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**THEME**

# *kinetics of Desert truffle Tyrosinase Inhibition by Euphorbia guyonianus hydromethanolic extract*

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***2019/2020***

## *Dedication*

*To the special people in my life, their willingness to help, the knowledge and support they provided, to the ones who always make me feel smart and unique I owe thanks to my dear parents*

*BenbehazSlimane and ChouchaFatiha*

*You are the light to my dark nights.*

*To my sister khedidja and brother mohamed I appreciate you and thank you for your encouragement and aid.*

*To my Brother in law Mechikel Ahmed yacine I am very grateful for your help.*

*To all relatives,friends and others who in one way or another shared their support,either morally, financially and physically,thank you*

*Last but not least to my beloved nephew Iyad*

***B.Messaouda***

# *Dedication*

*There are not enough words to describe how thankful I am to all of you. Parents, sister brother and professor I dedicated my precious master memory to each one of you.*

*Dear my father Abdelhamid and my mother Khadidja*

*Thank you for your endless amounts of love, thank you for giving me the opportunity to do what I want. Thank you for all your support I promise to make you proud of me*

*Dear my sister Wafa :*

*My sister, you are my inspiration, the one that makes me believe I can be the best person. You are adorable, you are the one I will keep holding on to. Thank you sister to blessed our family with a little angel Sally.*

*Dear my brother Ahmida:*

*A brother is priceless, a brother is a rare gem, whether he is older or younger than you.Thank you my brother you been always ready to play superhero for me to make me happy, you simply amazing and important person in my life.*

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## *LIST OF ABBREVIATIONS*

**CuA\CuB:** copper A\copper B

**Cu°:** reduced copper

**DMSO:** Dimethyl sulfoxide

**DOPA:** dihydroxyphenyl alanine

**DQ:**Dopaquinone

**Edeoxy:** Deoxy-tyrosinase

**EC:**Enzyme commission

**Emet:** Met-tyrosinase

**Eoxy:** Oxy-tyrosinase

**ESI:** Enzyme-substrate-inhibitor

**His:** Histidine

**IC50:** Half maximal inhibitory concentration

**IUB:** International union of biochemistry

**KI**: Inhibitor constant

**Km:**Michaelis constant

**MCOs:** Multi-copper oxidases

**MOE:** Molecular operating environment

**MW :** Molecular Wight

**PPO :** polyphenol oxidase

**$R^2$:** Linear regression

**SD:** Standard deviation

**SDS**:Sodiumdodecyl sulfate

**T:** Tyrosinase

**UV:** Ultraviolet

**Vmax:** Maximal Velocity

**VOCs:** Volatile organic compounds

# *Summary*

# INTRODUCTION

Tyrosinase, also called polyphenol oxidase, is a multifunctional copper-containing metalloenzyme present in a broad variety of organisms. It catalyses the ortho-hydroxylation of monophenol molecules and can also catalyse the oxidation of ortho-diphenolic to produce ortho-quinones.

This enzyme is essential in the melanogenesis process, and also plays critical roles in the browning of food and in the development of insects (Chai et al., 2015).

Tyrosinase (E.C.1.41.18.1) is a common copper containing enzyme responsible for eye, skin, inner ear and hair melanisation as well as browning in fruits and vegetables, also known and reported under various names (polyphenoloxidase, phenolase, catechol oxidase, monophenol oxidase, O-diphenol oxidase and ortho-phenolase) based on substrate specificity (Gul Guven et al., 2017; Nuruddin and Al-Jasabi., 2016) due to its activity this enzyme has been considered as an important target for developing therapeutic agents for pigment disorders. Therefore, its inhibitors can be attractive in cosmetics and medicinal industries as depigmentation agents and also in food and agriculture industries as anti-browning compounds (Samaneh et al., 2019).

Due to the importance of tyrosinase inhibitors,searchers have been using plants from the entire world in order to develop new potent natural tyrosinase inhibitors which are safe but The Algerian flora specifically the Saharan diversity have never token the same attention. The species living in this environment usually offer molecules with activities interesting biological compounds. These compounds include to a large extent secondary metabolites, which have been particularly successful in therapeutics, one of the largest family found in this environment is Euphorbiaceae.

The Euphorbiaceae family is known to be cosmopolitan: of great botanical and chemical diversity. It offers several plants of economic interest. (Manga., 2004), and other widely used in traditional medicine. Among the

species representing this family in the Algerian Sahara, *Euphorbia guyoniana Boiss and Reut*.

Of our knowledge, *Euphorbia guyonianus* have been proved to be an efficient plant in various activities anti-inflammatory, anti-diabetic, anti-bacterial (Boumaza et al.,2018) and vasodepressor effects (El-Bassuony and Asian.,2007;Aiyelaagbe et al.,2007;Barla et al .,2006) but there is no studies concerning the effect of *Euphorbia guyonianus* extract on tyrosinase activity.

Thus, with the objective of contributing in the valorisation of plants growing in our Sahara, we proposed to study inhibitory effect of the hydromethanolic extract from the aerial parts of the species *guyonianus tithymlus* (*Euphorbia guyoniana Boiss & Reut*) harvested in the region of Ghardaïa on the tyrosinase activity which leads sometimes into variety of undesirable effects particularly enzymatic browning in food provoking a decreasing of consumers acceptability and resulting a marketing lost .

The present manuscript has been organized in different chapters describing the successive stages of this study as follow:

The first chapter concerns a bibliographical reminder as accurate as possible on truffle, *Euphorbia guyoniana Boiss & Reut* and tyrosinase.

In the second chapter, we will describe the experimental procedures involved in this study. The third chapter is devoted to a discussion of the experimental results obtained. A brief summary of the results and the prospects leading to further studies are presented in the last chapter.

# CHAPTER II

## *Euphorbia guyonianus*

## II.1 History

The botanical name Euphorbia derives from the Greek Euphorbus, physician of king Juba II of Numidia (52-50 BC - 23 AD). Euphorbus believed to have used *Euphorbia resinifera* latex to cure ailments for example, when the King had a swollen belly (Lovell., 1998; Van Damme., 2001).In 1753, Carolus Linnaeus, the great taxonomist, assigned the name Euphorbia, to the entire genus in the physician's honor (Ashwani K., 2009).

## II.2 Definition of Euphorbia

Euphorbia is a very large and diverse genus of flowering plants in the spurge family (Euphorbiaceae) (Karimi et al., 2016). It contains at least 2,000 species (Bruyns et al., 2006). These species were distributed throughout the world. Its exceptional diversity of growth forms and near cosmopolitan distribution has attracted human interest since ancient times.

## II.*3 EuphorbiaguyonianaBoiss&Reut*

*Euphorbia guyoniana* (Boiss. and Reut.) belongs to the large family of Euphorbiaceae is an endemic Saharan plant growing in sandy and desert habitat (Quezel and Santa., 1963), in the northern Sahara in Algeria, Tunisia, Libya and Morocco. It is a shrub 30–100 cm high and locally known as Lebbina (Bellakhdar.,1997). This genus is rich in secondary metabolites. The Phytochemical examinations of *E. guyoniana* revealed presence of triterpenes (Lima et al., 2003), diterpenes (Shi et al., 2005), macrocyclicditerpenes (Redei et al., 2003), steroids (Tanaka et al., 1999) and aromatic compounds (Oksuz et al., 2002).

### II.3.1 Systematic botany:

According to classical botanical classifications, euphorbiaceae are classified in dicots and *Euphorbia guyoniana* are ranked as follows

Reign: Plantae-plants

Phylum: Magnoliophyta

Sub-branch: Angiosperms

Class: Magnoliopsida-dicotylidones

Order: Malpighiales

Family: Euphorbiaceae

Genus: *Euphorbia*

Species: *Euphorbia guyoniana*

Botanical name: *Euphorbia guyonianaBoiss. &Reut*.but according to the latest classification *Euphorbia guyonianustithymlus* (*Euphorbia guyonianaBoiss&Reut*) (Klotzsch and Garcke ).

### II.3.2 Distribution

The genus Euphorbia features several species, distributed throughout in the whole world. Certain species are found in India, more precisely in the North and West (Dorsey et al.,2013). This genus is meeting in West and East Africa where is used in the traditional way by the population to treat various diseases. It is seen in Europe and America.

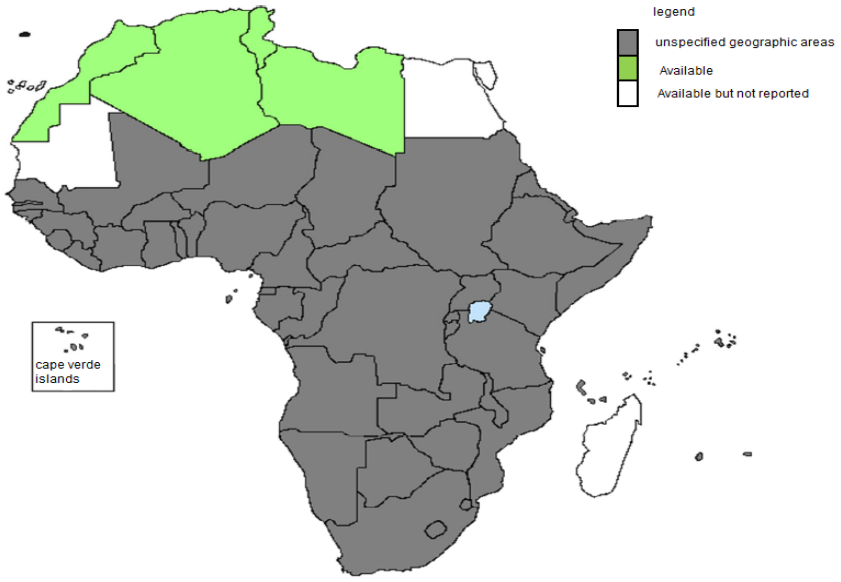



**Figure 1**:Geographical distribution map of Euphorbia guyoniana in the northern Sahara according to African plants data bases (SmaraO.,2014).

### II.3.3 Description and Morphology

The morphological diversity in this genus (euphorbia) includes geophytes, herbs, shrubs, understory and canopy trees, and an array of succulent and xerophytic forms. Despite this vast vegetative variation, the entire genus is united by a distinctive morphological synapomorphy, the cyathiuma pseudanthial inflorescence that looks superficially likes a typical dicot flower (Steinmann and Porter., 2002; Horn et al., 2012).

The plants are annual or perennial herbs, woody shrubs, or trees with caustic, poisonous milky latex (Bruyns et al, 2006). The roots are fine or thick and fleshy or tuberous. Many species are more or less succulent, thorny, or unarmed. The main stem and mostly also the side arms of the succulent species are thick and fleshy, 15–91 cm (6–36 in) tall. The deciduous leaves may be opposite, alternate, or in whorls. In succulent species, the leaves are mostly small and short-lived. The stipules are mostly small, partly transformed into spines or glands, or missing.

All flowers in the Euphorbiaceae are unisexual, and they are often very small in size. In Euphorbia, the flowers are reduced even more and then aggregated into an inflorescence or cluster of flowers known as a "cyathium" (plural cyathia). This feature is present in every species of the genus but nowhere else in the plant kingdom. Whereas most other large genera of plants differ in features of the flowers themselves, Euphorbia varies instead in features of the cyathium, which can show amazing modifications in different groups within the genus.

For *guyonianaBoiss&Reut* The flowers and cyathium are seen from afar, we can see either small yellow balls the flowers or small green balls the fruits. The non -fleshy erect stems, starts from the ground as many stems. The leaves are narrow and alternate, quickly drying out and often absent on the flowering branches. There is a single terminal cyathium that is very small less than 2 mm. The seeds without wattles, blackish and provided with longitudinal gray ribs, gland of the cyathium get rounded, without tip (Smara ., 2014).

**Figure 2**:fruit and flower of *Euphorbia guyonianaBoiss. &Reut.*

### II.3.4 Toxicity of *Euphorbia guyonianaBoiss. &Reut*

The latex of *euphrbiaguyoniana* generate redness on the skin, erythema or phlycten .this latex is very irritating to the eyes ,it causes by simple contact, even stealthy, intense tearing .At higher doses ,**s**erious eye damage occurs up to

blindness. Eyesight disturbances are often accompanied by cough, rhinitis with runny nose, laryngitis and burning lips. Once absorbed, the latex causes more or less severe symptoms of gastroenteritis and inflammation of the mucous membranes of the tube digestive (Bellakhdar., 1997).

Latex is an emulsion with a diversified composition that includes alkaloids, terpenoid compounds, poly-mericsubstances, such as resins and gums, starch, oils,and a large number of proteins and enzymatic activities (Han et al. 2000; Kekwick 2001; Ko et al. 2003).

The presence of Euphorbone (C15H24) and diterpenene esters (phorbol esters), a complex mixture of acylated and acetylated derivatives of phorbol,ingenol and myrsinol in 12 and 13 (or the reverse) makes Euphorbiaceae responsible for severe bulbous dermatitis on the skin, labial lesions and pharyngeal edema by ingestion.Eye accidents can be severe (lesions of the corneal epithelium) (Champy.,2008).

### II.3.6 Utilizations

The *Euphorbia guyoniana Boiss & Reut* latex is used by locale people to attack warts and to extirpate the spines. They apply it locally on poisonous bites and stings.The present study gives a first indicative overview of other Euphorbia species used for health and wellbeing around the world .*euphorbia tirucalli* : each part of this plant can be used as a treatment the juice of the stem is purgative and carminative; useful in gonorrhea, whooping cough, asthma, dropsy, leprosy, enlarged spleen, dyspepsia, jaundice, colic, tumours and stone in the bladder (Rahman.,2013), the root scrapings mixed with coconut oil are taken for stomach-ache (Gupta.,2013),The young branches can be roasted then chewed to relieve a sore throat ,and the latex is used as an application to warts, epilepsy, toothache ,hemorrhoids, snake bites (Panchal.,2017).*Euphorbia milii* : widely used in folk medicine for the treatment of warts (South Brazil), cancer

and hepatitis (china) , It has been reported that *Euphorbia milii* possesses antifungal and antinociceptive property, acts as natural molluscicide.*E.hirta*is used in the treatment of gastrointestinal disorders (diarrhea, dysentery, intestinal parasitosis, etc.), bronchial and respiratory diseases (asthma, bronchitis, hay fever, etc.),the Decoction of fresh herbs is used as gargle for the treatment of thrush.

Root decoction is also beneficial for nursing mothers deficient in milk. Roots are also used for snake bites (Kumar, Rashmi, and Dinesh., 2010) *Euphorbia calyptrate* and *E. retusa* it used in Algerian Sahara for treatment of infantile eczema, warts and trichiasis (Lahmadi et al., 2019).

# CHAPTER I

# TRUFLES

## I.1 History:

According to some historians, the first appearances of the truffle date back to the Mesopotamian. In the 4th century the genus *Terfezia* was created by tulasne and tulasne (1851).And gathered all the African truffles under the term of *TerfezialeonisTul.* in 1892,while several other species and varieties of *Terfezia*, particularly citing the genus *Tirmania*, have also been described by Chatin (Fortas et al.,1992).

## I.2 Definition:

Truffles were mentioned in the ancient world by the Romans and Greeks, and many studies have taken place to obtain further information and data about several varieties of desert truffles from different locations (Iddison., 2016).

The term “desert truffles” is used to refer to edible hypogeousmacrofungi, which grow in arid zones of the Mediterranean. Occasionally, some truffle genera found in semi-arid zones, such as *Terfezia* and *Tirmania* (the most abundant), Tuber, Picoa, Delastria, and Loculotuber. They grow naturally after rainfall during the season of Al-Wasm in arid and semi-arid districts of Saudi Arabia, Iraq, Kuwait, Qatar, Bahrain, United Arab Emirates, Syria, Jordan, Palestine, Lebanon, Egypt, Iran, Turkey, Tunisia, Algeria, Libya, and Morocco. Al-Wasm provides rainfall of more than 200 ml in September to October in the Arabian Peninsula, and is important for the early growth and appearance of desert truffles. The number of truffles increases depending on the amount of rainfall, as in other countries. (Iddison.,2016).

In the Arabian region, the common name of the desert truffle, in Kuwait: Fagaa, Kami. Morocco, Algeria and Tunisia: Terfez ,Terfesse, or Terfos in Kabylia .Egypt: Terfas .Qatar, Iran and Saudi Arabia: Zoubaydi “Eterfes”, but the classic Arabic word is “al-kamah', or “kame”. Most desert truffles collected from the Arabian Desert belong to 2 genera, *Terfezia* sp. and *Tirmania* sp. (Bokhary.,1987).

Desert truffles have been used as food and medicine. Originally, these types of organisms were associated with Mediterranean region and were first recorded as poem in Egyptian temples as follows: “Without leaves, without buds, without flowers. Thus, they were considered as food and medicine for royalty, and that no normal citizens were allowed to consume this precious food. During Greek and Roman eras, they were imported from Libya and sold in southern part of the European continent. In the southern part of African continent, the nomadic people of Kalahari Desert used truffles for millennia (Trappe., 1990).

**I .3 Classifications**

Truffles are Eumycetes, wich means they are real mushrooms with a cell membrane .their thallus is made up of saptate filamentous mycelium (Seteptomyctes).Their dissemination takes place by immobile spores (characteristic of amastigomycota).(Rouissat et al.,2012) within amastigomycetes, truffles are ascomycota, mushrooms producing spores from a meiosis in sac-like cells (ascus).Unlike the basidomycota (Paris mushroom, porcini.....), spores form outside the basidia ,Truffles are part of the pezizomycetideae order.

Based on the morphometric studies of their spore ornamentation, these desert truffles cannot be well distinguished into individual species (Al-Qarawi and Mridha 2012). However, convincing delimitation between different species and taxa has been achieved based on molecular studies and gene sequencing.

Trappe.,1979 transferred some of families from Tuberales to Pezizales and amended some families in Pezizales to include related hypogeous fungi,and included *Terfezia* in the Terfeziaceae and *Picoa* in the Balsamiaceae, based on a comparative morphological study.

The most known species are: *Terfezialeonis, Terfeziaclavery, Tirmanianivea , Tirmaniapinoyi , Tirmanialeptoderma,Tubermelanobosporum , Tuber brumale , Tuber aestivum(uncnatum)* and *Tuber uncinatum*: each one of them has its own specific criteria (Calonge et al.,1995;Pegler.,2002).

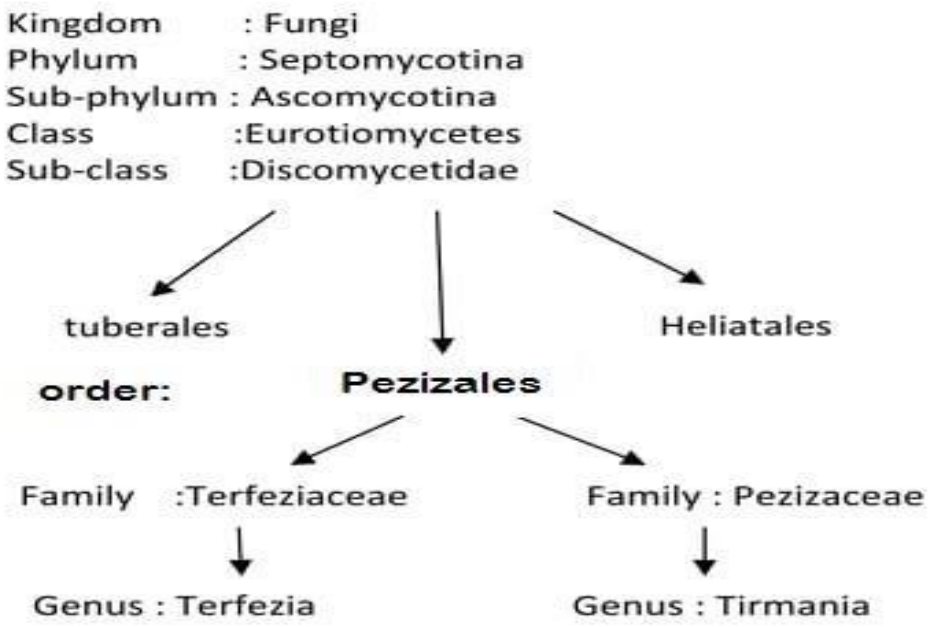



**Figure 3**:Position of the terfesse in the classification of ascomycetes (Trappe.,1979; Delma.,1989).

The truffles are classified according to the following standards: peridium, gleba, ascus, Ascospores(Trappe.,1979).he most known species are: *Terfezialeonis, Terfeziaclavery, Tirmanianivea , Tirmaniapinoyi , Tirmanialeptoderma,Tubermelanobosporum , Tuber brumale , Tuber aestivum(uncnatum)* and *Tuber uncinatum*: each one of them has its own specific criteria (Calonge et al.,1995;Pegler.,2002).

## I.4 Morphology

Truffles have no stalk and no gills with firm, dense, and woody feature (O'Donnell et al.,1997). Truffles are aromatic, wrinkled and have bruised, lobed potato-like appearance. Tuber species can generally be distinguished on the basis of their fruit bodies and mycorrhizae (Bertini et al., 1998). Their gleba has a more or less clear coloring; their surface is of a variable color between black, white and brown, of various or smooth shape (Fortas et al.,1992; Chellal., 1995).

## I.5 Ecology

In Algeria, desert truffles develop in semi-arid and arid zones , which are characterized by hot, dry summers, coldwinters .they grow in heterogeneous soils of the sandy texture, moderately calcareous (10.19 %), slightly alkaline (7.87%), with low organic matter (0.86 %) and slight phosphorus content (Loizides et al .,2012; Bradai et al., 2015) . The development of these fungi is closely related to the stormy winter rains as well as the density of the cover and host plants. The truffles develop in depth of about 5 cm below the ground following the rains in the beginning of spring then in period of droughts. The harvest of truffles begins during March and April in the depressions, the wades beds, and the desiccations where the annual helianthemums grow (Fortas et al,. 1992).

## I.6 Life cycle

The truffle's life cycle has been a mystery since the ancient civilizations. After thousands of years of uncertainly, man finally found the right answers at the beginning of the 19$^{th}$ century, Although until today , some exact processes, like spontaneous truffle spore germination , are still unknown, but despite that , we can state that the exact reproduction phases or cycles are well known and worth commenting . The Ascomycota comprise a single class, the Ascomycetes, characterized by a asexual reproduction mainly by conidia (not known in truffles) and a sexual reproduction by different methods.

Ascospores are released after the fruitbody breakdown and germinate in the soil; a heterocaryotic mycelium is formed, which makes contact with the host plant, leading to the formation of mycorrhizal roots. The fruitbody is formed only after the establishment of the mycorrhiza (Fortas et al., 1992), where the plant host provides the sugar produced by photosynthesis to the fungi and receives an exchanges of water and soil nutrients such as nitrogen and phosphorus(truffles are heterotrophic).

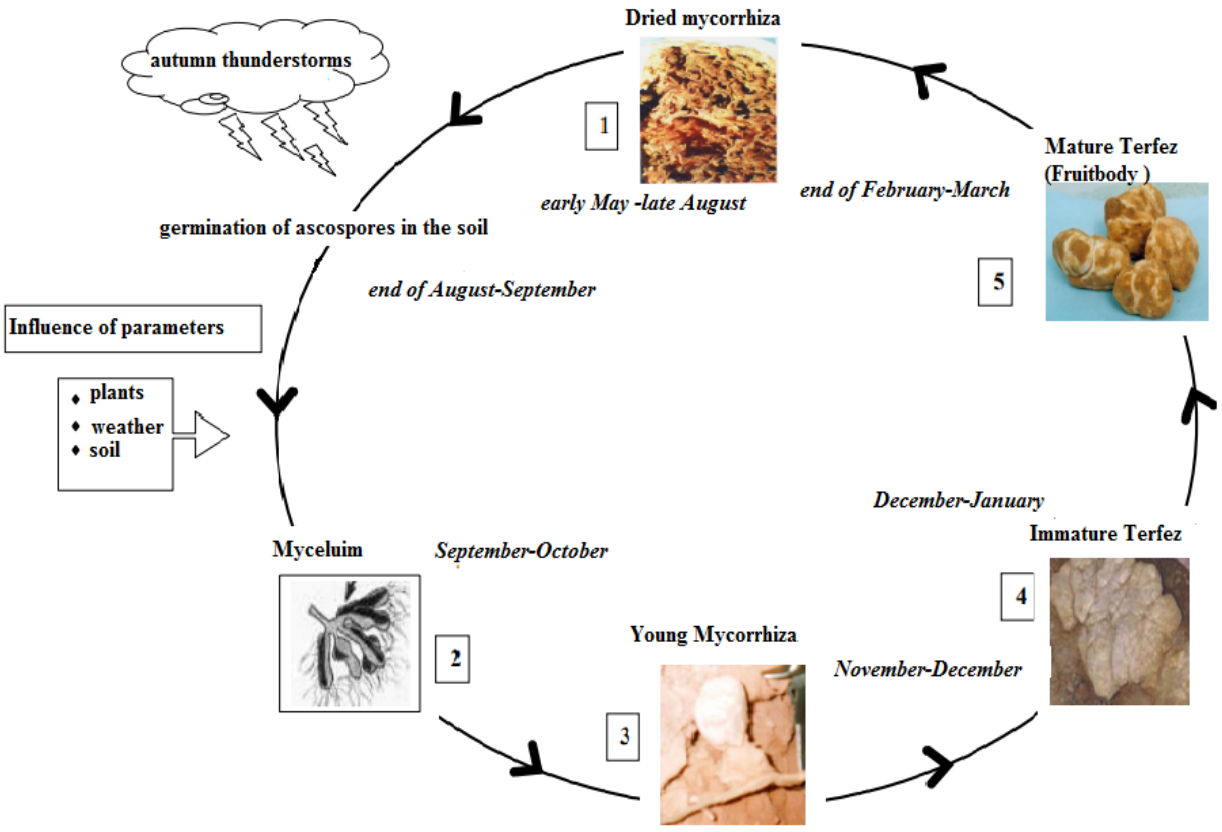



**Figure 4:**Main stages of Terfez's life cycle (Bradai., 2006).

## I.7 Chemical composition

Researchers discovered that the nutritional contents of truffles varied from species to species. they are a rich source of crude fiber (7-13%), proteins (20-27%), fat (from 3 to 7.5%),ascorbic acid (5.2%) and minerals(phosphorus,

calcium, magnesium, potassium, sodium, iron, zinc, fiber, sulfur, chlorine, and silicone), such a composition partly explains its different bio-activities including antioxidant , antiviral activities , antimicrobial, hepatoprotective, antimutagenic and anti-inflammatory (Wang et al.,2011;Spivallo et al.,2011).

Gioacchini and al (2005) identified 36 volatile organic compounds (VOCs) including alkanes, alcohols, esters, aldehydes, ketones, terpenes, etc.

widely distributed in six different species of white and black truffles (*T. magnatum, T. borchii, T. dryophilum, T. aestivum, T. mesentericum and T. Brumale*).

**I.8 Geographical distribution of truffles in Algeria:**

The geographic distribution of Terfès is in the Mediterranean region, the semi-arid and arid zones or even with a sub-Saharan climate. Approximately between the 28$^{th}$ and the 45th degree of north latitude (Chatin., 1891; Trappe., 1990).

In Algeria, we can find the three (03) genuses:

•Terfezia: brown truffles (*Terfeziaclaveryi, Terfeziaboudieri, Terfeziaarenaria ,Terfizialeonis*)

• Tirmania: whitle truffes (*Tirmaniapinoyi, Tirmanianivea*)

• Picoa: Black truffles (*Picoalefebvrei, Picoajuniperi* and *Picoacarthusian a*).

These species are found in the regions of Bechar (Kenadssa, Taghit, Tabelbala, Abadla, Beni Abbes), Tindouf, Timimoune, Ouargla (OuedMya), Touggourt, Tamanrasset (Hoggar Mountains), Ghardaïa, Biskra, Batna, Djelfa, Saida, Boussaâda, Mécherai, Naama, Laghouat (Chatin., 1891 ; Fortas., 1990 ; Fortas and Chevalier.,1992).

**I .9 Multiple uses of truffles**

The role of truffles in ethno-medicine of Bedouins is documented in Islamic literature (Mandeel and Al-Laith., 2007). Truffles are rich in therapeutic

compounds with anti-inflammatory, antioxidant, antiviral, antimicrobial, anti-mutagenic, anti-carcinogenic and hepatoprotective bioactivities.

Desert truffles are edible, seasonal and socio-economically important fungi (Al-Laith., 2010).

As model for investigation of enzymatic adaptation: Truffles show both morphological, physiological and biochemical adaptations to poorly oxygenated area and are good models for investigation of enzymatic adaptation to microaerobic conditions. Extraction of tyrosinase from *Terfezia leonis* Tul(gouzi and al., 2012).

According to Rougieux (1963), Terfezia has stimulating action on telluric microflora, especially on azotobacter.

**I .10 *Terfezia leonis* Tul**

This specie has a brownish black surface and a pinkish white gleba with a diameter between 3 and 10 cm. it weights between 8 to 10 grams, the tapered part of the truffle constitutes a base which is sunk into the ground and would be in relation to the mycelial cord that attaches to the host plant. The asques of *terfezia leonis* has a spherical shape and contain 3 to 6 spores. They have an average diameter between 25 and 31 ụm and a spherical shape accompanied by a spine in surface (Caloxge and Lawrynowic.,1977; Azeddine, 1996; Fortas, et al., 1992).

# CHAPTER III

# *Tyrosinase*

## III.1History

The first reported interest in Oxidases was in 1856 when Schoenbein identified the presence of an oxygen-activating agent in Boletus luridus mushrooms. This agent provided a blue pigment because of aerobic respiration for some materials in the plant (Whitaker., 1995). tyrosinase was prepared for the first time simultaneously from potato by Kubowitz (1938) and by Keilin and Mann (1938) from d'Agaricusbisporus. the first serious study of this enzyme followed the large -Boswell and whiting Were among the first attempting to show that polyphenol oxidase (tyrosinase) can function as a respiratory enzyme (Boswell et al.,1938) .it was the first enzyme purified by affinity chromatography (Lerch,. 1981). Recently a clear image of this enzyme started to emerge (Jolivet et al., 1998).

## III.2 Definition

Tyrosinase (EC 1.14.18.1) also known as polyphenol oxidase is a multifunctional copper-containing enzyme which exists widely in mammals, plants, insects and microorganisms .It catalyzes two distinct reactions both of which are essential for biosynthesis of melanin, namely the hydroxylation of L-tyrosine to L-dopa (monophenolase activity) and also the subsequent oxidation of L-dopa to dopaquinone (diphenolase activity) in the presence of oxygen molecular. Thus, is directly associated with plant browning (Gandia-Herrero et al., 2003), cuticle formation (Guerrero and Rosell.,2005), and skin pigmentation (Olivares,Solano,.2005). Tyrosinase is also involved in Parkinson's disease (Asanuma et al., 2003). Tyrosinase is used in predictive biomarker for melanoma cancer and variety of skin disorders (Chen., 2020) .

## III.3 Classification

Tyrosinase is an oxidoreductase oxygenase (papa et al,. 1994). The classification and specificity of this enzyme are somehow still ambiguous (Eicken et al.,1999).

In the international union of biochemistry (IUB) classification of tyrosinase has therefore been given two entries: as EC 1.14.18. 1-monophenol monooxygenase, also known as tyrosinase. and EC 1.10.3.2 p-diphenol: 02 oxydoréductase. This classification only differentiates the two activities cresolase and catecholase, from the same enzyme. (Mayer.,1987; Zawistowski et al., 1991).

Nowadays, it is generally accepted that PPO (PPO: monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase; EC 1.14.18.1) is enzyme that catalyzes two different reactions in the presence of oxygen molecular: hydroxylation of monophenols to o-diphenols (activity monophenolase, cresolase or hydroxylase) and the oxidation of o-diphenols to o-quinones (diphenolase, catecholase or oxidase activity) (Granata et al.,2005).

## III.4 Activity

Tyrosinase (EC 1.14.18.1) has dual functionality that causes monophenolase activity (hydroxylation of monophenols to o-diphenols) and diphenolase activity (oxidation of diphenoles to o-quinones) (Fairhead and Meyer., 2012).

- The polyphenol oxidases group involve two types of enzymes: o-diphenol oxidase (catechol oxidase, tyrosinase, phenolase, polyphenol oxidase) and monophenol oxidase or cresolase, the two types are a phenol-oxygen oxidoreductase (Mayer and Harel.,1991).

### III.4.1Monophenoloxidase activity (EC 1.14.18.1)

This reaction is catalyzed by monophenols oxidases or monoxygenases which are responsible of Monophenols' hydroxylation into o-diphenols. This enzyme is also called tyrosinase due to his major monophenolic substrate in animal reign “tyrosine” or cresolase because of its capability of using cresol as a substrate. monophenol oxidase activity is generally understudied in plants because the hydroxylation reaction is much slower than the oxidation reaction which forms quinones and initiates browning reactions. However, it has been known for a long time in mushrooms (Varoquaux.,1978) and most recently in apples (Nicolas et al.,1994).

### III.4.2. Cathecoloxidase activity (EC 1.10.3.1)

Catalyzes exclusively the oxidation of o-diphenolicsubstrats to o-quinone by o-diphenoloxidase activity (catecholase or catecholoxidase ) in the presence of oxygen. The Quinones produced are highly reactive compounds usually undergo in aqueous solution polymerization and non-enzymatic reactions forming heterogeneous pigment: melanin or react with amino acids and proteins to produce colored compound (Dicko et al., 2002; Cho and Ahn., 1999; Burton., 1994).

## III.5 Molecular structure and active site

Although tyrosinase was one of the first discovered monooxygenases, its crystallographic structure has not been yet elucidated. However, it can be assumed that tyrosinases, hemocyanin and catechol oxidases share similar binuclear copper sites (Claus and Decker.,2006). Due to multiple sources of tyrosinase its structural properties are diverse in nature along with their distribution in tissues and cells, so no common protein is observed across all species (Mayer., 2006; Jaenicke and Decker., 2003). The difference is observed

in primary structure, size, in post modification sites like active site and in glycosylation mechanism.

Garcia-Borron and Solano (2002) described the tyrosinases active site as a hydrophilic sphere delimited by a four-helix bundle containing the six imidazole residues. The hydrophilic sphere would be located in a hydrophobic shell which is aromatic and formed by highly conserved residues. The configuration of the tyrosinases active site would essentially be maintained by electrostatic or cations-π interactions.

Tyrosinase takes α -helical structures with the core of the enzyme, which is formed by a four-helix-bundle (α2, α 3, α 6, and α7 helices). The catalytic binuclear copper center is lodged in the helical bundle. Each of the two copper ions in an active site is coordinated by three His residues.

The coupled binuclear copper site consists of two coppers, CuA and CuB (figure 5). With each copper atom coordinated with three conserved His residues (Jaenicke and Decker., 2003). In catechol oxidase from *Ipomoea batatas* CuA is coordinated with His-118,His-109, and His-88,are located in the middle of α2 the residues His-118,His-109 are at the beginning and in the middle of α3 whereas CuB is coordinated with His-240, His-244, and His-274. The CuA and CuB, present in the holo-enzyme is the site at which tyrosinase interacts with both molecular oxygen and its phenolic substrates (klabunde et al., 1998).

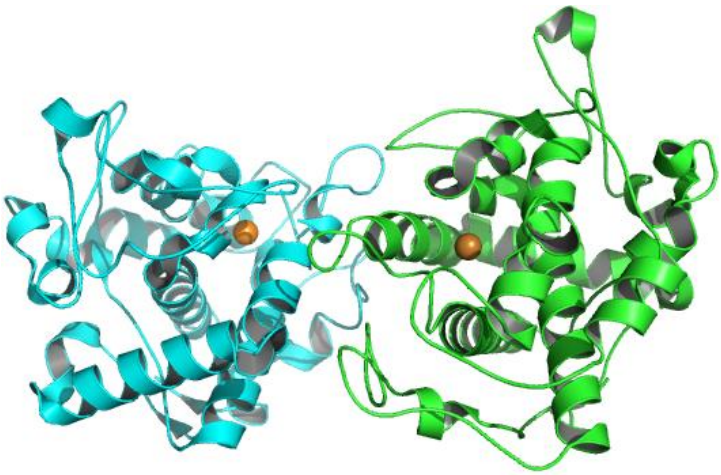


**Figure 5**:Three-dimensional structure of Tyrosinase from *Bacillus megaterium* (PDB ID: 3NQ0), the two copper atoms are shown as orange spheres (Harrat and benbahaz., 2020).

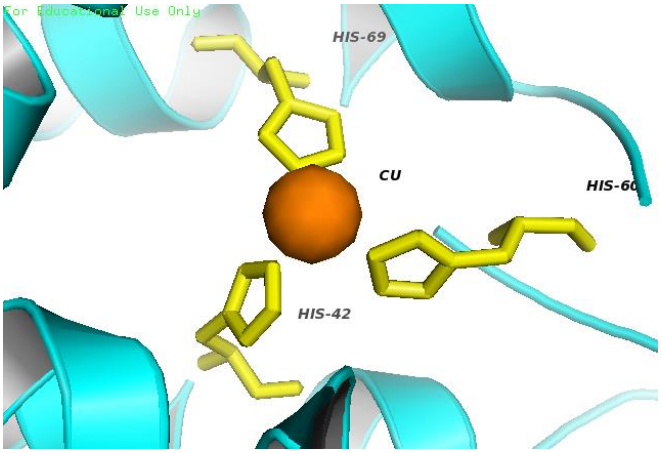



**Figure 6**: The active site of Tyrosinase from *Bacillus megaterium*,chain B of 3NQ0,the copper atom Cu B shown as orange sphere coordinated by three residues His are colored yellow, shown as sticks(Harrat and benbahaz.,2020).

## III.6 Reaction characteristics

The catalytic mechanism of tyrosinase was first studied in detail by Solomon and al.Solomon proposed a mechanism for both the cresolase and catecholase activities of tyrosinase.

The most accepted proposed reaction mechanism slightly refines former models (Lerch., 1983), and describes a common catalytic site for the two activities with threedifferent forms of the enzyme, called met, oxy and deoxy, according to the absence ⁄ presence of oxygen and the oxidation state of the copper ions [Cu(II)⁄Cu(I)].(Kanteev et al.,2015).

Native tyrosinase occurs primarily as met-tyrosinase, which cannot bind oxygen and in which both copper atoms have the CuII oxidation state. Two electron reduction of met-tyrosinase gives deoxy-tyrosinase in which the copper atoms are in the CuI oxidation state. Deoxy-tyrosinase binds dioxygen to give oxy-tyrosinase.

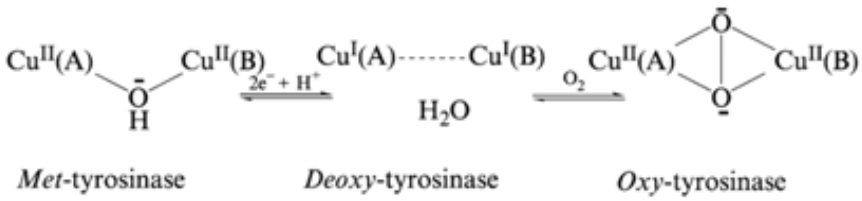



**Figure 7**:Tyrosinase oxidation states (Itoh.,2003)

In the monophenolase activity (oxidation of L-tyrosine to L-DOPA), deoxytyrosinase (Edeoxy) binds to oxygen and form oxy-tyrosinase (Eoxy) and then to L-tyrosine, which is further catalyzed to LDOPA. In this process, the enzyme is finally recycled as Edeoxy for further dioxygen binding.

In the diphenolase activity (oxidation of L-DOPA to DQ), the regeneration of oxytyrosinase drops only one oxygen atom to form met-tyrosinase (Emet), where the two close copper centers are bridged by an aqua (hydroxo) ligand. Tyrosinase occurs mainly as the Emet form, which cannot oxidize phenols, e.g., tyrosine, and needs to be reduced to Edeoxy by L-DOPA before the tyrosine oxidation initiated. This explains the higher affinity of Emet to L-DOPA as compared to other forms, and thus Emetis considered as an important target for

the discovery of tyrosinase inhibitors (Favre et al., 2014). After catalyzing L-DOPA, Emet loses the oxygen atom to regenerate Edeoxy form. Catechol oxidation is based on the phenolic oxidative mechanism that results in a reduction of copper to Cu° and tyrosinase deactivation (Ramsden et al., 2014; Munoz-Munoz et al., 2010).

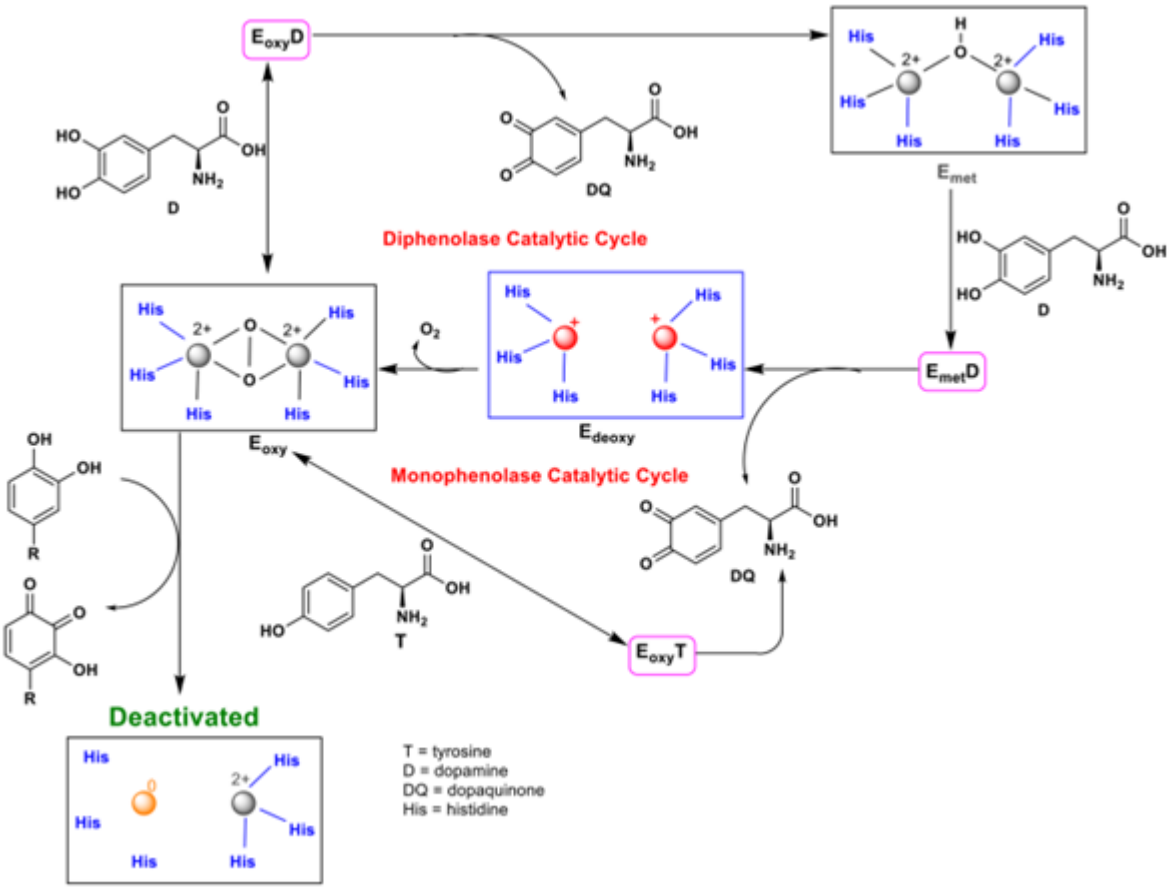



**Figure 8**:Catalytic cycles of tyrosinase: monophenolase and diphenolase (Pillaiyar and al.,2018*).*

## III.7 Source and localization

Tyrosinase can be produced and extracted through number of organisms such as prokaryotes, Fungi, Vascular plants, Arthropods, Amphibians, insect's and mammals (Claus and Decker.,2006).and certain number of microorganisms

(Burton.,1994).Bacteria: Rhizobium (Liu et al., 2005), Campylobacteriumthermophilus (McMahon et al.,2007), Bacillus thuringiensis and Pseudomonas putida(Matoba et al.,2006).Plants: Monastrell grape and apple (Janovitz-Klapp et al.,1989), Sunflower seed ( Janovitz-Klappet al.,1989),Portulacagrandifora ( Raymond et al.,1993).Fungi: Agaricusbisporus (Strothkamp et al.,1976), Amanita muscaria (Mueller et al.,1996), lentinulaboryana (Defaria et al.,2007),Tyrosinases can be purified from these sources and studied for specific functioning. They are located at the plastid membranes (chloroplasts for leaves and fruit, amyloplasts or leucoplasts in the parenchyma of reserve (potato tuber or carrot roots) (Steffens et al.,1994).

More precisely, ultrastructural studies associated with cytochemistry and immunohistochemistry have suggested that tyrosinases are linked to thylakoids membranes (Dry et al.,1994).

## III.8 Role

Tyrosinase is the key enzyme in melanin biosynthesis and in the undesired enzymatic browning of fruits and vegetables (Whitaker.,1995; Fenoll et al.,2004).

The role of PPO in browning phenomena is so well documented, that it need not be discussed further. A major difficulty is always to determine whether PPO is the direct reason for browning or whether the browning reaction is a secondary result of other metabolic events (Zhou et al., 2003). In fungi and vertebrates, tyrosinasecatalyzes the initial step in the formation of the pigment melanin from tyrosine.

A wide variety of plant tyrosinase be haviors has been described and reviewed (Mayer and Harel., 1991; van Gelder et al., 1997). A molecular oxygen-scavenging (regulation) function in the chloroplast and role in photosynthesis proposed (Kuwabara and Katoh.,1999).

A resistance role against microbial, viral infections, herbivores and bad weather conditions (Martinez and Whitaker.,1995).

Tyrosinase catalyzes the oxidation of phenolic compounds into quinones which are a bactericides and fungicides (Zinkerage.,1986). The involvement of tyrosinase in the metabolism of betalains was provided (Gandía-Herrero., 2005) but the physiological function of tyrosinase in the secondary metabolism is yet to be fully determined.

In insects, tyrosinase is acknowledged to be a beneficial enzyme with functions of sclerotization, melanin synthesis and parasite encapsulation (Steffen and al.,1998). Consequently, it is an important enzyme due to the defensive mechanism of insects. it is an essential enzyme for the beneficial coloring of our food (Whitaker and lee.,1995).by its hydroxylase activity tyrosinase is involved as well in the biosynthesis of phenolic compounds (Zawistowski et al.,1991).

## III.9 Application

As there is a great request for different enzymes in the industries, tyrosinase is one of significant commercial enzymes. Microbial tyrosinases are highly promising enzymes for the pharmaceutical and food bioprocessing technologies. However, various industries like paper, chemical, textile, mining, coal and petroleum produce waste waters that consist of the phenols and its derivatives. Tyrosinase is capable of oxidizing the phenols into insoluble substances that can be eliminated by precipitating or filtration.

**Table 1**: Application of tyrosinase in different industries.

| Fields | Application | References |
|---|---|---|
| Food industry | In cereal processing , to improve the baking in order to make better the volume and crumb structure of breads | Facio.,2011 |
| | In dairy processing ,to cross link various dairy proteins | Selinheimo.,2008 |
| | In meat processing , for improvement of the gelation | Selinheimo2008 |
| Medical fields | As prodrug, in immunoassays and antibody microarrays, to produce L-DOPA and to treat neurological problems. | Selinheimo,2008 ;Valipour and Buhan,2016 ;Zaidi et al.,2014 |
| Textile industry | To modify the wool fibers and production of different dyes. | Selinheimo.,2008 ;Valipour and Buhan.,2016 |
| Cosmetic industry | As self tanning agent | Selinheimo,2008 ;Valipour andBuhan,2016 |
| Environmental significance | As biosensors to detect the toxic phenolic compounds. | Selinheimo,2008;Singh N.and singh J.,2002 |

Beside that tyrosinase is also used as a defences ystem for certain plants to prevent the insect's and microorganisms attack by the formation of impervious

skin (Gelder et al., 1997). Synthetic melamine's has implementations as a form of protect agent against radiations (UV, X ray, gamma ray).

### III.10 Tyrosinase inhibitors:

tyrosinase plays an important role in the melanogenesis and enzymatic browning.

tyrosinase inhibitors can act by several mechanisms: reducing agents causing chemical reduction of dopaquinone such as ascorbic acid, which is used as a melanogenesis inhibitor or inhibition of tyrosinase activity; the Specific tyrosinase in activators such as mechanism-based inhibitors. They can inhibit tyrosinase activity by inducing the enzyme catalyzing "suicide reaction".the Nonspecific enzyme inactivators such as acids or bases, which non-specifically denature the enzyme, thus inhibiting its activity. Specific tyrosinase inhibitors such as most compounds the compounds reversibly bind to tyrosinase and reduce its catalytic capacity (Parvez and al., 2007; Chang., 2009).

The simple phenols such as hydroquinone and its derivatives,deoxyarbutin and its derivatives, 4–(6-Hydroxy-2-naphthyl)-1, 3-bezendiol, resorcinol (or resorcin) and 4-n-butylresorcinol, vanillin and its derivatives have been reported in the scientific literature as possible phenolic inhibitors of the tyrosinase. Chen et al. have found the alkylhydroquinone 10'(Z)-heptadecenylhydroquinone, isolated from the sap of the lacquer tree *Rhus succedanea*, can inhibit the activity of tyrosinase and suppress melanin production in animal cells (Samaneh et al., 2019).

### III.11 Tyrosinase Activators

Polyphenol oxidase activity has been activated by a variety of treatments or agents such as proteases, urea, fatty acids, polyanmines, divalent cations, acid

and basic shock, and anionic detergents such as SDS However, PPO properties vary widely between species.

Plant PPOs are generally expressed in a latent (enzymatically inactive) form, which contains a catalytically active domain shielded by a C-terminal domain. The latent form can be activated by limited proteolysis, acidic pH, fatty acids, or detergents (Mayer., 2006) Recently, it has been reported that the latent form can spontaneously activate during the first weeks of storage, generating an active form with a molecular weight of 38 kDa (Derardja et al., 2017, Kampatsikas et al., 2019).

# *CHAPTER IV*

# *Materials and methods*

### IV.1.1 Material

Brown Algerian desert truffles of the genus *Terfezia* (*Terfezia leonis* Tul.) were purchased from the local market of Laghouat (Algeria) during spring 2020. *Terfezia leonis* Tul. ascocarps were rinsed several times with distilled water to eliminate any trapped pockets of soil, freshly harvested and kept for several days in the refrigerator (-5°C) before extracting.

### IV.1.2 Reagents

L-Tyrosine and dimethyl sulfoxide (DMSO) were purchased from and sigma aldrich (St. Louis, MO, USA) and Fluka (Madrid, Spain), respectively. Sodium dodecyl sulfate (SDS), sodium acetate and acetic acid were obtained from Merck (Darmstadt, Germany). The remaining reagents were of analytical grade.

### IV.2.1 Plant collection, authentication and preparation of hydromethanolic extract

Plant *Euphorbia guyoniana* was collected from Ghardaïa region (Zone of wadi Drine) during February 2020 (fig 9). The samples were authenticated by Dr.Abdellah KEMASSI at the Laboratory of Mathematics and Applied Sciences (University of Ghardaïa), the voucher specimens were prepared and deposited at the Biological and Agronomical Sciences Laboratory of Laghouat University. The aerial parts of plants were shade dried to constant weight prior to pulverizing with an electric blender to fine powder (figure10) .

**Figure 9:** *Plant Euphorbia guyoniana*.

**Figure 10**: Plant *Euphorbia guyoniana* during drying.

10 g of condensed powder were extracted at room temperature with 100 ml of methanol-water (7:3, v/v) for 24 h. After maceration, the mixture were filtered twice through filter paper and concentrated under reduced pressure at 45°C to remove methanol. The combined organic extracts were evaporated to dryness using a rotary evaporator at 45°C.The remaining aqueous solutions were defatted three times with petroleum ether (3:1, v/v) to remove lipids and pigments. The extract was evaporated at 45°C in an oven. The residue was stored at 4°C until use.

### IV.2.2 Preparation of tyrosinase crude extract

Peridium from truffles (*Terfezia leonis* Tul.) (150 g) was homogenized in 350 ml of 0.05 M phosphate buffer (pH 7.0), using a Waring commercial blender for 5 min. The resulting homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged for 10 min at 4,500 rpm. The supernatant constituted the tyrosinase crude extract for this study and was kept frozen at -10°C until use.

### IV.2.3 Tyrosinase activity assay

Tyrosinase activity of the truffle homogenized supernatants was measured spectrophotometrically. Tyrosinase activity was determined at 30°C in a medium containing 1 ml of 1 mM L-tyrosine in 0.05 M sodium acetate buffer (pH 5.0) and 30 µL of enzyme solution, by recording dopachrome production (ε = 3600 $M^{-1}.cm^{-1}$) at 475 nm. The steady-state rate ($v_0$, µmol/l/min) of enzymatic activity was calculated from the linear zone of the product accumulation curve after the lag period.

All activity analyses performed in this work were carried out in triplicate, and the averages of data were considered. The error bars represent standard deviation (SD).

### IV.2.4 Effect of hydromethanolic extract of Euphorbia guyonianus on tyrosinase

To determine the effect of hydromethanolic extract of *Euphorbia guyonianus* on tyrosinase of desert truffle (*Terfezia leonis* Tul.), cresolase activity was measuredin the standard reaction medium in the presence or absenceof the plant extract at varying concentrations of L-tyrosine ranging from 0.15-0.5 mM .Tyrosinase activities were measured at two constant plant extract concentrations. Inhibition patterns were determined from double reciprocal plots of velocity versus initial substrate concentration. The inhibition constants were

obtained from the second plots of the kinetic parameters against the concentration of plant extract. The inhibition constant $K_I$ is a quantitative measure of inhibitorpotency for reversible inhibitor, which is the reciprocal of the enzyme-inhibitor affinity.

To determine the inhibitor concentration that reduced the enzyme activity by50% ($IC_{50}$), regression analysis graphs were drawn by using percent inhibition values by a statistical package programme on a computer. $IC_{50}$ value was determined from a plot of residual activity against the hydromethanolic extract of *Euphorbia guyonianus* concentration (0-1 mg/mL).

### IV.2.5 Molecular docking study of tyrosinase interacting with two different ligands :

To Predict how a protein (enzyme) interacts with small molecules (ligands).which plays a major role in the dynamics of that protein, which may enhance or inhibit its biological function. In the present study, we performed docking of tropolone and the new inhibitor condidat obtained from *Euphorbia guyonianus* (Kaempferol) into the active site of the mushroom tyrosinase Tyrosinase from *Bacillus megaterium* (PDB ID: 3NQ0) *in silico* using MOE docking programe. The crystal structure of tyrosinase (PDB ID: 3NQ0) was obtained from the Protein Data Bank (PDB, http://www.pdb.org) and searched for tyrosinase residues that would bind to tropolone and Kaempferol.

### IV.2.6 Data analysis

Linear and non-linear regressions fitting was performed by application of the Table CurveTM 2D v2.03 (Copyright © 1989–1994. AISN Software) , software MOE (Copyright © 2019)and SigmaPlot Version 12.0 (Copyright © 2011 Systat Software, Inc.) software.

# *CHAPTER V*

# *Results and discussion*

## V.1 Extraction of tyrosinase (*Terfezia leonisTul*)

The Extraction of truffle tyrosinase (*Terfezialeonis*Tul) is carried out according to the protocol described by Gouziet al., (2013) which is easy method, fast and allowed to get a dark brown crude extract. An unusual and intriguing characteristic of the enzyme is its ability to exist in either a latent and/or an active form (Mayer and Harel., 1979). Latent tyrosinases can be activated by different treatments such as detergents (Swain et al., 1966;Moore and Flurkey., 1990; Marques et al., 1995; Nellaiappan and Sugumaran., 1996), alcohols (Asada et al., 1993) and proteases (King and Flurkey.,1987; Robinson and Dry, 1992; Chosa et al., 1997) and by acid and base shock (Kenten.,1958),fatty acids (Sugumaran and Nellaiappan, 1991),The anionic detergents, SDS has widely use as PPO activator from several vegetables.(Angeletonand Flurkey.,1984; Sanchez–ferreret al., 1993; Jimenez and Garcia-carmona.,1996; Sojoet al.,1998., Gouziet al., 2012, 2013).

## V.2 The initial velocity of L-tyrosine oxidation:

Under the experimental conditions used, we followed the rate of the reaction by pursuing the increase in absorption at 475 nm (using a spectrophotometer), the initial linear rate of product formation is called the initial velocity ($v_0$).

We notice that the absorbance increases at a linear rate (figure11) which means more and more product is formed. However, the absorbance may eventually approach a plateau as the reaction slows down and fewer products are formed.

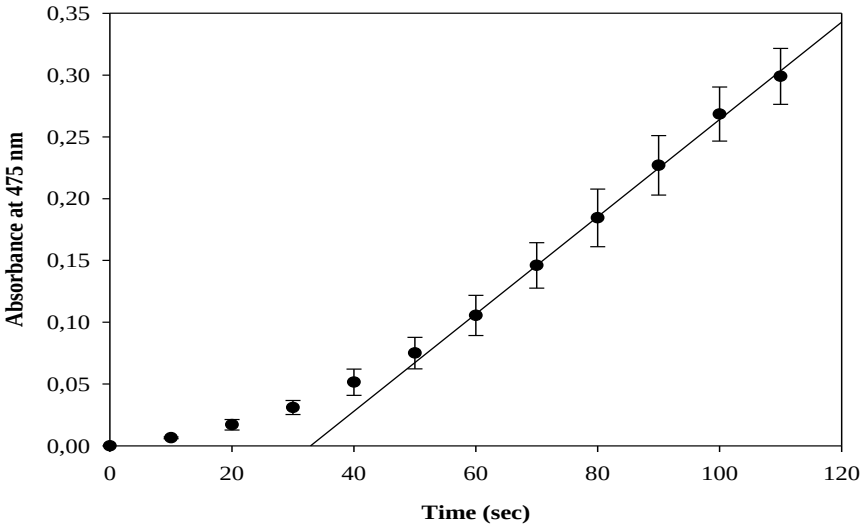



**Figure 11**. Determination of the initial velocity of L-tyrosine oxidation by desert truffle (*Terfezia leonis* L.) tyrosinase (V0) (L-tyrosine at 1 mM, SDS at 4 mM, enzyme extract 30 µL, sodium acetate at pH 5.0-0.05 M, temperature 30°C, volume of reaction mixture 1.0 mL).

## V.3 Effect of hydromethanolic extract of *Euphorbia guyonianus* on tyrosinase

The natural products of great structural diversity are still a good source for searching such inhibitors, thereby motivating to explore biologically active compounds from the highly diverse plants. However, in this study, the inhibitory activity of hydromethanolic extract obtained from *Euphorbia guyonianus* against desert truffle tyrosinase was evaluated.

### V.3.1 Determination of IC50

The present data revealed that the hydromethanolic extract of *Euphorbia guyonianus* significantly inhibited the desert truffle tyrosinase activity (inhibition ≥ 50 %). It can be suggested that this tyrosinase activity inhibition may be attributed to the richness of this plant in flavonoids and phenolic compounds The beneficial effects of *E. guyoniana* are due to its high content of secondary compounds, such as terpenoids (Haba et al., 2007), alkaloids, and flavonoids (Boudiar et al., 2010).

In fact, several works reported that phenolic compounds mainly flavonoids and tannins have an inhibitory activity on tyrosinase. The flavonoids, which are based on a common three-ring nucleus comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone ring in the middle, could inhibit tyrosinase activity by the interaction of the flavonoids with the copper ions in the catalytic domain of the enzyme. Furthermore, condensed tannins have also been reported to exhibit strong free radical scavenging activity (Chen et al.,2014).

Various families of chemical compounds have been isolated from species belonging to the Euphorbiaceae family, including alkaloids 1,5-diphenyl-3-styryl-2-pyrazoline 1 (De Nazaré et al., 2005;Boudiar et al.,2010 ), Aglycones and glycosylated flavonoids have been reported from many species(Pan et al.,2012; Nishimura.,2013) such as kaempferol,kaempferol 3-O-glucoside, kaempferol 3-rutinoside, quercetin, quercetin 3-O-glucoside, and rutin were isolated from the aerial parts of *Euphorbia guyoniana* (Boudiar et al.,2010 ).cyanogenic compounds (Hunsa et al.,1995), gallic tannins 1, 2, 6 tri-*O*-galloyl-β-D-glucose(samara and al.,2014),ellagic acid (Mavar et al. 2004),saponins (Tripathi and Tiwari 1980) and terpenes (Mazoir et al. 2008). In contrast, very few studies have been conducted on Euphorbia guyoniana.

Ahmed et al (2006) isolated and characterized two novel jatrophanediterpenes (Guyonianin A, Guyonianin B) diterpenes from the aerial part of the plant.these compounds are obtained in the pure state by the often combined use of different chromatographic methods.

As shown in Figure 12, there was a dose-dependent increase in the percentage inhibition against tyrosinase activity.

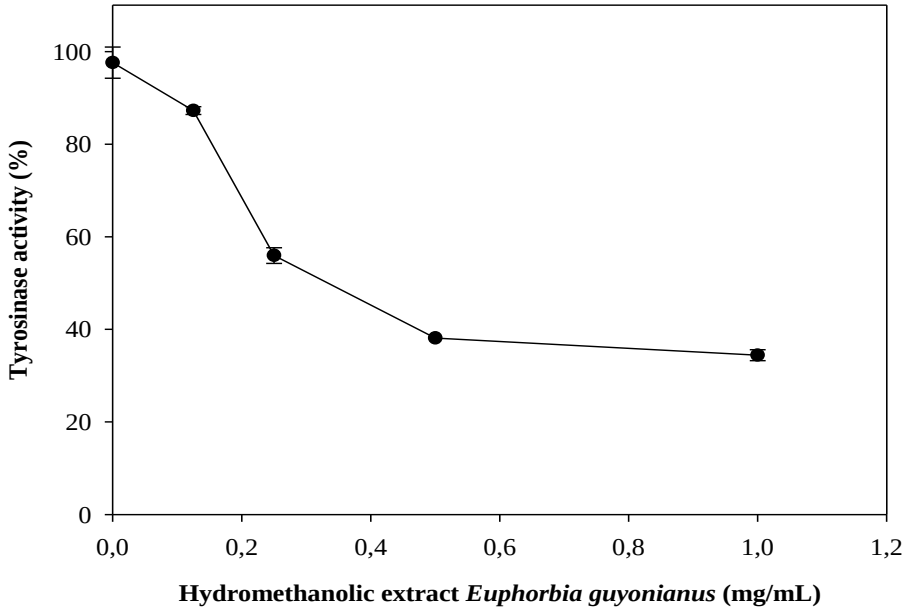



**Figure12**: Effect of hydromethanolic extract of *Euphorbia guyonianus* on tyrosinase activity (L-tyrosine at 1 mM, SDS at 4 mM, enzyme extract 30 µL, sodium acetate at pH 5.0-0.05 M, temperature 30°C, and volume of reaction mixture 1.0 mL).

## V.4 Kinetic mechanism of Truffle tyrosinase inhibition by *Euphorbia guynianus*extract:

Kinetic analysis of the hydromethanolic extract from *Euphorbia guyonianus*, against tyrosinase enzyme was performed using Lineweaver-Burk plots to determine the type of inhibition. These results were presented in Figure 13 and Table 2.

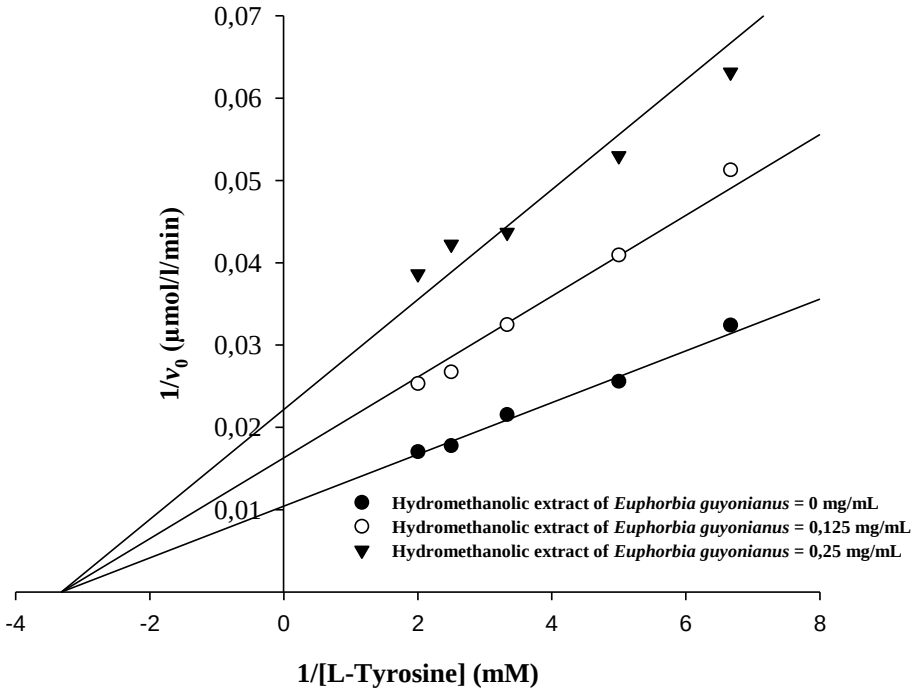



**Figure13:**The lineweaver-Burk graph of the non-competitive tyrosinase extracted from truffles (*Terfezia Leonis*Tul) activity inhibition , on L-tyrosinase by different concentration of *Euphorbia guyonianus* extract: 0(●), 0.125(o) and 0.25(▼).

**Table 2:** IC 50 and kinetics parameters of *Terfezia leonis* Tul. tyrosinase inhibition by hydromethanolic extract of *Euphorbia guyonianus*.

| Plant extract (mg/mL) | $V_{max}$ (µmol/l/min) | $K_m$ (mM) | $K_I$ (mg/mL) | $R^2$ | Inhibition type | $IC_{50}$ (mg/mL) |
|---|---|---|---|---|---|---|
| 0 | 96.03±7.23 | 0.30±0.04 | | 0.9860 | | |
| 0.125 | 71.81±5.13 | 0.39±0.05 | 0.22±0.01 | 0.992 | Noncompetitive | 0.28±0.01 |
| 0.25 | 34.57±1.72 | 0.30±0.03 | | 0.989 | | |

The presence of the hydromethanolic extract of *Euphorbia guyonianus* decreased $V_{Max}$value, but $K_M$ value remained unaltered. This indicated that these extracts inhibited tyrosinase enzyme in non-competitive manner (Figure 1). Which is a specific type of enzyme inhibition characterized by an inhibitor binding to an allosteric site; meaning the inhibitor shares the same affinity for both enzyme and enzyme-substrate complex. Resulting in decreased efficacy of the enzyme. The inhibitor has the same affinity for both the enzyme and enzyme-substrate complex. Binding of the inhibitor to the enzyme or enzyme-substrate complex inactivates the enzyme, disallowing the production of its end product. Inactivation of the enzyme decreases the maximum rate of the reaction (Vmax). The Michaelisconstant ($K_m$) remains unchanged as the active site is not competed for by the inhibitor.

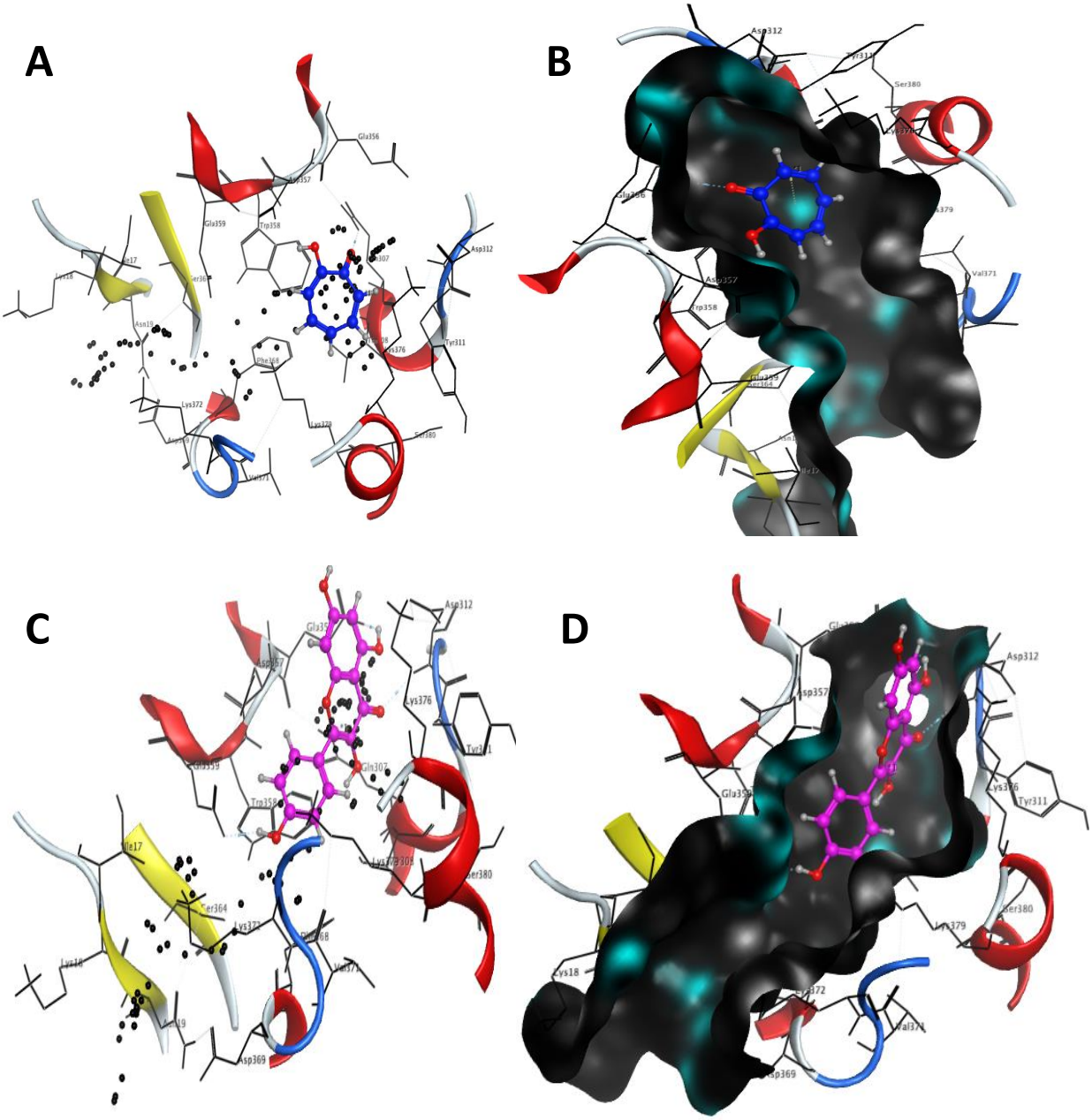
According to this type of inhibition, we can suggest that the active components in these extracts do not compete with the substrate to bind to the active site, rather the inhibitors bind to a separate site on the enzyme to delay the conversion of substrate to product.

The equilibrium constant for inhibitor binding with free enzyme, $K_I$, was obtained from a plot of the apparent maximal velocity ($V_{Max}$) versus the concentration of the plant extract. As seen from Table 1, the inhibitor constants ($K_I$) for hydromethanolic extract using L-tyrosine as a substrate, was 0.22 mg/mL.

The value of IC50 determined for tyrosinase inhibition by our plant of interest (0.28 µg/ml) was lower than the one obtained from *Ambrosia maritima L.* (0.48 µg/ml) (Muddathir et al .,2017), which means that *Euphorbia guyonianus* has the higher inhibitory effect.

## V.5 Molecular Docking Study

Molecular docking study was performed by using MOE. The 3D structure of *Bacillus megaterium* tyrosinase (PDB ID: 3NQ0) and the 3D structure of tropolone , Kaempferol were retrieved from the RCSB Protein Data Bank .



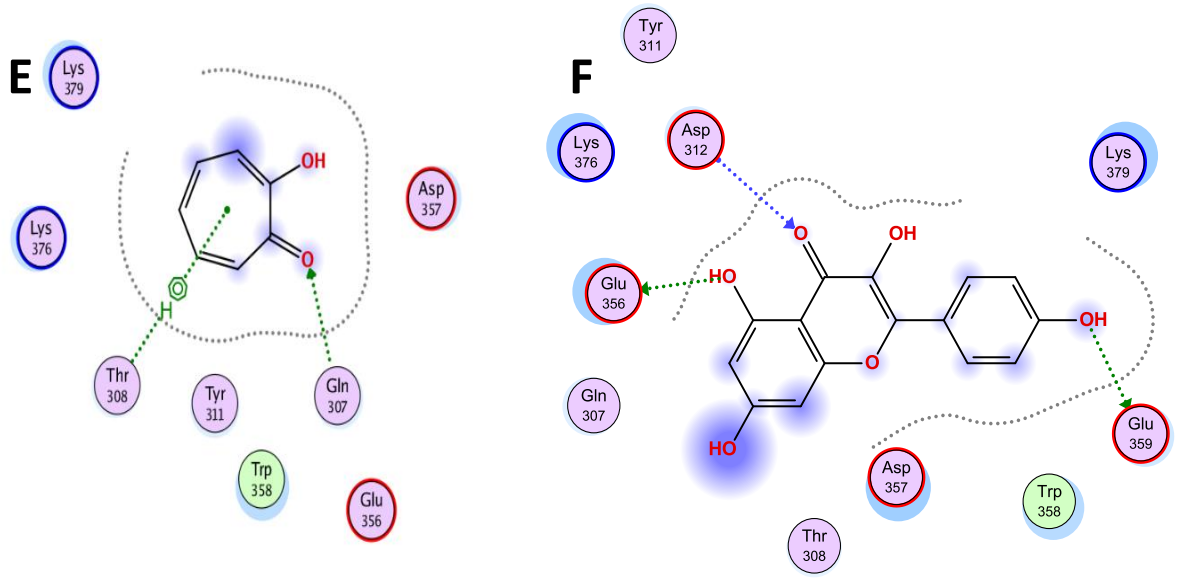



**Figure 7 :** Computational prediction of the structure for tyrosinase and docking simulation with tropolone and Kaempferol. Predicted 3D structure of Tyrosinase from *Bacillus megaterium* 3NQ0. Tyrosinase- tropolone complex (A, C, and E); tyrosinase- Kaempferol complex (B, D, and F).

**Table 3** : Results of docking experiments of Tropolone and Kaempferol with the tyrosinase (PDB ID: 3NQ0).

| Ligand | Receptor | Interaction Distance E (kcal/mol) | | Interaction energy (Kcal\mol) |
|---|---|---|---|---|
| **Tropolone** | **GLN 307** | **3.20** | **-1.1** | **-3.78 (RMSD : 2.70 A°)** |
| | **THR 308** | **4.83** | **-0.6** | |
| **Kaempferol** | **GLU 356** | **2.75** | **-1.2** | **-4.75 (RMSD : 1.52 A°)** |
| | **GLU 359** | **2.97** | **-2.7** | |
| | **ASP 312** | **3.56** | **-0.7** | |

These docking studies yielded crucial information concerning the operation of the inhibitor in the binding pocket of tyrosinase. Ligand–enzyme interaction analysis found that the interaction binding residues of Kaempferol (GLU356,GLU359, ASP,312 ) were more important than those of tropolone (GLN307, THR308) in the active site, also on the other side the interaction energy of kaempferol is lower than tropolone ($RMSD_k$ =1.52 A° < $RMSD_T$= 2.70 A°:lower RMSD represents good binding pose) meaning that kaempferol has highly effective tyrosinase inhibiton-activity, which is more potent than the commercial agent tropolone.

# *Conclusion*

The results of this work consolidate the fact that the field of polyphenols remains a very interesting field open to research and that plant extracts remains an important source for the subsequent identification of bioactive natural products. The hydromethanolic extract of *Euphorbia guyonianus* act as inhibitor for desert truffle tyrosinase when L-tyrosinase was used as substrate. The plant extract contains hydrophilic compounds which can binds on others binding site rather than the active site of substrate fixation causing a decrease of enzymatic activity. This non competitive inhibitors not affected the affinity of enzyme toward its substrate but decrease its catalytic activity.

From these results, it may be concluded that this hydromethanolic extract may selectively inhibit the first step of the melanin synthesis which involved the hydroxylation of L-tyrosine to o-diphenols (monophenolase activity). Consequently, these results suggested that the *Euphorbia guyonianus* extract is able to inhibit tyrosinase activity as well as melanin production for treatment hyperpigmentation and can be used also for controlling enzymatic browning. Future studies are needed to isolate and identify the bioactive metabolites from this plant in order to develop new tyrosinase inhibitors.

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***Abstract.*** The flora of South Algeria is relatively rich in medicinal plants and represents an important component of traditional medicine. In this study, hydromethanolic extract of *Euphorbia guyonianus* was evaluated for it inhibitory effect on desert truffle tyrosinase activity and to established its inhibition mechanism. The hydromethanolic extract of this plant inhibit strongly enzymatic oxidation of L-tyrosine in a dose-dependent manner ($IC_{50}$ = 0.28 mg/mL) at pH 5 and 30°C. Lineweaver-Burk plots of the different concentrations of L-tyrosine in the absence and presence of hydromethanolic extract of *Euphorbia guyonianus* showed a noncompetitive inhibition of desert truffle tyrosinase. This study suggests that this plant could potentially be used for the isolation and identification of the effective new tyrosinase inhibitors that can be used for hyperpigmentation treatment and as antibrowning agent.

**Key words**: Euphorbia guyonianus, hydromethanolic extract, truffle, tyrosinase, inhibition mechanism.

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إن النباتات في جنوب الجزائر غنية نسبيا بالنباتات الطبية وتمثل عنصرا هاما من عناصر الطب التقليدي. في هذه الدراسة، تم تقييم مستخلص الهيدورمانوليك من يوفوبيا غيونيانوس من حيث تأثيره المثبط على نشاط التيفوروسيناز الكمأة الصحراوية، وإنشاء آلية تثبيط لها. يعمل مستخلص الهيدانوليك من هذا النبات على منع الأكسدة الإنزيمية القوية للتيروسين L-tyrossine بطريقة يعتمد عليها الجرعة (IC50 = 0.28 ملغ/مل) عند درجة الحموضة 5 و30 درجة مئوية أظهرت قطع من Lineweaver-Burk من التركيزات المختلفة للالتيفروسين L-tyrosine في غياب وجود مستخلص من الهيدومثانوليك Euphorbia guyonianus تثبيطًا غير تنافسي لتخدير الكمأة الصحراوية التيفروسيناز. تشير هذه الدراسة إلى إمكانية استخدام هذه النبتة لعزل مثبطات التيفروسيناز الجديدة الفعالة التي يمكن استخدامها لعلاج زيادة الصبغات وكعامل مضاد للتحميص.

**الكلمات الرئيسية** : يوفوبيا غيونيانوس ، مستخرج هيدأثانوليك ، الكمأة ، التيفروسيناز ، الية التثبيط .

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La flore du sud de l'Algérie est relativement riche en plantes médicinales et représente une composante importante de la médecine traditionnelle. Dans cette étude l'extrait hydrométhanolique d'Euphorbia guyonianus a été évalué pour son effet inhibiteur sur l'activité de la tyrosinase de la truffe du désert et pour établir son mécanisme d'inhibition. L'extrait hydrométhanolique de cette plante inhibe fortement l'oxydation enzymatique de la L-tyrosine en fonction de la dose (CI50 = 0,28 mg/mL) à pH 5 et 30 °C. Lineweaver-Burk trace les différentes concentrations de L-tyrosine en l'absence et la présence d'extrait hydrométhanolique d'Euphorbia guyonianus a montré une inhibition non compétitive de la truffe du désert tyrosinase. Cette étude suggère que cette plante pourrait potentiellement être utilisée pour l'isolement et l'identification des nouveaux inhibiteurs efficaces de la tyrosinase qui peuvent être utilisés pour le traitement d'hyperpigmentation et comme un agent antibrunissement .

**Mots clés** : Euphorbia guyonianus, extrait hydrométhanolique, truffe, tyrosinase, mécanisme d'inhibition.