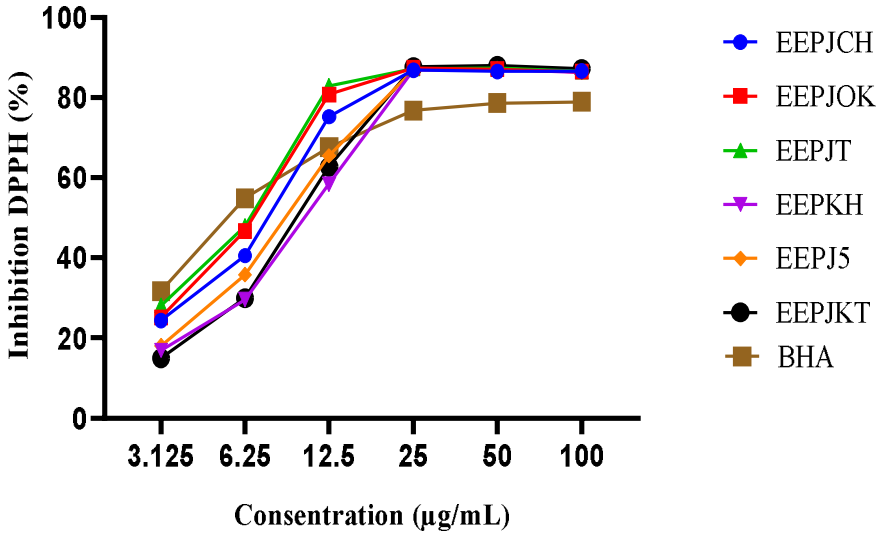


Figure .30 Percentage DPPH radical inhibition at different concentrations of propolis extracts and standard antioxidants

On the basis of IC_{50} values (Figure 31), the best activity was observed by EEPJT (6.78±0.24 µg/mL), which showed comparable activity ($p>0.05$) to EEPJOK (7.14±0.14 µg/mL), EEPJCH (7.89±0.24 µg/mL) and the standard antioxidant BHA (5.73±0.41 µg/mL), followed by EEPJ5 (8.95±0.75 µg/mL), EEPJKT (9.22±0.83 µg/mL) and EEPKH (10.33±0.04 µg/mL), which differ ($p<0.05$) from BHA.

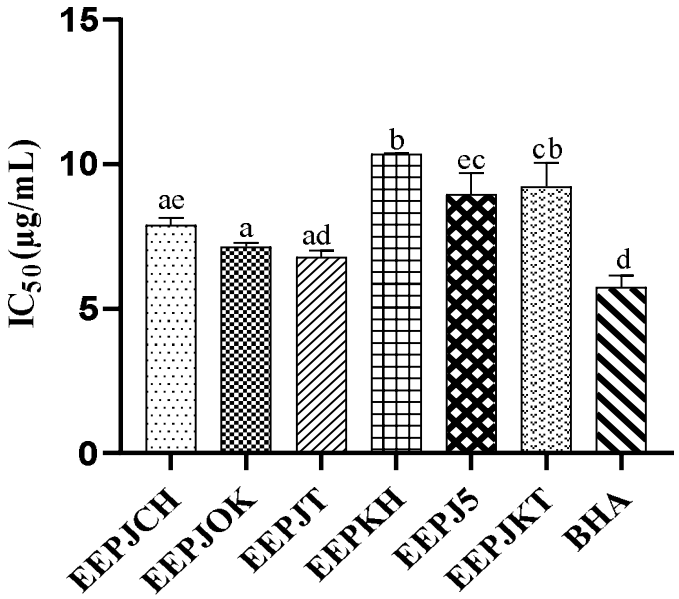


Figure 31. IC_{50} values obtained from propolis extracts and standard antioxidant for DPPH test

2.1.2 ABTS cation radical scavenging test

Another *in vitro* technique for estimating the scavenging capacity of propolis is that which uses the ABTS radical cation. Propolis extracts, as shown in figure (32), scavenge ABTS radical in a way that is proportional to the concentration of propolis extracts. Following, the standards antioxidants BHA and BHT, which had inhibition percentages of 94.20±0.90, 93.37±0.86, respectively at concentration 50 µg/mL and 95.39±2.62, 94.87±0.87 at concentration 100 µg/mL, respectively, while EEPTH showed the best percentage of inhibition with value of 91.12±0.20 at 50 µg/mL and 90.79±0.12 at 100 µg/mL, respectively. EEPPS inhibited ABTS radical with a percentage of 85.61±1.37 at the concentration 100 µg/mL, which considered the lowest inhibition value. A stationary phase was observed for other extracts as well as for antioxidant standards, indicating a complete reduction of free radicals to their non-radical form.

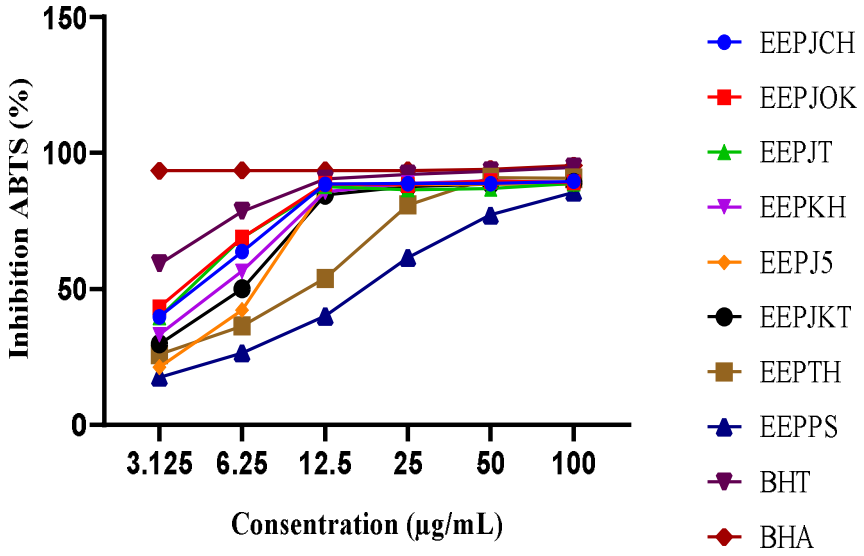


Figure 32. ABTS radical scavenging activity of propolis extracts and antioxidant standards

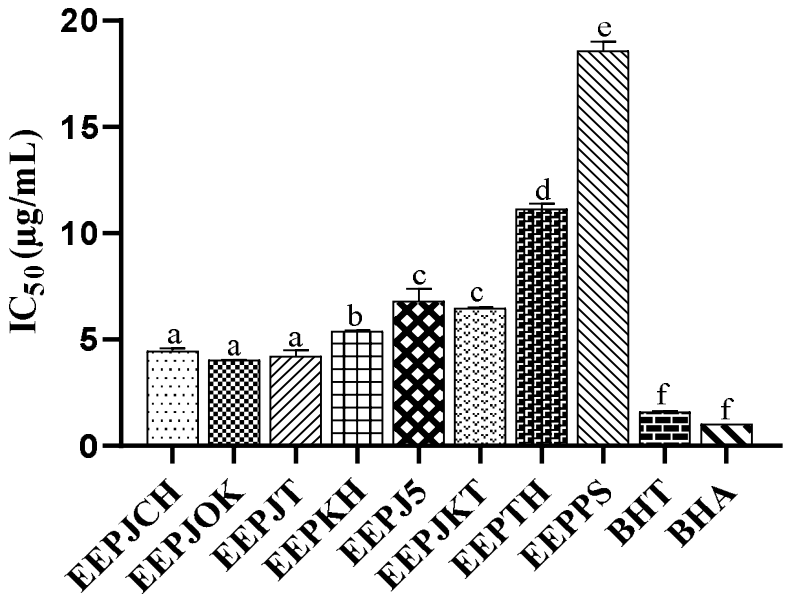


Figure 33. IC_{50} values obtained from propolis extracts and standard antioxidants for ABTS test

EEPJOK possesses the greatest ABTS scavenging capacity, with an IC_{50} value (4.02±0.02 µg/mL) ($p > 0.05$) similar to that of EEPJT (4.23±0.26 µg/mL) and EEPJCH (4.45±0.12 µg/mL), but less than those of BHT (1.59±0.03 µg/mL) and BHA (1.03±0.00 µg/mL) **(figure 33)**. EEPPS had the lowest activity, with a significant difference from other extracts and standard antioxidants with IC_{50} value of 18.55±0.44 µg/mL.

2.1.3 Galvinoxyl radical scavenging (GOR) activity

As illustrated in **figure 34**, propolis extracts have a dose-dependent effect. The scavenging activity at a concentration 100 µg/mL showed that EEPPS had the lowest radical scavenging capacity with value of 62.33±2.04 %, while the largest activity observed in EEPTH and EEPJ5 with a value of 76.91±0.56 % and 78.23±3.72 %, respectively, compared with antioxidant standards BHT (71.13 ± 0.74 %) and BHA (70.60 ± 0.10 %). A stationary phase was observed for extracts EEPJCH, EEPJOK, EEPJT, EEPJKT and EEPKH, EEPJ5, justifying a complete reduction of free radicals to their non-radical form.

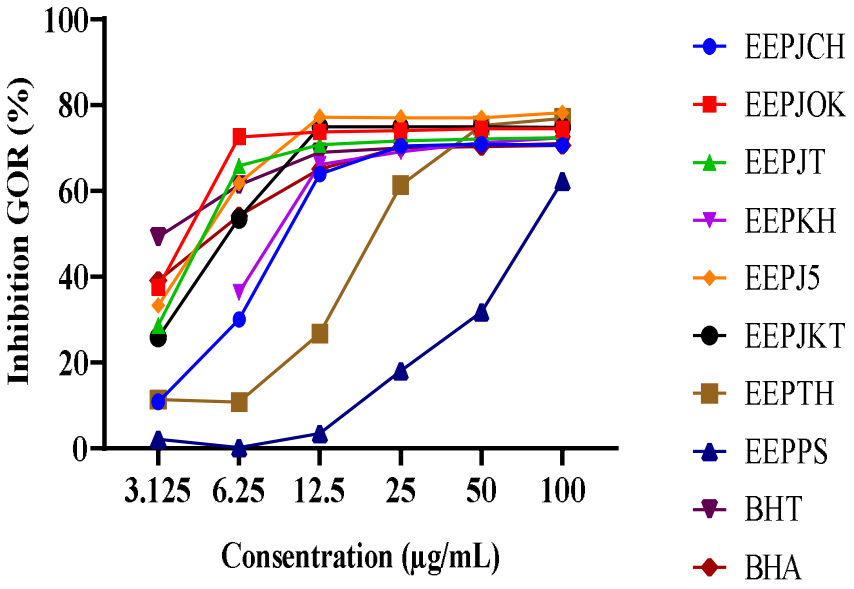


Figure 34. Percentage inhibition of GOR by propolis and antioxidant standards

Figure 35 demonstrates that EEPJT exicibited a strong IC_{50} value 3.61±1.02 µg/mL, which did not differ from other ($P>0.05$) standard antioxidants and propolis extracts, in contrast to EEPTH and EEPPS, which exhibited a significant difference ($P<0.05$) with IC_{50} values of 16.08±0.97 µg/mL and 76.25±6.27 µg/mL, respectively.

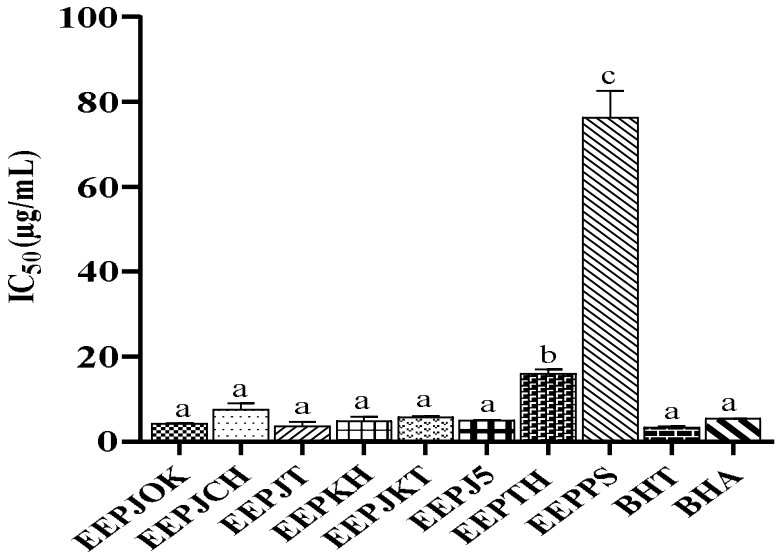


Figure 35. IC_{50} values obtained from the GOR test for propolis extracts and standard antioxidants

2.1.4 Cupric reducing antioxidant capacity

The ability of propolis extracts to reduce copper increases proportionally with extract concentration, as shown in **figure (36)**. EEPJOK exhibit the highest activity with absorbance value of 3.99±0.05, followed by EEPKH with absorbance value of 3.92±0.10 compared to the antioxidant standards BHT (2.04±0.14) and BHA (3.76±0.03).

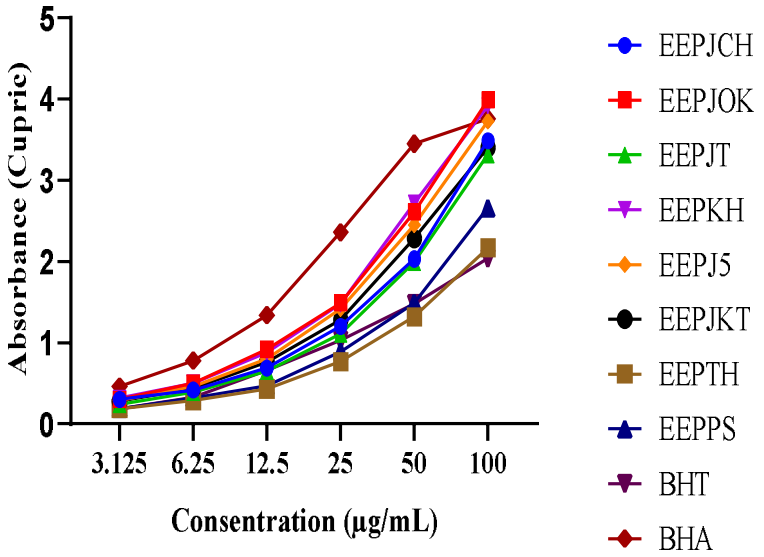


Figure 36. Cupric reducing capacity of propolis extracts and antioxidant standards

EEPKh demonstrated the strongest cupric reducing capacity with $A_{0.5}$ value 0.96±0.69 µg/mL as indicated in **figure 37**, in contrast to EEPTH, which showed the lowest activity with an $A_{0.5}$ value of 14.95±1.06 µg/mL.

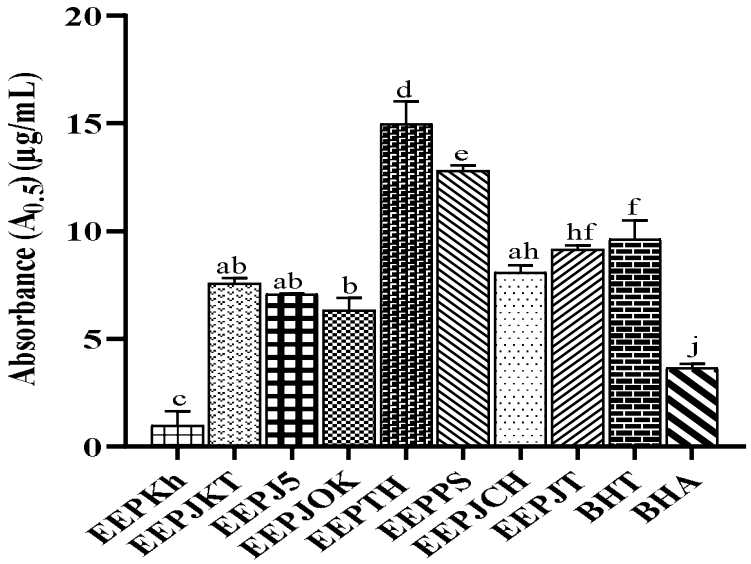


Figure 37. $A_{0.5}$ values obtained for propolis extracts and standard antioxidants from the Cupric reducing antioxidant capacity

2.1.5 Phenanthroline antiradical activity

BHT and BHA showed the greatest phenanthroline scavenging activity with absorbance values of 3.48±0.03 and 2.10±0.05, respectively, followed by EEPKh extract with an absorbance value of 1.68±0.00 at concentration 25 µg/mL **(figure 38)**. The lowest absorbance was observed for EEPPS with absorbance value 0.89±0.15.

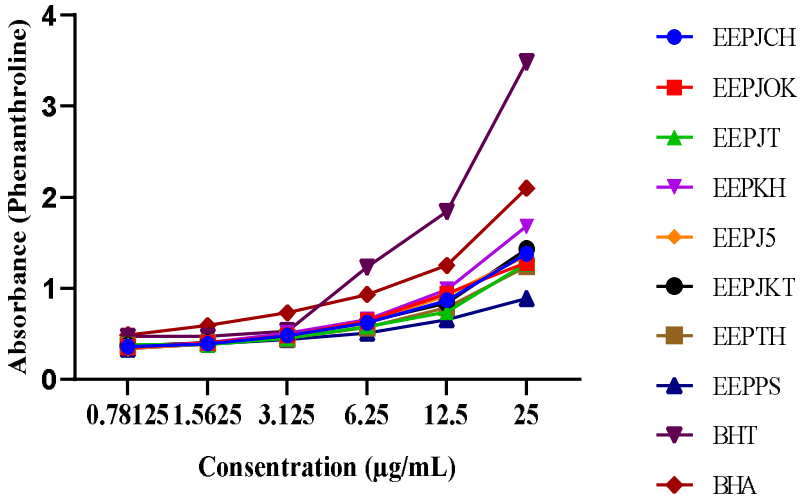


Figure 38. Phenanthroline inhibition capacity of propolis extracts and antioxidant standards

As shown in **figure 39**, EEPKh showed the best inhibition capacity for phenanthroline with $A_{0.5}$ value 0.51±0.19 µg/mL, which was close to that of BHA ($A_{0.5}$: 0.93±0.07) and differed significantly from that of other extracts and BHT.

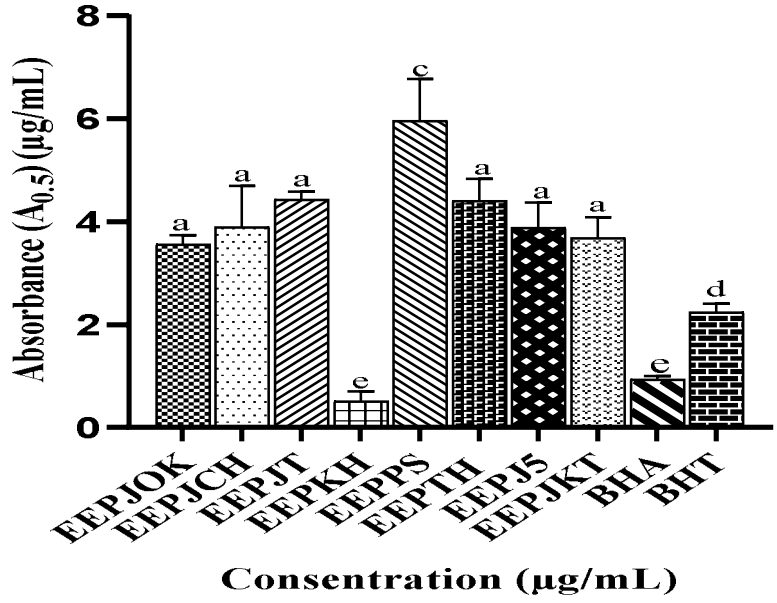


Figure 39. $A_{0.5}$ values obtained for propolis extracts and standard antioxidants from the phenonthroline test

2.1.6 Ferric reducing capacity of propolis

The reducing capacity of extract was exerted at 25 µg/mL by standard antioxidants with absorbance values of 2.02±0.23 for ascorbic acid and 1.74±0.07 for BHA, followed by EEPJT with an absorbance value of 0.97±0.17. Low absorbance was observed for EEPJKT and EEPJ5 with values of 0.17 and 0.27, respectively (**figure 40**).

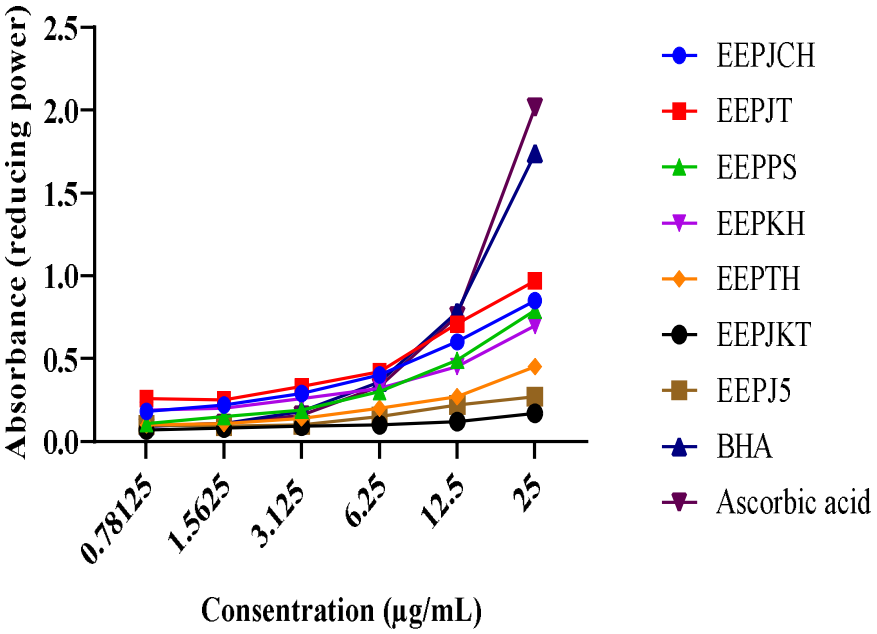


Figure 40. Reducing antioxidant capacity of propolis extracts

Among the propolis extracts, EEPKh showed the best activity, with an absorbance value of 1.19±0.66 µg/mL, that differ significantly ($p<0.05$) from other propolis extracts and BHA **(figure 41)**. EEPJOK, EEPJ5, EEPJKT and EEPTH showed $A_{0.5}$ value > 25 µg/mL.

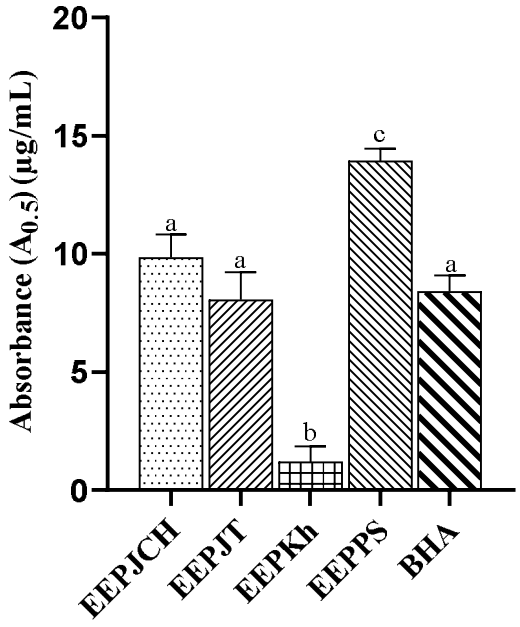


Figure 41. $A_{0.5}$ values obtained for propolis extracts and standard antioxidants from the reducing antioxidant test

Propolis has long been known for its several medical properties, most notably its antioxidant activity, which is connected to its chemical composition, namely polyphenolic components (ferulic acid, caffeic acid and caffeic acid phenethyl ester) **(Ahn *et al,* 2007)**. The results obtained suggest that all extracts exerted a powerful scavengers through their hydrogen donation or electron transfer capacity **(Rahman *et al*., 2015).**

Boulechfar et *al.* (2022) tested the ability of propolis extracts from El-Menia, Oum el Bouaghi, Collo and El-Harrouch regions to scavenge the DPPH radical, and their results revealed that propolis extracts exerted effective activity with IC_{50} values of 29. 06 ± 0.20 µg/mL, 42.02 ± 1.15 µg/mL, 41.33 ± 0.61 µg/mL, 22.24 ± 0.43 µg/mL and 22.32 ± 1.19 µg/mL, respectively. Furthermore, **Debab *et al.* (2017)** also confirmed the effectiveness of propolis harvested from the Mascara2 and Sidi Dahou regions versus the DPPH radical, where they revealed IC_{50} values of 0.045 mg/mL and 19.95 mg/mL respectively.

Propolis extract from Mila (Grarem) and Souk-Ahras regions scavenged 50 % of the ABTS radicals, with an inhibition concentration value of 10.08 ± 0.11 µg/mL, 7.05 ± 0.04 µg/mL and 3.11 ± 0.03 µg/mL, respectively ***(*Boulechfar *et al.*, 2019*;* Ouahab *et al*., 2023**).

Propolis extract collected from Cameroon showed an ability to reduce CUPRAC radicals, determined by an $A_{0.5}$ value of 6.50 ± 0.25 µg/mL **(Tamfu *et al.*, 2022)**. In addition, **Boulechfar et *al.* (2022)** declared that propolis originating from different regions of Algeria possessed a significant reduction capacity against CUPRAC and ferric radicals with an $A_{0.5}$ value ranging from 11.83 ± 0.12 µg/ml to 18.01 ± 2.15 µg/ml and from 31.46 ± 1.08 to 9.53 ± 2.93, respectively.

The results suggest that the polyphenolic compounds in propolis extracts, including caffeic acid, hesperidin, cynarin and naringin, may act as free radical scavengers **(Wilmsen *et al.*, 2005; Gülçin, 2006; Topal *et al.*, 2016)**. Propolis exerts different mechanisms of action against free radicals, which may include reducing free radical synthesis by inhibiting certain enzymes involved in their generation, scavenging free radicals, chelating metal ions such as iron and copper, or potentiating the action of other antioxidants, hydrogen atom transfer**,** transfer of a single electron**,** sequential proton loss electron transfer **(Freitas, 2015; Zeb, 2020)**.

Hydroxyl groups in the structure of phenolic compounds, among many factors, are responsible for the effectiveness of this compounds as antioxidant **(Shahidi *et al.*, 2010)**.

A number of factors have been identified as influencing the ability of extracts to quench and react with a variety of radicals, for example radical stereoselectivity, extract solubility, solvent polarity and functional groups found in bioactive components **(Mukherjee *et al.*, 2011)**.

3 Relationship between antioxidant activity and polyphenol contents

The scavenging capacity of propolis extracts is frequently related to the quantity and nature of the polyphenolic components contained in propolis. The relationship between the antioxidant capacity of propolis determined by different techniques and polyphenol content was explained by Pearson's correlation coefficient r **(Annexe 1) (Asem *et al.*, 2020)**. The results of the correlation study between antioxidant activity, TPC and TFC presented in Tables 5 and 6 show a positive correlation between these both factors. TPC and TFC derived from propolis extracts showed significant and strong ($p<0.001$, $p<0.0001$) positive correlation with CUPRAC, phenanthroline and reducing power assays. TPC and TFC of EEPPS showed a strong positive relationship with GOR ($p<0.0001$) and significant correlation with ABTS ($p<0.05$). Significant

correlations were also revealed between the phenolic and flavonoid contents of EEPTH and the GOR test ($p<0.05$). These results are also in agreement with those reported by **Syed Salleh *et al.* (2021)** who found that the polyphenols present in Malaysian propolis are closely associated with its antioxidant capacity. In addition, a significant correlation was also found between the antioxidant effect of Chinese propolis and its phenolic contents **(Peng *et al.*, 2023)**. The capacity of polyphenols and flavonoids to block oxidative stress is expressed by their ability to prevent the generation of free radicals via hydrogendonating ability of their hydroxyl groups and ability to donate electrons **(Yusop *et al.*, 2019)**.

Table 5. Relationship between antioxidant activity and TPC

Extracts (TPC)	DPPH	CUPRAC	ABTS	Phenanthroline	GOR	Reducing power
EEPJCH	0.6503^{ns}	0.9975****	0.5695^{ns}	0.9994****	0.6238^{ns}	0.9885***
EEPJOK	0.6082^{ns}	0.9887***	0.5519^{ns}	0.9886***	0.4218^{ns}	/
EEPJT	0.5977^{ns}	0.9961****	0.5518^{ns}	0.9967****	0.4701^{ns}	0.9875***
EEPKH	0.7116^{ns}	0.9835***	0.5924^{ns}	0.9998****	0.5965^{ns}	0.9985****
EEPJ5	0.6880^{ns}	0.9882***	0.5856^{ns}	0.9963****	0.5376^{ns}	/
EEPJKT	0.6977^{ns}	0.9871***	0.6141^{ns}	0.9987****	0.5387^{ns}	/
EEPTH	/	0.9956 ****	0.7971^{ns}	1.000****	0.8362*	/
EEPPS	/	0.9982 ****	0.8883*	0.9935****	0.9946****	0.9969****

^{ns} Non-significant correlation, $p \geq 0.05$

*Significant correlation, $p<0.05$

***Highly significant correlation, $p<0.001$

**** Highly significant correlation, <0.0001

Table 6. Relationship between antioxidant activity and TFC

Extracts (TFC)	DPPH	CUPRAC	ABTS	Phenanthroline	GOR	Reducing power
EEPJCH	0.6504^{ns}	0.9975****	0.5696^{ns}	0.9994****	0.6239^{ns}	0.9885***
EEPJOK	0.6099^{ns}	0.9890***	0.5535^{ns}	0.9876***	0.4231^{ns}	/
EEPJT	0.5979^{ns}	0.9962****	0.5519^{ns}	0.9967****	0.4702^{ns}	0.9874***
EEPKH	0.7114^{ns}	0.9834***	0.5922^{ns}	0.9998****	0.5963^{ns}	0.9985****
EEPJ5	0.6881^{ns}	0.9882***	0.5858^{ns}	0.9964****	0.5377^{ns}	/
EEPJKT	0.6976^{ns}	0.9871***	0.6141^{ns}	0.9987****	0.5387^{ns}	/
EEPTH	/	0.9956****	0.7971^{ns}	1.000****	0.8363*	/
EEPPS	/	0.9982****	0.8882*	0.9936****	0.9946****	0.9969****

^{ns} Non-significant correlation, $p \geq 0.05$

*Significant correlation, $p<0.05$

***Highly significant correlation, $p<0.001$

**** Highly significant correlation, <0.0001

4 Effect of propolis extracts on *Enterococcus faecalis* bacteria

4.1 Isolation and characterization of *Enterococcus faecalis*

Bile Esculin Azide Agar (BEA) revealed the presence of gray, spherical, small colonies with a brownish black coloration (**figure 42**), which can be considered as belonging to the *Enterococci* genus. These colonies were subcultured in BEA for purification before further microscopic, biochemical and physiological characterization in order to identify the *Enterococcus faecalis* species.

Microscopic examination of enterococcal isolates using a light microscope (X40 and X100) reveals non-motile, Gram-positive ovoid cocci presenting a single, paired and short chain **(figure 43)**.

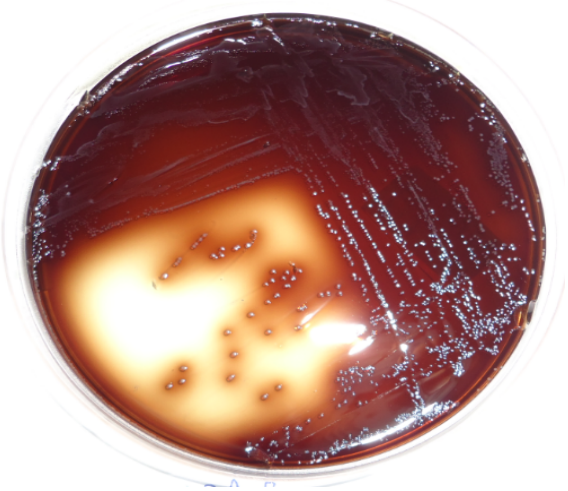


Figure 42. Macroscopic appearance of *Enterococcus* on BEA agar

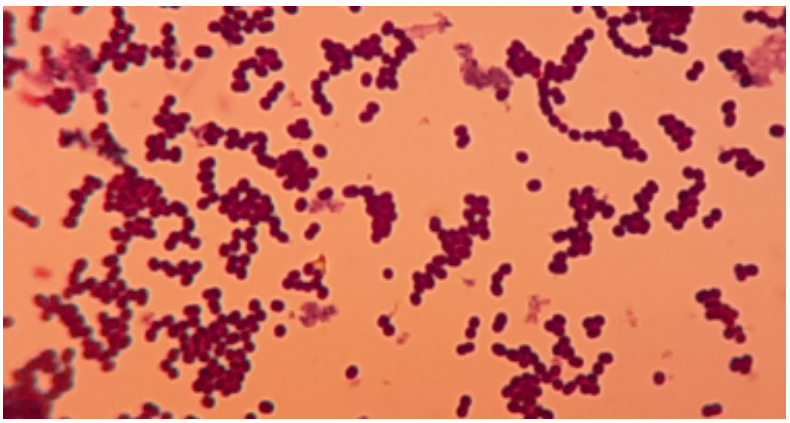


Figure 43. Microscopic observation of *Enterococcus* after Gram staining under microscope (100X)

Identification of the germs as *Enterococus faecalis* was confirmed by physiological and biochemical tests. The results indicated that isolates showed negative results for catalase and oxidase, positive growth on potassium tellurite agar and nutrient broth supplemented with 6.7 % NaCl and 0.5 % glucose **(figure 44)**, able to survive in a medium with a pH 9.6 and at 10 °C, 45 °C and 50 °C. Appearance of isolates on blood agar was non-hemolytic **(figure 45)**. Identification was completed by Chromagar orientation, on which *Enterococcus faecalis* appeared as small turquoise-blue circular colonies **(figure 46)**.

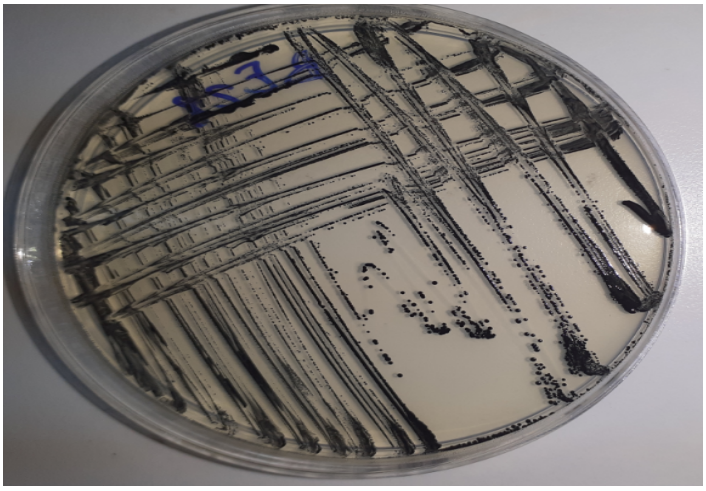


Figure 44. Macroscopic appearance of *Enterococcus faecalis* on potassium tellurite agar

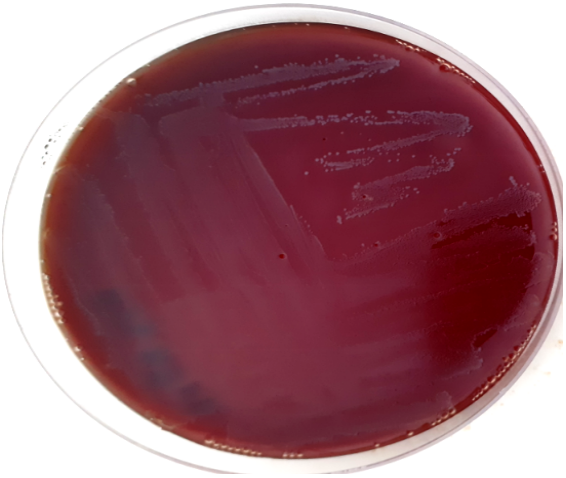


Figure 45. Appearance of *E.faecalis* on blood agar

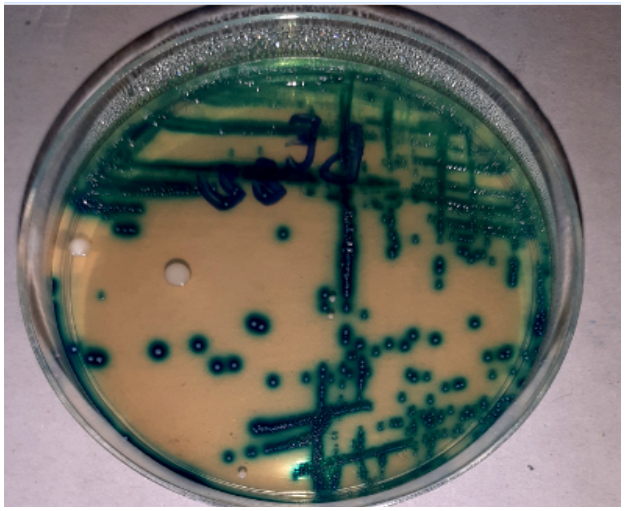


Figure 46. Appearance of *E.faecalis* on chromagar orientation

Enterococci develop under selective conditions, a parameter widely used for their isolation; several studies have shown that *Enterococcus faecalis* bacteria is cocci Gram-positive organized in pairs or chains, non-motile, non-hemolytic, negative to catalase and oxidase, and can develop at extreme temperatures between 10, 45 and 50°C, in an alkaline environment (pH 9.6) and in the presence of 0.04 % potassium tellurite **(Manero *et al*., 1999; Al-Sa'ady, 2019)**. The ability to reduce potassium tellurite and produce black colonies is a characteristic of the *E. faecalis* species **(Shekh *et al*., 2012)**.

Enterococci develop in the presence of 40 % bile salts and hydrolyze esculin, producing a black precipitate in bile-esculin agar. Bacteria decompose the esculin molecules and use the glucose generated as an energy source to liberate esculetin into the medium, which reacts with ferric citrate to give a phenolic iron complex, transforming the agar medium from dark brown to black **(Borah *et al*., 2016)**.

The capacity of enterococci to survive in a wide pH range can be attributed to their membrane durability and impermeability to acids and alkalis, whereas their temperature resistance can be explained by the lipids and fatty acids found in their membrane. The ability of enterococci to survive in extreme environments enables them to colonize a wide range of niches **(John *et al*., 2011)**.

4.2 Antibiotic sensitivity tests on *E.faecalis* isolates

Research on *E. faecalis* has revealed that this antibiotic-resistant pathogen can be the cause of a number of oral disorders, including tooth decay, endodontic and

periodontal diseases **(Souto *et al*., 2008; Kouidhi *et al*., 2011; Suliman Al-Badah *et al*., 2015)**. It is widespread in primary and secondary endodontic infections, with percentages of 54.83 and 71.42 respectively **(Prado *et al*. 2017)**. The high persistence of *E. faecalis* was associated to its various virulence factors including, their capacity to compete with other microbes, invading dentinal tubules and survive with poor nutrition **(Stuart *et al*., 2006)**. A number of researchers have documented the resistance of *Enterococcus faecalis* isolates from endodontic disease to antimicrobial drugs **(Endo *et al*., 2014; Barbosa-Ribeiro *et al*., 2016; Monteiro *et al*., 2021)**.

For this study, fourteen *E.faecalis* isolates were tested against eight antibiotics (penicillin G (10 µg), erythromycin (15 µg), tetracycline (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg) and amoxicillin (30 µg)) to determine their resistance profile. Results were interpreted on the basis of guidelines established by the Clinical Laboratory Standards Institute (CLSI 2020), as shown in **figure (47)**.

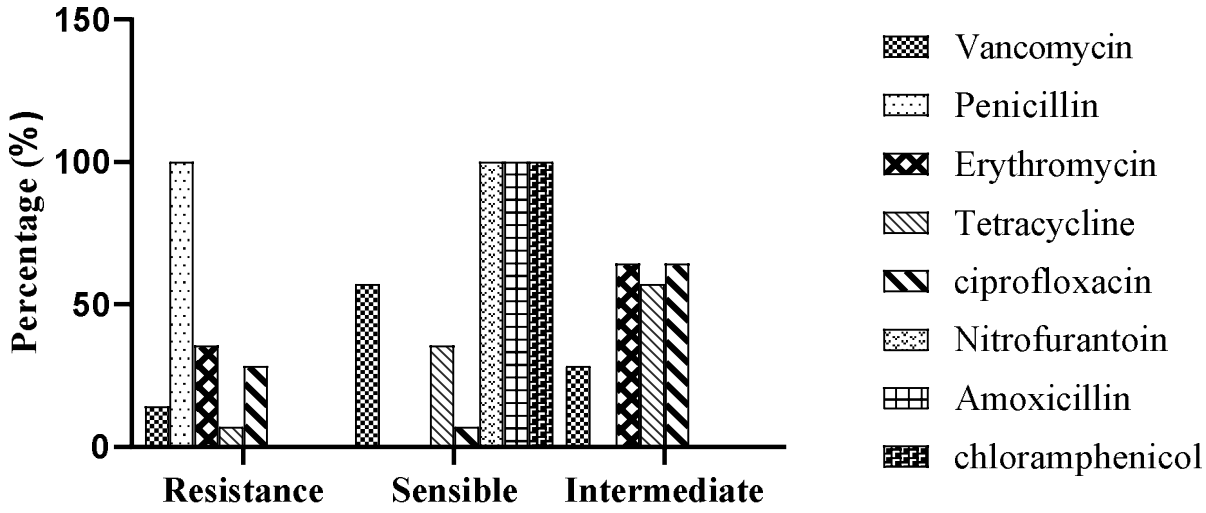


Figure 47. Susceptibility profile of *E. faecalis* isolates to different antibiotics

All clinical isolates of *E. faecalis* were 100 % sensitive to nitrofurantoin, chloramphenicol and amoxicillin, the latter being one of the most commonly used antibiotics in the treatment of different dental problems. Resistance to other antibiotics was observed, with percentages of 100 % for penicillin G, 35.71 % for erythromycin, 7.14 % for tetracycline, 14.28 % for vancomycin and 28.57 % for ciprofloxacin, respectively.

Benbelaïd *et al.* (2014), found that *Enterococcus faecalis* isolates from oral diseases were resistant to vancomycin, chloramphenicol, ciprofloxacin, erythromycin and tetracycline, but susceptible to amoxicillin.

Lins *et al.* (2013) revealed that penicillin G, erythromycin and vancomycin inhibited the growth of *Enterococcus faecalis* isolated from endodontic disease, and that only 70 % of isolates showed a high prevalence of resistance to tetracycline. While, **Patidar *et al.* (2013)** reported that 100 % of clinical endodontic *E. faecalis* bacteria were susceptible to amoxicillin and vancomycin, 61.5 % of isolates were resistant to chloramphenicol, 57.6 % to ciprofloxacin, 75 % to erythromycin and 65.3 % to tetracycline.

Bacteria use several strategies to develop resistance to antimicrobial drugs. Examples of these processes include active efflux pump, hyperproduction of the target enzyme, limiting antimicrobial access to target sites by altering cell membrane permeability, destruction of antibiotic, enzymatic transformation of antibiotic, alteration of target of antibiotic, acquire metabolic processes different from those repressed by the drug **(Van Hoek *et al.*, 2011)**.

In enterococci, antibiotic resistance mechanisms can be explained by intrinsic or acquired resistance through mutation of intrinsic genes or horizontal transfer of genetic information encoding resistance determinants **(Hollenbeck *et al.*, 2012)**.

For the antibacterial and antibiofilm activity tests, only four clinical strains of *E. faecalis* were selected, due to their resistance to different ATB (**Table 7**).

Table 7. Antibiotic resistance/susceptibility of *E. faecalis* isolates

***Enterococcus faecalis* isolates**	**Origin**	**Antibiotics**							
		P	**E**	**TE**	**VA**	**CIP**	**C**	**F**	**AML**
1 (BEF28)	**Dental abscess**	R	I	I	R	R	S	S	S
2 (BEF33)	**Intracanal**	R	R	R	I	I	S	S	S
3 (BEF17)	**Tooth decay**	R	R	I	S	I	S	S	S
4 (BEF34)	**Intracanal**	R	I	I	R	R	S	S	S

(S): Sensible**, (R):** Resistant **(I):** Intermediate

5 Antibacterial activity

5.1 Disc diffusion assay

The effect of propolis extracts on four *Enterococcus faecalis* endodontic pathogens was evaluated using the disk diffusion technique. Inhibition zone diameter values are presented in **Table (8)**. All propolis samples were effective against *E. faecalis* bacteria*;* it was observed that inhibition zone values increased with increasing extract concentration. At concentrations 20 mg/mL, EEPJKT demonstrated strong antibacterial activity against BEF33 strain, with inhibition value of 21.67±2.08 mm, followed by EEPJCH and EEPKh with the same inhibition diameter 20.33±0.57 mm against BEF34. EEPPS also showed the highest inhibition diameter 20±2 mm against BEF33. The negative control (DMSO) has no effect on bacteria.

In dentistry, propolis and its components appear to be a promising alternative for the treatment of oral infections such as tooth decay, pulpal infection and endodontic infection **(Uzel *et al*., 2005; Parolia *et al*., 2010; Zulhendri *et al*., 2021)**. In this experiment, the results revealed that propolis extracts were effective against *E. faecalis* bacteria.

Several studies have already confirmed the effectiveness of propolis extracts compared with other intracanal drugs against *E. faecalis* **(Madhubala *et al.*, 2011; Vasudeva *et al*., 2017)**. **Carbajal Mejía (2014)** revealed that the antibacterial effects of chlorhexidine (2%) and propolis against *Enterococcus faecalis* is comparable. On the other hand, **Elsayed *et al*.(2021)** examined the efficacy of propolis against *E. faecalis* and reported that propolis had a powerful effect on this bacterium.

However, **Madhubala *et al*. (2011)**, showed that propolis extract was more potent than a combination of three antibiotics in reducing *E. faecalis*.

Propolis exerts its action by interacting directly with bacteria cells or by stimulating the host cell's immune system. Several studies indicate that structural disruption of microbes is a potential strategy by which propolis exerts its antimicrobial effect **(Bouchelaghem, 2021)**. Some active substances in propolis have the ability to alter the cell wall and cytoplasmic membrane of bacteria; caffeic acid is an inhibitor responsible for inhibiting bacterial multiplication and growth, while flavonoids act on the bacterial membrane, modifying the permeability of the inner membrane of bacteria **(Wojtyczka *et al*., 2013)**.

5.2 Minimum inhibitory and bactericidal concentrations of propolis

The MIC and MBC of propolis extracts against *Enterococcus faecalis* isolates were determined using the microdilution method. MBC/MIC ratios were calculated to define the mechanism of action of propolis extracts on these bacteria. As shown in **table (9)**, MIC values range between 0.625 and >20, while MBC values range between 1.25 and >20. The lowest inhibitory and bactericidal concentrations were observed for EEPJCH and EEPJOK versus BEF34, BEF28 and EEPJT versus BEF33, BEF28 and EEPKh versus BEF33, with values of 0.625 mg/mL and 1.25 mg/mL, respectively.

Numerous studies have shown that propolis extracts have a good bactericidal capacity against *E. faecalis*, implying that they could be used as an alternative intracanal drug **(Arslan *et al*., 2011; Jahromi *et al*., 2012)**.

Antibacterial compounds are categorized as bacteriostatic agents when the MBC/MIC ratio > 4, and as bactericidal agents when the MBC/MIC ratio ≤ 4 **(Krishnan *et al*., 2010)**. The results of this study indicate that propolis extracts have a bactericidal effect against all *Enterococcus faecalis* bacteria.

The bactericidal effects of propolis appear to be due to multiple mechanisms, the destruction of bacterial membranes and cell wall structures, destruction of DNA-dependent RNA polymerase, arrest of bacterial cell growth and division, and inhibition of protein synthesis **(Parolia *et al*., 2021; Vadillo-Rodríguez *et al*., 2021)**.

Propolis acts on bacteria through various mechanisms, which may be linked to its different compounds such as various esters of caffeic acid, apigenin, rutin, luteolin, naringin, galangin, caffeic acid, cinnamic acid, quercetin **(Madhubala *et al*., 2011; Garg *et al*., 2014; Gutiérrez-Venegas *et al*., 2019)**.

Table 8. Effect of propolis extracts on *E. faecalis* bacteria

Extracts	Concentration (mg/mL)	*E. faecalis* strains			
		BEF17	BEF34	BEF28	BEF33
		inhibition zone diameter (mm)			
EEPJCH	20	17.67±1.53^{a}	20.33±0.58^{a}	19.5±1.80^{a}	16.67±2.08^{a}
	10	15.17±0.58^{b}	19±2^{a}	14±1.73^{b}	15±1.72^{b}
	5	14.33±1.15^{b}	18.33±0.58^{a}	13.33±1.53^{b}	-
	2.5	-	16.33±1.53^{a}	12±1^{b}	-
	1.25	-	-	-	-
	0.625	-	-	-	
EEPJT	20	18±1^{a}	14.33±1.15^{b}	14.67±0.29^{b}	15±0,5^{b}
	10	15±2.65^{a}	13.67±1.15^{a}	13.33±0.58^{a}	14.33±0.58^{a}
	5	14.67±0.58^{a}	12.33±0.58^{b}	11.67±1.53^{b}	-
	2.5	14±1^{a}	11±1.73^{b}	10±1^{b}	-
	1.25	13.67±0.58^{a}	-	-	-
	0.625	11.67±2.08^{a}	-	-	-
EEPJOK	20	15±1^{a}	14±1^{a}	16.67±2.08^{a}	17.33±3.06^{a}
	10	14.33±2.52^{a}	13±1.73^{a}	14±1^{a}	13±1^{a}
	5	13.67±1.53^{a}	12.33±2.31^{a}	13.33±2.08^{a}	-
	2.5	13.33±0.58^{a}	11±1^{b}	-	-
	1.25	12.33±2.08^{a}	-	-	-
	0.625	11±2.65^{a}	-	-	-
EEPKh	20	16±0^{b}	20.33±0.57^{a}	17±1^{ba}	16±1.73^{b}
	10	14.67±2.5^{ba}	18.67±1.52^{a}	16.33±0.57	14.33±0.57^{b}
	5	13±1.73^{bc}	17.67±2.30^{a}	15.67±0.57^{ba}	12.33±1.15^{c}
	2.5	12.33±1.52^{b}	16±1.73^{a}	15±0^{a}	-
	1.25	11±0^{b}	15.33±0.57^{a}	14±1^{a}	-
	0.625	10±0^{b}	14±1^{a}	10±0^{b}	-
EEPJ5	20	16.33±1.52^{a}	15.67±2.08^{a}	17±3^{a}	18.33±1.15^{a}
	10	16±1^{a}	15.33±1.15^{a}	15.67±3.05^{a}	18±1^{a}
	5	14.67±3.78^{a}	14.33±3.78^{a}	13.67±2.08^{a}	17.67±1.52^{a}
	2.5	13.33±1.52^{a}	-	8±1^{b}	16±3.60^{a}

	1.25	7.33±0.57[a]	-	-	11.67±1.15[b]
	0.625	-	-	-	10.67±0.57[a]
EEPJKT	20	19.33±1.52[a]	16.33±5.13[a]	14.67±1.15[a]	21.67±2.08[a]
	10	18.33±1.52[a]	16±2.64[a]	14.33±1.15[a]	18.33±2.08[a]
	5	17.67±2.08[a]	15.67±1.15[a]	13.33±2.30[a]	17.33±2.51[a]
	2.5	17.33±1.15[a]	10±0[c]	12.67±2.51[bc]	16.67±2.08[a]
	1.25	16.67±0.57[a]	-	-	9±1[b]
	0.625	15.33±0.57[a]	-	-	-
EEPPS	20	14.67±0.57[a]	16.33±3.21[a]	15.33±4.16[a]	20±2[a]
	10	14.33±0.57[a]	-	14±2.64[a]	19.33±0.57[b]
	5	13±1[a]	-	13.67±1.52[a]	18.33±2.08[b]
	2.5	9±1[a]	-	-	16.33±1.52[b]
	1.25	-	-	-	-
	0.625	-	-	-	-
EEPTH	20	17±1[a]	18.67±4.04[a]	14±2.64[a]	16.67±1.15[a]
	10	16.33±0.57[a]	17.33±3.78[a]	13.83±0.76[a]	-
	5	15±2.64[a]	16±1[a]	-	-
	2.5	14.67±0.57[a]	15.33±1.52[a]	-	-
	1.25	14.33±2.08[a]	-	-	-
	0.625	12±2[a]	-	-	-
DMSO	/	-	-	-	-

(-): no activity

Table 9. MIC, MBC and MBC/MIC of propolis extracts

Propolis extracts	*E.faecalis* isolates	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC
EEPKH	BEF34	2.5	5	2
	BEF33	0.625	1.25	2
	BEF17	5	10	2
	BEF28	5	10	2
EEPJOK	BEF34	0.625	1.25	2
	BEF17	20	>20	/
	BEF33	1.25	2.5	2
	BEF28	0.625	1.25	2
EEPJCH	BEF34	0.625	1.25	2
	BEF17	2.5	5	2
	BEF33	5	10	2
	BEF28	0.625	1.25	2
EEPJT	BEF34	1.25	2.5	2
	BEF17	2.5	5	2
	BEF33	0.625	1.25	2
	BEF28	0.625	1.25	2
EEPTH	BEF34	5	10	2
	BEF17	10	20	2
	BEF33	5	10	2
	BEF28	2.5	5	2
EEPPS	BEF34	5	20	2
	BEF17	2.5	5	2
	BEF28	10	20	2
	BEF33	10	20	2
EEPJ5	BEF34	1.25	2.5	2
	BEF17	>20	ND	2
	BEF28	2.5	5	2
	BEF33	1.25	2.5	2
EEPJKT	BEF34	10	20	2
	BEF17	>20	ND	/
	BEF28	20	>20	/
	BEF34	20	>20	/

ND: not determined

5.3 Biofilm inhibition potential of propolis extracts

The pathogenesis of *E. faecalis* is linked to its ability to create a biofilm, which is considered a critical step in endodontic infection. The presence or absence of virulence genes involved in biofilm development, and their expression levels, explain the difference in biofilm formation **(Liu *et al.*, 2020)**. Biofilm provides bacteria with an effective defense against the host defense system, which renders them more resistant to various anti-infective drugs and treatments **(Jhajharia *et al.*, 2015).**

Propolis extracts were tested for antibiofilm capacity against four *Enterococcus faecalis* isolates at MIC-MIC/16 levels. The MIC values obtained range between 0.625 and 20 mg/mL. The ability of propolis extracts to inhibit biofilm formation increases with propolis extract concentration, as shown in the figure (48, 49, 50 and 51). The results obtained for antibiofilm activity indicate that EEPKH, EEPPS, EEPJKT and EEPJT showed no significant difference ($P > 0.05$) in biofilm inhibition of *Enterococcus faecalis* isolate (BEF17) at MIC with percentage inhibition of 65.93±1.11, 64.14±1.79, 63.23±0.59 and 60.76±0.93, respectively. EEPJKT inhibited the biofilm formed by *Enterococcus faecalis* isolate (BEF28) with a percentage of inhibition of 61.51±0.26, which was comparable to that of EEPJ5 (58.15±2.23 %) at the same concentration. The biofilm formed by *Enterococcus faecalis* isolate (BEF34) was inhibited by EEPKh with a percentage inhibition of 60.19±0.69 %, which was significantly different ($P<0.05$) from other extracts, whereas EEPKh and EEPPS extracts showed no significant difference in biofilm reduction of BEF33 isolate (51.93±1.2 and 52±3.87, respectively).

A number of studies have shown that propolis extracts can reduce biofilm production in a variety of bacteria **(Ceylan *et al.*, 2020; Daikh *et al.*, 2020)**.

Mooduto *et al.* (2021) reported that propolis extracts effectively reduce *Enterococcus faecalis* biofilm, in particular *E. faecalis* extracellular polymeric substance (EPS). A study by **Dogan et *al.* (2014)** revealed that Turkish propolis has the ability to inhibit *E. faecalis*, with an inhibition value of 69.5% at 2 mg/mL.

Many researchers have reported that propolis, due to its various constituents such as caffeic and ferulic acids can inhibit the development of biofilm in bacteria **(Daikh *et al.*, 2020)**.

Propolis compound including apigenin and tt-farnesol have the ability to inhibit *E. faecalis* biofilm by disruption of the biofilm membrane and which results in a

decrease in the amount of polysaccharides in the biofilm, then releases the cellular contents of the biofilm **(Wahjuningrum *et al.*, 2014)**.

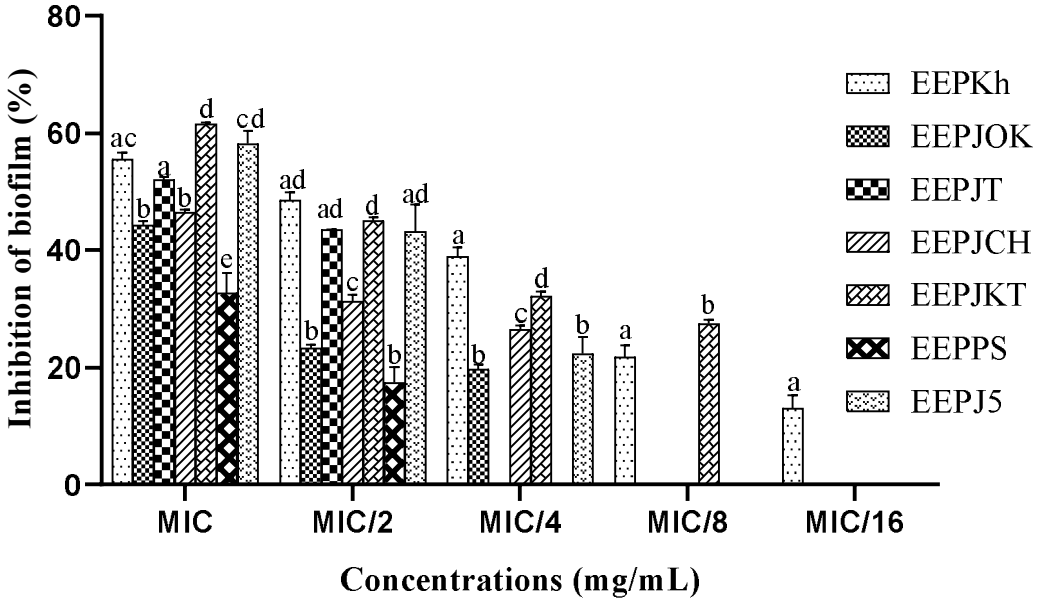


Figure 48. Percentage of antibiofilm capacity of propolis on *Enterococcus faecalis* biofilm (BEF28). Data are given as mean standard deviation (n=3). Letters varying in the columns represent statistically significant differences ($p<0.05$).

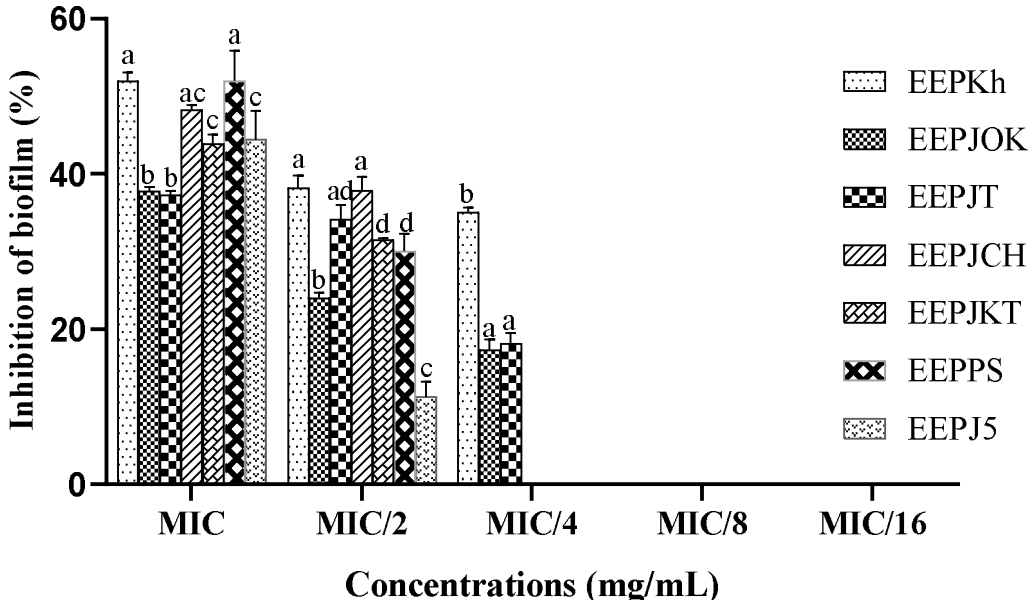


Figure 49. Percentage of antibiofilm capacity of propolis on *Enterococcus faecalis* biofilm (BEF33). Data are given as mean standard deviation (n=3). Letters varying in the columns represent statistically significant differences ($p<0.05$).

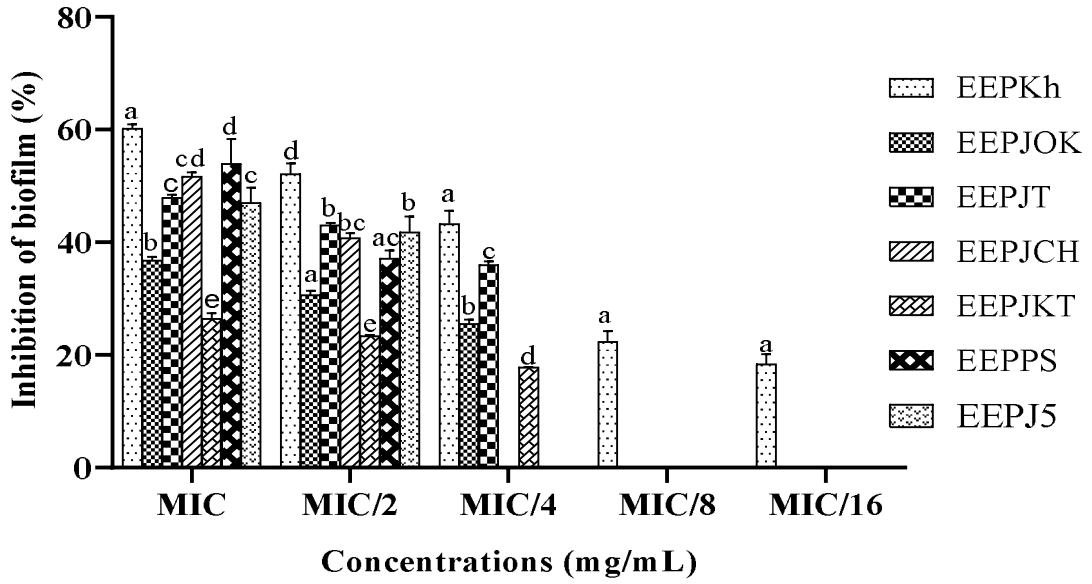


Figure 50. Percentage of antibiofilm capacity of propolis on *Enterococcus faecalis* biofilm (BEF34). Data are given as mean standard deviation (n=3). Letters varying in the columns represent statistically significant differences ($p<0.05$).

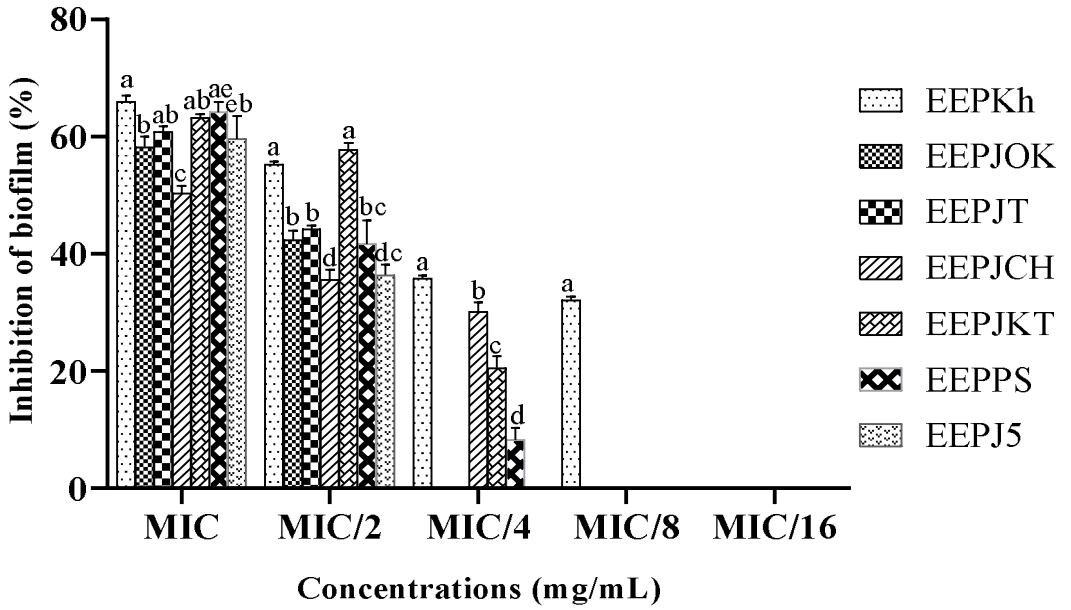


Figure 51. Percentage of antibiofilm capacity of propolis on *Enterococcus faecalis* biofilm (BEF17). Data are given as mean standard deviation (n=3). Letters varying in the columns represent statistically significant differences ($p<0.05$).

6 Quorum-sensing and violacein inhibition activity of propolis extracts

The QS communication mechanism between bacteria is based on signaling chemicals called autoinducers (N-acyl-homoserine lactone, oligopeptides), which are synthesized in response to an increase in bacterial cell density. It is involved in many pathogenic processes in microorganisms, including virulence factors, sporulation, biofilm development, toxin secretion, motility, resistance to medications, and damages the host **(Zhao *et al*., 2020)**.

6.1 Inhibition of quorum sensing in *Chromobacterium violaceum* (*CV026*)

The bacterium *CV026* produces violacein during growth on its medium plate in the presence of the hormone acylhomoserine lactone (AHL), This mutant strain is used to assess the disruption of quorum sensing by determining zones of inhibition ***(*Tamfu *et al*., 2022)**. the appearance of a creamy or yellowish ring around the well on plates stained with purple lawn staining produced by activated *CV026* bacteria is evidence of disrupted quorum sensing **(Tamfu *et al.*, 2020)**.

The inhibition of quorum sensing in the *CV026* bacteria was assessed at the MIC and the sub-MIC, as indicated in the table (10). All samples had MIC values of 20 mg/mL. The highest inhibition activity was obtained by EEPJT with an inhibition diameter of

13 mm at MIC, followed by EEPKH (11.16±0.29 mm), EEPJOK (11 mm), EEPPS (11 mm) and EEPJCH (10 mm). However, no inhibition diameter for EEPJ5, EEPTH, or the negative DMSO 10 % has been detected. There is no zone of inhibition at MIC/4 and below.

6.2 Violaceum inhibition in *chromobacterium violaceum* (*CV 12472*)

During growth, *Chromobacterium* naturally generates a purple pigment called violacein which, through a quorum sensing-mediated process, acts as an antioxidant, protecting the bacterial membrane from oxidative stress. The bacterial strain used for qualitative screening of the potential of inhibition of violacein biosynthesis is *C. violaceum 12472*; the disappearance or reduction of the violet color identifies the inhibition of violacein biosynthesis **(Tamfu *et al*., 2022)**.

After determining the MIC values of propolis extracts on *CV12472*, the ability of propolis to inhibit violacein production was tested at MIC and sub-MIC levels, as presented in Table 11.

Results revealed that EEPTH and EEPJT exhibited high inhibition efficacy (100 %) against *C. violaceum ATCC 12472* at MIC and sub-MIC, followed by EEPJKT, EEPKH, EEPPS, EEPJCH and EEPJ5 with the same percentage inhibition at MIC, with the exception of EEPJOK, which showed a percentage inhibition of 54.84±2.35. **Tamfu *et al*.(2022)** revealed that propolis from the village of Babanki (Cameroon) strongly suppressed violacein synthesis in *CV12472* with a value of 100±0.00% at MIC: 0.5 mg/mL and inhibited quorum sensing in *CV026* with a value of 18.0 1.0 mm at MIC: 0.25 mg/mL. **Ceylan and Halime (2020)** also showed that propolis extracts from Bodrum in Muğla, southwest Anatolia, possessed antiquorum-sensing capacity against *CV026* (12 mm) and *C.V ATCC 112472* (100 %) at MIC of 3.12 mg/mL.
Propolis from Bursa region (Turkey) has been shown to inhibit violacein synthesis and quorum sensing in both bacterial strains *CV12472* and *CV026*, respectively, with an inhibition percentage of 100 ± 0.01 at MIC: 12.5 mg/mL and 14.17±1.61 mm at MIC: 1.56 mg/mL, respectively **(Sorucu *et al*., 2021)**.
We can explain our results by hypothesizing that propolis extracts can inhibit the production of signaling molecules (AHL) as well as the reception of signals in *C. violaceum*, a promising approach to preventing bacteria from controlling their colonial behavior, expressing virulence and developing resistance **(Alain *et al*., 2022)**.

Several chemical components found in propolis, such as caffeic acid, pinocembrin, chrysin, and galangin, have been linked to quorum-sensing inhibition **(Kasote *et al.*, 2015; Savka *et al.*, 2015 ; Kharsany *et al.*, 2019).**

This chemical compounds of propolis seems to possess great ability to interfere with signal transmission and reception in *Chromobacterium violaceum*; consequently disturbing microbial communication (**Bulman *et al.*, 2011; Savka *et al.*, 2015)**. Propolis is a potential product for future anti-QS studies focusing on the isolation, identification and characterization of bioactive components, as it contains compounds that inhibit AHL-dependent QS regulation in bacteria **(Bulman *et al.*, 2011)**.

Table 10. Inhibition of violacein synthesis in *CV 12472* by propolis extracts

Samples	EEPTH	EEPJKT	EEPJT	EEPKH	EEPJOK	EEPPS	EEPJ5	EEPJCH
***CV12472* MIC (mg/mL)**	**2.5**	**0.625**	**1.25**	**0.625**	**10**	**1.25**	**1.25**	**0.625**
MIC	100±0.00	100±0.00	100±0.00	100±0.00	54.84±2.35	100±0.00	100±0.00	100±0.00
MIC/2	100±0.00	74.16±0.44	100±0.00	91.75±1.35	32.38±0.22	72.36±0.34	73.47±1.53	69.71±0.19
MIC/4	86.00±0.80	58.9±1.53	61.48±0.45	67.12±2.48	23.29±1.31	53.23±0.93	42.06±0.40	38.59±0.14
MIC/8	54.40±0.60	30.08±0.72	43.24±0.36	36.00±0.40	14.86±1.34	20.14±0.71	30.45±0.55	-
MIC/16	27.79±0.82	-	22.20±1.49	14.50±1.60	-	14.61±1.19	-	-
MIC/32	16.72±3.18	-	-	-	-	-	-	-
MIC/64	5.28±2.02	-	-	-	-	-	-	-

(-): no inhibition

Table 11. Quorum-sensing inhibitory effects of propolis extracts on *CV026*

Concentration (mg/mL)	Propolis extracts								
	EEPKh	**EEPJT**	**EEPJOK**	**EEPJCH**	**EEPPS**	**EEPJKT**	**EEPJ5**	**EEPTH**	**DMSO (10%)**
MIC (20 mg/mL)	11.16±0.29	13	11	10	11	ND	-	-	-
MIC/2	9.67±0.58	10	9	8	-	ND	-	-	-
MIC/4	-	-	-	-	-	ND	-	-	-
MIC/8	-	-	-	-	-	ND	-	-	-
MIC/16	-	-	-	-	-	ND	-	-	-

(-): no inhibition, **(ND):** not determined

7 The cytotoxic effect of propolis extracts

7.1 Brine shrimp lethality activity

The presence of toxic compounds in propolis extracts was revealed by brine shrimp lethality assay. The cytotoxicity of the extracts was expressed in terms of LC50 values, corresponding to 50 % mortality, which were estimated from the straight line obtained from plotting % mortality versus the logarithm of the sample concentrations. In this experiment, the percentage lethality of brine shrimp larvae increased with increasing concentration of propolis extracts. The results presented in **Table (10)** show that EEPPS, EEPTH and EEPKH have a percentage lethality of shrimp larvae between 10±0 and 46.67±5.77 at 50 and 400 µg/mL, respectively, while EEPJOK has a percentage lethality of shrimp larvae only at concentration 400 µg/mL with value of 43.33±5.77 %. No mortality was observed at concentrations less than or equal to 25 µg/mL, indicating that the product is not toxic to shrimp larvae.

Cytotoxic values were calculated as low in the range $500 \geq LC_{50} \leq 1000$ µg/mL, moderate in the range $100 \geq LC_{50} \leq 500$ µg/mL, strong in the range $0 > LC_{50} < 100$ µg/mL, and nontoxic in the range $LC_{50} > 1000$ µg/mL **(Fahad *et al.*, 2021)**.

All propolis extracts tested had LC_{50} values > 400 µg/mL, indicating moderate toxicity to shrimp larvae. The LC_{50} value for the standard, potassium dichromate was 21.11±3.74 % (table 9).

Tanvir *et al.* (2018) reported that Bangladeshi propolis extract had an LC_{50} value 57.99 µg/mL, indicating that it is toxic to brine shrimp nauplii. On the other hand, **Ngassapa *et al.* (2022)** examined 28 extracts of Tanzanian propolis and found LC_{50} values ranged from 7.75 - 1244.64 µg/mL, revealing variable levels of toxicity to brine larvae

The potential toxicity of propolis against shrimp nauplii can be explained by the presence of toxic compounds with larvicidal properties **(Boulechfar, 2023)**

Table 12. Toxic effect of propolis extracts on brine shrimp larvae

Propolis extracts	% of mortality						LC_{50} (µg/mL)
	Concentrations (µg/mL)						
	12.5	**25**	**50**	**100**	**200**	**400**	
EEPPS	0±0.00	0±0.00	10±0.00	16.67±5.77	16.67±5.77	43.33±5.77	**>400**
EEPKH	0±0.00	0±0.00	13.33±5.77	23.33±5.57	33.33±11.54	46.67±5.77	**>400**
EEPTH	0±0.00	0±0.00	13.33±5.77	23.33±5.57	26.67±5.77	36.67±5.77	**>400**
EEPJOK	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	43.33±5.77	**>400**
EEPJ5	ND	ND	ND	ND	ND	ND	ND
EEPJT	ND	ND	ND	ND	ND	ND	ND
EEPJCH	ND	ND	ND	ND	ND	ND	ND
EEPJKT	ND	ND	ND	ND	ND	ND	ND

ND: Not determined

Table.13 Toxic effect of potassium dichromate on brine shrimp larvae

Standard	Concentrations (µg/mL)				LC50 (µg/mL)
	10	**20**	**40**	**80**	
Potassium dichromate	0±0.00	50±10.00	80±0.00	100±0.00	21.11±3.47

Conclusion

Conclusion

This study focuses on the richness and type of phenolic and flavonoid compounds present in Algerian propolis from different regions: Ouana region (city Jijel), Chekfa-El milia region (city Jijel), Taxanna region (city Jijel), Kissir ouana region (city Jijel), Kaous region (city Jijel), Kherrata region (city Bejaïa), Bouteldja region (city El-Taref), Tamalous region (city Skikda), as well as their pharmacological properties.

The total phenolic and flavonoid contents in these ethanolic propolis extracts were detected using the folin-Ciocalteu and aluminum chloride colorimetric methods, which showed different amounts.

Different classes of phenolic and flavonoid compounds in propolis extracts, including 25 molecules were identified and quantified using the HPLC-DAD method. The most abundant compounds were caffeic acid, naringenin, chrysin, and hesperetin; however, there was a new phenolic compound identified in all propolis extracts which is Cynarin.

Antioxidant experiments including DPPH, ABTS, GOR, cupric, iron and phenanthroline assays showed that propolis extracts have strong antioxidant properties, measured by their ability to scavenge radicals and reduce ions, which showed a positive correlation with phenolic and flavonoid content.

Propolis extracts were tested for their antibacterial and antibiofilm potential using disk diffusion, microdilution and crystal violet assays against *Enterococcus faecalis* isolates; the results revealed that propolis extracts have potent antibacterial and anti-biofilm activity against *E. faecalis*.

Evaluation of the ability of propolis extracts to inhibit quorum sensing in *CV026* and violacein production in *CV12472* showed that the propolis extract from Taxana had the best ability to inhibit quorum sensing in *CV026*; propolis extracts inhibited violacein production in *CV12472* with a percentage of 100 % at MIC, and only propolis extracts from Bouteldja and Taxanna showed high inhibition efficiency at MIC and sub-MIC concentrations with a percentage inhibition of 100 %.

Cytotoxicity against brine shrimp larvae showed that propolis extracts from Bouteldja, Tamalous, Kherrata and Ouana regions are moderately toxic and show no toxicity at a concentration 25 µg/mL and below.

Propolis extracts have a very significant effect on *E. faecalis* and their biofilm, scavenge radicals and reduce ions, as well as its ability to block the quorum sensing and violacein production in *CV026* and *CV12472*, respectively. These findings indicate that propolis extracts have a promising future medical application, particularly in dentistry, to treat infections caused by *E. faecalis* and their biofilm, as well as to prevent the communication between bacteria carried out by the quorum-sensing system, in addition to the oxidative stress generated during the infection. Further research is needed on the isolation and chemical identification of the components of propolis, and the possible relationship between them and its biological activity like the mechanisms of action, molecular identification of these bacteria and description of antibiotic resistance genes, as well as *in vivo* toxicological studies on animals.

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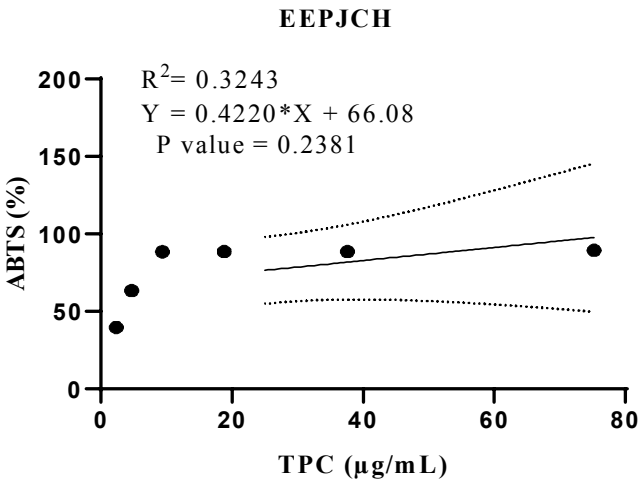
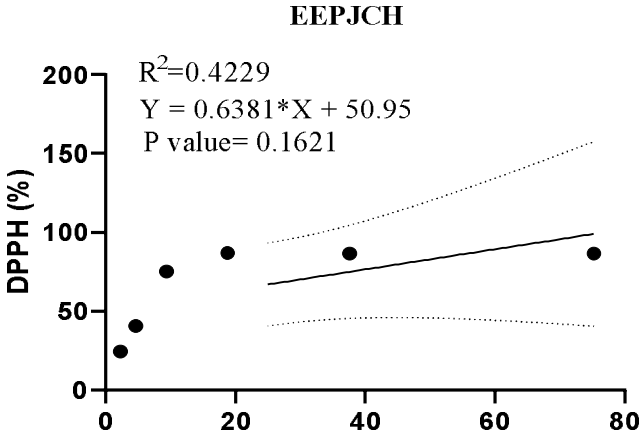
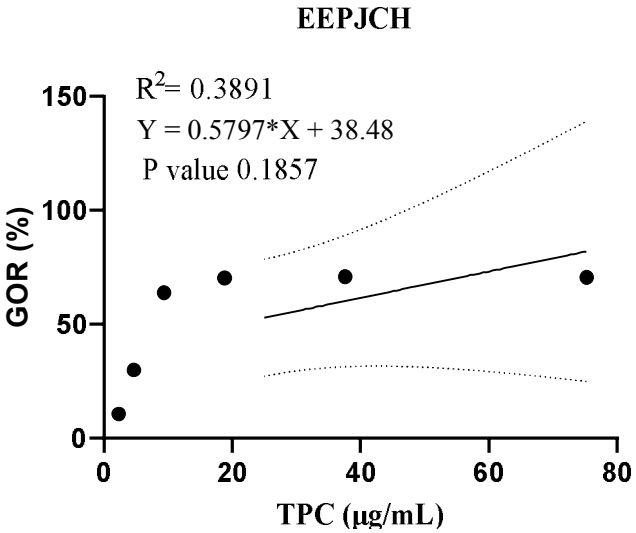
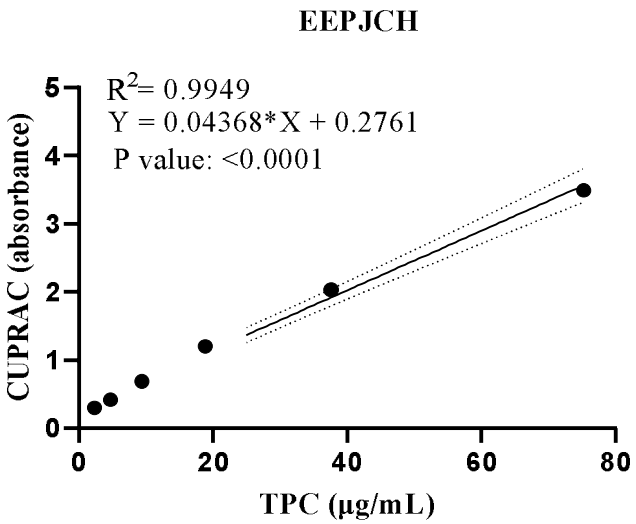
Annex

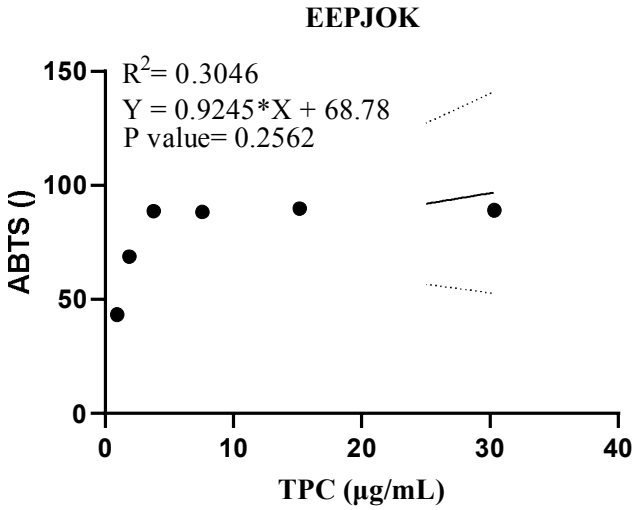
Annexe 1. Correlation between TPC, TFC contents and antioxidant activities

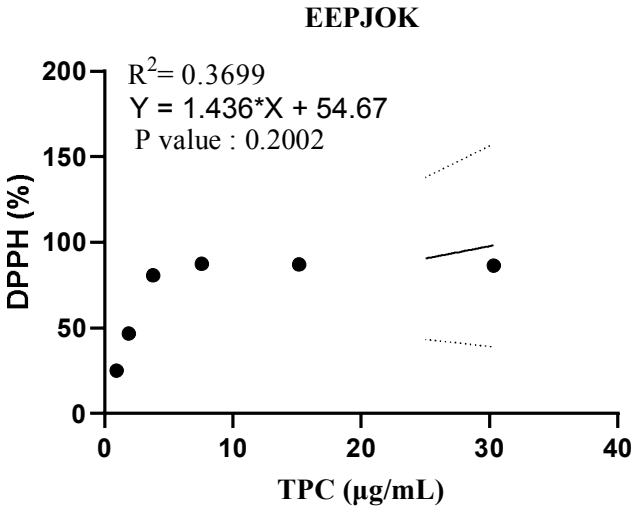


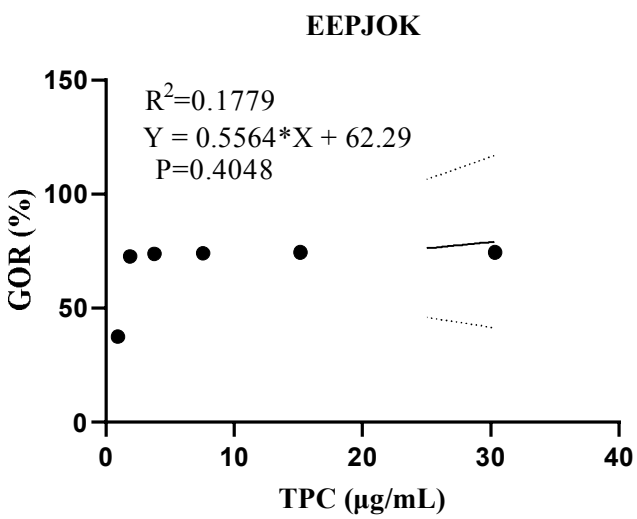


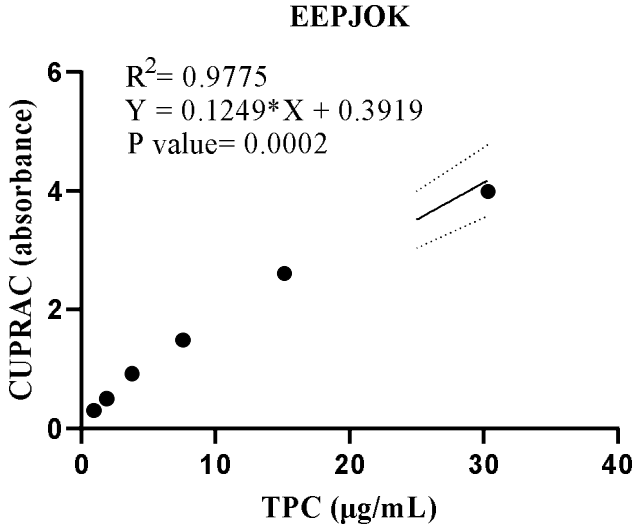


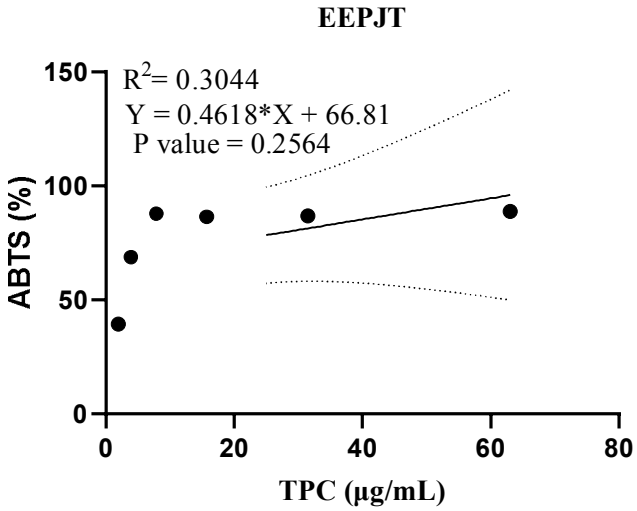


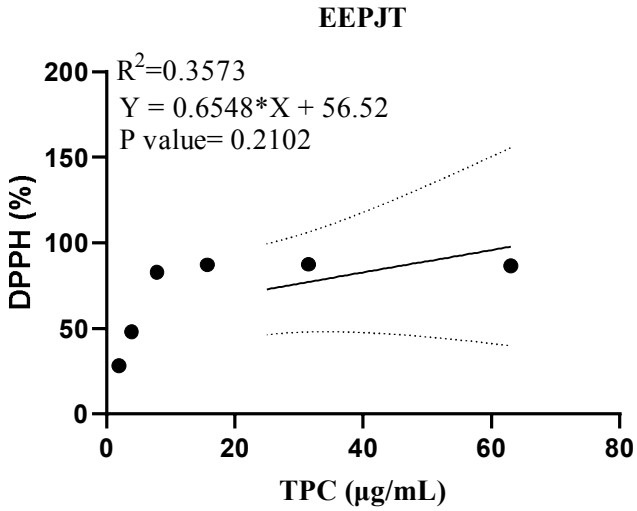


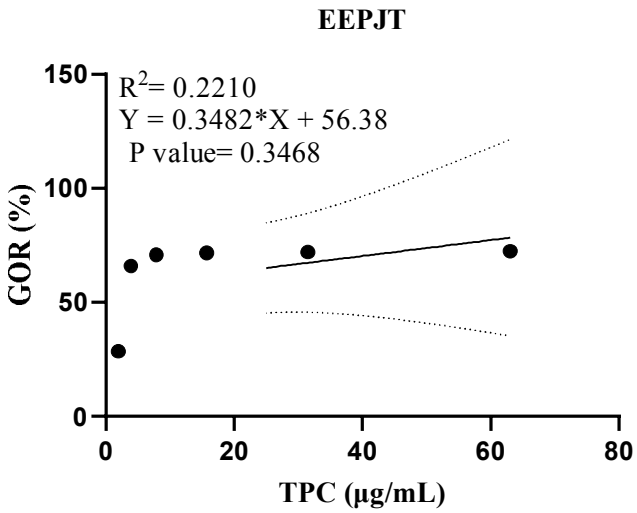


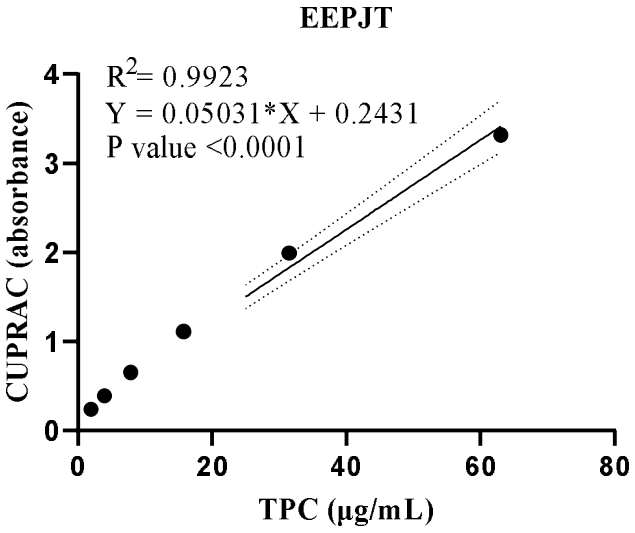

EEPJOK
R2=0.1779
Y = 0.5564*X + 62.29
P=0.4048
GOR (%)
TPC (µg/mL)

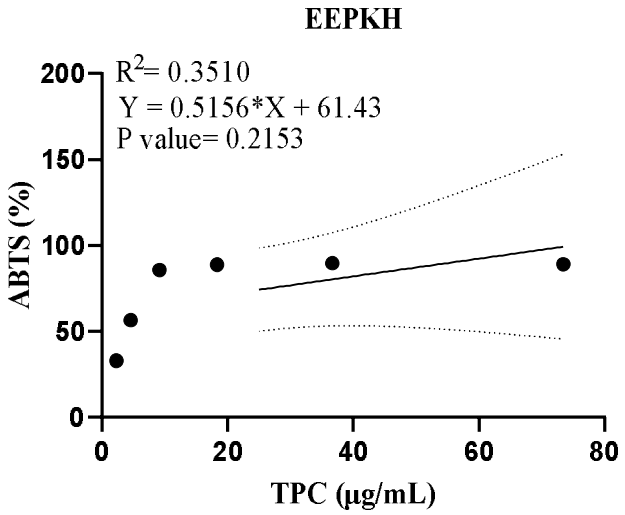

EEPJOK
R2= 0.9775
Y = 0.1249*X + 0.3919
P value= 0.0002
CUPRAC (absorbance)
TPC (µg/mL)


EEPJT
R2= 0.3044
Y = 0.4618*X + 66.81
P value = 0.2564
ABTS (%)
TPC (µg/mL)


EEPJT
R2=0.3573
Y = 0.6548*X + 56.52
P value= 0.2102
DPPH (%)
TPC (µg/mL)


EEPJT
R2= 0.2210
Y = 0.3482*X + 56.38
P value= 0.3468
GOR (%)
TPC (µg/mL)


EEPJT
R2= 0.9923
Y = 0.05031*X + 0.2431
P value <0.0001
CUPRAC (absorbance)
TPC (µg/mL)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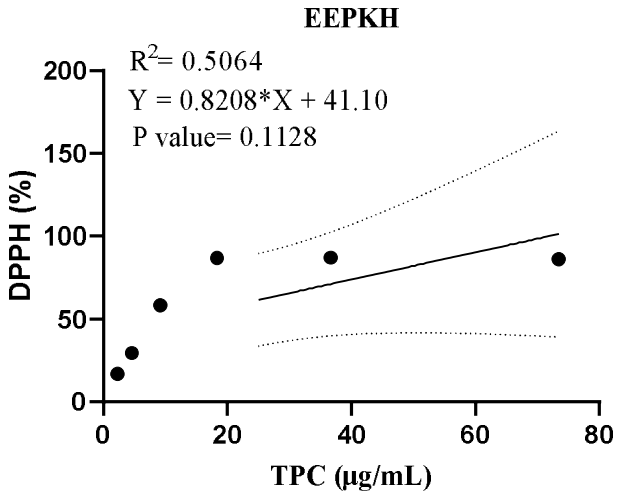
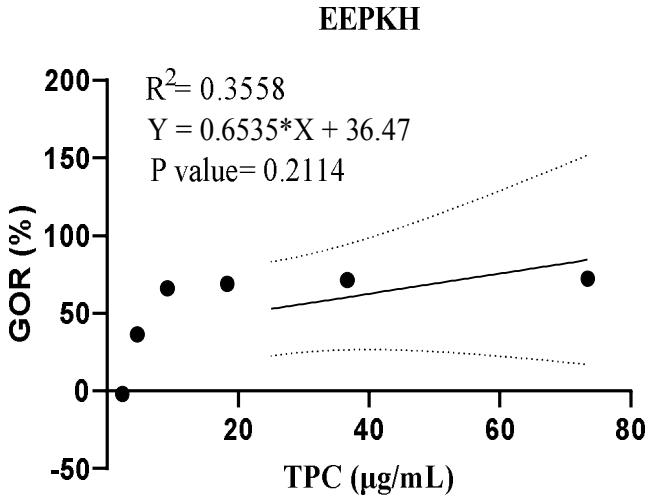
R2= 0.3510
Y = 0.5156*X + 61.43
P value= 0.2153
ABTS (%)
TPC (µg/mL)


EEPKH
R2= 0.5064
Y = 0.8208*X + 41.10
P value= 0.1128
DPPH (%)
TPC (µg/mL)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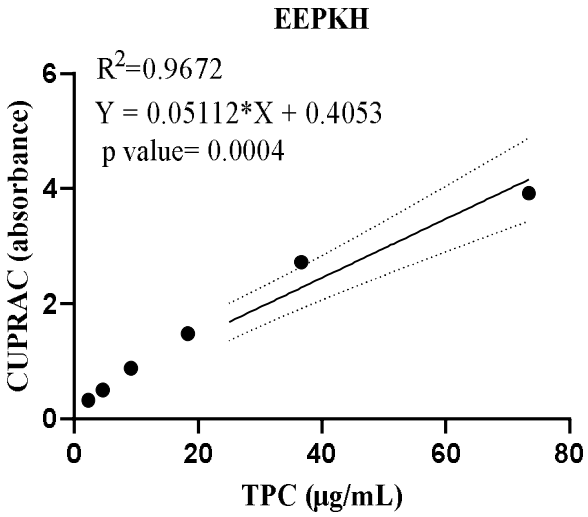
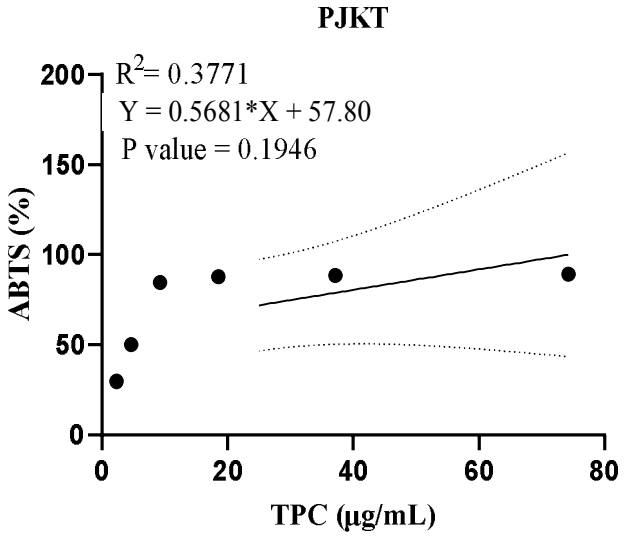
R2= 0.3558
Y = 0.6535*X + 36.47
P value= 0.2114
GOR (%)
TPC (µg/mL)


EEPKH
R2=0.9672
Y = 0.05112*X + 0.4053
p value= 0.0004
CUPRAC (absorbance)
TPC (µg/mL)


PJKT
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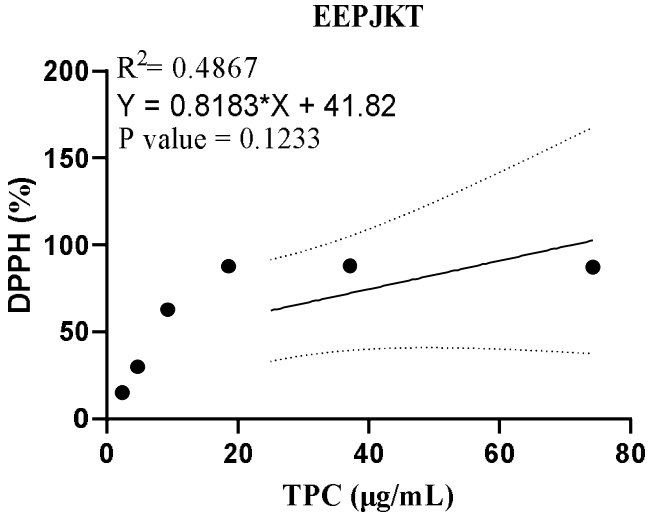
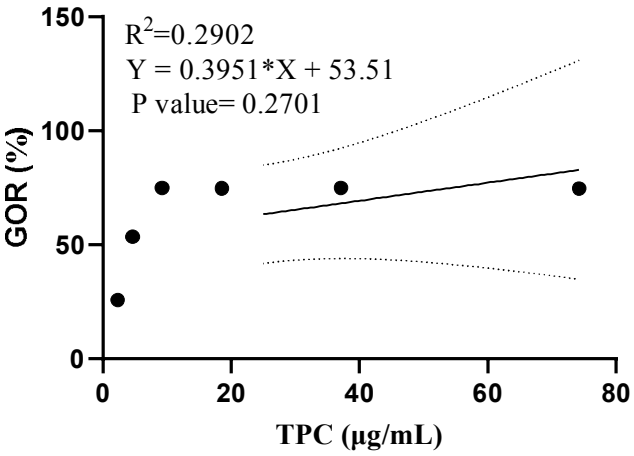
Y = 0.5681*X + 57.80
P value = 0.1946
ABTS (%)
TPC (µg/mL)


EEPJKT
R2= 0.4867
Y = 0.8183*X + 41.82
P value = 0.1233
DPPH (%)
TPC (µg/mL)

EEPJKT



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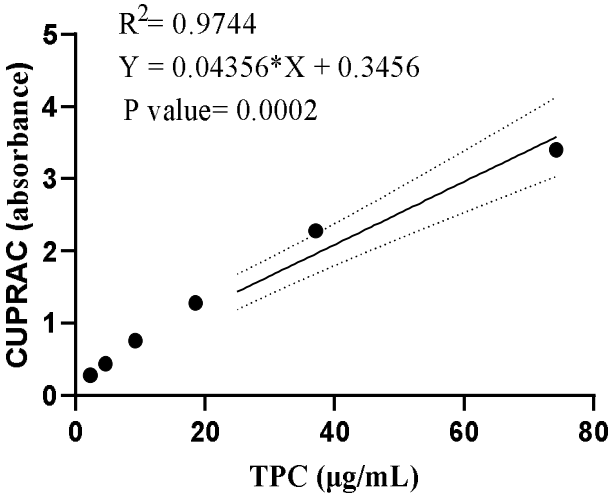
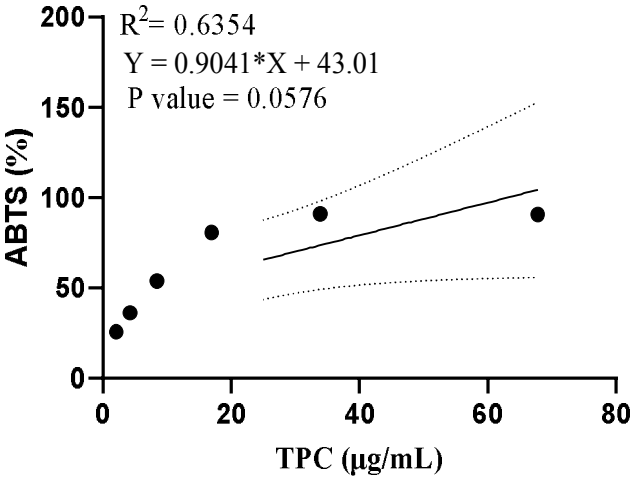


EEPTH



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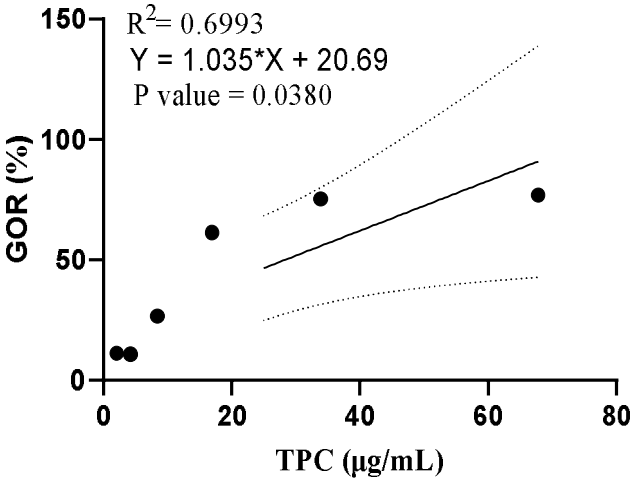

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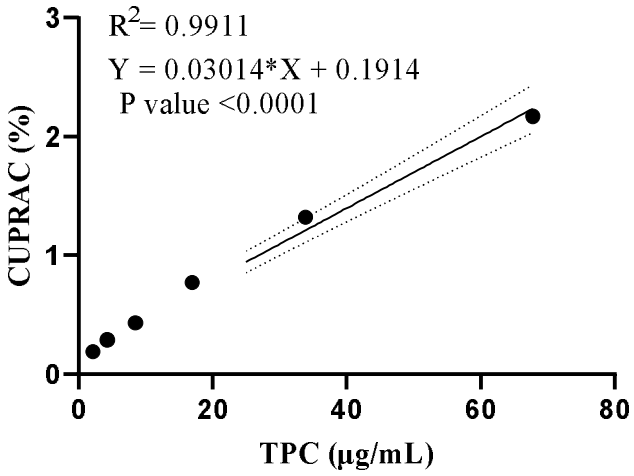

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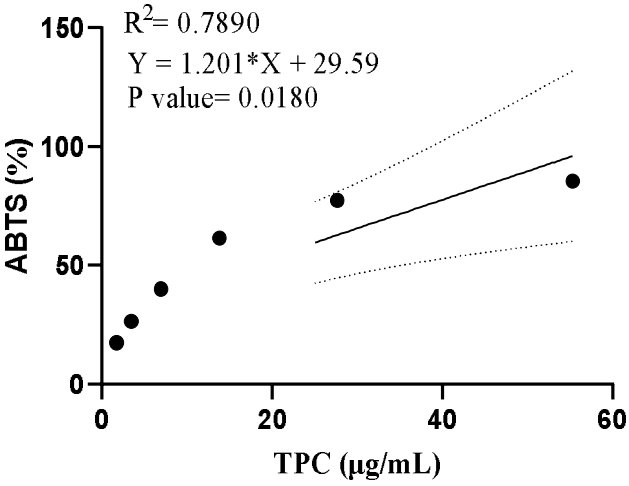

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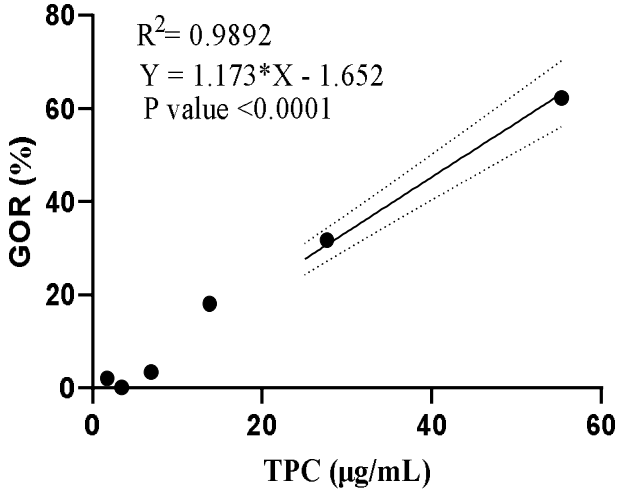

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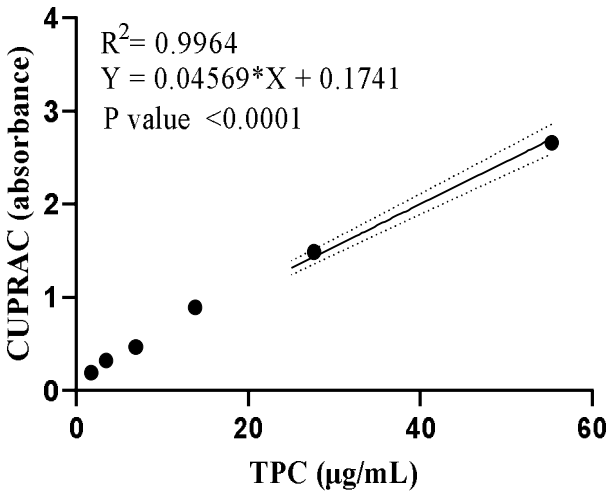

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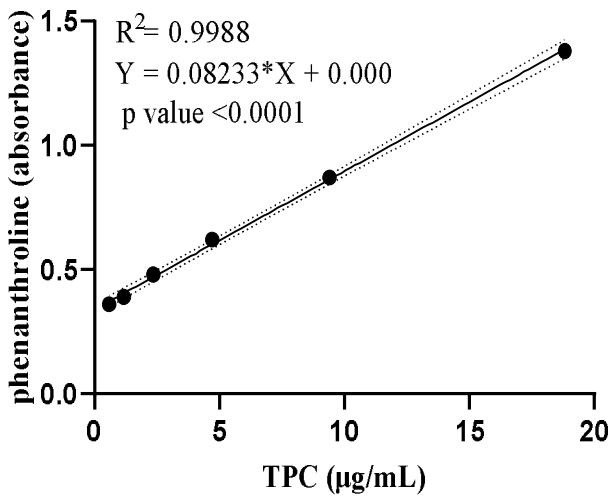

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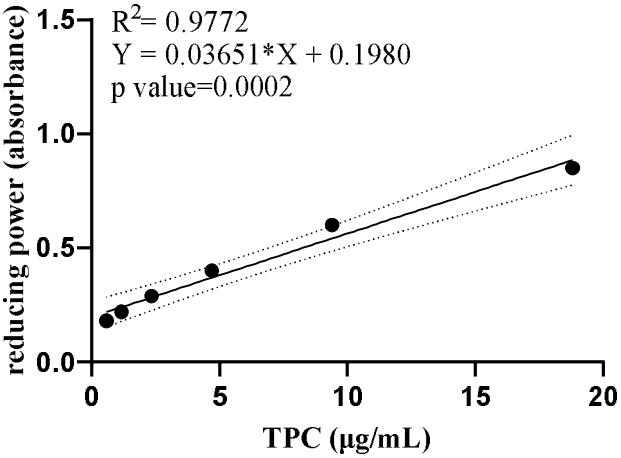

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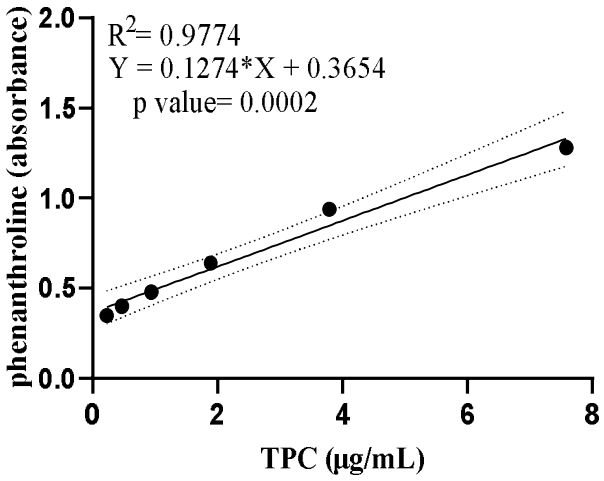

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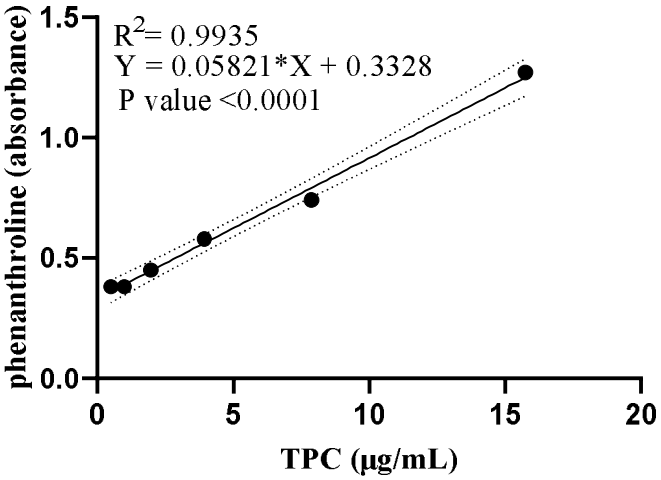

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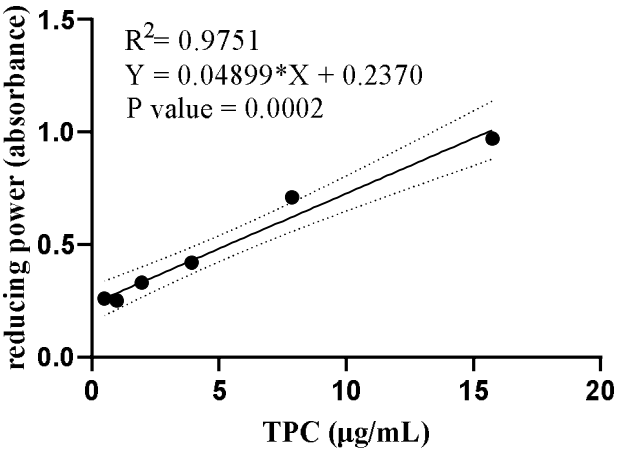

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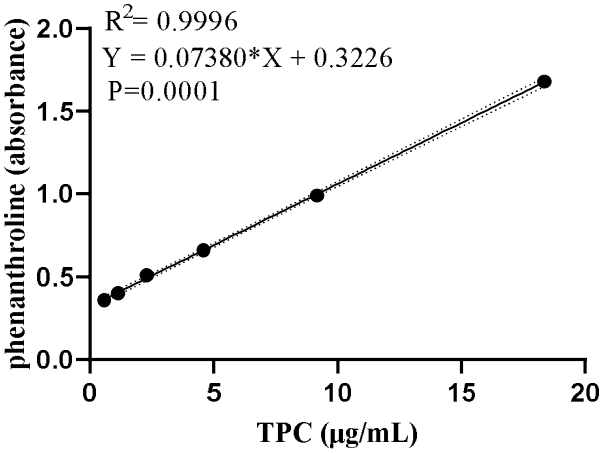

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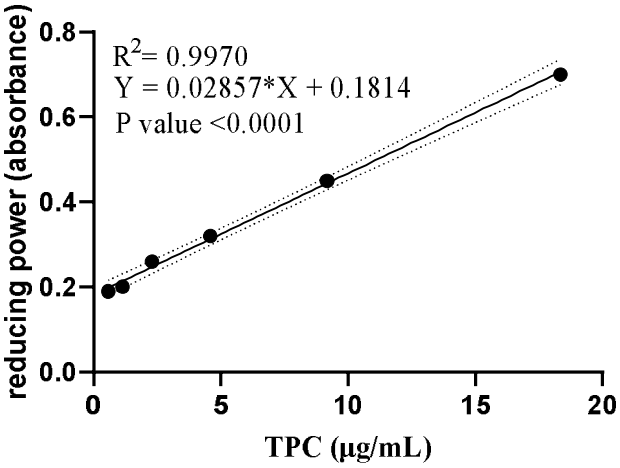
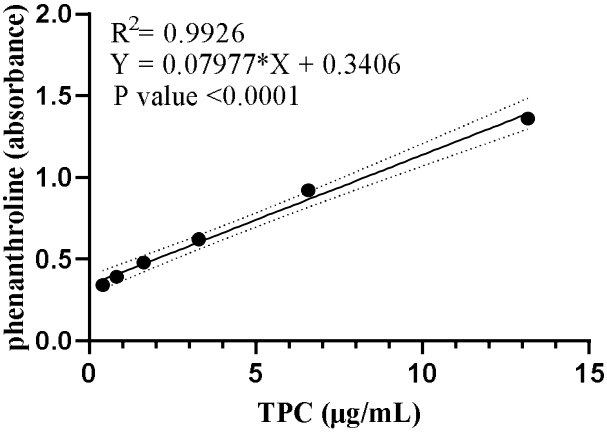


EEPJ5



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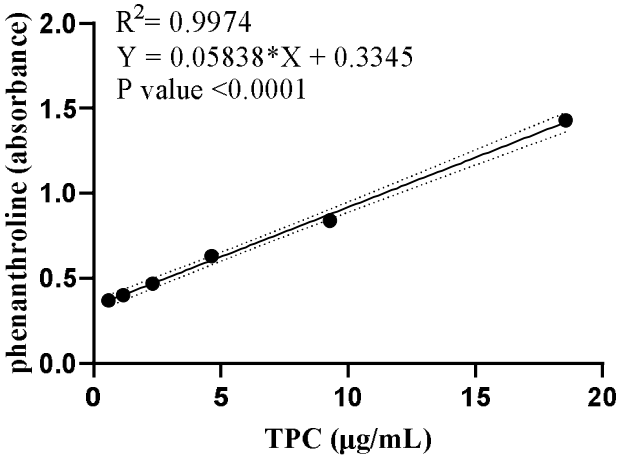

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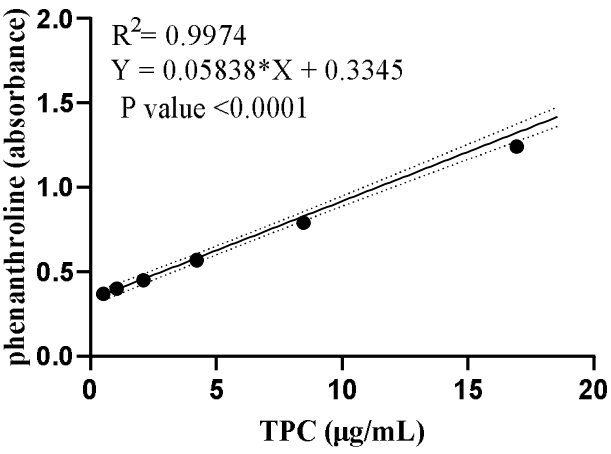
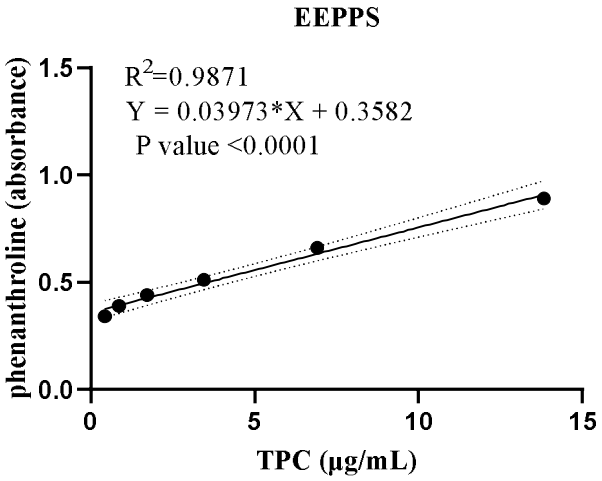
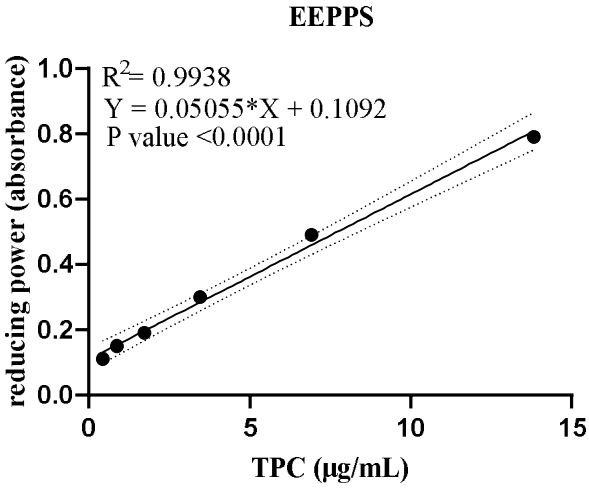
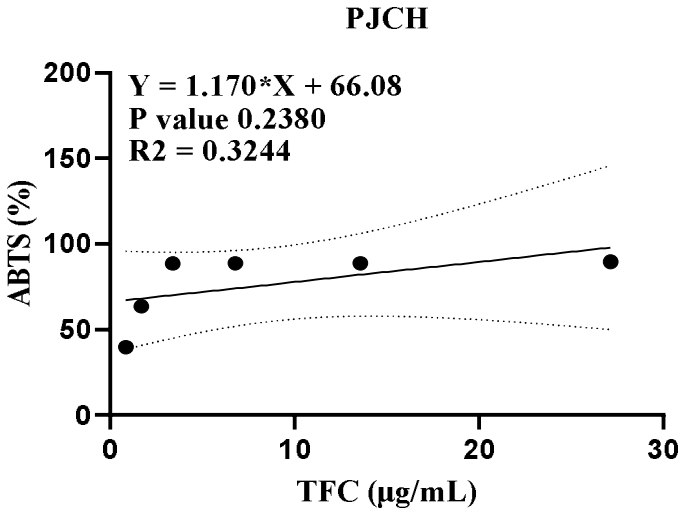
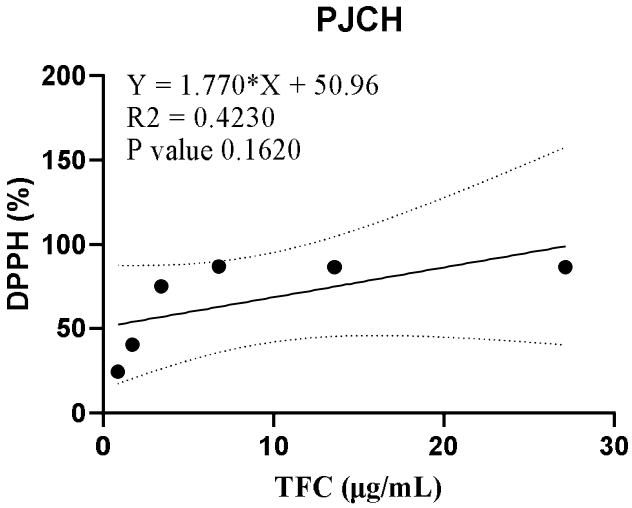
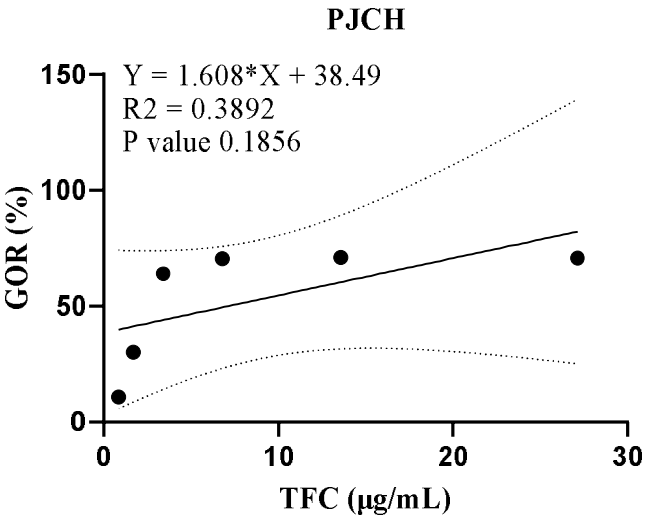


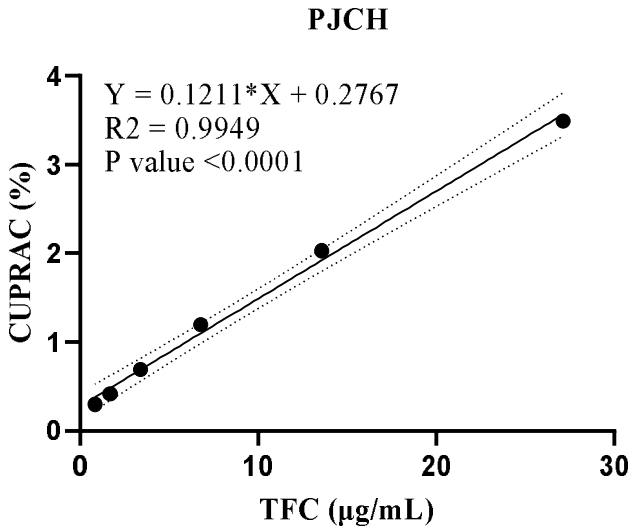

EEPPS
R^2=0.9871
Y = 0.03973*X + 0.3582
P value <0.0001
phenanthroline (absorbance)
TPC (µg/mL)

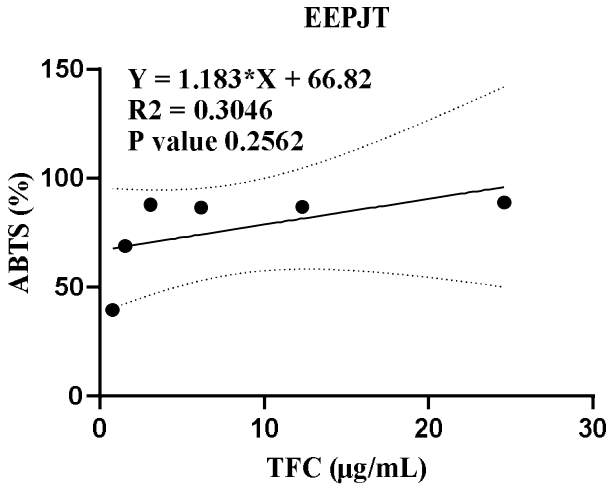

EEPPS
R^2= 0.9938
Y = 0.05055*X + 0.1092
P value <0.0001
reducing power (absorbance)
TPC (µg/mL)

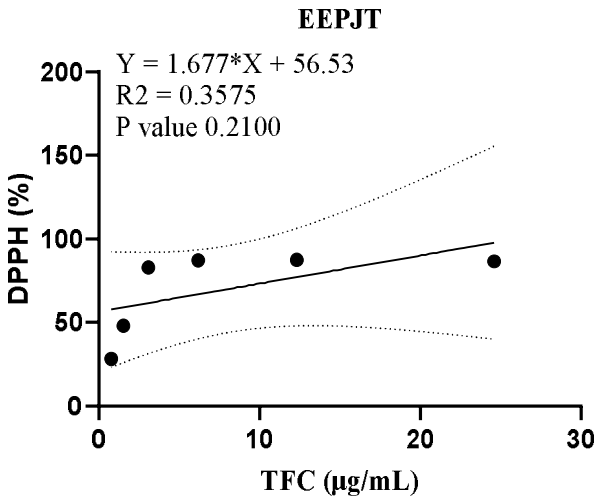

PJCH
Y = 1.170*X + 66.08
P value 0.2380
R2 = 0.3244
ABTS (%)
TFC (µg/mL)

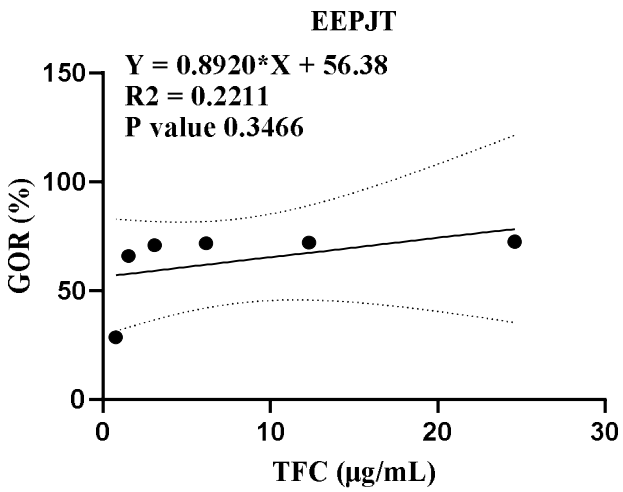

PJCH
Y = 1.770*X + 50.96
R2 = 0.4230
P value 0.1620
DPPH (%)
TFC (µg/mL)

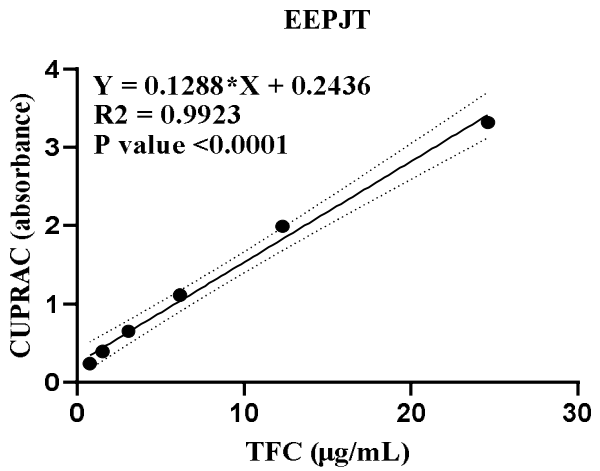

PJCH
Y = 1.608*X + 38.49
R2 = 0.3892
P value 0.1856
GOR (%)
TFC (µg/mL)

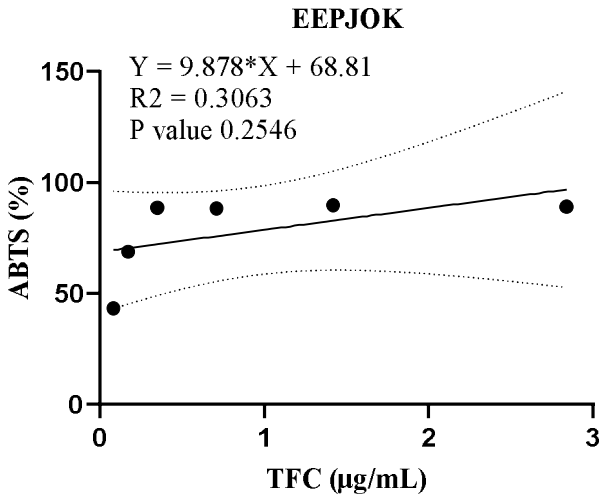

PJCH
Y = 0.1211*X + 0.2767
R2 = 0.9949
P value <0.0001
CUPRAC (%)
TFC (µg/mL)

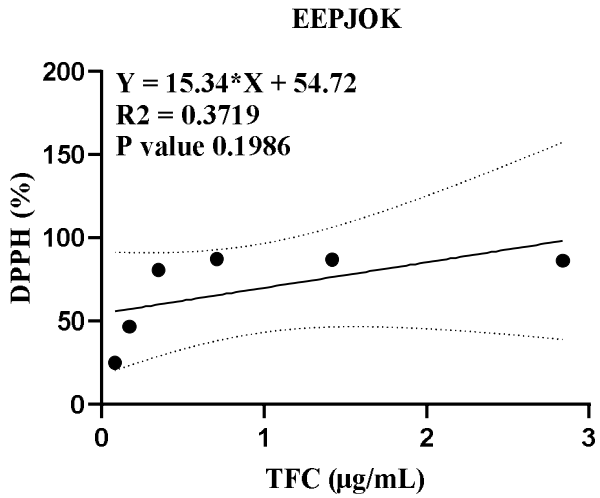

EEPJT
Y = 1.183*X + 66.82
R2 = 0.3046
P value 0.2562
ABTS (%)
TFC (µg/mL)


EEPJT
Y = 1.677*X + 56.53
R2 = 0.3575
P value 0.2100
DPPH (%)
TFC (µg/mL)

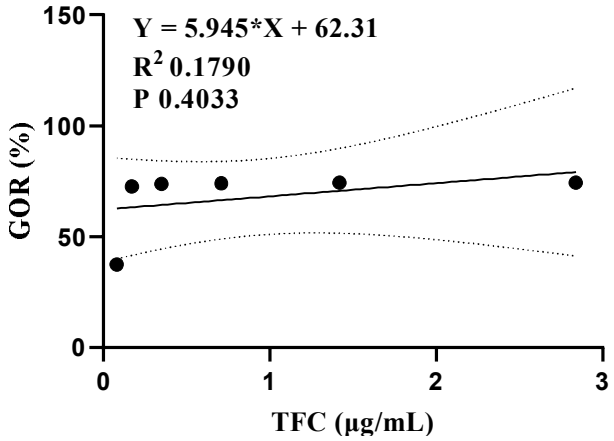

EEPJT
Y = 0.8920*X + 56.38
R2 = 0.2211
P value 0.3466
GOR (%)
TFC (µg/mL)


EEPJT
Y = 0.1288*X + 0.2436
R2 = 0.9923
P value <0.0001
CUPRAC (absorbance)
TFC (µg/mL)

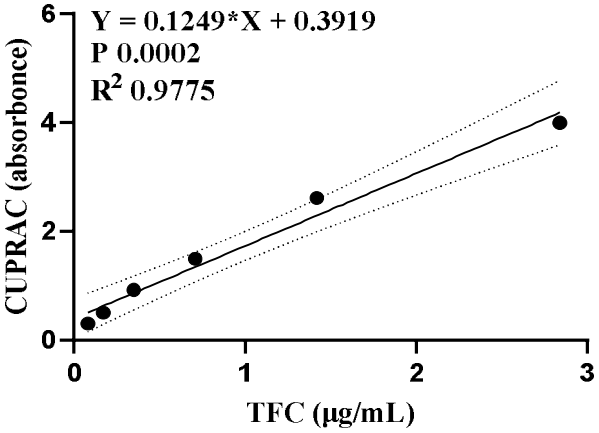

EEPJOK
Y = 9.878*X + 68.81
R2 = 0.3063
P value 0.2546
ABTS (%)
TFC (µg/mL)


EEPJOK
Y = 15.34*X + 54.72
R2 = 0.3719
P value 0.1986
DPPH (%)
TFC (µg/mL)

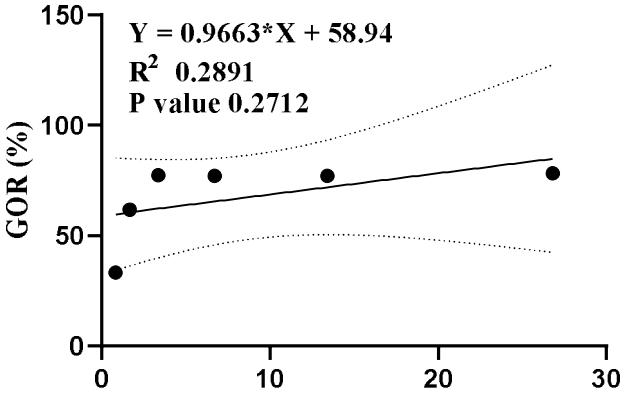
EEPJOK



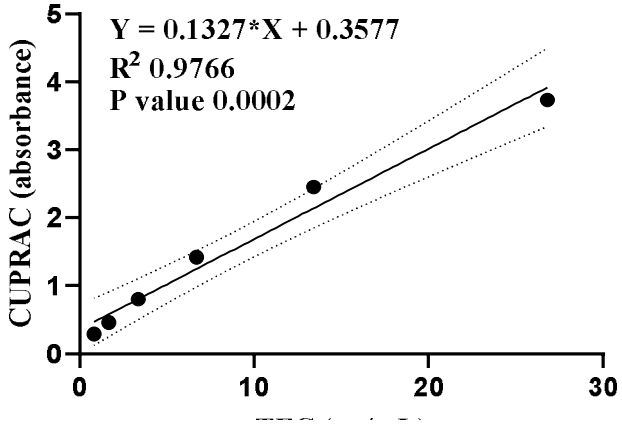
EEPJOK



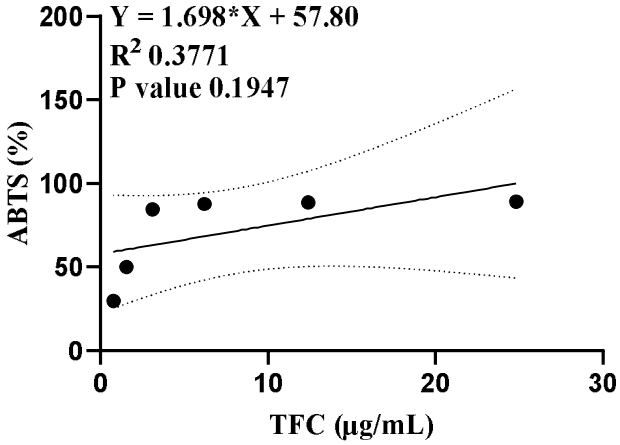
EEPJ5



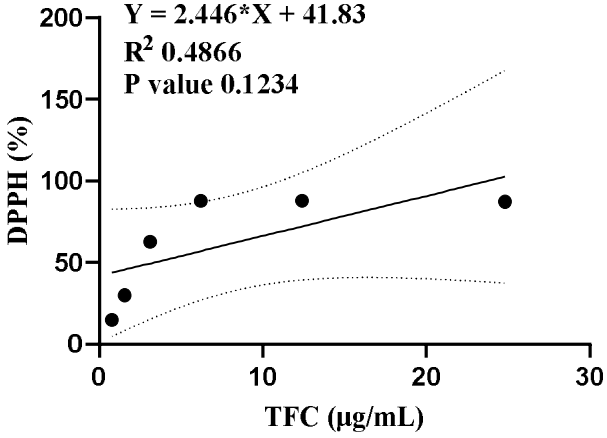
EEPJ5



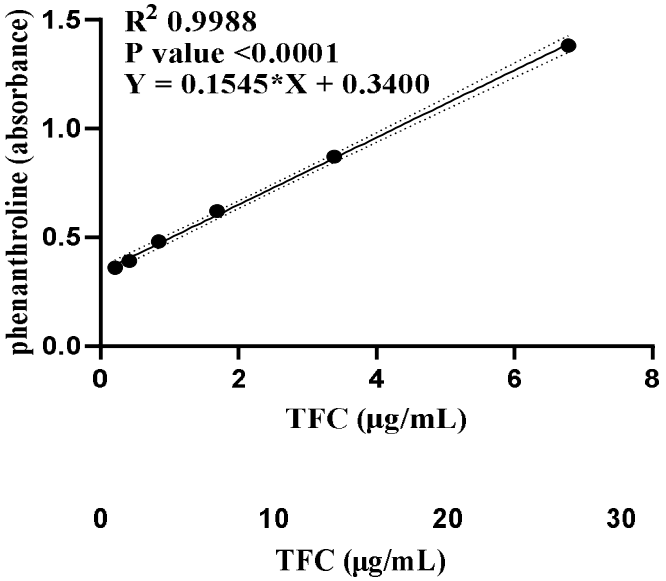
EEPJKT



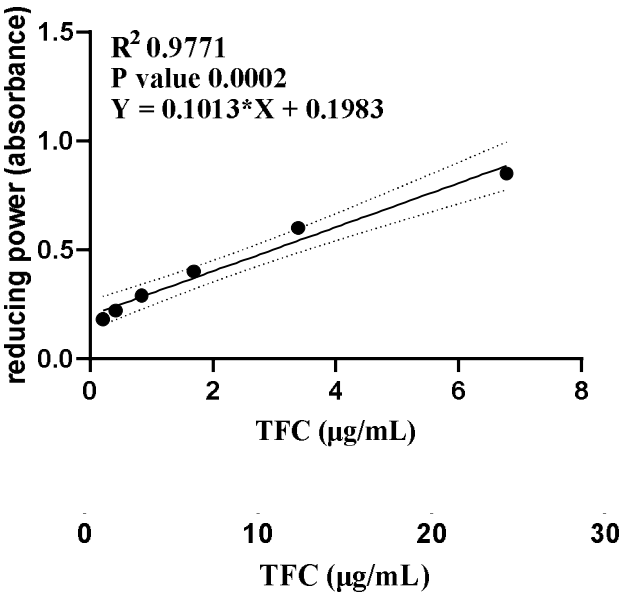
EEPJKT



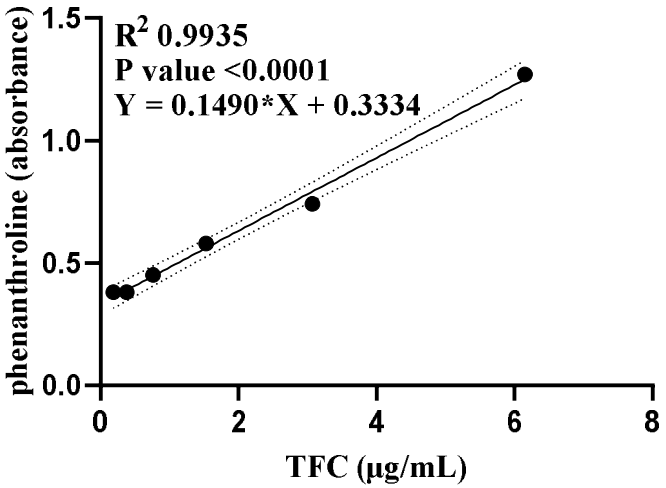
EEPJCH



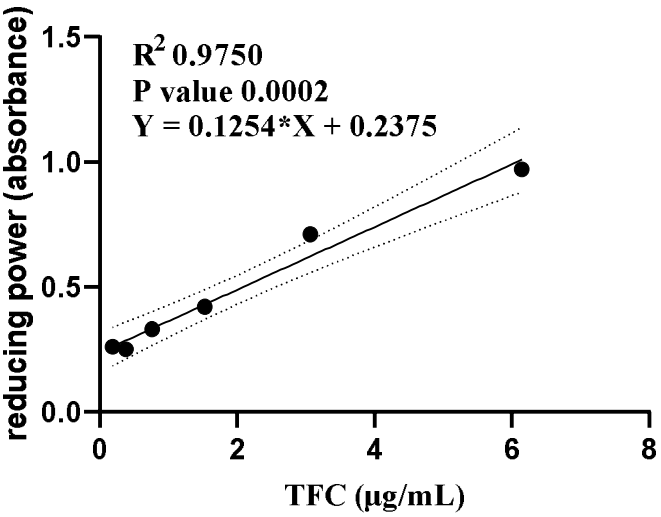
EEPJCH



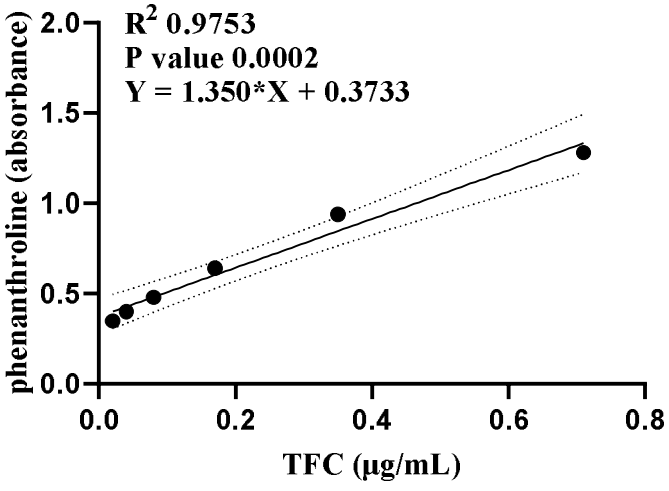
EEPJT



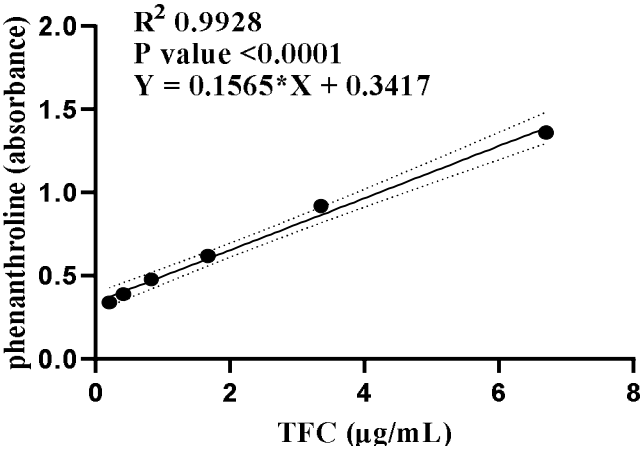
EEPJT



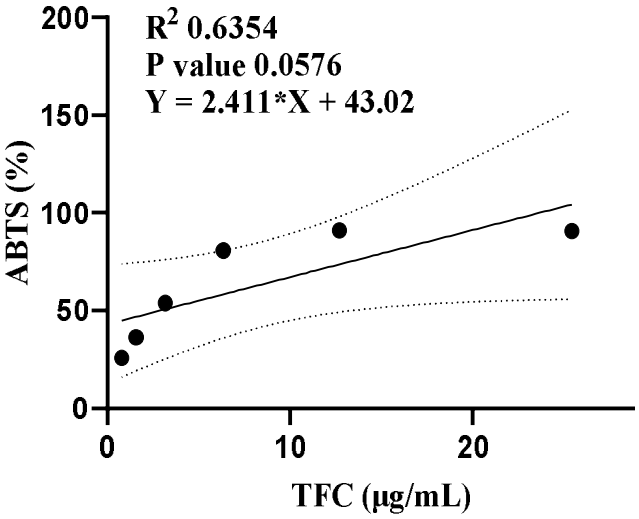
EEPJOK



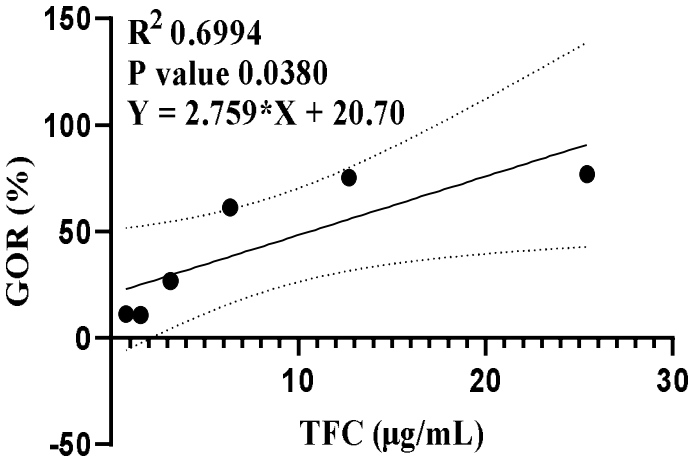
EEPJ5



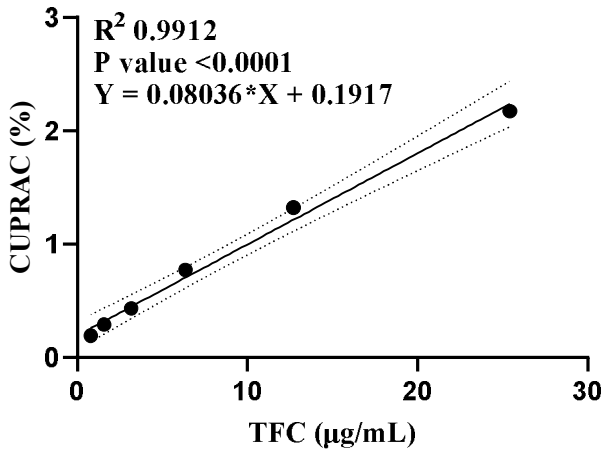
EEPTH



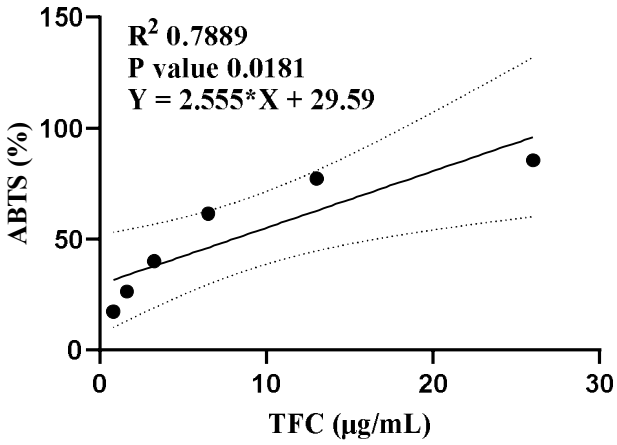
EEPTH



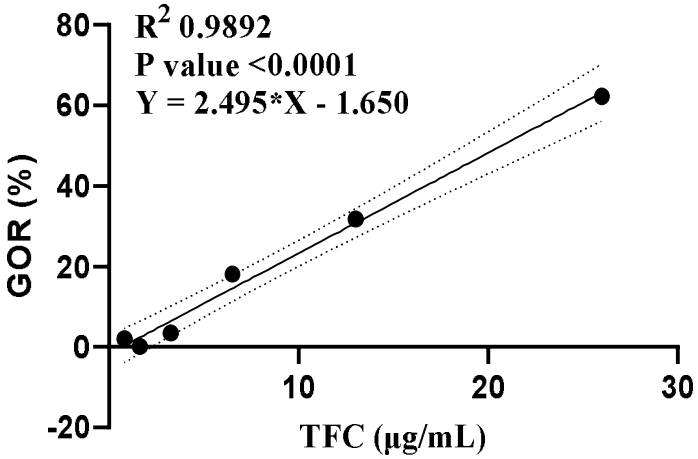
EEPTH



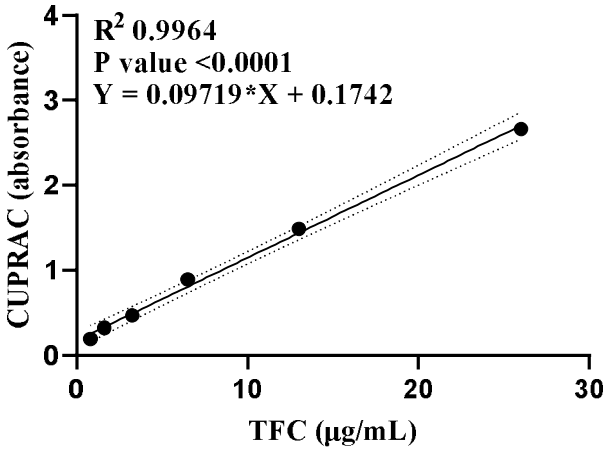
EEPPS

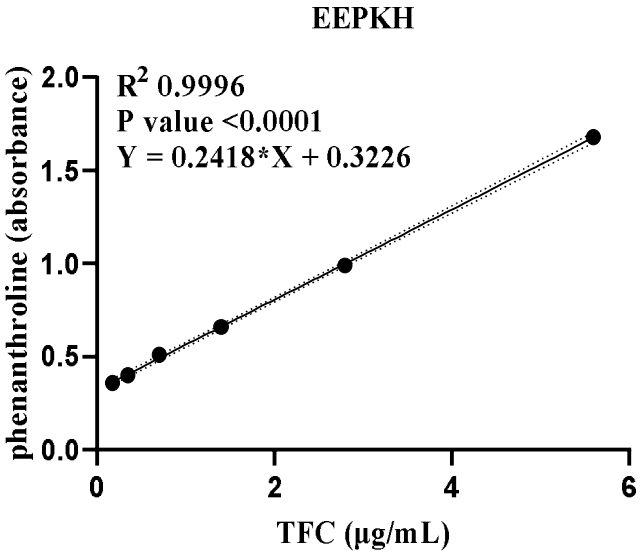


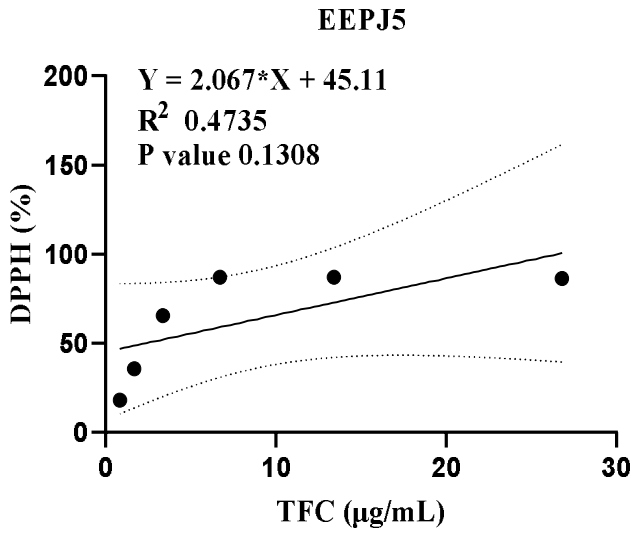
EEPPS

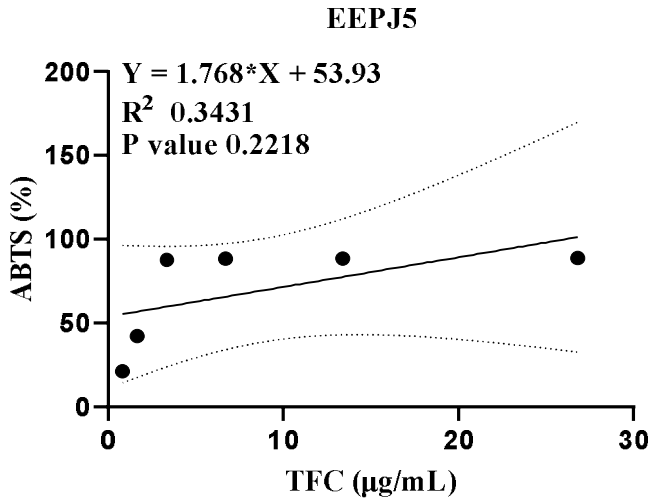


EEPPS




EEPKH
R2 0.9996
P value <0.0001
Y = 0.2418*X + 0.3226
phenanthroline (absorbance)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
TFC (µg/mL)


EEPJ5
Y = 2.067*X + 45.11
R2 0.4735
P value 0.1308
DPPH (%)
200
150
100
50
0
0
10
20
30
TFC (µg/mL)


EEPJ5
Y = 1.768*X + 53.93
R2 0.3431
P value 0.2218
ABTS (%)
200
150
100
50
0
0
10
20
30
TFC (µg/mL)