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Biological activities assessment of rhizome extracts from *Carthamus caeruleus* in Sétif region.

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This research work is dedicated to:

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My friends who encouraged and supported me,

To all the ***members*** *of my class.*

To all my ***teachers*** *since my first years of study,*

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To all those who I feel dear and whom I have forget to mention

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Dedication

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I dedicate this work to my dear parents, my dear father ***Rabah*** *and my dearest mother* ***Fadila*** *I thank them very much for all they have given for me, for their unwavering love, support, force and sacrifices. I am forever grateful for everything you have done with heartfelt appreciation.*

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ROUMAISSA

ABSTRACTS

ملخص

هذه الدراسة تهدف إلى تقييم الأنشطة البيولوجية والسمية لمستخلصات نبات القرطم الأزرق من منطقة سطيف وأظهرت نتائج واعدة . حيث أظهرت مستخلصات جذور نبات القرطم الأزرق التي تم الحصول عليها عن طريق الغلي والمنقوع وجود الصابونينات والبوليفينولات والسكريات المختزلة والتربينويدات والتانينات المكثفة والكينونات الحرة والتانينات المحللة بالماء. كما أظهرت مستخلصات المنقوع مستويات أعلى من البوليفينولات والفلافونويدات مقارنة بمستخلصات الغلي. وأظهر اختبار النشاط المضاد للأكسدة DPPH قدرة تثبيطية معتبرة ضد الجذر مع تركيز تثبيطي اقل لمستخلص الغلي. بالإضافة إلى ذلك أظهرت المستخلصات نشاطا متغيرا ضد سلالات ميكروبية معينة. فيما يتعلق بتجربة السمية، أظهرت مستخلصات النقع والغلي سمية قريبة من المتوسط اتجاه روبيان البحر لنوع الارتيمياساالينا مع تراكيز مميتة متقاربة لكل من مستخلصات النقع والغلي.

الكلمات المفتاحية : القرطم الأزرق،البوليفينول، مضادات الأكسدة، مضادات الميكروبات، السمية.

Résumé

Cette étude sur l'évaluation des activités biologiques et de la cytotoxicité des extraits de plantes de *Carthamus caeruleus* de la région de Sétif a révélé des résultats prometteurs. Les extraits de rhizome de *Carthamus caeruleus* obtenus par décoction et infusion ont montré la présence de saponines, de polyphénols, de sucres réducteurs, de terpénoïdes, de tannins condensés, de quinones libres et de tannins hydrolysables. Les extraits d'infusion ont présenté des niveaux plus élevés en polyphénols et en flavonoïdes par rapport aux extraits de décoction. Le test d'activité antioxydante a démontré une capacité inhibitrice significative contre le radical libre DPPH, avec des concentrations inhibitrices plus faibles (IC_{50}) pour l'extrait de décoction. De plus, les extraits ont montré une activité antimicrobienne variables contre certaines souches microbiennes. En ce qui concerne le test de cytotoxicité, les extraits de rhizome de *Carthamus caeruleus* ont montré une cytotoxicité prés de moyenne envers les nauplii d'*Artemia salina*, avec des concentrations létales (CL_{50}) assez similaires pour les extraits de décoction et d'infusion.

Mots clés : *Carthamus caeruleus*, polyphénols, antioxydant, antimicrobienne, cytotoxicité.

Abstract:

This study on the evaluation of the biological activities and cytotoxicity of *Carthamus caeruleus* plant extracts from the Setif region has revealed promising results. The rhizome extracts of *Carthamus caeruleus* obtained through decoction and infusion showed the presence of saponins, polyphenols, reducing sugars, terpenoids, condensed tannins, free quinones, and hydrolyzable tannins. The infusion extracts exhibited higher levels of polyphenols and flavonoids compared to the decoction extracts. The antioxidant activity test demonstrated significant inhibitory capacity against DPPH free radical, with lower inhibitory concentrations (IC_{50}) for the decoction extract. Additionally, the extracts exhibited variable antimicrobial activity against certain microbial strains. Regarding the cytotoxicity assay, the rhizome extracts of *Carthamus caeruleus* showed near average toxicity towards *Artemia salina* nauplii, with quite similar lethal concentrations (CL_{50}) for both decoction and infusion extracts.

Keywords: *Carthamus caeruleus*, polyphenols, antioxidant, antimicrobial, cytotoxicity.

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ABREVIATIONS

Abreviations

$Alcl_3$	Aluminum chloride
Asc A	Ascorbic acid
ATP	Adenosine triphosphate
BN	Nutritious broth
BSLA	Brine shrimp lethality assay
CH_3CO_2Na	Sodium acetate trihydrate
$CuSO_4$	Copper sulphate
DMSO	Dimethyl sulfoxide
DPPH	2,2'-diphenyl-1-picrylhydrazyl
$FeCl_3$	Ferric chloride
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
IC_{50}	The dose of extract necessary to cause an inhibition of 50%
LC_{50}	Lethal concentration
mg GAE/g	Milligram gallic acid equivalent per gram
mg QE/g	Milligram quercetin equivalent per gram
NA	Nutrient agar
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
TFC	Total flavonoids contents
TPC	Total phenolic contents

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Materials and methods

INTRODUCTION

Introduction:

The use of herbal remedies in traditional medicine for the treatment of human and animal diseases is very ancient. It has evolved throughout human history (Saffidine ,2015) and the vast majority of rural populations exclusively rely on medicinal plants for their healthcare needs (Saffidine, 2015). Despite the toxicity of chemical products, this is primarily due to the high cost of medicines prescribed by modern medicine and the remoteness and/or inadequacy of healthcare centers (Saffidine, 2015). Thus, it is estimated that two-thirds of current medications have a natural origin, either obtained through semi synthesis or by modifying a natural product (Newman *et al.*, 2007).

These plant compounds provide a valuable source of bioactive agents that can have diverse effects on human health (Saidi, 2018). Their biological activities including antioxidant, anti-inflammatory, antimicrobial, antiviral, antifungal, antiparasitic, anticancer, antidiabetic, cardioprotective, and many others generated by them increasing interest due to their therapeutic potential and applications in various fields, including medicine, pharmacology, and cosmetology (Shokoh *et al*., 2020). These therapeutic virtues of plants are experiencing a renewed interest to the use of modern analysis, screening techniques, such as chromatography, mass spectrometry, extraction techniques and advancements in these structural analysis methods for the discovery of new active compounds. These advanced techniques have facilitated the isolation, purification, and characterization of bioactive compounds from medicinal plants (Najmi *et al*., 2022).

Their active compounds can belong to different classes, including alkaloids, flavonoids, terpenoids, phenols, saponins, coumarins, lignans, and many others (Amlan, 2012). Each of these compound classes possesses a unique chemical structure and specific properties that confer particular biological activities (Karma *et al*., 2022). Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens, protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Kanti et Rizv,2009). The chosen plant for our study is *Carthamus caeruleus* L. It is known as blue Cardoon (Bowles *et al*., 2010) and rarely specie found in the *Mediterranean region* in moist clearings of forests, plains, stream banks, and especially in clayey soils (Meratate, 2016).

In this context, our study focuses on the evaluation of the biological activities of selected plants based on their use in traditional pharmacopoeia in the Setif region of Algeria. Our study is structured in a classical manner, divided into three parts.

- The first part is a literature review, providing a synthesis of data related to our research topic.
- The second part is the experimental section, which describes the methodological approaches, including quantitative and qualitative analysis of the extracts from our selected plant, as well as the evaluation of their biological activities and cytotoxicity assay.
- The final part consists of the discussion of the obtained results. Finally, a conclusion summarizing the main findings, along with future perspectives, concludes our manuscript.

REVIEW OF LITERATURE

1. *Carthamus caeruleus*

1.1. Morphological representation

*Carthamus caeruleus*belongs to the Asteraceae family. This family belongs to the Dicotyledons and comprises more than 1500 genera and over 25000 species. The genus *Carthamus* includes 14 species of annual and perennial herbaceous plants, including *Carthamus caeruleus* L (Guignard, 1994). *Carthamus caeruleus* is an annual herb with a simple branched and erect stem, ranging from 0.2to 0.6m in height. The stem is also slightly hairy. The rhizome of plant consists main horizontally root and vertically secondary roots. The leaves are glabrous or and strongly veined, with an oval contour. This plant is characterized by solitary and blue-violet capitula at the top of the stem and branches, which are globular in shape. The flowers are blue, although some varieties produce violet flowers. *Carthamus caeruleus* is highly attractive to insects. The fruit is an achene that is noticeably shorter, glabrous and whitish. The seeds of Carthamus caeruleus are generally small and produced in large quantities**(**Dahmani,2018) (Figure 1).

Figure 1: Different parts of *C. caeruleus* (Original, 2023).

1.2. Biological cycle

The biological cycle of *C. caeruleus* plant includes germination as the first stage. Then, the seeds of *C. caeruleus*isusually germinate in spring when environmental conditions are favorable. After germination, the plant develops roots and leaves, and its stem begins to elongate. This phase of vegetative growth allows the plant to strengthen and prepare for reproduction. Its flowering step typically occurs in summer, specifically between the months of May and June. During this period, the plant produces colorful flowers that contain the reproductive organs (Mihoub *et al*., 2017).

This is followed by the pollination stage, which can occur in different ways. It can be through insect pollination (entomophily) or through wind pollination (anemophily). After pollination, the flowers transform into fruits containing the seeds. Once the seeds are mature, they are dispersed in the environment by various dispersal mechanisms. Some seeds may enter a period of dormancy, remaining inactive for a variable period before germinating **(**Dahmani, 2018) (Figure 2).

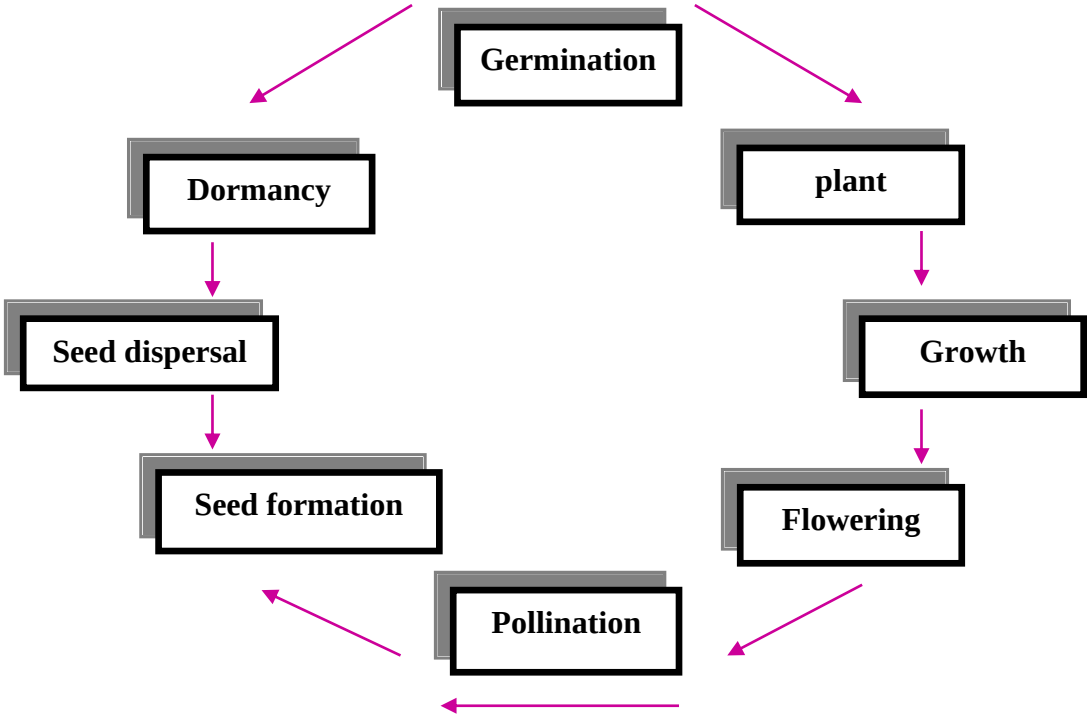


Figure 2: Biological cycle of *C. caeruleus* plant (Original, 2023).

1.3. Geographic distribution

The geographic distribution of the plant varies across regions. This plant is found in different areas of the world, including Europe, North and South Africa, Asia, as well as North and South America (Dahmani *et al*., 2019). In terms of habitat, *C. caeruleus* is mainly grows in humus-rich and light soils. It is commonly found in forest, fields, and well-fertilized gardens (Baghiani *et al*., 2010).

In Algeria, for example, this species has been collected in regions such as Bouira, Tizi-Ouzou, Telemcen, Setif, and Boumerdes. It should be noted that the precise geographic distribution of *C.caeruleus* may vary depending on the specific environmental conditions of each region **(**Boumerfeg, 2010) (Figure 3).

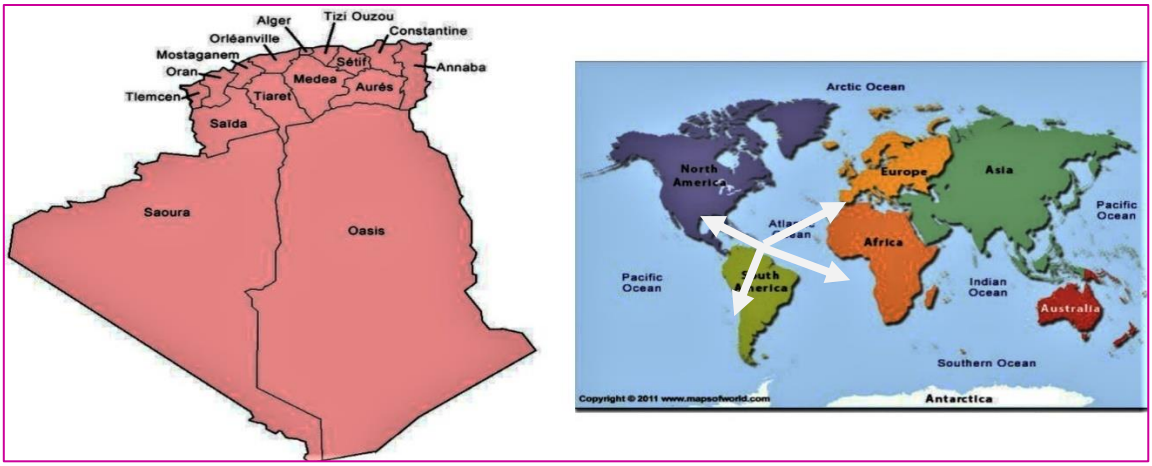


Figure 3: Distribution of *C. caeruleus* plant (Dahmani, 2019).

1.4. Taxonomic position of *C. caeruleus*

The genus *Carthamus* of the family Asteraceae, including blue Carduncelle or kendjar, which has many scientific names; *Carthamus caeruleus* L., *Kentrophyllum caeruleum* L., *Carduncellus caeruleus* L., *Onoborma caerulea* L., and *Carthamus tingitanus*. However, the local noun is Gares Mars (Boumerfeg *et al*., 2010). According to Quezelet Santa (1963), *Carthamus caeruleus* is classified as follows:

Kingdom: Plantae

Sub-family: Carduoideae

Tribe: Cardueae

Sub-tribe: Centaureinae

Family: Asteraceae

Gender: *Carthamus*

Species: *Carthamus caeruleus*

1.5. Previous studies

Some previous studies of different parts of *C. caeruleus* extracts are realized by various authors are summarized in Table 1.

Table 1: Previous studies of different parts of *C. caeruleus* extracts**.**

Used part	Preparation method	Studied Activity	Results	Traditional uses	References
Rhizomes	Aqueous extract	In vivo anti-inflammatory activity of aqueous extract of *C. caeruleus* rhizome against carrageenan-induced inflammation in Mice	-The effectiveness against paws edema induced by carrageenan	-Inflammation -Burns -Wounds -Healing properties	(Amari *et al.*,2020)
	Infusion decoction extracts (cream)	Ethnobotanical study, phytochemical characterization and healing effect of *Carthamus coeruleus* L. rhizomes	-healing properties of skin burned,	Traditional cream, which contributes to the disappearance of scars generated by burning.	(Benhamou et Fazouane, 2013)
	Aqueous extract	Study of the anti-inflammatory and healing properties of the rhizomes of C. Caeruleus L. (Asteraceae) harvested in the Region of Tipaza	-Reduction of carrageenan edema volume of 87.34%. -Healing of wounds at the end of 14 days.	Cicatrization the injuries resulting from burns (cream prepared with milk)	(Benmansour, 2020)
Roots	Methanolic extract	Gas chromatography coupled to mass spectrometry characterization, anti-inflammatory effect, wound-healing potential, and hair growth-promoting activity of Algerian *C. caeruleus* L (*Asteraceae*)	-An excellent reduction of inflammation. - A wound-healing property. - An interesting hair-promoting growth activity.	-Inflammation -Healing properties	(Dahmani, 2018)

2. Secondary metabolites

2.1. Definition

Secondary metabolites are compounds that are characteristic of a range of species. These compounds generally do not play a direct role in growth, development, and reproduction, but they often serve as defense chemicals against predators, pathogens, or environmental stresses. Some examples of secondary metabolites include alkaloids, terpenoids, and phenolic compounds. Alkaloids are nitrogen-containing compounds found in plants, such as morphine and caffeine. Terpenoids are a diverse group of compounds derived from isoprene units, including essential oils and certain pigments. These secondary metabolites contribute to the medicinal and pharmacological properties of many plant species (Karbab, 2020).

2.1.1. Phenolic compounds and classification

Phenolic compounds are a large and diverse group of secondary metabolites found in plants. They are characterized by the presence of a phenol ring with one or more hydroxyl (OH) groups attached. Phenolic compounds are widely distributed in nature and are known for their various biological activities and health benefits (Boizotet, 2006). Phenolic compounds can be classified into different subclasses, including flavonoids, phenolic acids and tannins. Each subclass has its own unique chemical structure and biological properties (Kanti et Rizv, 2009).

2.1.1.1. Phenolic acids

Phenolic acids are classified into two main types: benzoic acid derivatives and cinnamic acid derivatives, based on the C1-C6 and C3-C6 backbones, respectively (Figure 4). These phenolic acids can only be released or hydrolyzed through acid or alkaline hydrolysis, or by enzymes (Chandrasekara et Shahidi, 2010).

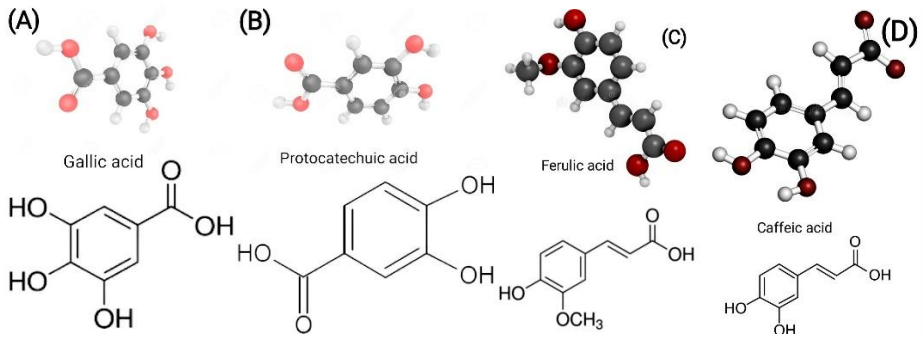


Figure 4: Phenolic acids of **(A, B)** benzoic and **(C,D)**cinnamic acids (Chanforan, 2010).

2.1.1.2. Flavonoids

Flavonoids are characterized by their chemical structure consisting of a 15-carbon skeleton with two aromatic rings (A and B rings) connected by a three-carbon bridge (C ring) (Figure 5) (Pietta, 2000). There are several subclasses of flavonoids, including flavones, flavonols, flavanones, flavanols, anthocyanins, and isoflavones (Figure 5). Each subclass has its own unique chemical structure and biological activities (Bruneton, 1999).

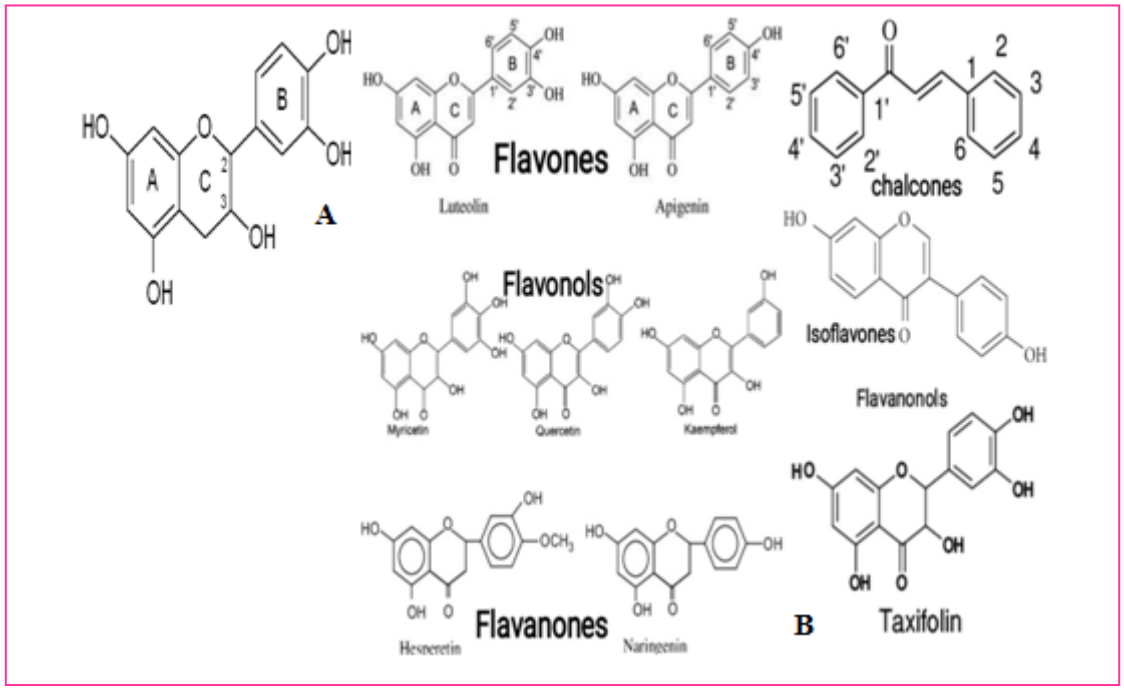


Figure 5: Basic structures of flavonoids and the main classes of flavonoids (Abedini, 2013).

2.1.1.3. Tannins

Tannins are defined as water-soluble phenolic compounds with a molecular weight ranging from 500 to 3000, which have the property of precipitating gelatin and other proteins and staining with ferric salts (Chung *et al*., 1998). Chemically, tannins are classified into two categories: hydrolysable tannins and condensed tannins (Figure 6), which differ in their chemical structure and biogenetic origin (Schofield *et al*., 2001).

- **Hydrolysable Tannins:** These tannins are esters of gallic acid or ellagic acid with glucose or a polyol. They can be further classified into gallotannins and ellagitannins (Derwich *et al*., 2010)**.**

- **Condensed Tannins:** These tannins are polymers of flavan-3-ol units, primarily catechin and epicatechin (Hammoudi, 2014).

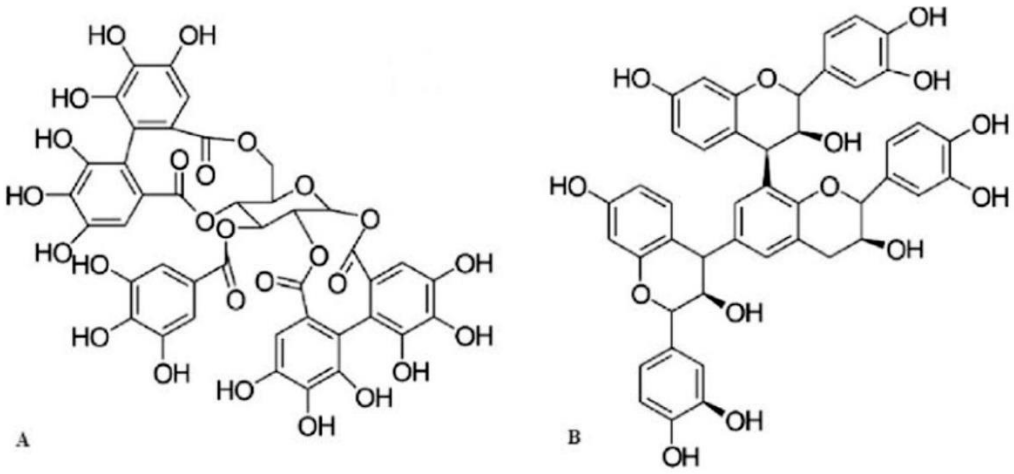


Figure 6: Chemical structure of hydrolysable **(A)** and condensed tannins **(B)** (Favier, 2003).

3. Biological activities

3.1. Antioxidant activity

3.1.1. Free radicals

The free radicals are highly reactive molecules that possess one or more unpaired electrons in their chemical structure. There are different types of free radicals, such as hydroxyl radicals ($OH^{\cdot}$), superoxide radicals ($O2^{\cdot -}$), peroxide radicals ($ROO\cdot$), nitric radicals ($NO^{\cdot}$), and many others (Favier, 2003). These free radicals are naturally produced within our bodies during normal metabolic processes. For example, the mitochondria, which are the powerhouse of the cell, produce free radicals during the production of adenosine triphosphate (ATP).

Additionally, certain enzymatic systems, such as peroxidase and xanthine oxidase, can generate free radicals as part of their normal functions (Aurousseau, 2002). Also, these free radicals can originate from external factors such as exposure to harmful environmental agents such as air pollution, ultraviolet radiation from the sun, toxic substances like pesticides and chemicals, smoking, alcohol, and certain drugs (Tamer, 2003). This wide variety of physical, chemical and metabolic factors lead to a common expression known as oxidative stress (Gabriele *et al*., 2017).

3.1.2. Oxidative stress

The oxidative stress is the result of an exaggeration the production of oxygen-derived radicals and the powerlessness of the body's antioxidant defense system to detoxify and neutralize these harmful molecules (Walker *et al*., 1992). The oxidative stress can be caused by others factors, including chronic inflammation, cardiovascular disease, diabetes, neurodegenerative diseases, and cancers (Favier, 2006).

Thus, the unstable nature and highly reactivity of free radicals can interact with other molecules in the body, including lipids, proteins, and DNA (Lobo *et al.*, 2010). The excessive production of ROS can cause damage to cellular components, including lipids, proteins, and DNA, leading to cellular dysfunction, tissue damage (Figure7) and development and progression of various diseases (Favier, 2006).

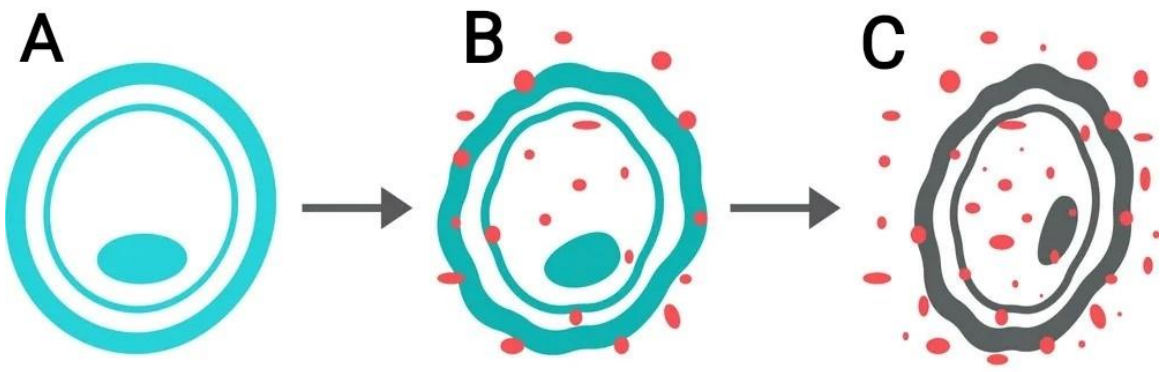


Figure 7: Damage to healthy cells caused by an attack of free radicals. **(A)** Normal cell; **(B)** Free radicals attacking cells; **(C)** Cell with oxidative stress (Birben *et al*.,2012).

3.1.3. Defense systems

The body has a defense system that includes antioxidants, enzymes that scavenge and neutralize reactive oxygen species (Lobo *et al.*2010).Thereby, these various defense systems can protect the cells and tissues from oxidative damage. And play a crucial role in maintaining the balance between reactive oxygen species production and neutralization(Lobo *et al.*,2010).Firstly, the antioxidants are compounds presents in low concentrations relative to the oxidizable substrate and can inhibit the oxidation process (Al-mamary *et al*., 2002; Vansant, 2004).

These molecules include vitamins E and C, as well as plant-derived polyphenols no synthesized by the body and must be obtained through diet (Favier, 2003).The antioxidants act by binding to free radicals and reacting with them in a controlled manner. They donate an

electron to these free radicals, convert them into stable forms (Figure 8) and render them harmless (Fettah, 2019). The modes of action of antioxidants can also include inhibition of enzymes, and chelation of trace metals responsible for ROS production (Swaran, 2009). Secondly, the enzymes are highly effective defense systems, because they have the ability to work continuously. This line of defense consists of superoxide dismutase, catalase, and glutathione peroxidase (Favier, 2003).

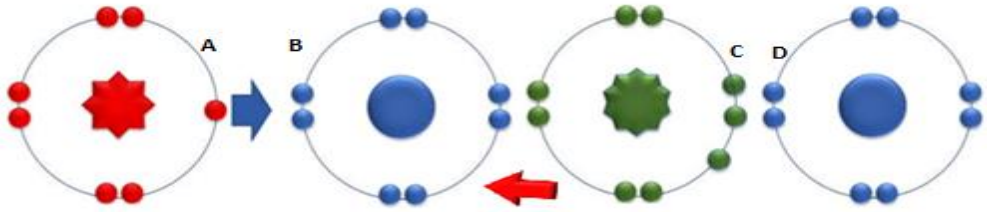


Figure 8: Free radicals neutralized by antioxidants. **(A)**Instable free radical with electron unpaired; (**B)** Antioxidant molecule donates electron to free radical**)**; **(C)** Antioxidant molecule; **(D)** Stable atom with electron paired (Gopalan *et al*., 2022).

3.2. Antibacterial activity

3.2.1. Bacteria

Humans live in an environment colonized by a large number of ubiquitous microorganisms. These microorganisms include bacteria, viruses, fungi, and protozoa (Khiati, 1998).Bacteria are unicellular prokaryotic microorganisms that can be observed under an optical microscope and can have different shapes(Ricci *et al*., 2005). However, some bacteria can also cause infections in plants and animals(Khiati, 1998). It is well-known that the treatment of bacterial infections relies primarily on the use of antibiotics. However, the widespread consumption of antibiotics has led to the selection of multidrug-resistant strains (Figure 9), highlighting the need to direct research towards new alternatives, particularly plants, which have always been a source of inspiration in medical research (Ali-Shtayeh *et al*., 1998).

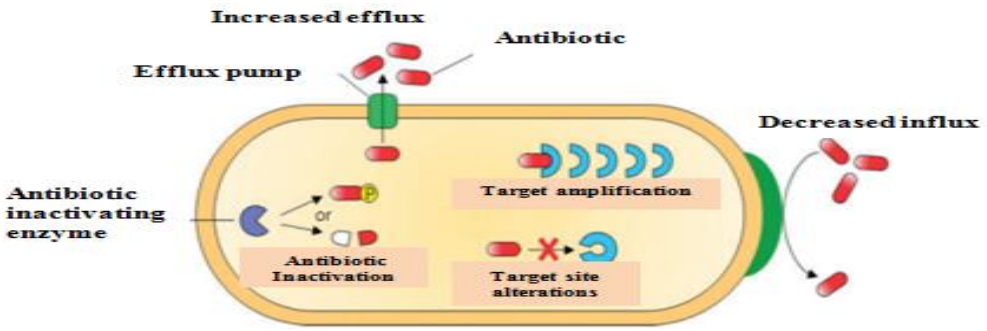


Figure 9: Diagrammatic representation of antibiotic resistance mechanisms utilized by bacteria (Alav *et al*., 2018).

3.2.2. Antimicrobial substances

3.2.2.1. Antibiotics

They are defined by Turpin and Velu as any chemical compound, produced by a living organism or synthesized, with a high chemotherapeutic coefficient, whose therapeutic activity is manifested at very low doses and in a specific manner, by the inhibition of certain vital processes, against viruses, microorganisms, or even certain cells of multicellular organisms(Subhasree *et al*., 2009). According to their mode of action, antibiotics can be divided into two main groups: bacteriostatic antibiotics, which inhibit the growth of bacteria, and bactericidal antibiotics, which kill bacteria (Walsh, 2003).

Bacteriostatic antibiotics slow down or inhibit bacterial growth, allowing the body's immune system to eliminate the infection. Bactericidal antibiotics, on the other hand, directly kill the bacteria, leading to their death. The choice between bacteriostatic and bactericidal antibiotics depends on various factors, including the type of infection, the severity of the condition, and the specific bacteria involved (Walsh, 2003).There are several classifications of antibiotics, which are based on their spectrum of activity target, or chemical family (Cohen et Jacquot, 2001).

3.2.2.2. Antimicrobial agents

Recently, the great adverse effects of synthetic molecules and conventional antibiotics used to combat bacterial infections give the importance in the discovery of new biologically active substances of plant origin and provide an alternative in primary healthcare systems. In particular, many researchers are interested in medicinal plants for their richness in natural molecules such as polyphenols, flavonoids and tannins, which possess antimicrobial activities(Vaou *et al*., 2021). Therefore, the exploitation of new bioactive molecules with limited or nonexistent side effects from natural sources and their adoption as a therapeutic alternative to synthetic molecules have become top priorities for scientific research and the food and pharmaceutical industries (Prasad et Seenayya, 2000).

However, in general, antimicrobial agents act by specifically target in microorganisms and disrupting their vital functions, resulting in their inability to reproduce, spread, or cause infections (Milane, 2004). Additionnally, some antimicrobial agents work by disrupting the cell wall of bacteria, leading to their rupture and death (Vaou *et al*., 2021). Others targets including the inhibition of specific metabolic pathways (protein or nucleic acid synthesis) and the binding to specific targets inside microorganisms, such as enzymes that are crucial for

their survival (Vaou *et al*., 2021). Thus some antimicrobial agents stimulate the body's immune system to enhance its ability to fight infections by the activation of specific immune cells or increase antibody production **(**Kempf, 2009).

3.2.3. Description of microorganisms tested.

3.2.3.1. Bacteria

3.2.3.1.1. *Escherichia coli*

Escherichia coli, also known as "coli bacillus," with rod-shaped cell, measuring approximately 2-3 µm in length and 0.6 to 0.7 µm in diameter. *E. coli* belongs to the family *Enterobacteriaceae* (Nolan *et al*., 2013, Hufnagel *et al*., 2015).

3.2.3.1.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is non-sporulating and possesses 1 to 2 flagella. These bacteria synthesize two main types of pigments: pyocyanin, which is blue, and pyoverdine, which is yellow-green. They are known to be resistant to multiple antibiotics. (Van Delden et Iglewski, 1998).

3.2.3.1.3. *Staphylococcus aureus*

Staphylococcus aureus belongs to the family *Micrococcaceae*, with a diameter of 0.5 to 1.5 µm. They are facultative anaerobes, non-sporulating and non-capsulated (Dworkin et Falko,2006).

3.2.3.1.4. *Bacillus subtilus*

Bacillus subtilus is a aerobic bacterium fast-growing, with rod-shaped cells. Typically *B. subtilus* measures 2–6 µm in length and just less than 1 µm in diameter. The optimal growth temperature is about 30-35 C°. Alternatively, they can form bacterial biofilms (Errington et Aart, 2020). The classification of each bacteria strains is summarized in (Appendices1).

3.2.3.2. Fungus

3.2.3.2.1. *Aspergillus flavus*

Aspergillus flavus is a fungus that grows rapidly on classical media (malt agar and Sabouraud agar) at 22-25°C.Its optimal growth temperature is 37°C. This fungus forms fluffy to powdery colonies, initially white, then yellow, and finally greenish-yellow. The reverse side can be colorless, pinkish, or dark brown-red (Tabuc,2007).

3.2.3.2.2. *Aspergillus niger*

Aspergillus niger is an ascomycete fungus known to reproduce only through asexual spores (Wadman *et al*., 2009). The asexual cycle is the main means of cellular dispersion and protects the fungal genome under unfavorable conditions, and the production of secondary metabolites is frequently associated with these developmental processes (Ward *et al*., 2006).

3.2.3.2.3. *Aspergillus brasiliensis*

Aspergillus Brasiliensis is a fungus and one of the most common species in the genus *Aspergillus*. This common food contaminant is ubiquitous in soil and is also frequently reported in enclosed spaces such as industrial sites. *A. brasiliensis* rarely causes disease in humans unlike other Aspergillus species (Patterson *et al*., 2016).

3.2.3.3. *Candida albicans*

Candida albicans is most commonly associated with the majority of pathological manifestations in humans. It is usually found in a saprophytic state in the human digestive tract and can also be found in the vulvovaginal mucosa or mouth through contiguity. However, *C. albicans* is rarely found on the skin (Bohm *et al*.,2017).

3.3. Cytotoxicity assay

Research on the cytotoxicity of medicinal plants aims for understanding their therapeutic potential, particularly in the field of cancer. This evaluation is essential to lead to the isolation and purification of potentially useful cytotoxic compounds for the development of new anticancer drugs or other areas of medicine (Greenwell et Rahman, 2015). Additionally, this can lead to determine the plant extracts, which have toxic effects on cells. However, further studies are needed to deepen our knowledge of the cytotoxicity of medicinal plants and explore their potential use in the development of new treatments.

3.3.1. *Artemia salina*

The biological model is presented by *Artemia salina* to evaluate the cytotoxicity effect of medicinal plants extracts (Sandeep *et al*., 2019). *Artemia salina* is known as the brine shrimp and process various characteristics make them a significant species in both natural ecosystems and aquaculture, where it serves as a valuable food source (Epole *et al*., 2020).

3.3.1.1. Taxonomy

According to (Linnaeus, 1758), *Artemia salina is* classified as follows:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Branchiopoda

Order: Anostraca

Family: Artemiidae

Genus: Artemia

Species: *Artemia salina*

3.3.1.2. Geographical distribution

This crustacean inhabits in biotopes with tropical, subtropical, or temperate climates (Emslie, 2003). The brine shrimp is able to tolerate temperature and salinity fluctuations, along with its flexible feeding habits, contributes to its successful colonization of diverse aquatic habitats. In Africa, specifically in the Maghreb region, it can be found in chotts, sebkhas, or mellaha (Leach, 1819). It is found abundantly in the salt pans of western Algeria due to its strong reproductive capacity (Kara *et al*.,2004). The different countries around the world that host this natural resource are summarized in the (Table 2).

Table 2: Countries hosting *Artemia* worldwide (FAO 2023).

Continents	Countries
Africa	Algeria, Egypt, Kenya, Libya, Madagascar, Morocco, Mozambique, Niger, Senegal, Tunisia, South Africa
North America	Canada, USA (Arizona, California, Nevada, Hawaii, Nebraska, New Mexico, Texas, North Dakota, Utah, Washington)
Central America	Bahamas, Mexico, Martinique, Antilles, Puerto Rico, Dominican Republic, Saint Martin.
Oceania	Australia (East and South), New Zealand.
South America	Bolivia, Brazil, Chile, Colombia, Ecuador, Peru, Venezuela.
Asia	China, India, Iraq, Iran, Palestine, Japan, Turkey.

3.3.1.3. Characteristics of brine shrimp

3.3.1.3.1. Anatomy

The adult Artemia body is devoid of a carapace but clearly segmented (Khalil, 2017). It can be divided into three parts (Figure10):

- **Head**

The head contains a median naupliar eye, a pair of stalked lateral eyes, and a simple ring-shaped brain structure around the mouth. At its anterior part, there is a pair of curved antennae and a second pair of antennae, more developed. On the head, there is the oral cavity, represented by a wide labrum, a pair of mandibles, and two pairs of maxillae. These various parts constituting the oral cavity are connected to the esophagus, which leads to the stomach(Khalil, 2017).

- **Thorax**

The adult Artemia body contains 11 pairs of phyllopods, all similar in structure, play a role in feeding, swimming, and respiration and processed a long digestive tract (Haddag, 1991).

- **Abdomen**

The abdomen is composed of 8 segments, with the first two supporting the external genital organs, hence the name "genital segments." The last 6 segments are almost cylindrical, with the last segment having two appendages at its distal end called furca (Khemakhem, 1988).

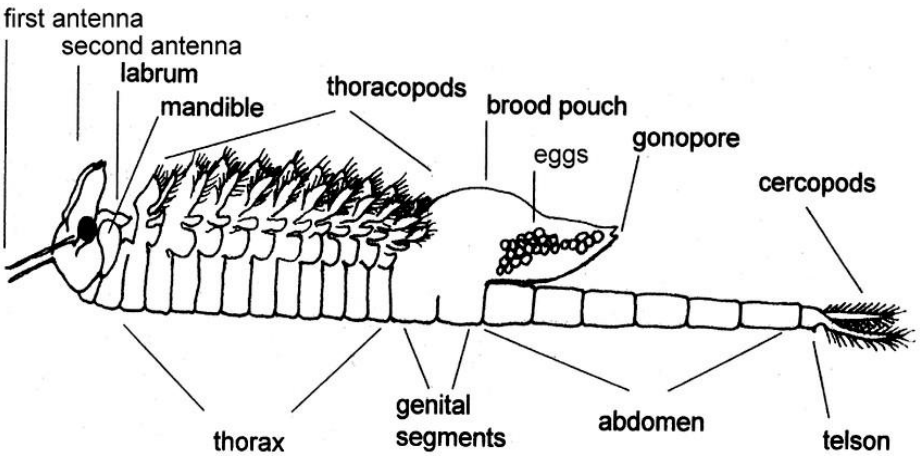


Figure10: Lateral view of an adult female fairy shrimp showing its structure (Timms, 2015).

3.3.1.3.2. Reproduction

Artemia salina exhibits both sexual and asexual modes of reproduction. The reproductive strategy depends on the environmental conditions and the specific strain of *Artemia* (Sorgeloos *et al*., 1986). The mode of reproduction in *Artemia* is influenced by environmental factors(Aloui, 1998).

- **Sexual Reproduction**

In bisexual strains of Artemia, sexual reproduction occurs through mating between males and females. The mating process involves the male grasping the female in the riding position (Figure 11), where he holds onto her between the uterus and the last pair of thoracopods. This mating position can be maintained for a prolonged period. During sexual reproduction, fertilization takes place, and the female produces cysts containing fertilized eggs. These cysts can then develop into nauplii, which are the early larval stages of *Artemia* (Sorgeloos, 1980).

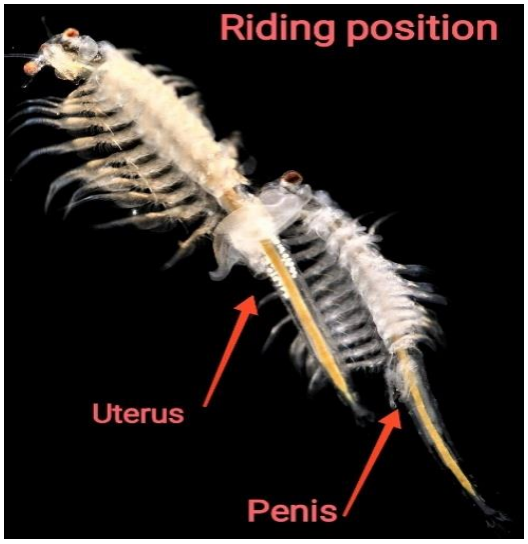


Figure 11: Pair of *Artemia salina* in mating position (Lavens *et al*., 1996).

- **Asexual Reproduction**

The females in parthenogenetic populations are capable of producing viable cysts without mating with males. This mode of reproduction, known as parthenogenesis. The produced cysts can develop into nauplii directly, without the need for fertilization (Sorgeloos, 1980).

3.3.1.3.3. Biological life cycle

The biological life cycle of *A.salina*, also known as brine shrimp, consists of several distinct stages (Figure12). According to (Lavens et Sorgeloos, 1996). There are the main stages of its life cycle:

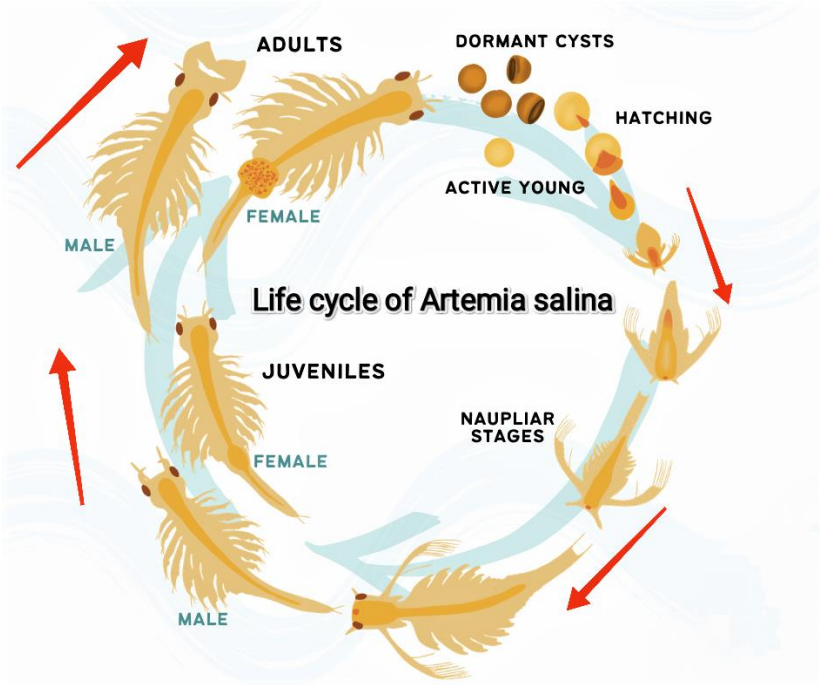


Figure12: Biological life cycle of *Artemia salina* (Abatzopoulos *et al*., 2010).

- **Eggs**

The life cycle of Artemia salina begins with the laying of eggs. The eggs are laid by adult females under favorable conditions, including high salinity and appropriate temperature.

- **Cyst**

The eggs hatch to form larvae that develop inside a protective shell called a cyst. Artemia salina cysts are extremely resistant and can survive in unfavorable environmental conditions for a long period.

- **Hatching**

Under optimal conditions, the cysts hatch, releasing nauplii larvae, which are the first developmental stages. Hatching can be triggered by stimuli such as dilution of saltwater, temperature increase, or chemical signals.

- **Metamorphosis**

As the nauplii develop, they undergo several stages of metamorphosis. They molt several times and develop new structures, including more complex appendages, before becoming adults.

- **Adult**

After several developmental stages, the nauplii reach the adult stage (Figure 13). Adult Artemia salina have a shrimp-like appearance, with an external carapace and numerous pairs of limbs Males are distinguished by two large spines on their head, used for grasping the female during mating and a pair of penises on their abdomen. Females only have a uterus in

their abdomen, which can contain up to 200 cysts. (Dhont et Van, 2003; Abatzopoulos *et al*., 2010). A sexual dimorphism in males, the antennae form a large claw with two spines, while in females, they are much smaller (Abatzopoulos *et al*., 2010).

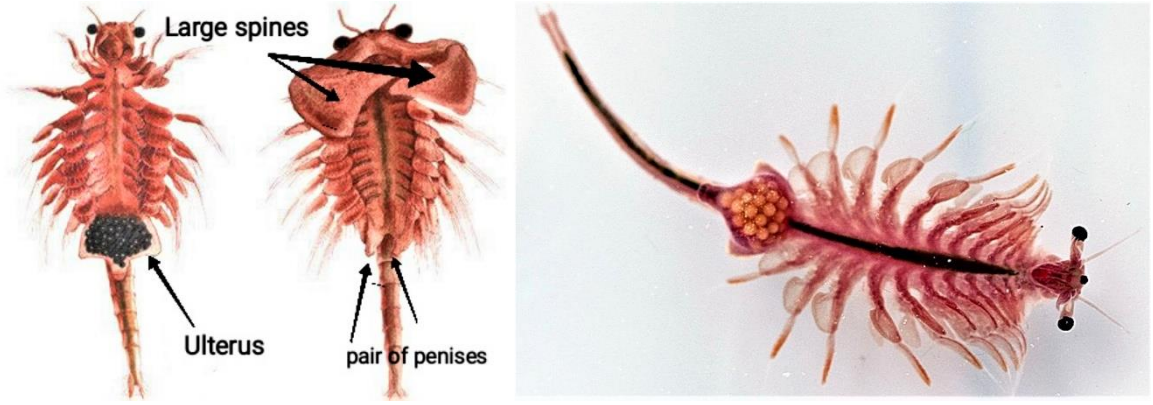


Figure 13: Adult forms of Artemia (Abatzopoulos *et al*., 2010).

3.3.1.3.4. Other characteristics (Size, color, feeding)

A.salina it's a small aquatic phyllopod crustacean, which that exceed generally around 8-12 mm, but it can reach up to 20 mm (Figure 14) (Trigui, 2017). The size of male adults Artemia are usually smellers than females, swim faster and eat less. (Trigui,2017). The males Artemia are less colorful. Their coloration ranges from milky white to blue-green, brick red, and vermilion, depending on their diet and the environment (Trigui, 2017).

In terms of feeding, *A. salina* is a filter-feeding organism that is non-selective in its diet, feeding particles smaller than 60µmsuch as detritus, protozoa, microalgae, and bacteria and using it's antennae and thoracopods (Dhont et Vanstappen, 2003; Abatzopoulos *et al*., 2010). These particles are then transferred to the labrum, where a viscous secretion surrounds them before the maxillae and mandibles transport them into the esophagus (Dhont et Vanstappen, 2003). This feeding behavior enables Artemia to adapt to different aquatic environments and efficiently utilize available food resources (Abatzopoulos *et al*., 2010)). This organism can also withstand long periods of drought (Abatzopoulos *et al*., 2010).

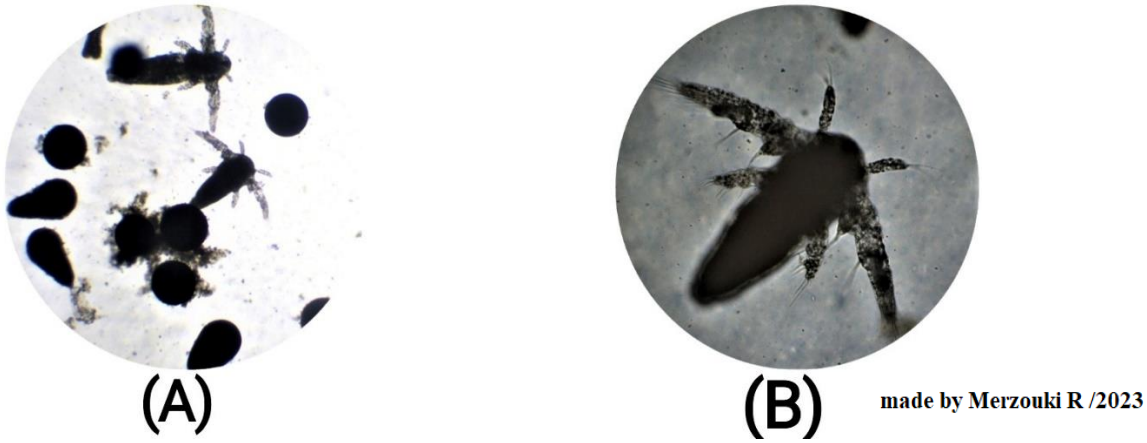


Figure 14: *Artemia salina* under an optical microscope. **(A):** magnification power×10; **(B):** magnification power ×40 (Original 2023).

MATERIALS AND METHODS

1. Materials

1.1. Plant material

Unpeeled rhizomes of *C. caeruleus* was harvested from Hamama area (Ain Roua, Setif) in February 2023 before flowering. Then, the rhizomes were cleaned, dried, crushed and sieved (Figure 15). Finally, the resulting powder is stored in a glass jar covered with aluminum foil.

Figure 15: Preparation of plant material. **A)** collecte of rhizomes; **B)** drying**; C)** crushing; sieving (Original, 2023).

1.2. Microorganisms strains

Microbial cultures and their respective reference used in this study are mentioned in Table3. The four bacterial strains (*Bacillus subtillus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), fungal strain (*Aspergillus brasiliensis*) and yeast (*Candida albicans*), belonged to the American Type Culture Collection (ATCC) were supplied by Doctor Benserradj Ouafa, University Center of Mila. Additionally, *Aspergillus niger* and *Aspergilus flavus*, was isolated from gains of wheat. Bacterial strains were routinely grown in nutrient agar at 37° for 24h, with the exception of fungal strains and *Candida albicans*, for which the growth temperature was set to 30° on Sabouraud dextrose agar for 7days and 48h, respectively.

Table 3: Microorganisms strains used in study.

Microorganisms	Species	References	Pathogenicity
Bacilli bacteriaGram$^+$	*Escherichia coli*	25922ATCC	Gastroenteritis
	Pseudomonas aeruginosa	27853ATCC	Nosocomial infection
Cocci bacteriaGram$^+$	*Staphylococcus aureus*	06538 ATCC	Nosocomial infection
BacillibacteriaGram$^+$	*Bacillus subtillus*	6633ATCC	Pneumonia, nosocomial infection and dermatitis
Fungi	*Aspergillus niger*	Isolated	Mycotoxin and aflatoxin productions, Aspergillosis, lung infections, allergic reactions
	Aspergillus brasiliensis	0392ATCC	
	Aspergillus flavus	Isolated	
Yeast	*Candida albicans*	0443ATCC	Mucocutaneous candidiasis

1.3. Brine shrimp eggs

Brine shrimp (*Artemia salina)* lethality assay is an important tool for the preliminary cytotoxicity assay of plant extracts, based on the ability to kill a laboratory cultured larva (Figure 16). These nauplii were supplied by Doctor Manallah Ahlem, University Center of Mila and used as a biological assay to assess the toxicity of tested *C. caeruleus* extracts.

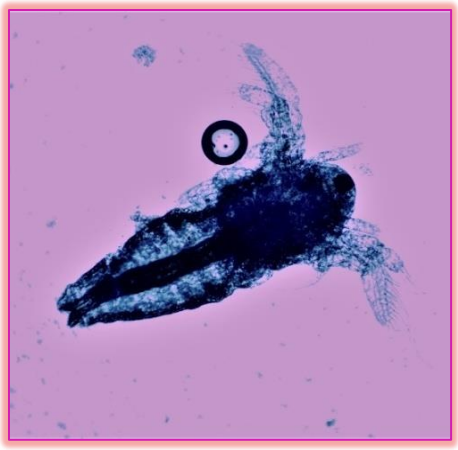


Figure 16: Microscopic view of shrimp larvae taken during cytotoxicity experiment (Original, 2023).

I.4. Materials

The materials and different equipment used in this study are summarized in (Appendices2).

I.5. Chemicals and reagents

The chemicals and reagents used for phytochemical screening and the determination of total polyphenols and flavonoids, as well as those used for the evaluation of antioxidant, antibacterial, antifungal, and cytotoxic activity, they are as follows: Acid ascorbic, 2,2'-diphenyl-1-1-picrylhydrazyl (DPPH),aluminum chloride ($Alcl_3$), ammonium hydroxide(NH_4OH), concentrated HCl, copper sulphate ($CuSO_4$), ethanol, fehling's reagent, ferric chloride ($FeCl_3$), folin-ciocalteu reagent, gallic acid($C_7H_6O_5$), magnesium, quercetin($C_{15}H_{10}O_7$), sodium acetate trihydrate (CH_3CO_2Na), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium hydroxide (NaOH),sulfuric acid (H_2SO_4)and microbiological materials.

2. Methods

2.1. Study area

Hamama region (Ain Roua) is located in the northwest of Algeria, less than 28 km from the capital city of Setif and characterized by a cold and dry climate in winter and in summer, respectively. Ain Roua region is a partially mountainous, high plateau area and contains many sources of water (Figure 17).

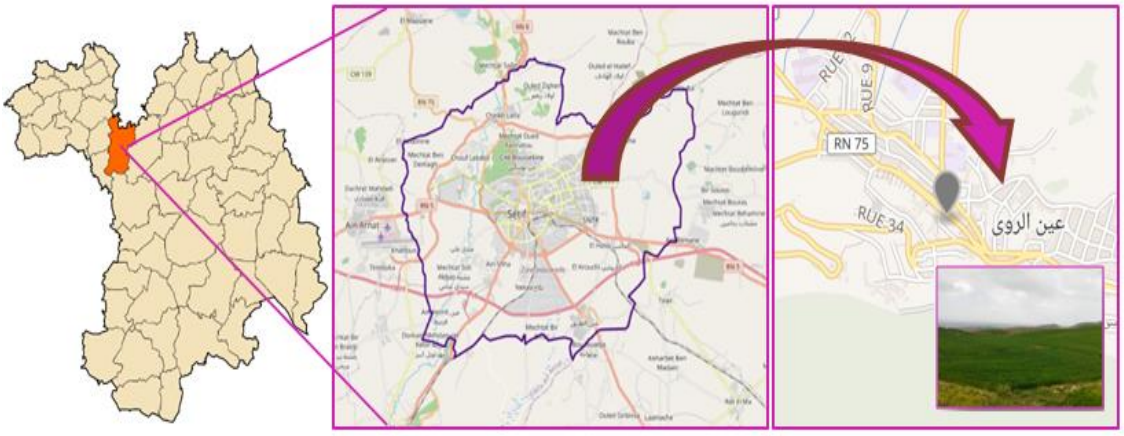


Figure 17: Geographical location of the area study.

2.2. Extraction

2.2.1. Preparation of the aqueous extract by decoction

The preparation of plant extracts was carried out according to the method of Martins *et al*. (2014). Decoction extract was prepared by boiling 20 g of dried rhizomes in 200 mL of distilled water at heating plate, for 5min. Then the mixture was filtered through filter paper and dried to obtain a dry extract (Figure 18).

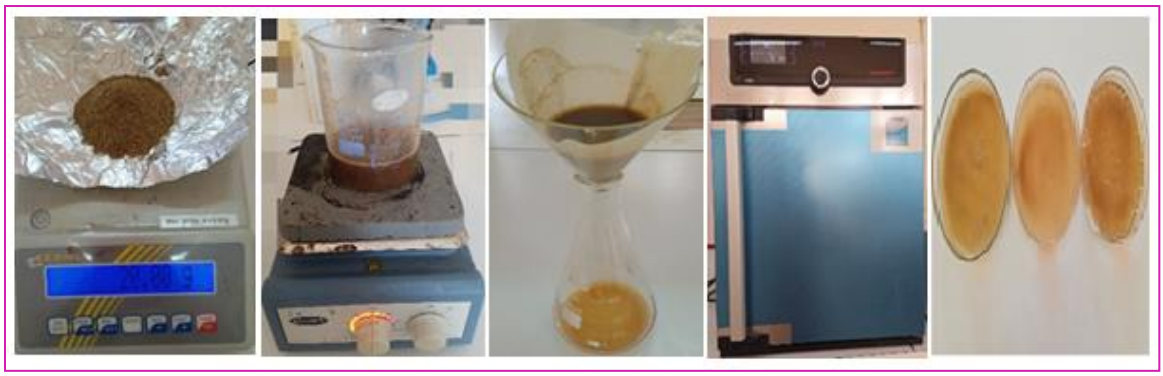


Figure 18: Steps of the decoction method (Original, 2023).

2.2.2. Preparation of the aqueous extract by infusion

The preparation of the infusion extract was carried out according to the method of (Martins *et al*, 2014). Infusion extract was prepared by mixing20 g of dry sample in 200mL of boiled distilled water and left to stand at room temperature during 5 min. Then the mixture was filtered and dried to obtain a dry extract (Figure 19).

Figure19: Steps of the infusion method (Original, 2023).

II.3. Determination of extraction yield

The percentage of extraction yield for each extract was calculated by the following formula as the ratio of the mass of the dried extract to the mass of the ground plant sample.

% Yield= Weight of extract obtained / Total weight of the sample *

2.4. Phytochemical evaluation

2.4.1. Qualitative phytochemical analysis

The presence of different phytochemical compounds in extracts of this plant is detected by specific reagents and based on coloring and/or precipitation reactions (Amari *et al.*, 2023).

- **Detection of phenolic substances:** the characterization of polyphenols is based on a reaction with ferric chloride ($FeCl_3$). About 500µLof the extract, a drop of 2 % alcoholic solution of ferric chloride is added. The appearance of blackish-blue or more or less dark green color is the sign of the presence of polyphenols.
- **Detection of flavonoids:** 500 µL of each extract was treated with few drops of concentrated HCl and a quantity of magnesium turnings. The appearance of a red or orange color confirms the presence of flavonoids.
- **Detection of condensed tannins:** to 500 µL of each extract is added some quantity of CH_3CO_2Na, followed by a few drops of an aqueous solution of 2% $Fecl_3$. The reaction is positive if a blue -black color appears.
- **Detection of hydrolysable tannins:** Tannoids are polymers of polyphenols. These latter have been demonstrated by the iron chloride reaction. To 1 mL of crude extract, a few drops of a 2% ($FeCl_3$) aqueous solution are added. The appearance of a blue-black indicates the presence of tannoids or true tannins respectively.
- **Detection of free quinones:** to a volume of 500 µL of each extract, a few drops of 1% NaOH are added, the appearance of color that turns yellow, red, or purple indicates the presence of free quinone.
- **Detection of anthraquinones:** 1250µL of each extract was added to 650 µL of NH_4OH to 10 %. After shaking, the appearance of a red ring confirms the presence of anthraquinones.
- **Detection of saponins:** mix 25 mg of each extract with 2 mL of distilled water, the mixture is vigorously shaken. The formation of persistent foam for 30 min confirms the presence of saponins.
- **Detection of terpenoids:** mix 500µL of each extract with 300 µL of H_2SO_4, the mixture is shaked. The presence of terpenoids is revealed by the appearance of red-brown color.
- **Detection of reducing sugar:** in test tubes, is introduced 1mL of Fehling's reagent, and heated in a water-bath at 70°C. The formation of brick red precipitate confirms the presence of sugar.
- **Detection of proteins:** in teste tubes is introduced 500 µL of each extract, followed by the addition of 250 µL of 20 % NaOH, after shaking. Drops of a 2%aqueous solution of $CuSO_4$ are added reddish tint, indicates a positive reaction.

2.4.2. Quantitative phytochemical analysis

2.4.2.1. Determination of phenolic compounds

The total phenolic in extract s is estimated using the method of Folin-Ciocalteu method (Karbab *et al*., 2020). In tubes, a volume of 100 µL of each extract was added to the 500 µL of Folin-Ciocalteu reagent for 4min, 400 µL of a 7.5 % sodium carbonate solution was added. Then, the tubes are shaked and incubated for 2h. The absorbance is determined at 765 nm (Figure 20).

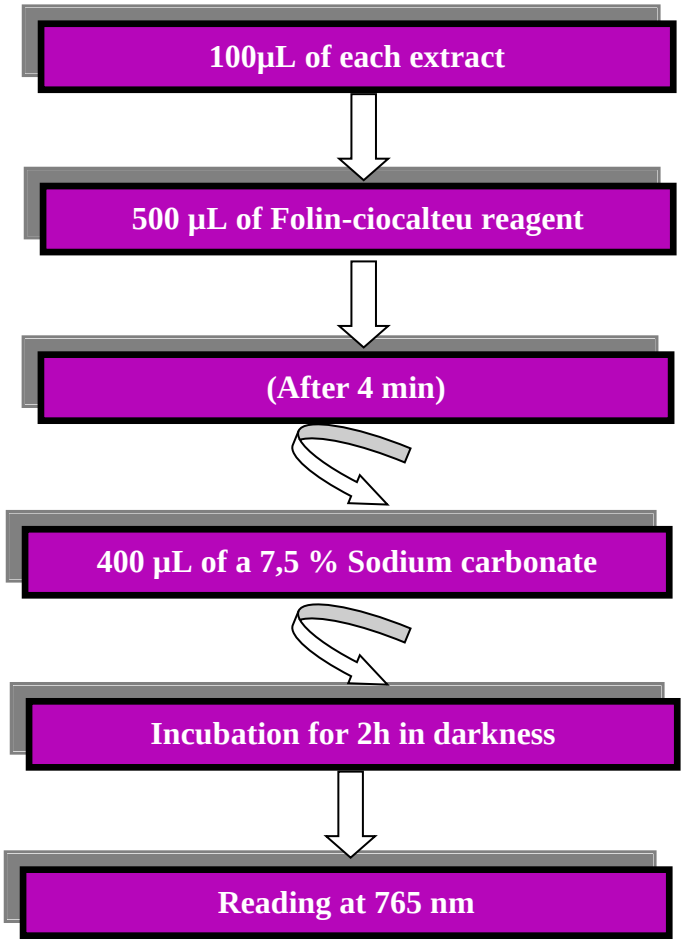


Figure 20: Total polyphenols protocol.

Polyphenolic content was expressed as µg gallic acid equivalent (GEq)/mg dried extract. The number of total polyphenols in different extracts was determined from a standard curve of gallic acid ranging from 10 to 160 µg/mL (Figure 21).

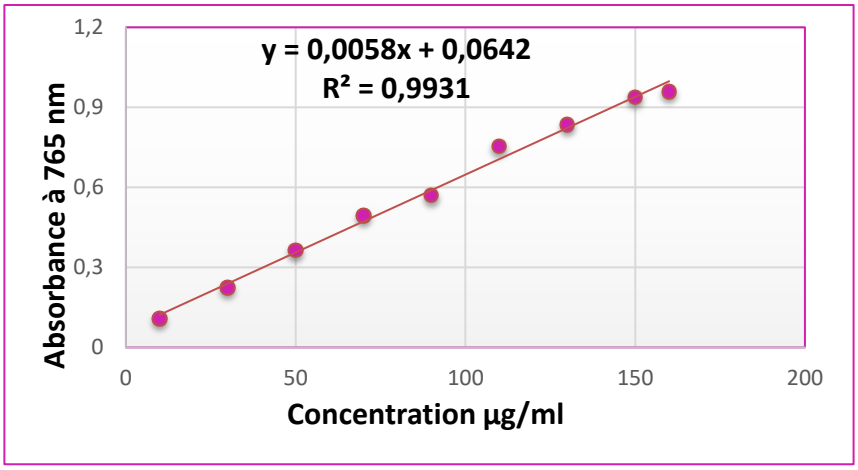


Figure 21: Standard curve of gallic acid

2.4.2.2. Determination of flavonoids

The total flavonoids content was evaluated by the method of aluminum chloride ($Alcl_3$) (Karbab *et al*., 2020)**.** A 500 µL of each extract was added to 500 µL of the solution of $Alcl_3$ (2% in ethanol). After 10 min of incubation, at room temperature, the absorbances are measured at 430 nm (Figure 22).

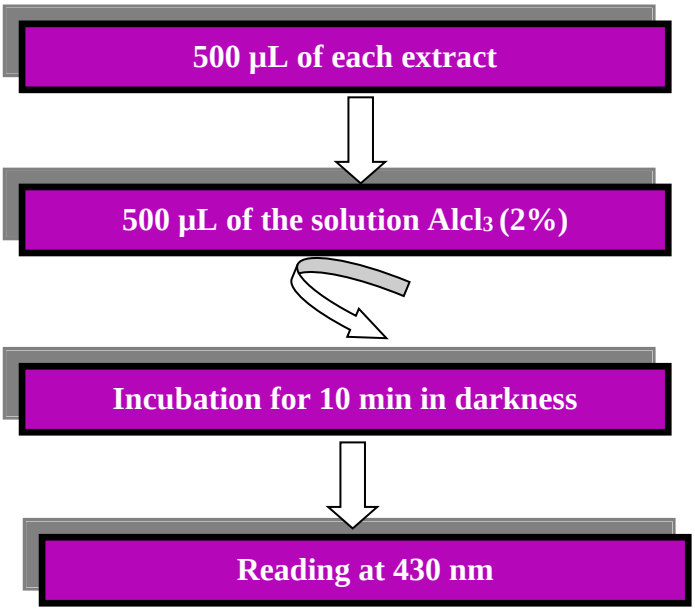


Figure 22: Total flavonoids dosage protocol.

Total flavonoids were reported as µg of quercetin equivalent (QEq)/mg dried extract. The number of total flavonoids in different extracts was determined from a standard curve of quercetin ranging from 2,5 to 40 µg/mL (Figure 23).

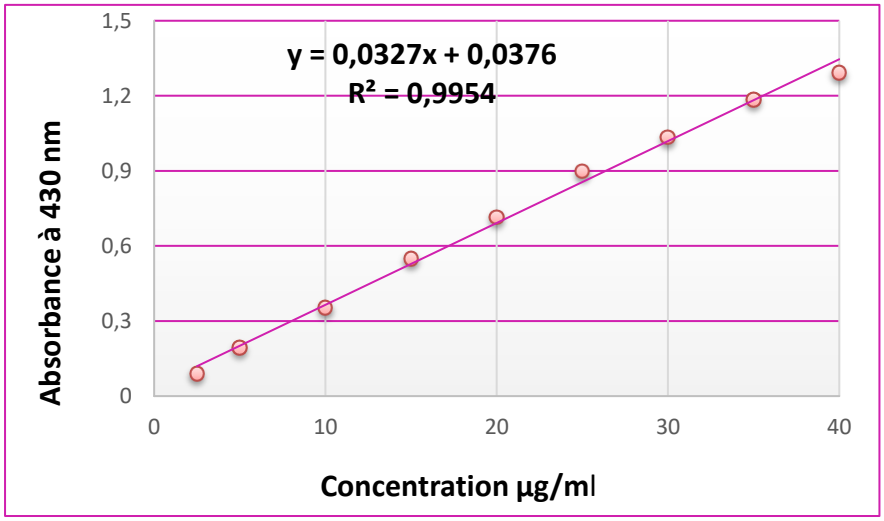


Figure23: Standard curve of quercetin.

2.5. Biological activities

2.5.1. Antioxidant activity

2.5.1.1. DPPH radical scavenging assay

Free radical scavenging capacity of the extracts was assessed using the 2,2'-diphenyl-1-1-Picrylhydrazyl (DPPH) assay by measuring the decrease in the DPPH maximum absorbance at 517 nm **(**Boutellaa *et al*.**,** 2019**).** In this method, 200µL of various concentrations of the extracts/standard were mixed with 800 µL of DPPH solution in ethanol. Absorbance of sample was measured at 517 nm after a 30 min of incubation in the dark at room temperature (Figure 24). Ascorbic acid was used as positive control. For each extract, we determined the IC_{50} value, which is the concentration of the tested sample required to reduce 50% of DPPH radical (Bouras et Houchi, 2013).

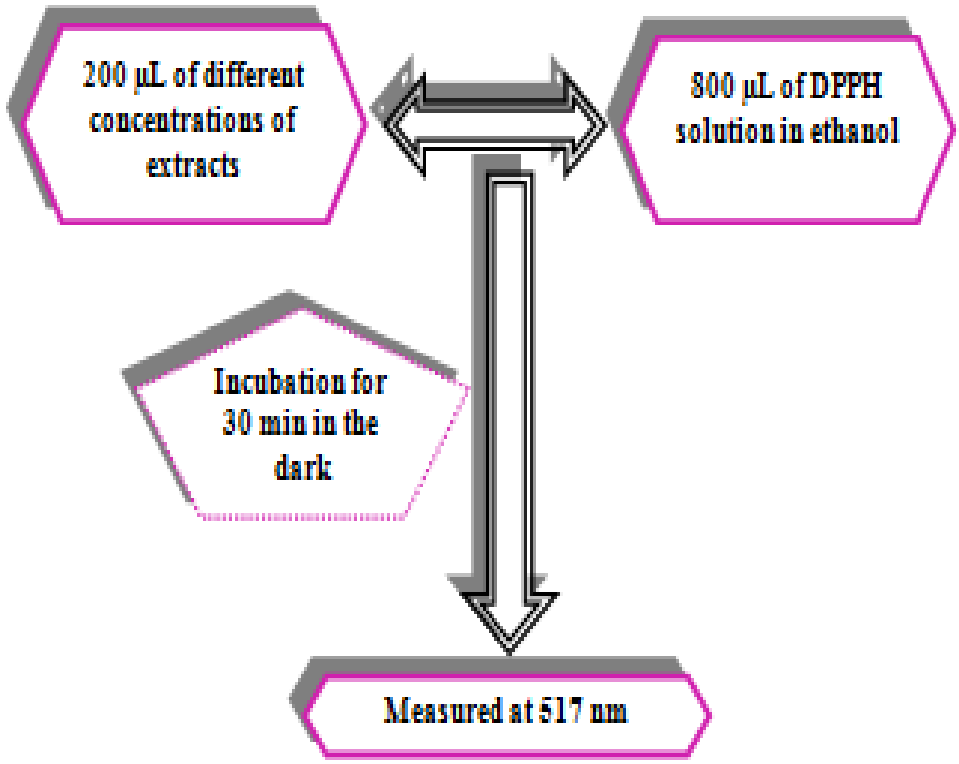


Figure 24: DPPH radical scavenging assay.

The scavenging capacity was calculated according to the following formula. Where A blank = absorbance of the solution except the tested sample or standard, and A test= absorbance of the tested samples or standard.

I% = (A blank – A test /A blank) * 100

2.5.2. Antimicrobial activity

For determined the sensitivity of the bacteria, fungi and yeast, agar disk-diffusion method was employed (Yaici *et al*., 2019).

2.5.2.1. Preparation of culture media and samples tested

Under aseptic conditions, sterile Mueller Hinton and Sabouraud agar (Appendices 3) were poured into sterile petri dishes (90mm). Then, these petri dishes were dried for 30 minutes at room temperature. Three dilutions were prepared: 1/2, 1/4, 1/8 (v/v) in DMSO (dimethyl sulfoxide). This choice was taken by the majority of authors because the DMSO solvent has no antimicrobial activity.

2.5.2.2. Microbial inoculums preparation

- **Bacteria cultures**

For each bacterial strain, stock culture was prepared in petri dishes containing nutrient agar (NA) and incubated to obtain a young and well-isolated bacterial culture. After 24 hours, bacterial colonies were taken from the stock cultures using a platinum loop and transferred to tubes containing 9 mL of sterile physiological saline solution, to obtain a cell density equivalent to 0.5 McFarland (10^6 CFU/mL). This bacterial cell density was obtained using a spectrophotometer and adjusted to a value between 0.08 and 0.1at 625nm (Falleh *et al*., 2008).

- **Fungi cultures**

At the center of plate containing Sabouraud medium, each fungal strain was placed and incubated until mycelial growth and sporulation occurred. After 7 days, fungi strains were suspended in sterile physiological saline solution to obtain a spore suspension of 5×10^6 spores/mL, measured using a spectrophotometer and adjusted the absorbance determination at 625nm between 0.08 and 0.1(Surapuram *et al*., 2014).

- **Yeast culture**

Yeast suspension was prepared from a petri dish containing a young culture (18-24 h) and the optical density determination was adjusted to a value between 0.08 and 0.1 at 625nm (Yazdani *et al*., 2012).

2.5.2.3. Inoculation and disc deposition

Within 15 min of adjusting the density of microbial suspensions, a swab sticker was dipped into the suspension and spread over the entire surface of the agar (Mueller Hinton Agar for bacteria or Sabouraud Agar for fungi and yeast) in three repetitions to achieve a uniform distribution of the microbial inoculum. Then, sterile forcep was used and sterile discs (5mm of diameter) were placed on the previously inoculated agar with the microorganism tested. The discs were then impregnated with the different dilutions of the extracts (1/2, 1/4, and 1/8; v/v). Each disc contains 10 µL of each concentration, while other discs were impregnated with 10 µL of DMSO. Gentamicin (0.120 mg/mL) is used for bacterial strains, while Mycozan, Fongenal, and Verten (1mg/mL) were used for fungal strains as a positive control.

2.5.2.4. Incubation and interpretation of results

The Petri dishes are incubated in an incubator at 37°C for 18 to 24 hours for bacteria and at 30°C for three-seven days for yeast and fungi strains, respectively. Then the

antimicrobial activity was determined by measuring the diameter of the inhibition zone produced around each disk (Figure 25).

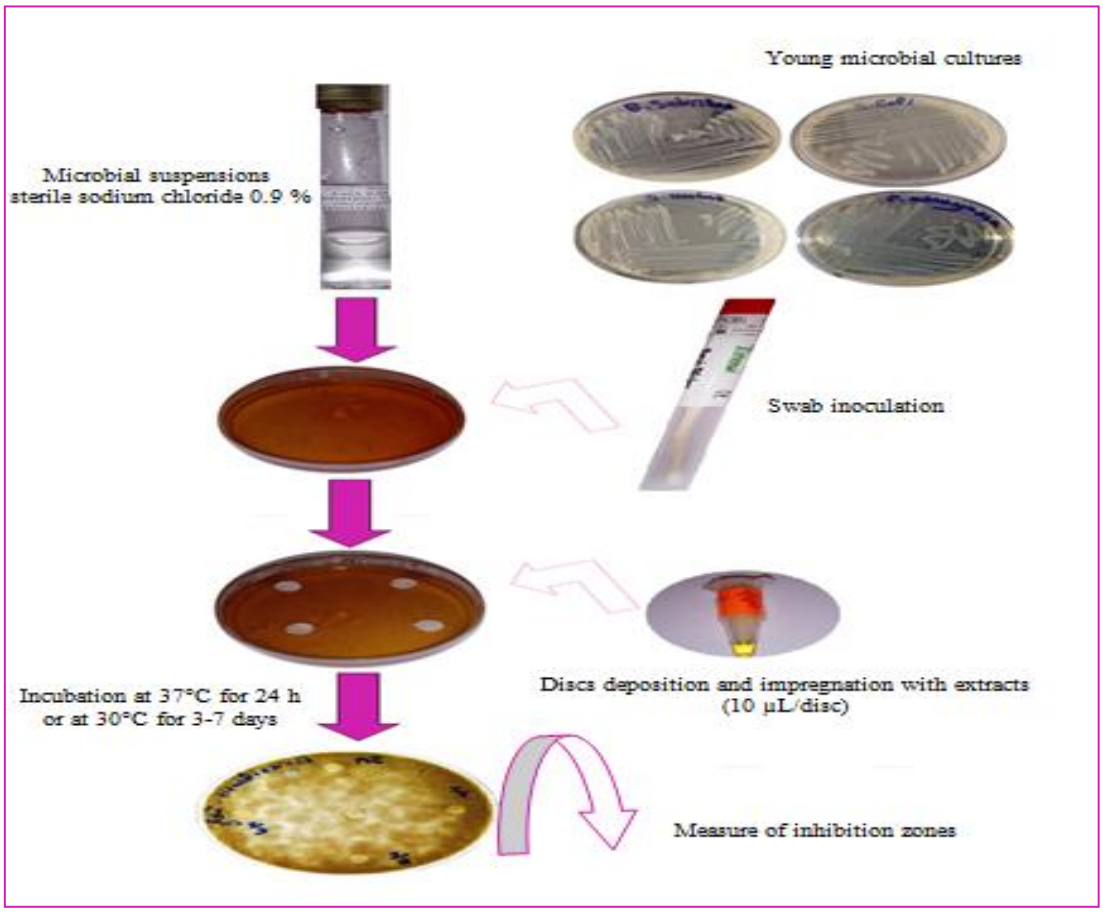


Figure25: Agar disk-diffusion method (original 2023)

2.5.3. Cytotoxicity assay

Medicinal plants are a good source of novel therapeutic drugs, due to the phytochemicals molecules. Artemia, commonly known as brine shrimp, is a tiny halophilic invertebrate belonging to class Crustacean, which plays an important role in saline aquatic and marine eco-systems.

Besides its usage in aquaculture, it is also highly valued for its application in toxicity detection and it is used in areas such as ecology, physiology, ecotoxicology, aquaculture and genetics. Furthermore, Artemia based lethality assay (brine shrimp lethality assay, BSLA) is rapid, convenient and low cost. Presently, brine shrimp lethality assays are enormously employed in research and applied toxicology (Ntungwe *et al*., 2020).

BSLA (*Artemia salina*) test has been developed for toxicity, testing various concentrations of extracts from the plant *C. caeruleus* according to the method of (Quazi *et al*., 2017). The nauplii were exposed to different concentrations of plant extracts for 24 h. The number of mobile nauplii was calculated for the effectiveness of the extract. It is a simple, cost effective and require small amount of test materials.

2.5.3.1. Hatching of *Artemia salina*

In order to prepare Artemia salina, we mixed water with some grams of table salt and pouring it into the rectangular container. Then, the tip of an air pump airline was placed at the bottom of the container to maintain proper aeration. Then, some grams of Artemia salina eggs was added in the water surface level of the container and mixed them with the water. Next, a light (60–100-watt bulb) was switched on a few inches away from the container. After 20-24 hours, the nauplii were hatched. Additionally, the eggs and nauplii were observed during this all time. After 24 h, we collected the hatched nauplii, separated It from the empty egg and transferred 10 nauplii to a petri dish using a syringe (Figure 26).

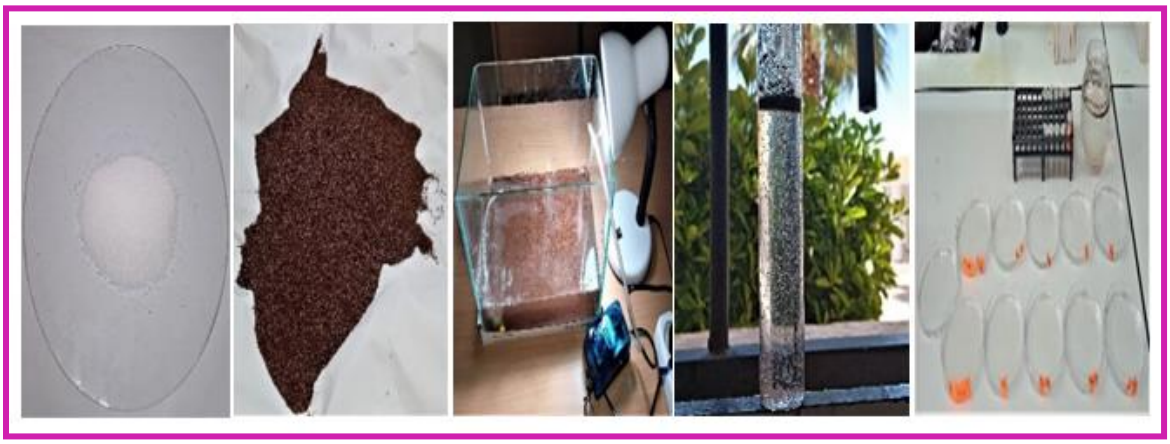


Figure 26: Steps of hatching and collected of BSLA (Original, 2023).

2.5.3.2. Toxicity testing

The nauplii were exposed to different concentrations of *Cc* extracts and. Then, the number of survivors and dead nauplii was counted and the percentage of death after 24 hours was calculated (Figure 27). The concentration of killing fifty person of larvae (LC_{50}) was determined from the graph.

Figure 27: Toxicity testing of extracts against *Artemia salina.* (Original, 2023).

The percentage of lethality of the nauplii for each concentration and negative control was calculated using the following formula:

%Death = Number of dead nauplii/ Number of dead nauplii + Number of live nauplii x 100

2.5.3.3. Microscopic observation of *Brine shrimp*

The biological study of BSLA involves optical microscopic observation to identify and count individual *Artemia salina* organisms based on their developmental stage (Figure 28).

Figure 28: Optical microscopic observation of BSLA (Original, 2023).

RESULTS AND DISCUSSION

1. Extraction yields

The extracted yield is defined as the ratio of the amount of plant material extracted with respect to the amount of plant material used. The table shows the extraction yield of *C. caeruleus* by the decoction and infusion method. The yield of each extract is shown in Table 4.

Table 4: Extraction yield of *C. caeruleus* rhizomes extracts

Extracts	**Decoction extract**	**Infusion extract**
weight of the extract	3.90	4.20
Yield extraction (**%**)	19.05	21

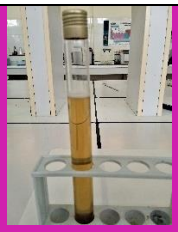
According to the results of Table, the variability of extraction yield depends on the extraction processes. Their yields decreased in the following order: Infusion> Decoction extract*;* which ranged from 19.05 for decoction to 21% for infusion extract. The results obtained show that the highest yield is mentioned by the infusion extract. The extraction yield of *C. caeruleus* extracts obtained in this study is larger to those obtained by the previous scientific report of Saffidine *et al*. (2013) with a value ranging of 15.2-16.2 % from alcoholic extracts of the same plant, collected in same region. The variation in content may be due to several factors in particular the degree of maturity of the *C. caeruleus,* the interaction with the environment (climate, soil), the time of harvest and method of extraction. Additionally, this could be attributed to the extraction solvent employed (Saffidine *et al*,. 2013).

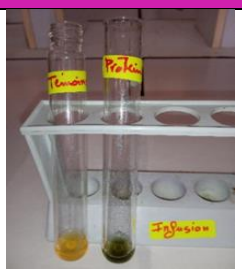
2. Phytochemical evaluation

2.1. Qualitative phytochemical analysis

The phytochemical screening results can gain insights into the chemical composition of the plant extract and its potential pharmacological effects and medicinal use. The results of phytochemical screening provide also information about the presence or absence of various classes of secondary metabolites in a plant extract include polyphenols, flavonoids, hydrolysable tannins, condensed tannins, quinones, anthraquinones, terpenoids, alkaloids, saponins, sugars and proteins. It is important to note that further analysis and characterization of these metabolites are usually required to identify and quantify specific compounds of interest. The phytochemical screening of the roots of *C. Caeruleus* was mentioned in (Table 5).

Table 5: Phytochemical screening of different extracts from *C. caeruleus* rhizomes.

Metabolites Results	**Extracts**			
	Decoction	**Infusion**	**Decoction**	**Infusion**
Saponins	+++	+++		
Polyphenols	+++	+++		
Flavonoids	-	-		
Terpenoids	++	++		
Anthraquinones	-	-		
Reducing sugars	+++	+++		

Free quinones	+++	+++	 
Condensed tannins	+++	+++	 
Hydrolysable tannins	+++	+++	 
Proteins	-	-	 

(-) absent; (++) presence; (+++) strong presence.

The results obtained from the phytochemical analysis of the unpeeled rhizomes of *C. caeruleus* revealed the richness of plant on saponins, phenolic, free quinones, condensed tannins, hydrolysable tannins, reducing sugars, and presence of the terpenoids, for the tow extracts. On other hand, flavonoids, anthraquinones, proteins are negative. Terpenoids, tannins, saponins, quinones, proteins and glycosides detected in plant extracts were in agreement with those obtained by Dahmani *et al*. (2018) and Amari *et al*. (2020) from the rhizomes aqueous extracts from C. *caeruleus* harvested at Sidi Lakhder, Mostaganem region (Amari *et al*. 2020) and from the methanolic extracts of the same plant harvested at Draâ El Mizan, TiziOuzou region (Dahmani *et al*,. 2018), in which the presence of flavonoids

compounds were also reported. The flavonoids absence in infusion and decoction extracts is also contradict the strong presence of them in aqueous extract from C. *caeruleus* harvested at Baghlia, Algeria, which is indicted by Benhamou and Fazouane. (2013).

This difference can be explained by the climatic factors such as rainfall, temperature, and wind strength, as well as the biological nature of the soil in each region (Saffidine *et al*,.2013). Additionally, the availability of nutritional elements that also play an important role in the growth of different parts of the plant and the presence of certain biological substances (Dahmani *et al*., 2018). However, it can also be attributed to the nature of the solvent used during extraction and the extraction method employed (Dahmani *et al*., 2018).

The presence of these secondary metabolites in the studied plants explains their strong therapeutic power. Therefore, these results justify the wide use of this plant in traditional medicine by the local population. Indeed, the saponins, phenolic compounds, free quinones, condensed tannins, hydrolysable tannins, terpenoids possess several beneficial properties including antimicrobial, antioxidant, anti-inflammatory, anti- ulcer and many others (Umesh et al., 2018).Several biological effects have been attributed to them such as tannins, with their ability to form complexes with proteins, exhibit anti-diarrheal, antibacterial, and antifungal properties and contribute on strengthen blood vessels (Daing *et al*., 2017; Usman *et al*., 2018), also, saponins with their antioxidant, antifungal, antiviral, immunostimulant, hypocholesterolemic, and anticancer properties(Francis *et al*., 2002).

2.2. Quantitative phytochemical analysis

2.2.1. Determination of total polyphenols and flavonoids

Quantitative tests were performed to determine the concentration of the phytochemicals in the extracts. Polyphenols and flavonoids were quantified using spectrometric methods. The total phenolic contents (TPC) and total flavonoids contents (TFC) of decoction and infusion extracts of the unpeeled rhizomes of *C. caeruleus* were evaluated employing the Folin-Ciocalteu's reagent and aluminum chloride respectively. Results are shown in (Table 6).

Table 6: Polyphenols and flavonoids contents of decoction and infusion extracts of *C. caeruleus*.

Extracts	TPC (µg GAE/mg extract)	TFC(µg QE/mg extract)
Decoction extract	**27,46±1,34**	**6,29 ±1,56**
Infusion extract	**33,67 ± 0,85**	**6,92 ± 0,28**

The data are expressed as the mean ± standard deviation of triplicate samples

The data of Table 5showed that the amount of phenolic and flavonoid compounds varied significantly with the different extracts. It is noted that the total phenolic content is (33.67 ± 0,85 mg GAE/g extract) for the infusion extract is higher than that of the decoction extract, which is (27.46±1,34 mg GAE/g E).

Our results in terms of phenolic compounds and flavonoids were compared with those reported by various authors, which used in their studies the same plant, variable extraction methods, changeable vegetal parts and different extraction solvents, such as Dahmani *et al* .(2018) and Amari *et al*.(2020)**,** using the methanolic extract, which were registered values of 57.91±0.57 and 13.08 ± 0.22 (mg GAE/g extract), respectively for phenolic compounds, at the same time as the value of 5.02 ± 0,55 (mg QE/g extract) was indicated by the study of and Amari *et al*.(2020)for flavonoids content. Thus, Arroudj, *et al*.(2017), who used the hydromethanolic, ethanolic and aqueous extracts of *C. caeruleus* for their biological activities exhibited the values rangedfrom11.80 to 17.56 (mg EAG/g extract) for phenolic compounds and others values ranged from3.48 to 4.40 (mg QE/g extract) for flavonoids.

Additionnally, Baghiani *et al., (*2010) using it several sub-fractions such as methanol, ethyl acetate, chloroform and aqueous extracts showed the values ranged between10.35-75.71 (µg EAG/ mg extract) and between 1.50-9.98 (µg QE/ mg extract) for phenolic compounds and flavonoids, respectively . It is clair that the total phenolic and flavonoids of plant extracts obtained by previous studies showed very low quantity, which agree with the results obtained by our study.

This difference may be attributed to the extraction method, the harvesting region, as well as the type of solvent used. In fact, the solubility of phenolic compounds depends on their degree of polymerization, their interaction with other constituents, and the type of solvent employed (Bentoura *et al*., 2021). The phenolic content of a plant also depends on a number of intrinsic and extrinsic factors, particularly climatic conditions, maturity at harvest, and storage conditions (Dahmani *et al*., 2018).

3. Biological activities

3.1. In vitro antioxidant activity

3.1.1. DPPH radicals-scavenging assay

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants (Miri, 2015).The IC_{50} free radical inhibitory values of DPPH in aqueous extract for the *C. caeruleus* represented in Table 7. Many plants extracts exhibit efficient antioxidant properties due to their phytoconstituents. In the present study, reduction of DPPH radical observed at 517 nm by the decoction and infusion extract of *C. caeruleus*.

Table 7: IC_{50} values for the studied samples

Studied samples	**Decoction extract**	**Infusion extract**
IC_{50}mg /ml	0.19 ± 0,02	0.16 ± 0,02

Through the table we find that the infusion extract detected the highest radical scavenging activity (0.16 ± 0.02mg /mL), which represented the lowest power comparing to IC_{50} indicated the ascorbic acid which is equal to (0.04±0.001mg/mL). Our extracts may have a medium activity antioxidant. According to the study carried out by Baghiani *et al., (*2010), the best 50% inhibition of DPPH radical was obtained with chloroform fraction, which exhibited notable antioxidant potential (IC_{50} at 53.26 ± 1.74 µg/mL) followed by methanol extract (IC_{50}, at 187.38 ± 2.36 µg/mL),whereas the aqueous and ethyl acetate fractions showed a weak activity with an IC50 of (271.12 ± 3.67 µg/mL) and (291.87 ±4.98 µg/mL), respectively. Our results disaccorded with those obtained by Baghiani *et al*. (2013) for DPPH radical scavenging assay.

3.2. Antimicrobial activity

We conducted antimicrobial activity against 8 strains of microorganisms, including 4 bacteria (2 Gram-positive and 2 Gram-negative), 3 fungal strains and one yeast.The results of the diameters of inhibition zones of antibiotic and extracts from the rhizomes of *C.caeruleus*are shown in the Table8, illustrated also in Figure 29, 30 and 31.

3.2.1. Antibacterial activity

Through the obtained results, the *B. Subtilus* strain was found to be sensitive to the decoction and infusion extracts of *C. caeruleus* rhizomes, with an inhibition zones diameter of 9.31 ± 0.43 and 11.89 ± 4.39 mm, respectively. However, *B. Subtilus* is the most sensible strain to infusion extract.

Our results are agree with those showed by previous studies of Saffidine *et al*. (2013); Dahmani *et al*. (2018), those have reported that the growth of *B. subtilus* is sensible to various extracts of *C.caeruleus*. On the other hand, The results of previous Algerian study established by Dahmani *et al*. (2018) about the antibacterial activity of the methanolic extract of aerial parts of *C. caeruleus* from Algerian provenance against the growth this gram positive strain exhibited similar inhibition zones diameter of 11.33 mm. Thus, Saffidine *et al*. (2013) indicated that the antibacterial power of roots methanolic extracts of *C caeruleus* extracts against *B. subtillus* exhibited maximum inhibition value of 20 ±1.5 mm.

Likewise, sensitivity to the tested extract was noted only in the decoction extract for the *S. Aureus* with value of 15.02 ± 0.02 mm. In the previous study of Saffidine *et al*. (2013), the S. *aureus* strain exhibited a high sensitivity to the aqueous fraction with an inhibition zone diameter of 20 mm and to the ethyl acetate fraction with an inhibition zone diameter of 7 mm. Furthermore, the study of Laoufi *et al*. (2018) noted the bacterial potential of methanolic extract against *Staphylococcus pneumonia* with a maximum inhibition zone diameter of 24.67 mm. In general, significant and variable antibacterial effects were obtained with the extracts on Gram-positive (Figure 29). The sensitivity of Gram positive bacteria is reported by Chi and Holo. (2018).

Moreover, all extracts of plant appear to be more active against Gram-positive bacteria than Gram-negative bacteria. This behavior is not surprising because Gram-negative strains

possess intrinsic resistance, which is related to the nature of their outer membrane (Boudjema *et al*., 2018; Bakli, 2020).

The difference in the structure of the bacterial cell wall plays an important role in the susceptibility of bacteria. According to several authors, Gram-negative bacteria have an outer membrane composed of lipopolysaccharides and that limits the diffusion of hydrophobic compounds (Bakli, 2020). Additionally, the periplasm contains enzymes, which are able of breaking down foreign molecules introduced from the outside, which generally makes these bacteria less sensitive to plant extracts compared to Gram-positive bacteria (Bakli,2020).

Indeed, this outer membrane makes their surfaces highly hydrophilic, while the lipophilic ends of lipoteichoic acids in the cell wall of Gram-positive bacteria facilitate the penetration of hydrophobic compounds (Bakli,2020), such as tannins that can reach the cytoplasmic membrane and disrupt proton motive force, active transport, and coagulation of cellular content (Bakli, 2020).

The decoction and infusion extracts were noted the absence of antibacterial activity against *E. coli* Gram negative bacteria(Figure 29). On the other hand, Saffidine and his collaborators. (2013) observed inhibition zones diameter of 9 and 11 mm, by the aqueous and ethyl acetate fraction. Another study, realized by Dahmani and his collaborators. (2018) showed inhibition zone diameter of 12.67 mm, by the methanolic extract. For the second bacteria Gram negative, including *P. aeruginosa*, our results showed also inexistence of zone of inhibition diameter. These findings are consistent with those of Saffidine *et al*. (2013) and (Dahmani *et al*. (2018).

The results showed also that all the bacterial strains were very sensitive to the used standard as gentamicin (0.12 mg/mL). The largest inhibition zone was noted by *S.aureus* (35.18 ± 0.20mm), followed by *P. aeruginosa* and *E. coli*, with the respective zones of 32.48 ±3.08 and 32.73 ± 1.57 mm. It is also noted that *B. subtilus* strain is the less sensitive to the antibiotic compared to other strains, who showed the smallest inhibition zone of 30.06 ± 1.05mm. The following (Figure 31) revealed the effect of Gentamicin on tested bacterial strains. Our results accorded with those obtained by Saffidine et al. (2013) for *S.aureus, P. aeruginosa* strains.

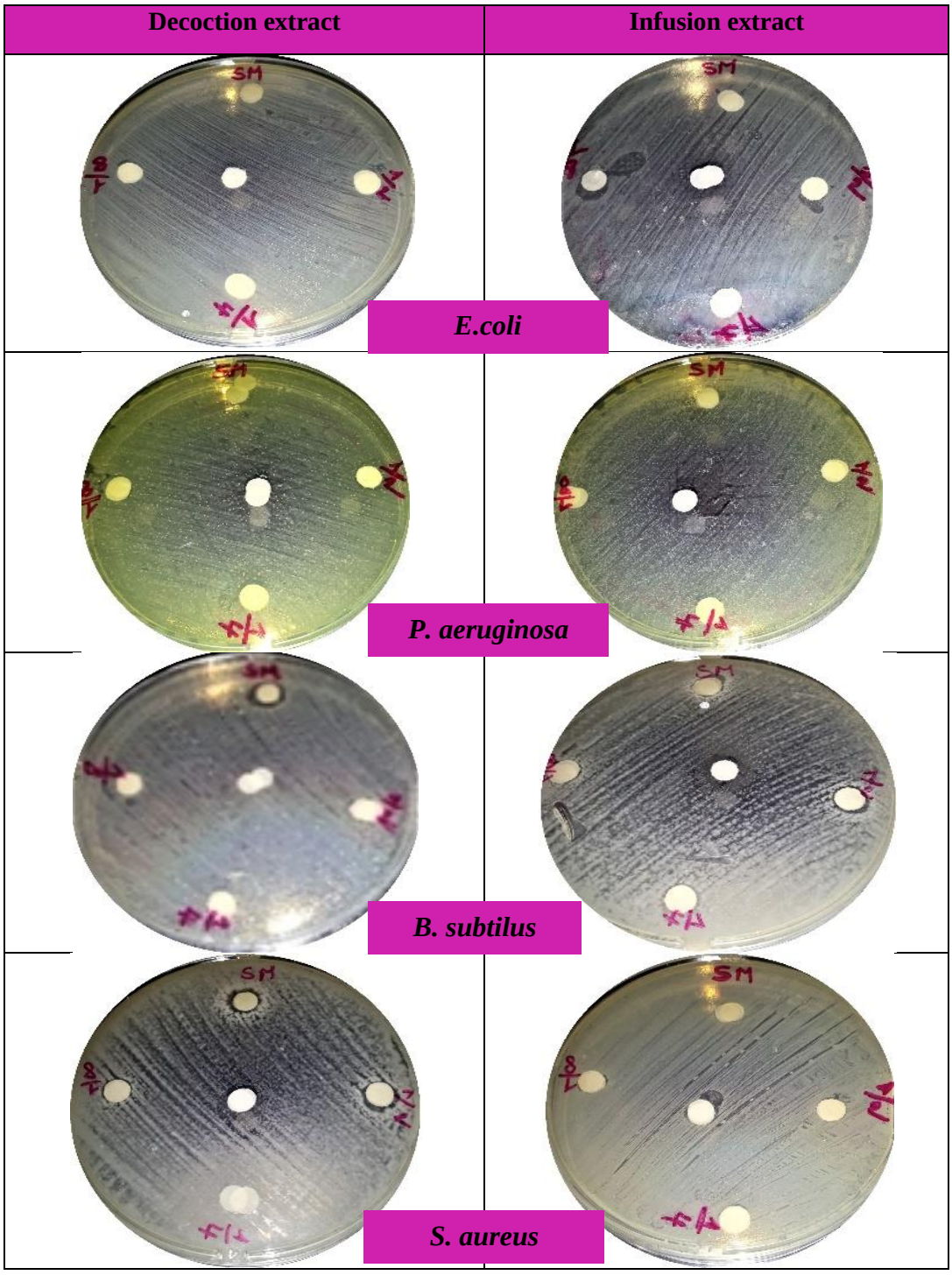


Figure 29: Antibacterial activity of extracts against bacterial strains (Original, 2023).

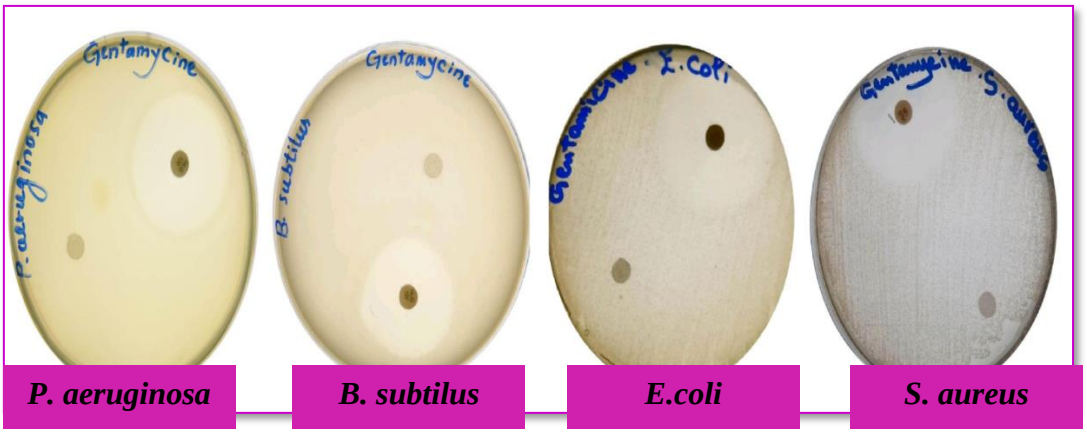


Figure 30: Antibacterial activity of gentamicin against bacterial strains (Original, 2023).

3.2.2. Antifungal activity

The results of the antifungal tests of the extracts from the rhizomes of *C. Caeruleus* L. are shown in the Figure 32. Through the results obtained we find that the microorganisms' strains of *A. brasiliensis, A. niger, A. flavus* and *C. albicans* were all resistant to the extracts of decoction and infusion of the rhizomes of *C. caeruleus* L plant.

The results obtained by previous study of Saffidine *et al*. (2013) indicated the absence of antifungal activity against *C. albicans*, using the aqueous and it presence using ethyl acetate fraction of *C. caeruleus* L plant, with inhibition zone diameter of 25 mm. Another study realized by Laoufi *et al*. (2018) indicated that the *A. niger strain* was sensible against methanolic extract with a inhibition zone diameter 10 mm. Our results accorded with those obtained by Saffidine *et al*. (2013) for C. *albicans*.

The results showed also that all the fungal strains were very sensitive to the used standard as vorten (1 mg/mL). The largest inhibition zone was noted by (29.32 ± 4.75 mm), followed by *A. niger* and A. *flavus*, with the respective zones of17.17 ± 1.84 and 12.76 ± 1.32 mm. It is also noted that *C. albicans* strain is the less sensitive to the antibiotic compared to other strains, who showed the smallest inhibition zone of 8.81 ± 0.08mm. The following (Figure) revealed the effect of vorten on tested fungal strains. Regarding to the others antifungal used, Mycozan, Fongenal were showed no zone of inhibition against *A.brasiliensis*, *A. flavus*, *A. niger* and *C. albicans*.

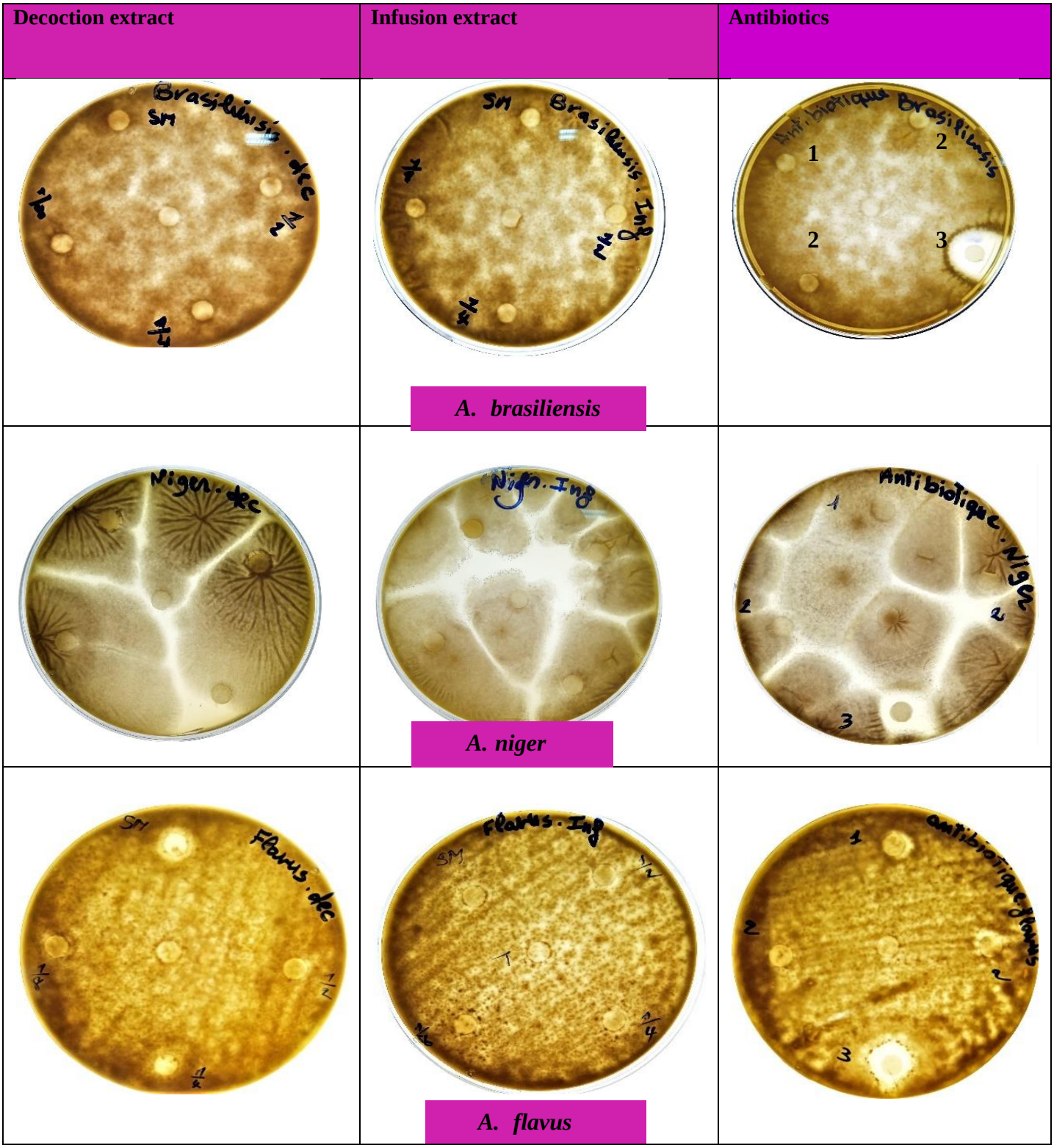


Figure 31: Antifungal activity of extracts and antibiotics. 1) Mycozan; 2) Fongenal; 3) Verten (original, 2023).

Table 8: Diameter of inhibitions zones of C. *caeruleus* extracts against tested microbial strains.

Extracts	Concentration (mg/mL)	Microorganisms' strains/ diameter of zone (mm)							
		E. coli	***P. aeruginosa***	***B. subtilus***	***S. aureus***	***A. brasiliensis***	***A. niger***	***A. flavus***	***c. albicans***
Decoction	100	/	/	9.31 ± 0.43	15.02 ± 0.02	/	/	/	/
	50	/	/	8.48 ± 0.68	9.04 ± 0.06	/	/	/	/
	25	/	/	6.35 ± 0.50	8.45 ± 0.64	/	/	/	/
	12.5	/	/	6.2 ± 0.28	7.24 ± 0.33	/	/	/	/
Infusion	100	/	/	11.89 ± 4.39	/	/	/	/	/
	50	/	/	8.5 ± 0.70	/	/	/	/	/
	25	/	/	7.35 ± 0.91	/	/	/	/	/
	12.5	/	/	6.66 ± 0.47	/	/	/	/	/
Gentamicin 0.12		32.48 ± 3.08	32.73 ± 1.57	30.06 ± 1.05	/	ND	ND	ND	ND
Mycozan 1		ND	ND	ND	ND	/	/	/	/
Fongenal 1		ND	ND	ND	ND	/	/	/	7,49±0,24
Verten 1		ND	ND	ND	ND	29.32 ±4.75	17.17 ± 1.84	12.76 ± 1.32	8,81±0,08

3.3. Cytotoxicity assay

❖ **Mortality percentage and fifty lethal concentration**

BSLA can be used to check the toxicity of various plants extracts. This method is very simple, rapid, inexpensive and attractive method. The present investigation was carried out to determine the toxicity against *A. salina* by using different tested extracts (Banti *et al*., 2021). The number (N) of survivors and vials was calculated after24 h. For each dilution, the percent mortality(%M) of brine shrimp was intended at concentrations of 100, 50, 12.5 and 6.25 mg/mL. The results were shown in (Table 9). The extract was safe up to 12.5 and 6.25 mg/mL, exhibiting zero percent mortality. No mortality was found in negative control. Thus, the percent mortality of the brine shrimp larvae increased with increase in concentration of extract.

Table 9: Cytotoxic of different extract the rhizomes of *C. caeruleus* plant

Extracts	Concentration (mg/mL)	N of live nauplii	N of dead nauplii	% M	LC_{50}(µg/ mL)
Decoction	100	7	3	30	**565,8**
	50	7	3	30	
	25	9	1	10	
	12.5	10	0	0	
	6.25	10	0	0	
Infusion	100	8	3	27.27	**636,2**
	50	9	3	25	
	25	9	1	10	
	12.5	10	0	0	
	6.25	10	0	0	
Negative control	/	10	10	0	/

After 24 h, the lethal concentration (LC_{50}) for each tested extracts was calculated by regression equation (Figure 33). The lethal concentration (LC_{50}) value is the concentration of the extract, signified by the produced death in half (Bravalia *et al*., 2012).

The cytotoxicity effect was quite safe if the LC_{50} value was higher than 1000 µg/mL and which suggests that may be used as non cytotoxic drugs. Thus, if it much lower than 1000 µg/mL which suggests that it is quite toxic and may find use as a cytotoxic drug (Kamanja *et al*., 2018). In the other hand, if LC_{50} value ranges of 100 - 500 µg/mL are considered as medium toxic (Suneka et Manoranjan, 2021). It revealed that decoction extract is near to moderate toxic towards *Artemia salina* leach cells with LC_{50} of 565.8 µg/mL, followed by the infusion extract with LC_{50} of 636.2 mg/mL. The extracts showing a near average LC_{50} (> 1000) and hence further investigations would also be needed.

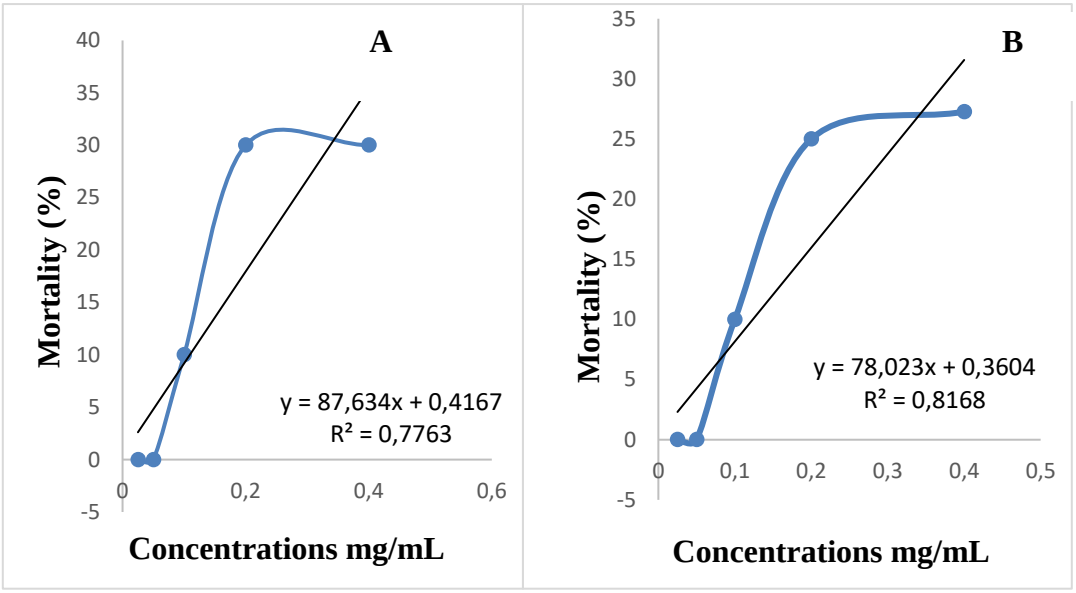
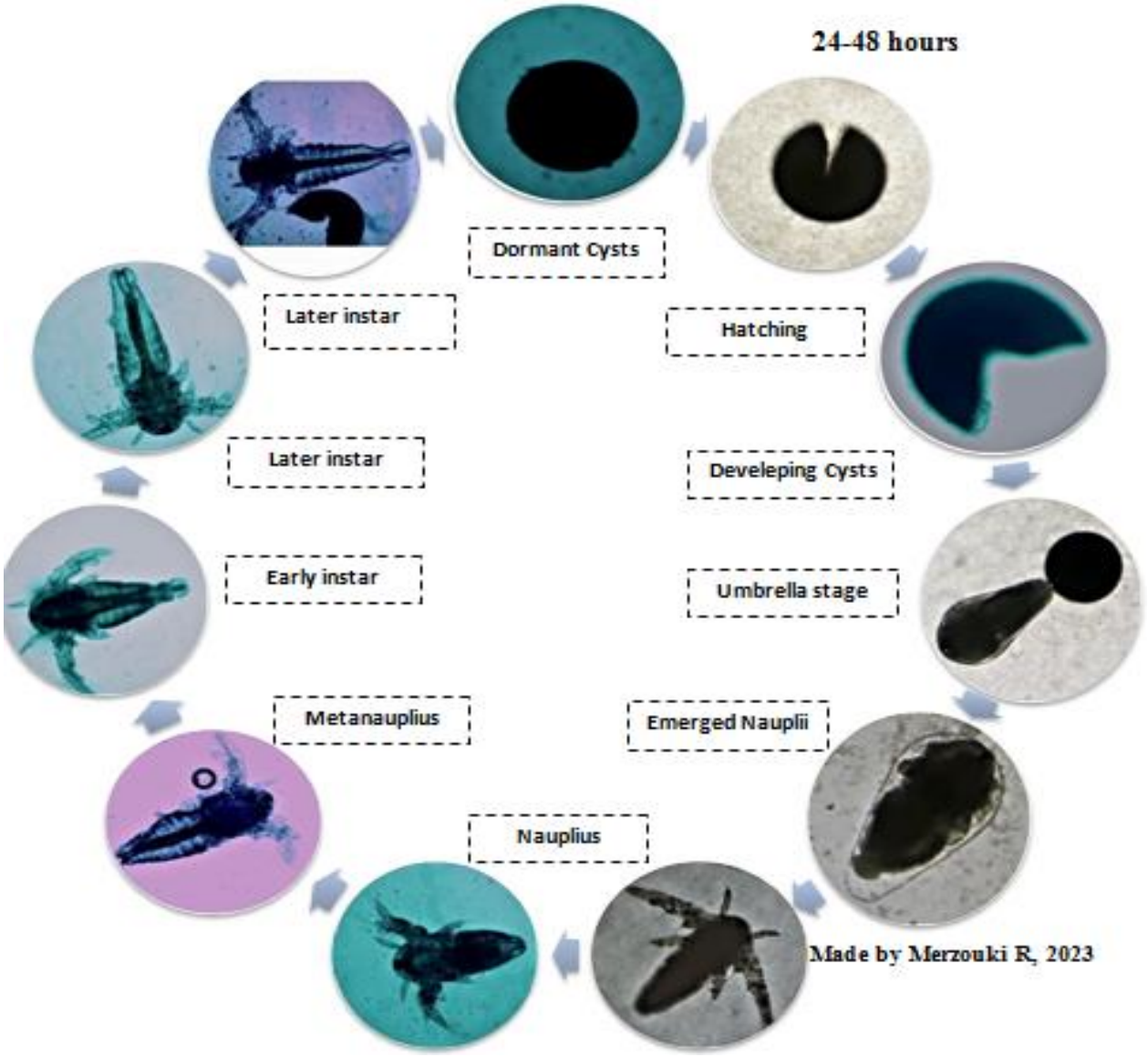


Figure32 : Cytotoxic effect of decoction **(A)** and infusion **(B)** extracts against *Artemia salina*.

❖ **Microscopic observation of Brine shrimp *Artemia salina***

According to the results observed under microscopic, the life cycle of nauplii refers to the early developmental stages of various aquatic organisms, particularly crustaceans. Here is a general overview of the life cycle of nauplii, which is shown in figure 34.

A.salina begins its life cycle as cysts, which are dormant eggs with a protective outer shell. These cysts can withstand extreme environmental conditions such as desiccation and high salinity. When the conditions become favorable, such as the presence of water and appropriate salinity, the cysts hatch into nauplii. Hatching is triggered by the rehydration of the cysts (Piper, 2018).



Figur 33 : Life cycle of *Artemia salina* development from Egg to later instar (Original, 2023).

The Nauplii are the first larval stage of *A. salina*. They are small, translucent, and possess simple appendages for swimming and feeding. Nauplii feed on microscopic algae and organic matter in the water (Piper, 2018). The nauplii of the brine shrimp are considered as a simple and suitable model system for acute toxicity tests. The nauplii feature a higher sensitivity to toxic agents compared to the adult Artemia (Banti *et al*., 2021).

Next and after several molts, the nauplii develop into meta nauplii. Meta nauplii have more advanced appendages and begin to resemble adult Artemia. Finally, the meta nauplii

continue to molt and grow, gradually developing into juvenile Artemia. During this stage, they become more active and develop more complex structures, including fully formed appendages (Piper, 2018).

CONCLUSION

Conclusion

The most dangerous diseases that affect human life are pathogenic microorganisms and radicals, which make them interesting to investigate in order to find solutions for these diseases through the discovery of antioxidants and natural antimicrobial agents from active principles of medicinal plants.

This study aimed to evaluate the biological activities and cytotoxicity of *Carthamus caeruleus* rhizome extracts from the Setif region. Two extraction methods, decoction and infusion, were used.

Phytochemical analysis revealed the richness of plant on saponins, phenolic, free quinones, condensed tannins, hydrolysable tannins, reducing sugars, and presence of the terpenoids, for the tow extracts. On other hand, flavonoids, anthraquinones, proteins are negative.

Total polyphenols were quantified using the Folin-Ciocalteu colorimetric method. The results showed That the infusion extract of the roots had polyphenols content of approximately 33.67 ± 0.85 µg GAE/mg, while the decoction extract displayed a content of 27.46 ± 1.34 µg GAE/mg. Flavonoids were quantified using the aluminum trichloride ($AlCl_3$) method, revealing a concentration of 6.29 ± 1.56 µg QE/mg for the decoction extract and 6.92 ± 0.28 µg QE/mg for the infusion extract, the infusion extracts exhibited higher levels of polyphenols and flavonoids compared to the decoction extracts

The decoction extract of rhizomes exhibited the better antioxidant activity by 2,2-diphenyl-l-picrylhydrazyl (DPPH) scavenging test (IC_{50} =190 µg/mL) compared to that indicated the ascorbic acid which is equal to (0,04±0,001mg/mL) with low antioxidant effect. This low activity is related to the presence of different secondary metabolites.

Furthermore, results showed that the extracts exhibited the inexistence of antimicrobial power against certain pathogens include fungi, yeast and bacteria. The *C. caeruleus rhizomes* extract showed highest antibacterial activity especially against *S. aureus*

and *B. luteus* with diameter of inhibition zones of 15.02 and 11.89 mm, respectively, which noted only in the decoction extract. Additionally, all the bacterial strains were very sensitive to the used standard as gentamicin and the largest inhibition zone was noted against *S. aureus* of 35.18 ± 0.20 mm.

It is also noted that all fungi and yeast strains were resistants to the extracts of decoction and infusion of the rhizomes of *C. caeruleus* and the largest inhibition zone was noted against *A. brasiliensis* of 29.32 ± 4.75 mm, noted by used standard as vorten (1 mg/mL).

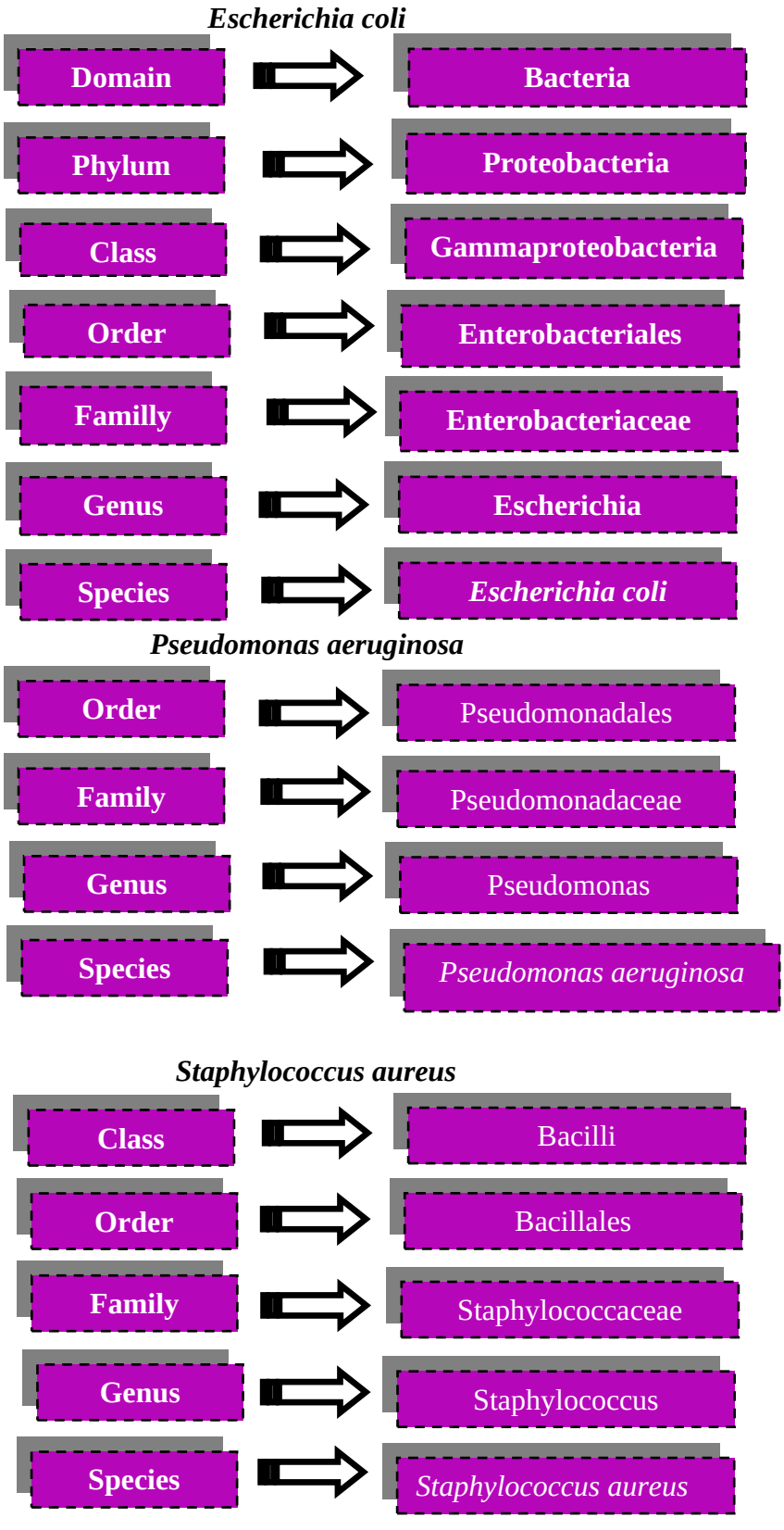
For the brine shrimp cytotoxicity potential of the plant extracts at concentrations 100, 50, 12,5 and 6.25 mg/mL, the extract was safe to 12,5 and 6.25 mg/mL, exhibiting zero percent mortality. Thus, The present study showed that the extracts of selected plant have active compounds with cytotoxicity against *Artemia salina*.. It revealed that the lowest value was obtained by infusion extract with LC_{50} of 636.2 mg/mL.

These finding suggest that *Carthamus caeruleus* rhizome extracts could be considered as a potential source of beneficial bioactive compounds for traditional medicine or the development of new drugs. However, further studies are required to explore the mechanisms of action.

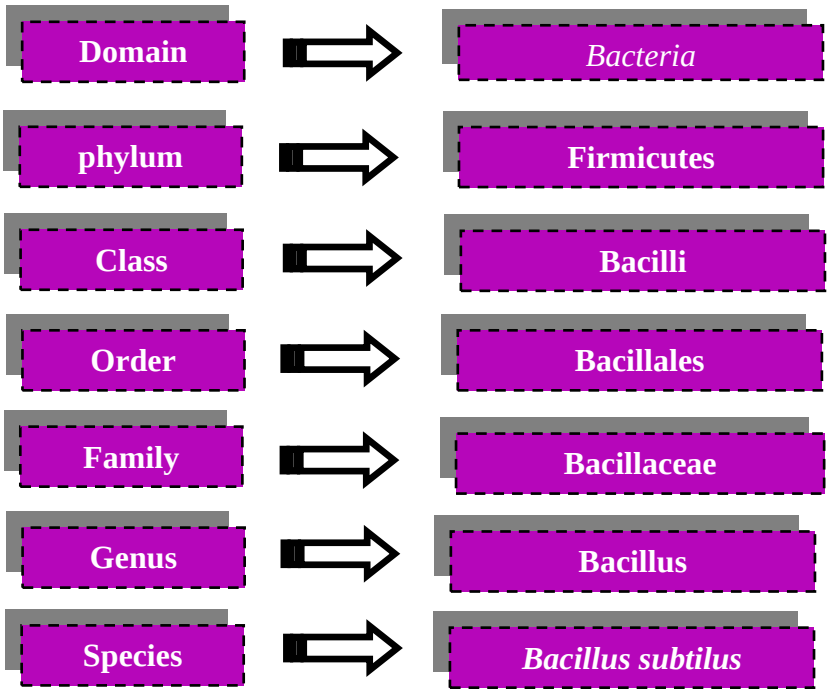
APPENDICE

Appendice1

Classification of tested bacteria

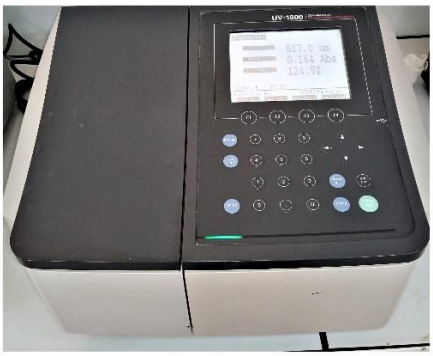
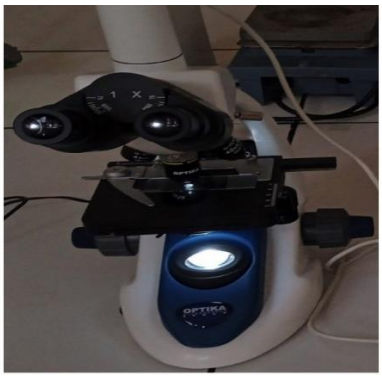


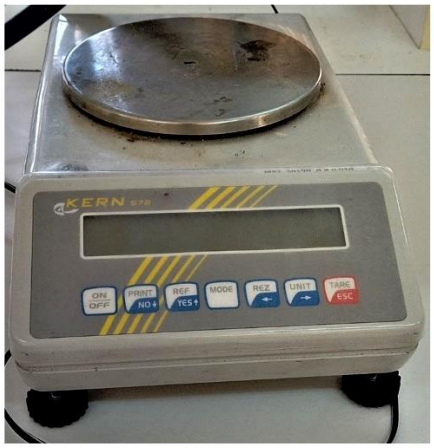
Bacillus subtilus



Appendice 2

Materials and different equipments

	
Microwave oven	**Spectrophotometer**
	
Oven	**Optical microscope**
	
Pressure balance	**caliper**

	
Balance	**Bunsen burner**
	
Magnetic stirrer	**Vortex**
	
Autoclave	**Water bath**

Appendice3

Steps of antibacterial activity evaluation

The first day

- **Preparation of culture media**

38 grams of Muller Hinton agar were added to 1 liter of distilled water, 65 grams of Sabouraud agar powder were added to 1 liter of distilled water, and the solutions were placed on a heat source until its reached boiling. The solutions were then poured into sterile bottles, which were placed in an autoclave for 1 hour and a half.

Figure 34: Preparation of culture media.

- **Reactivation of strains**

The reactivation of strains was performed in sterile BN-containing tubes and then incubated at 37°C for 24 hours to optimize their growth.

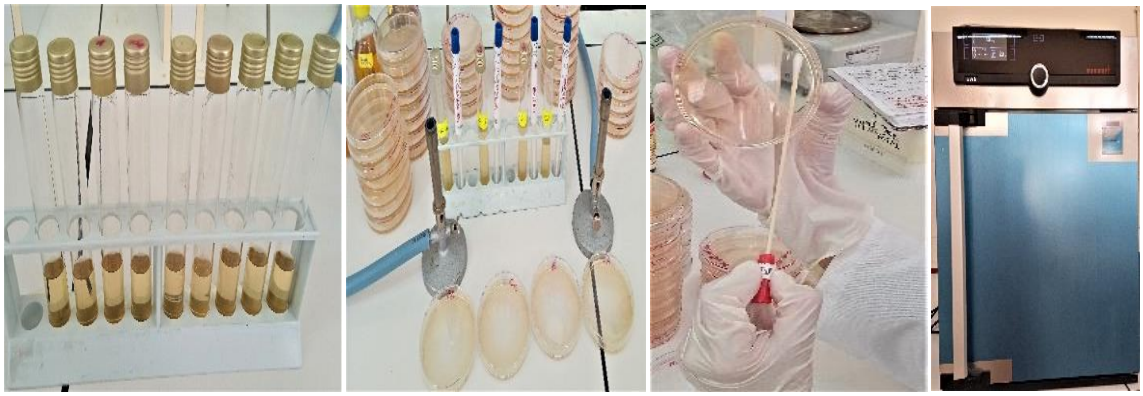


Figure 35: Reactivation of bacterial strains

The second day

- **Transplanting of microbial strains**

Bacterial species were streaked using the streaking method and then incubated at 37°C in the incubator to obtain colonies that will be used for inoculum preparation. the fungal cultures were prepared to be used in the antimicrobial assay. Inoculate the fungal into a nutrient Sabouraud agar and incubate them 7 days at the 30C° temperature to allow for sufficient growth.

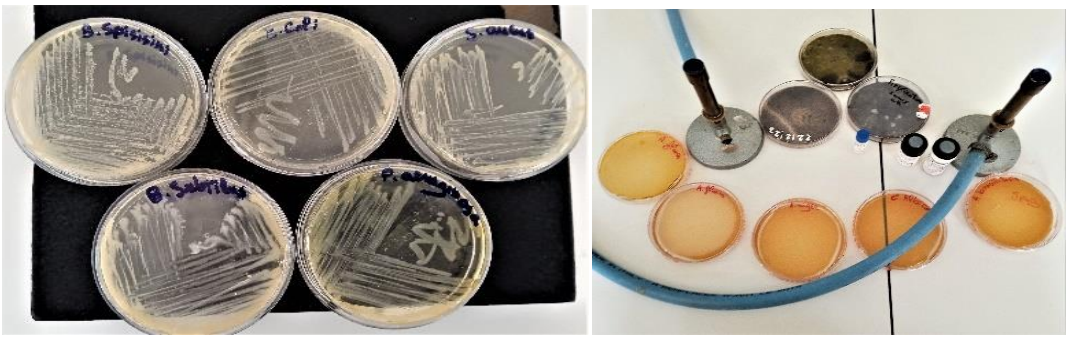


Figure 36: Transplanting of microbial strains

- **Preparation of plant extracts**

A series of dilutions (1/2, 1/4, and 1/8) of the extracts in dimethyl sulfoxide (DMSO) was performed. The extracts from the rhizomes of Carthamus *caeruleus* were dissolved in sterile DMSO to prepare different concentrations using three successive half dilutions (1/2, 1/4, 1/8). The concentration of the stock solution was 100 mg/ml.

Figure 37: Preparation of plant extracts.

The third day

- **Inoculum preparation**

Using a platinum loop, pick a few well-isolated and identical colonies of each bacterial strain to be tested. Transfer the loop into 9 ml of sterile physiological saline solution (0.9% NaCl). The bacterial suspension is thoroughly mixed until homogeneous, and its

absorbance should be between 0.08 and 0.10 at 625 nm. The inoculum can be adjusted by adding more culture if it is too weak or sterile physiological saline solution if it is too strong. After 7 days, several well-isolated colonies were picked using a platinum loop and inoculated into 10 ml of sterile physiological water to obtain an optical density of approximately 0.08 to 0.1.

Figure 38: Preparation of bacterial inoculum

- **Inoculation and disc deposition**

Inoculation and disc deposition were performed as follows: A sterile swab was dipped into the bacterial suspension and then streaked across the entire surface of the agar (MH). This process was repeated three times, rotating the petri dish 60° each time. Finally, the swab was rubbed around the edge of the agar surface. Sterile Waltman paper discs with a diameter of 6 mm were placed onto the inoculated agar surface. Each disc was impregnated with 10 µL of each extract at different concentrations. Discs impregnated with 10 µl of DMSO were used as negative controls, and Gentamicin (1 µl/disc) was used as a positive control. Each test was repeated triplicate.

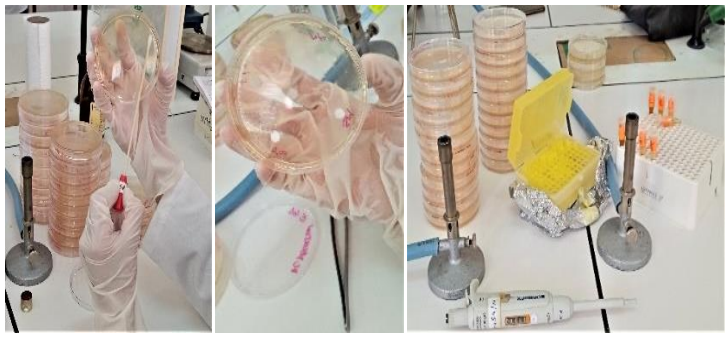


Figure 39: Inoculation and disc deposition

- **Incubation and measure**

Once the discs are placed on the agar, the Petri dish needs to be incubated at 37°C for 18 to 24 hours. After overnight incubation, the diameter of each inhibition zone (including the disc diameter) is measured in mm and recorded.

Figure 40: Incubation and measure of inhibition zones diameter on (mm)

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