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THESIS IN VIEW OF OBTAINING THE MASTER DEGREE IN BIOLOGY
Option: Biochemistry

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The theme:

Contribution to study the antioxidant,cytotoxic and antimicrobial activities of umbelliferone (coumarin)

Before the jury committee, composed of:

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Academic Year 2020-2021

Thanks:

Our thanks go first to God for giving us the strength and courage we needed to complete this work.

To Pr. Abdelkarim Berroukch for having supervised us.Your guidance has been very beneficial to us for the realization of this work, your rigor and way of working, allowed us to be more attentive and critical in our work. Thank you for your patience in correction of this brief.

We hope we have lived up to your expectations.

Our thanks also go to the members of our memory jury.Thank you for having us it is such honor for us.

Dedication

To the most adorable person in my life and in my world my only parent:
Mother, this job is yours.
Thank you for having supported me morally and materially to this day.
Here is the culmination of your many nights of prayers wisdom and generosity for your little girl. Dear mother, this work is the fruit of your efforts.
To my brother, the road is hard and still long, it would take courage and a lot of luck, god bless you.
Without forgetting to thank my second family:
Amina, Dallel, and Wadjila, and all my friends and those from my online game specially my Zhbill for their moral support for having my back all through bad times.
I would simply like to tell them that I will l always love them with all my heart. God bless you.
To all my teachers also who were more like parents for us and guided us to only success.

Fatima

Dedication

With the help of God Almighty, who has set me on the path of my life, I was able to realize this work and with a huge pleasure, an open heart, and immense joy, that I dedicate my work

To my dear parents

To my brothers

To my family

To my teachers

To my friends

And to all the people who have encouraged me or helped me throughout my studies.

Chikh

Abstract:

Umbelliferone is a 7-hydroxycoumarin that's pharmacologically active. It is found in *Rutaceae* and *Apiaceae* families *(Umbelliferae)*and is extracted using methanol.

UMB is used as a sunscreen agent and optical brightener in textiles. It's synthesized chemically using the Pechmann condensation reaction from resorcinol and formyl acetic acid. Biosynthetically it's synthesized using the phenylpropanoid pathway. Umbelliferone is a coumarin derivate with improved biological activities like antibacterial and antifungal activities and also showed good inhibitions of DPPH, hydroxyl, superoxide anion. Other reported activities are anti-inflammatory, Antidiabetic effect, and cytotoxic activities.

Résumé:

L'umbelliférone est un 7-hydroxycoumarine qui est pharmacologiquement active. Il se trouve dans les familles des *Rutacées* et des *Apiacées (Ombellifères)* et est extrait à l'aide de méthanol.

L'UMB est utilisé comme agent de protection solaire et azurant optique dans les textiles. Il est synthétisé chimiquement en utilisant la réaction de condensation de Pechmann à partir du résorcinol et de l'acide formyl acétique. Biosynthétiquement, il est synthétisé en utilisant la voie des phénylpropanoïdes. L'ombelliférone est un dérivé des coumarins avec des activités biologiques améliorées telles que des activités antibactériennes et antifongiques et a également montré de bonnes inhibitions de la DPPH, de l'hydroxyle et de l'anion superoxyde. D'autres activités rapportées sont des activités anti-inflammatoires, antidiabétiques et cytotoxiques.

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List of Abbreviations :

AS: Angelicin synthase

B8M: Bergapten -8-monooxygenase

C2H: Cinnamic acid 2-hydroxylase

C4H: Cinnamic acid 4-hydroxylase

CS: Columbianetin synthase

4CL: 4-coumarate-CoA ligases

CO2H: 4-coumaric acid 2-hydroxylase

C2'H: P-coumaroyl-CoA 2'-hydroxylase

DBU: 1, 8-diazabicyclo undec-7-ene

DDU-7: Dimethylallyl diphosphate umbelliferone transferase

GSK3β: Glycogen synthase kinase-3β

hMAO: Human monoamine oxidase

M: Monooxygenases

MS: Marmesin synthase

MMP: Matrix metaloproteinase

OMT: Omethyltransferase

PAL: Phenylalanine ammonia-lyase

PDB: Protien data base

P5M: Psoralen-5-monooxygenase

P8M: Psoralen-8- monooxygenase

PPO: Polyphenol oxidase

PPARγ: Major nuclear receptor in adipogenesis

PS: Psoralen synthase

PT: prenyltransferase

SHC: Squalene-hopene cyclase

TAL: Tyrosine ammonia-lyase

Umb: Umbelifferrone

X5M: Xanthotoxin-5-monooxygenase

GENERAL
INTRODUCTION

During their development, plants can be subjected to various aggressions. Abiotic factors related to weather and climate (storms, wind, snow, drought ...) and biotic factors (viruses, bacteria, fungi, animals and other plants) are real threats to the survival of plants. These plants have various defense systems to cope with these stresses. For example, the external tissues of plants are composed of lipidic layers such as cutin for the aerial parts and suberin for the underground parts, providing protection against attacks and water loss **(1)**. Some plants also have a Bark or spines protecting them from herbivorous animals. Despite these passive means of protection, plants remain vulnerable and subject to attack by a variety of stressors. The smallest (viruses, bacteria and fungi) can enter the plant through mechanical wounds. Some fungi use natural "openings" and manage to cross the epidermis of the plant through the stomates, during the germination of spores **(1)**.

Herbivorous animals, on the other hand, can feed on the leaves, thus strongly threatening the survival of the plants To protect themselves, in addition to physical barriers, plants have developed defense systems based on the production of more or less toxic molecules. These natural compounds are derived from the plant's metabolism and are called secondary or specialized metabolites. They were first mentioned as "endproducts" by Czapek who considered that they originated from the metabolism of nitrogen following "secondary modifications" **(2)**. Subsequently, in the 1980s, they were defined as "natural products, generally of plant origin, which are not directly involved in the primary biochemical activities that enable the growth, development and reproduction of the organism in which they are produced" **(3)**. Although the term "secondary metabolite" was originally coined because of a lack of understanding of their role in the plant, because their synthesis varies greatly from one plant to another and because these molecules are rarely remobilized after their accumulation **(4)**, a great deal of research is now showing their fundamental roles in plant development. In fact, we are now talking more and more about "specialized metabolism"**(1).**

The specialized metabolites can be classified into four main groups (Figure1):

-Terpenoids, alkaloids, heteroids, and phenolic compound **(5)**. Terpenoids belong to the hydrocarbon class and have one or more isoprene units with five carbon atoms. There is a great diversity of terpenes with more or less complex structures and with different roles in the plant. For example, we find carotenoids such as β-carotene (**6),** some phyto-hormones such as

abscisic acid **(7),** sterols such as cholesterol **(8)** and their derivatives or volatile terpene compounds such as limonene, present in citrus essences **(1).**

-Alkaloids are generally heterocyclic molecules, having at least one nitrogen atom often bonded to a proton, which gives them a slightly basic pH in solution, hence the name alkaloid **(5).** Unlike othersecondary metabolites, alkaloids are a class of very heterogeneous compounds that can be divided into four groups: alkaloids derived from amino acids (e.g. nicotine), thosederived from purine (e.g: caffeine), those derived from terpenes (e.g:solanine), and alkaloids with the nitrogen in a polyketide moiety as in conine (**9**). Some of these molecules allow the plant to defend itself against insects, bacteria, and viruses (for review, see (**10**), and the bitterness of most alkaloids isthought to act as a deterrent to herbivores (**9**). In addition, some alkaloids are drugs that can sometimes have therapeutic uses for humans such as morphine or codeine (**11**).

- Heterosides are molecules resulting from the condensation of a sugar with a non-carbohydrate molecule at a carbon, oxygen, sulfur or nitrogen atom. There are different families such as saponosides (glycosylated triterpenes), cardiotonic heterosides (differentiated from saponosides by the presence of a lactone ring and rare carbohydrates) and cyanogenic heterosides which, once hydrolysed, release toxic hydrocyanic acid (**5**). Glucosinolates were once thought to be related to sulfur-containing heterosides, due to their heteroside-like structure. They are widely reported in the Brassicaceae, but there are other plant families capable of producing them (**12**). Overall, heterosides allow the plant to defend itself against herbivores, thanks to repellent properties and a greater or lesser toxicity(**1**).

-Phenolic compounds also called polyphenols or phenylpropanoid derivatives phenylpropanoids have a basic structure composed of a hydroxylated aromatic benzene ring: phenol. There is a great diversity of polyphenols with different structures and roles. They can be complex molecules, such as lignin involved in the structure of the plant, or simpler molecules, involved in interactions with the environment. For example, flavonoids are involved in ultraviolet (UV) protection and pollinator attraction through anthocyanin pigments (**6**). Other phenolic compounds are synthesized for the protection of the plant against aggressors such as stilbenes (**13**), furocoumarin, and coumarins (**14**). This last class of compounds on which our theme on umbelliferone is a one of coumarins derivate.

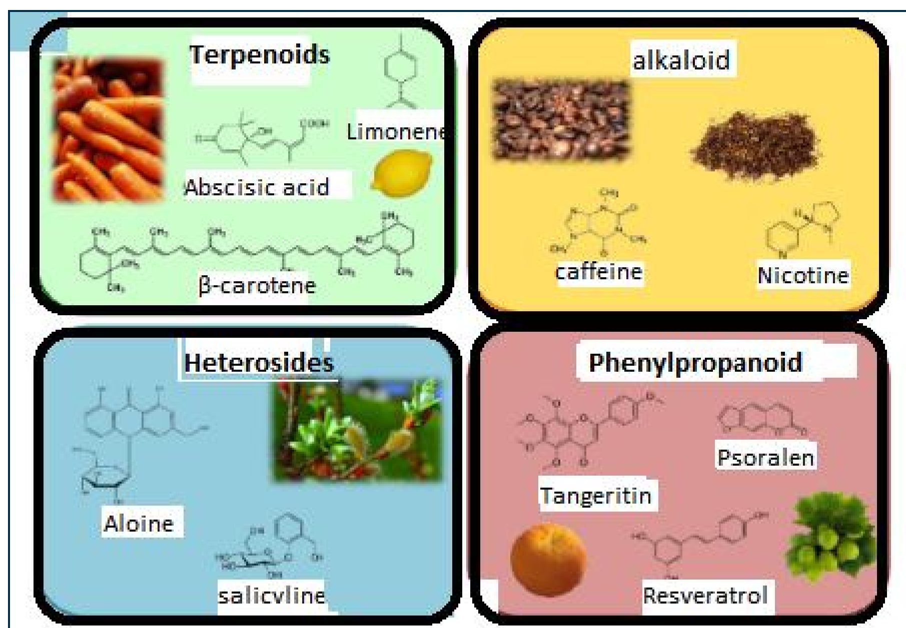

Figure 01: Classes of secondary metabolites and some examples of their natural compounds(15)

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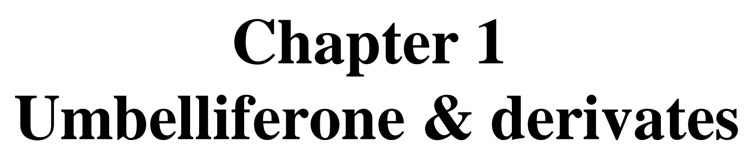

Chapter 1
Umbelliferone & derivates

I.Umbelliferone

I.1.Generalities:

Umbelliferone is a widespread natural organic compound of the coumarin family.Produced by the combination of a benzene ring with a pyran, having a ketone function in α position with respect to oxygen.

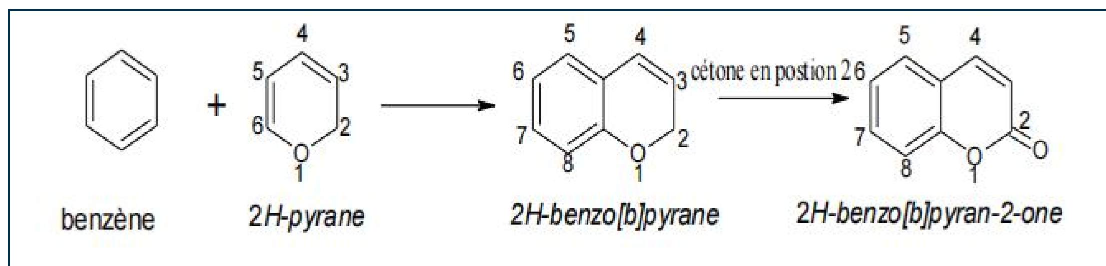


Figure 02: Combination of a benzene ring with a pyran

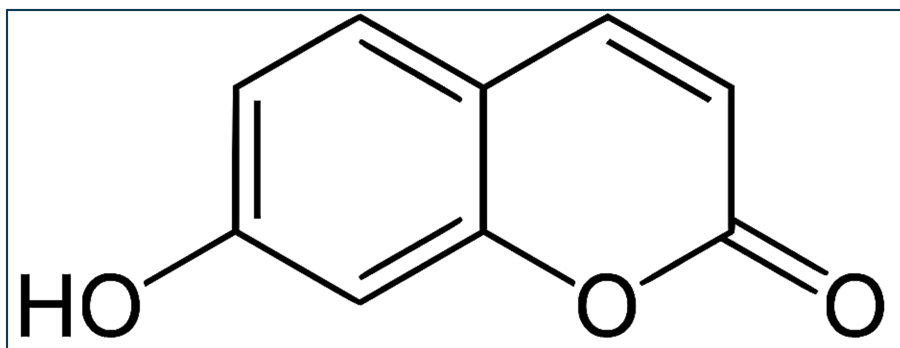


Figure 03: Chemical structure of Umbelliferone (17)

(https://pubchem.ncbi.nlm.nih.gov/compound/5281426)

- **Brute formula:** $C_9H_6O_3$
- **Mr:** 162,14 g/mol

I.2. Chemical names:

IUPAC name: 7-hydroxychromen-2-one

Other:

7-hydroxycoumarin

7-hydroxycoumarin sulfate

7-hydroxycoumarin, 14C-labeled

I.3. Physicochemical proprieties:

Umbelliferone yellowish-white crystals are slightly soluble in hot water, but have good solubility in ethanol The optimized geometry is planar and the OH group lies on the same plane as the whole molecule -Organoleptic characteristics (anything that can excite a sensory receptor).

Umbelliferone occurs as yellowish orthorhombic crystals. These molecules give off a pleasant odor generally bitter taste Coumarin is largely soluble in ethanol, chloroform and oils, while it is soluble in small amounts in boiling water and slightly soluble in water at 20°C.Free coumarins are soluble in alcohols and organic solvents, such as ether or chlorinated solvents

I.3.a. Solubility:

Umbelliferone yellowish-white crystals are slightly soluble in hot water, but have good solubility in ethanol chloroform and oils, while it is soluble in small amounts in boiling water and slightly soluble in water at 20°C.The optimized geometry is planar and the OH group lies on the same plane as the whole molecule Organoleptic characteristics (anything that can excite a sensory receptor). These molecules give off a pleasant odor generally bitter taste

I.3.b. Melting point:

Umbelliferone are characterized by a high melting point, given their important structure. The melting point of umbelliferone is 224-227°C.

I.3.c. Thermal stability:

Umbelliferones are relatively stable molecules at very high temperatures. This stability decreases when they are exposed to UV radiation. The different physico-chemical properties are due to the different substituents carried by the nucleus which give the possibility to separate them and to characterize them by different analysis techniques (TLC, GC and HPLC)

I.4. Distrubution of Umbelliferone in the plant kingdom:

Today, there are more than 1300 different coumarins **(18)** described in plants, but also in fungi and bacteria. They were originally discovered in the tonka bean (Dipteryx odorata, family *Fabaceae)* which contains many of them.

Figure 04: Tonka bean **(19)**

source: https://www.healthysupplies.co.uk/tonka-beans-sussex-organic-50g.html)

Umbelliferone was discovered in the family *Apiaceae* / *Umbelliferae*.

The *Umbelliferae* are a family of phanerogamous plants (from the Greek phaneros, visible, and gamos, union of the sexes) is the plants with feurs and seeds. dicotyledonous/ dialypetal, which contains *Parsley*, Carrot, Chervil, etc., and has for essential character the arrangement of its flowers in umbels, which earned its name. It contains only herbaceous plants.

	Plants	Localisation	Reference
umbelliferone	*Anethum graveolens*	Fruits and roots	Steck, W *et al*., 1969
	Angelica archangelica	Fruits and roots	
	Levisticum officinalis	Fruits and roots	
	Ruta graveolens	Roots	Lièvre, K. 2004

Table 01: Distribution of umbelliferone in the plant kingdom

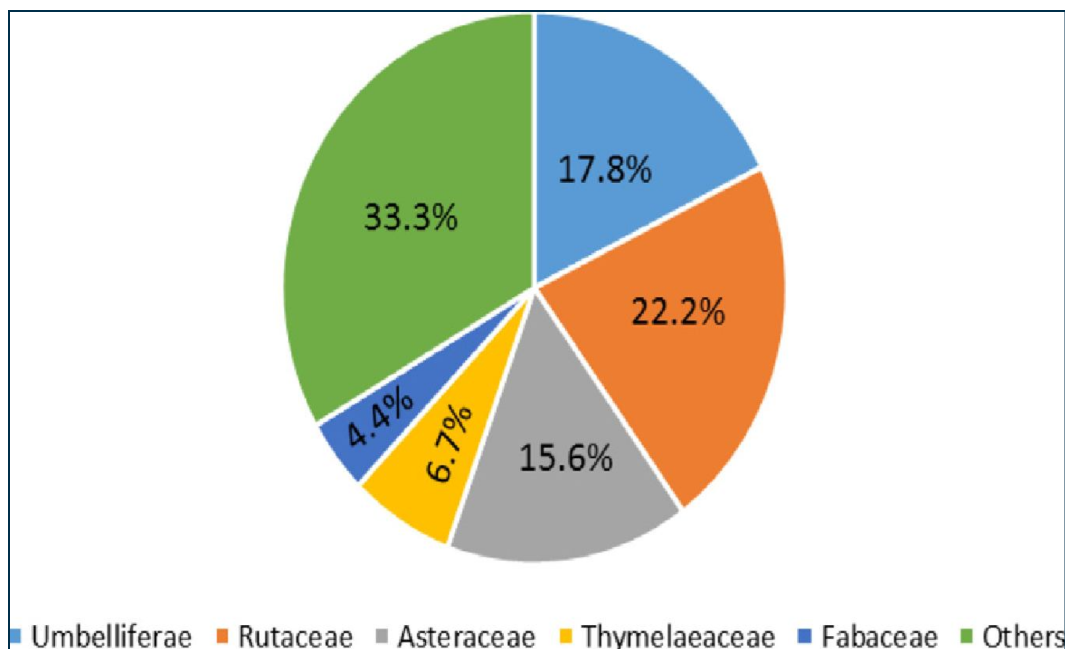


Figure 05: Distribution of umbelliferone in the plant kingdom(20)

I.5. Biosynthesis of Umbelliferone:

Phenolic compounds are synthesized from the three aromatic amino acids: phenylalanine, tyrosine and tryptophan. The latter are themselves derived from the shikimate pathway whose precursors are erythrose-4-phosphate and phosphoenol-pyruvate from the pentose phosphate and glycolysis pathways respectively **(5)**.In the case of coumarins and furocoumarins, they are derived from phenylalanine (Figure 06), which is first converted to cinnamic acid by the removal of ammonia (NH3) molecule, catalyzed by phenylalanine ammonia-lyase (PAL) **(5)**.

Following the action of PAL, cinnamic acid can be ortho-hydroxylated to lead to coumarin or para-hydroxylated to umbelliferone and furocoumarins. The ortho-hydroxylation of cinnamic acid by cinnamic acid 2-hydroxylase (C2H), has not been characterized from a molecular point of view to date. This step would lead to o-coumaric acid (Figure 06), which under neutral or acidic conditions is unstable and lactonizes spontaneously to give coumarin **(14)**. Para-hydroxylation of cinnamic acid leads to p-coumaric acid. This step is performed by cinnamic acid 4-hydroxylase (C4H), a cytochrome P450 of the CYP73A family initially identified in Jerusalem artichoke and later in dozens of other plants including Ruta graveolens .

P-coumaric acid is then converted to p-coumaroyl-CoA (Figure6) by the action of 4-coumarate-CoA ligase (4CL) **(15)**. Many secondary metabolitessecondary metabolites are

derived from p-coumaroyl-CoA, such as coumarins andfurocoumarins **(14)**, flavonoids monolignols or stilbenes . The pcoumaroyl- CoA is then converted to 2',4'-dihydroxycinnamoyl-CoA (Figure 06) by hydroxylation performed by an oxoglutarate-dependent dioxygenase (p-coumaroyl-CoA 2'-hydroxylase, C2'H) that has been identified in Ipomoea batatas (Matsumoto et al., 2012) . This enzyme also allows the formation of scopoletin from feruloyl-CoA. The 2', 4'-dihydroxycinnamoyl-CoA then undergoes spontaneous lactonization to form umbelliferone **(14)**

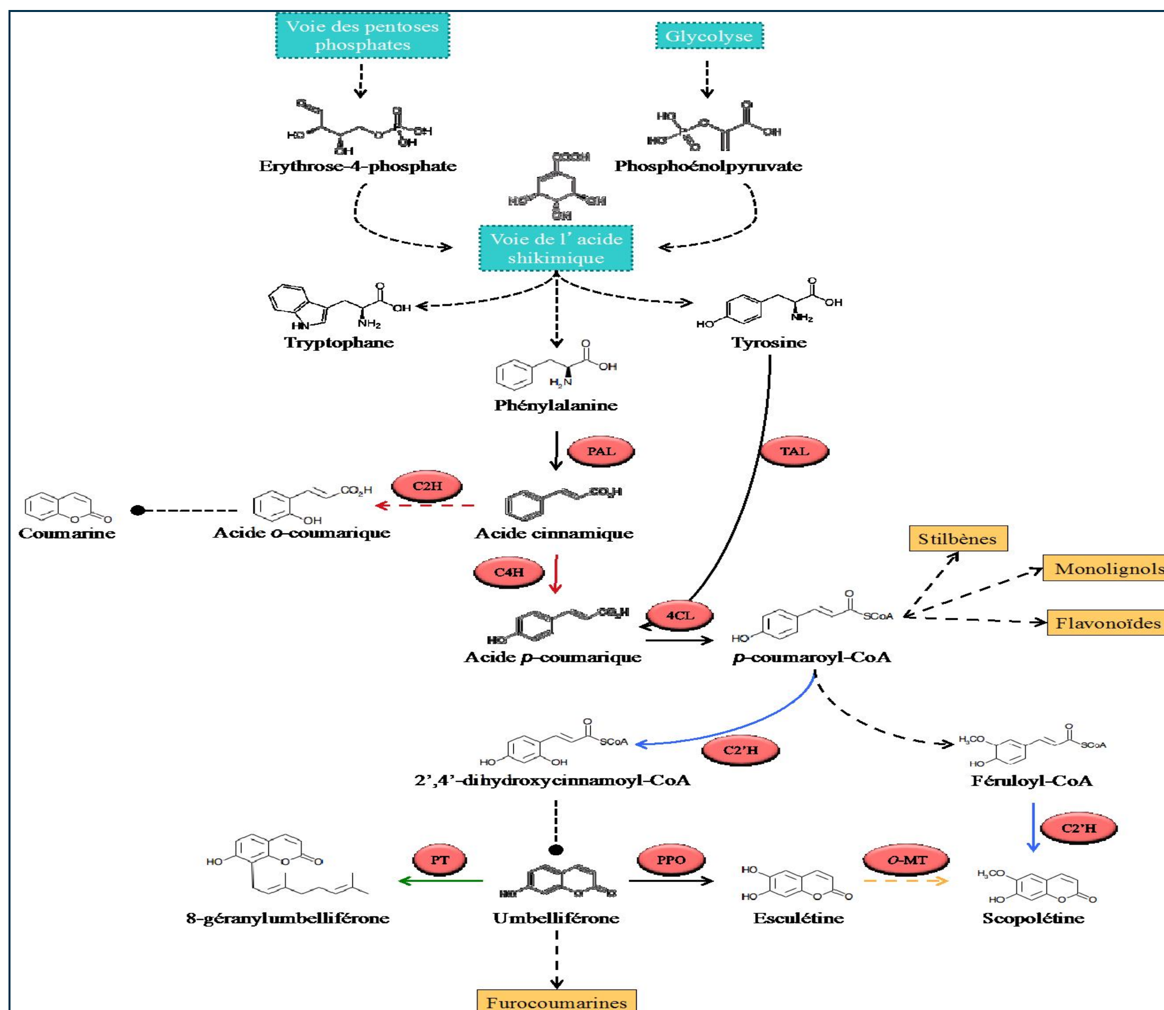


Figure06: Simplified biosynthetic pathway of Umbelliferone**(14)**

PAL: phenylalanine ammonia-lyase **TAL**: tyrosine ammonia-lyase **C2H:** cinnamic acid 2-hydroxylase **C4H:** cinnamic acid 4-hydroxylase **4CL:** 4-coumarate-CoA ligases

CO2H: 4-coumaric acid 2-hydroxylase **C2'H:** p-coumaroyl-CoA 2'-hydroxylase

PPO: polyphenol oxidase **OMT:** Omethyltransferase **PT:** prenyltransferase

I.6. Method of synthesis of Umbelliferone:

I.6.a. Synthesis of umbelliferone by the Pechman reaction:

Among the simplest and most widely used methods for the synthesis of umbelliferone we find Pechmann's reaction. Conventionally, the process consists of the condensation of a phenol with a β-ketoester. In the presence of the various reagents and gives good yields of umbelliferone substituted in position 4. Several acid catalysts were used in this reaction including H2SO4, HClO4, and P2O5 CF3COOH. In other methods, researchers have used ionic liquids and irradiation by, but these methods also generate strongly acidic by-products and / or they use very expensive, non-recurring agents recently, a number of heterogeneous catalysts such as Nafion-H, HBETA zeolite, Amberlyst 15, montmorillonite clay, silica with sulfuric acid, alumina, and ultrasonic irradiation were used in the Pechmann condensation(**21**).

Over the past few years, the Science and Technology Branch have shifted to environmentally friendly resources, natural products and reusable catalysts. For example, natural biopolymers are attractive candidates in the search for catalysts to solid support. Copolymers, especially starch and its derivatives, have the following characteristics unique, which make them attractive alternatives in organic or inorganic carriers for conventional catalysts for applications.

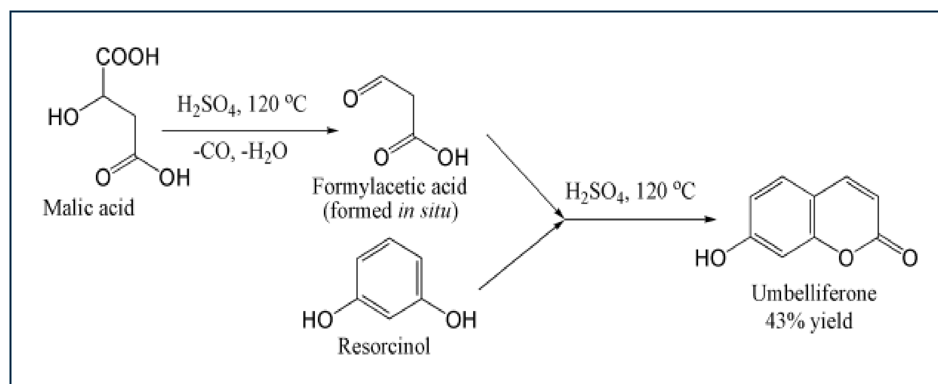


Figure 07: Pechmann condensation(**22**)

I.7. Therapeutic activities:

Umbelliferone has been shown to exhibit various pharmacological activities against various health-related conditions, including conditions related to pro-oxidants and reactive oxygen species such as inflammation, degenerative diseases, microbial infections and cancer cells.

I.7.a.Antioxidant activity:

Antioxidants are classified according to their mode of action: free radical scavengers, metal ion chelators, oxygen scavengers in closed systems.

Polyphenols, naturally present in foods or formed during processing, are considered to be free radical scavengers .

I.7.a.1. Free radical scavenging:

Polyphenols have antioxidant properties due to their ability to scavenge free radicals and reactive oxygen species, the process is radical They interfere with the oxidation of lipids and other molecules by the rapid donation of a hydrogen atom to free radicals according to a mechanism proposed as early as 1976 by Sherwin: the antioxidant formally gives up hydrogen radical, which may be an electron transfer followed, more or less rapidly, by a proton transfer, to give an intermediate radical. It is stabilized by its conjugated mesomeric structures (23) **(Sökmen et al., 2012**).

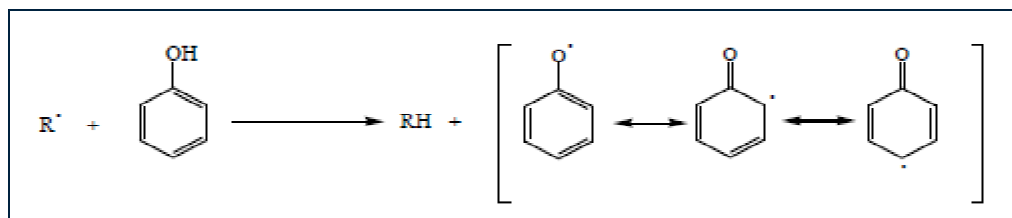


Figure 08: Mechanism of action of phenolic antioxidants(24).

A study reported 63.6% inhibition of membrane reactive free hydroxyl radical exhibited by umbelliferone on a site-specific deoxyribose degradation assay **(24)**

Umbelliferone inhibited superoxide anion formation on an assay based on the oxidation of nicotinamide adenine dinucleotide (NADH) by phenazine methosulphate showing IC50 value of 150 lM **(24)**

I.7.a.2. Chelation of metal ions:

Metal ions are necessary for the functioning of biochemical and physiological cellular processes. biochemical and physiological processes, but in some cases and when their mechanism of action is not well controlled these same ions can be the cause of lipid peroxidation, oxidative stress, or tissue injury, for example, Cu2+ is a stimulator of LDL peroxidation. **(25) Tiwari, 2001)**

Phenolic compounds with catecholate and gallate groups can inhibit metal-induced oxygen radical formation either by coordination with Fe2+ and enhancing auto-oxidation of Fe2+ or by inactive complex formation with Cu2+, Fe2+, or Cu+ with relatively weak interaction **(26)**.

Umbelliferone caused the reduction of the Fe3+/ferricyanide complex to the ferrous form monitored by the formation of Perl's Prussian blue and bound Fe2+ ions**(26)**

I.7.a.3. Enzymatic inhibition:

Due to the presence of multiple umbelliferone functionalities, it interacts with proteins so strongly, that precipitation of protein-polyphenol complexes frequently occurs, which is the basis for their use in the leather tanning process **(27) Handique & Baruah, 2002**). UMB-protein interaction phenomena have been extensively studied in vitro.To clarify the mechanism of action of the inhibitory activity of umbelliferone.

A study has shown Umbelliferone was assessed for the protection of b-carotene from linoleic acid-induced bleaching. Linoleic acid is capable of producing aqueous free radicals by generating hydrogen peroxide. The presence of umbelliferone in the M.glabra MEOH extract fractions helped to neutralize hydroperoxide and inhibited the oxidation of b-carotene**(28)**

These authors found that the mechanism of inhibition of linoleic acid by umbelliferone was not due to complexation or oxidation of Fe2+, but rather to irreversible inhibition resulting from covalent bonds between the enzyme and UMB**(29)**

I.7.a.4. Othereffects:

Umb play a role like a radioprotector against radiation-induced free radicals for most important cellular targets DNA and membranes **(30)**

UF supplementation decreased hepatic lipid peroxide and activated antioxidant enzymes levels in high-fat-fed mice**(29)**

I.7.b. <u>Anti-diabetic Properties:</u>

Since ancient times, there have been theories of banishing diseases with natural products. Aegle marmelos Corr. belonging to the Rutaceae in which marmesin (furocoumarin)

is mainly located, is widely used against diabetes in Indian Ayurvedic medicine.The effect of the extract from Aegle marmelos fruit is more effective than glibenclamide at a dose of 250 mg/kg. Coincidentally, as traditional Chinese medicine, umbelliferae and oleaceae were prescribed to remedy Xiaoke lesion, namely diabetes.With the development of extraction and separation technology, many active ingredients have been separated and purified (Figure 8)(**31**).

Due to the structural complexity of the molecules purified from plant extracts, it is difficult to ascertain whether the coumarins component is the active site. Umbelliferone (1), a simpler variant of coumarin, brings blood glucose level to near normal level. In Ramesh's study, elevated insulin level was detected with amelioration of plasma glucose elevation. All the evidences therefore indicate that coumarins are of antihyperglycaemic ability. Osthole (8), which is totally chemically different from thiazolidinedione in structure, significantly activates both PPARα and PPARγ in a dose-dependent manner. Scopoletin (3) reactivates insulin-stimulated Akt phosphorylation in insulin-resistant hepatocytes and upregulates PPARγ expression in adipocytes. A recent study reported that scopoletin inhibit advanced glycation endproducts (AGEs) production by activating Nrf2(Ser40) phosphorylation and PTP1B expression, reducing accumulation of AGEs in the livers and promoting Akt phosphorylation.Fraxetin, subjected to cortex fraxini coumarins **(5),** shows antihyperglycaemic effect by altering the activity of key enzymes of carbohydrate metabolism. Aculeatin **(11),** an active compound in T. asiatica, can enhance both differentiation and lipolysis of adipocytes, which are useful for the treatment of lipid abnormalities as well as diabetes.Recently, a number of researchers have generated synthetic coumarin derivatives, for example, osthole-derived N-hydroxycinnamides, which have more powerful antidiabetic activity than osthole(**9**). Some derivatives

also possess more potent antioxidant activity, which both directly and indirectly benefits diabetic physiology. Some other derivatives are designed and synthesized as phosphatase inhibitors to alter the insulin signalling pathway. More intriguingly, GLP-1 modified with coumarin derivative was stabler in plasma and long lasting in vivo(**31**).

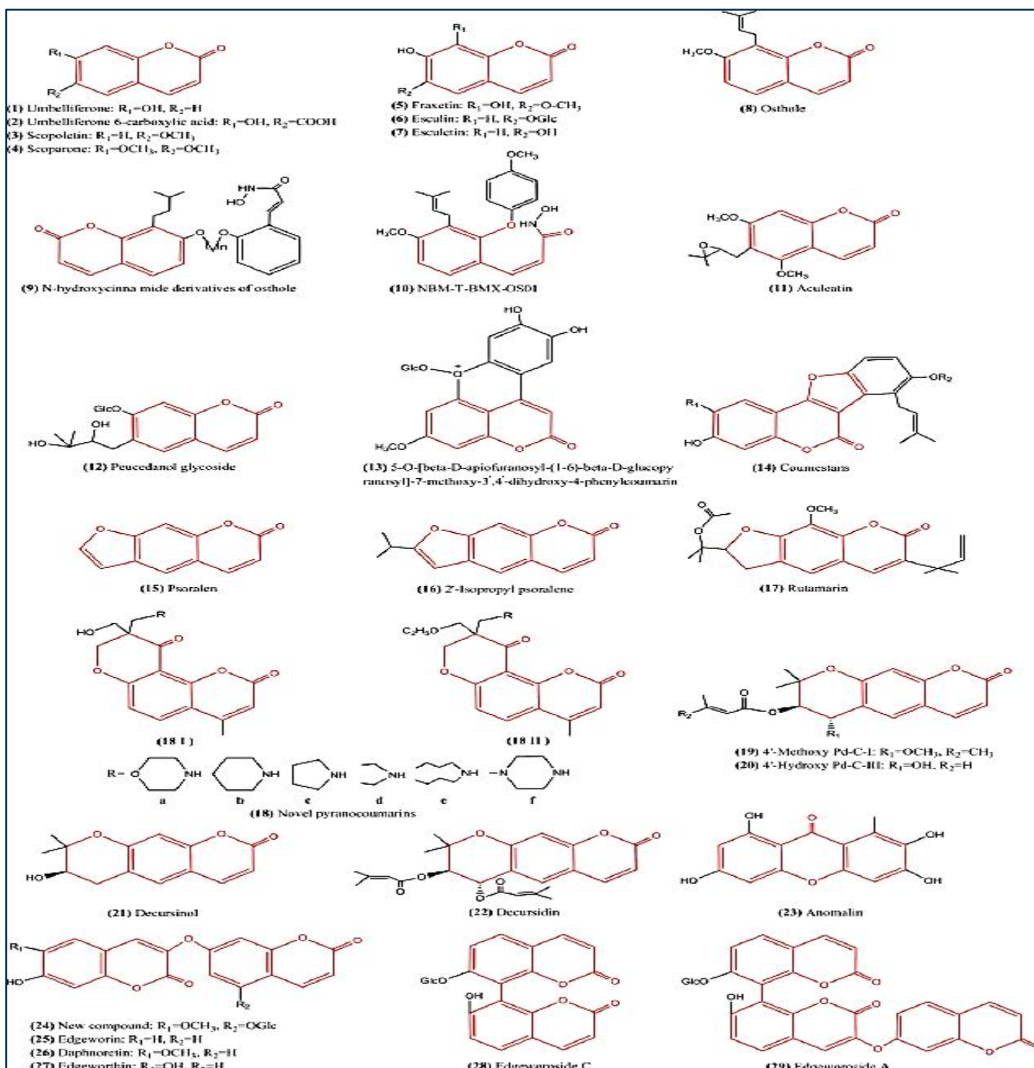


Figure 09: Chemical structures of coumarins **(32)**

(***J Pharm** Pharmacol*, Volume 69, Issue 10, October 2017, Pages 1253–1264**)**

Umbelliferone is a simple form of coumarin, which is mainly extracted and separated from Citrus aurantium and Aegle marmelos Correa. Oral administration of umbelliferone at doses of 30 mg/kg can lower both fasting blood glucose level and glycated haemoglobin type A1c (HbA1c) of diabetic rats induced by streptozocin (STZ) independently of body weight and insulin secretion.Jarinyaporn et al. reported that intraperitoneal injections of glucose tolerance (IPGTT) was significantly increased after umbelliferone treatment both in mice on high-fat diet and in STZ co-induced type 2 diabetic rats. The results of liver pathological section by haematoxylin and eosin staining showed reduced fat accumulation in the liver. Furthermore, expression of peroxisome **(33).**

Proliferator-activated receptor γ (PPARγ) and surface GLUT4 in adipose tissue was upregulated, suggesting that umbelliferone could enhance insulin sensitivity and promote GLUT4 translocation through activation of PPARγ. In type 1 diabetes, oxidative stress is an

important factor which worsens pancreatic β-cell dysfunction.Umbelliferone can help cells resist oxidative stress by facilitating biosynthesis of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT). Kumar et al. discovered that umbelliferone glucopyranoside had the same bioactivities as umbelliferone.In another perspective, Ramesh discovered that umbelliferone reduced production of misfolded collagen by inhibiting hydroxyproline level, which would be helpful to treat collagen-induced diabetic nephropathy and diabetic polyneuropathy **(33)**

I.7.c. Anti-microbial properties:

In addition to persistent hyperglycemia, diabetic patients generally have numerous complications, including periodontitis, which is an inflammatory illness of supporting tissues of teeth. Certain specific microorganisms are considered responsible for the progression of this disease. Periodontitis is a multifarious infectious disease, which is a consequence of the interaction of diverse bacteria, mostly due to bacterial biofilms .This bacterial biofilm formation is considered as a problematic health concern, responsible for antibiotic resistance, and it is a consequence of quorum sensing (QS, cell-cell signaling). Biofilm formation is a mutual group behavior that encompasses various bacterial populations entrenched on a self-produced extracellular matrix. The mutual performance of bacteria (biofilm) is mainly executed by QS, which is a cell-cell communication mechanism that coordinates gene expression in reply to population cell density. It has been well established that a change in biofilm life style of a bacterial population is directed by QS as the population density reaches a certain threshold level. Dentistry is facing a great problem of antibiotic resistance that could be specifically due to emergent bacterial biofilms. Various antibiotics, which are associated with side effects, are generally used against this problem. Thus, a quest to look for new treatment options is obvious; and traditional medicinal plants provide a promising option in this regard.The *Ferula narthex* Boiss. (*Apiaceae)* exudate is frequently used by local healers for antibacterial and analgesic effects in the oral cavity. The exudate of this plant is a rich source of umbelliferone derivatives, which have been reported to possess interesting antimicrobial properties**(34)**

Figure 10: Plant Image for Ferula narthex BOISS**.**

Umbelliferone has been reported to be active against *Brucella species*. However, from the examination of the effects of umbelliferone and other natural coumarins on the growth of Gram-positive bacteria (*Staphylococcus aureus, Micrococcus lysodeicticus*, and *Bacillus megatherium*) and Gram-negative bacteria (*Escherichia coli, Aerobacter aerogenes* and *Serratia marcescens*), Dadak and Hodakll reported that umbelliferone (5 x 10^{-3}M) is completely inactive against all of these microorganisms. Gram-negative bacteria were not affected by any of the coumarins they examined**(35)**

However, ostruthin strongly inhibited growth of the Gram-positive bacteria at concentrations of 10-□M to 2.5 x 10-□ M, and it was also effective against the yeast *Saccharomyces cerevisiae*. These authors suggested that the antibacterial activity of ostruthin requires both the free 'I-hydroxyl group and the long geranyl side-chain, since methylation of the hydroxyl resulted in considerable loss of activity. In contrast to this observation, however, it has been reported13 that the 7-methoxycoumarin, osthol, is very active against some species of Gram-positive bacteria. **(35)**

I.7.d. Anti-fongical properties:

The resistance of plants to disease may be due to the presence of preformed natural fungicides, or to the synthesis of some composants in the plant tissue in response to fungal or viral infection. Although it has been suggested that some coumarins, including umbelliferone andfurano-coumarins, play important fungicidal roles in plants, the activities of these substances against a wide variety of bacteria, yeasts and molds have not been extensively

studied(13), and little is known of the effects of structural variations within this class on their microbial inhibition. In the case of fungi it has been shown that coumarin inhibits growth of the yeast *Saccharomyces cerevisiae* and at higher concentrations (100-1000 ppm) inhibits or retards germination of the spores of *Aspergillus niger*and *Penicillium glaucum*(36)

Chakraborty et aL6p7 studied the activity of umbelliferone (7-hydroxycoumarin) and of sixteen other natural coumarins at concentrations of 2×10^{-2} M to $2 \times 10^{-□}$ M against *Aspergillus niger* and *Curvularia lunata* and found that umbelliferone was virtually inactive against these fungi at lower concentrations(37)

I.8. Synthesis of Umbelliferone Derivates:

I.8.1.7-hydroxy-4-méthylcoumarine (HM):

I.8.1.a. Generalities:

It is a heterocyclic compound derived coumarin type umbelliferone.

- **Brute formula:**$C_{10}H_8O_3$
- **Mr:** 176.17 g/mol

- **Chemical structure:**

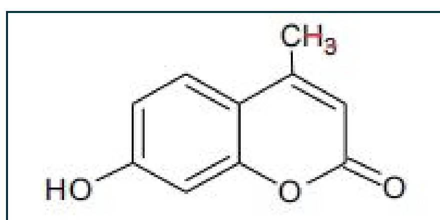


Figure 11: Chemical structure of hymecromone.

I.8.1.b. Chemical names:

7-hydroxy-4-methylcoumarin.

4-methylbeliferone.

INN: hymecromone.

ND: CANTABILINE®: HEPATOTROPIC.

I.8.1.c.Reaction:

Pechmann's synthesis results from the interaction between a phenol (resorcinol) and an α-β-ketoester (ethyl acetoacetate), this reaction requires a catalyst, in this case sulphuric acid is used, the reaction balance is as follows:

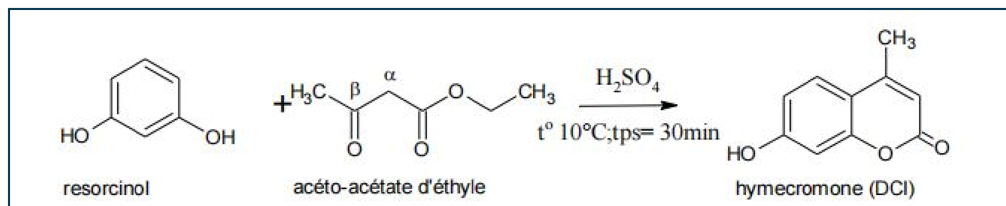


Figure 12: Pechmann reaction

I.8.1.d. Pharmacological profile:

I.8.1.d.1 Indication:

Symptomatic treatment of pain related to dyspeptic disorders**(38)**.

I.8.1.d.2. Contraindication:

Sensitivity to hymecromone**(38)**.

I.8.1.d.3. Warnings and precautions for use:

Do not use in cases of bile duct obstruction and severe hepatocellular **(38)**

I.8.1.d.4 Interaction:

The data available to date do not suggest the existence of clinically significant interactions**(38)**.

I.8.1.d.5. Pregnancy and lactation:

Experimental data in animals have not shown a teratogenic effect. In the absence of data in the human species, it is preferable not to prescribe it to pregnant women**(38)**.

I.8.1.d.6. Undesirable effects:

A few cases of diarrhoea and, exceptionally, skin reactions of allergic origin**(38)**.

I.8.1.d.7. Pharmacodynamics:

Drug with hepatobiliary (hepatotropic) action.

Hymecromone has a spasmolytic effect on the extra-hepatic bile ducts.

The mechanism of this action is musculotropic, without atropic effects**(38)**.

I.8.1.d.8. Pharmacokinetics:

The maximum blood concentration is reached between 2 and 3 hours after oral intake.The plasma half-life is about 1 hour(**38**).

Excretion is by the urinary route in the form of glycuro and sulphoconjugate derivatives.

I.8.1.e. Other effects:

Experimental use of 4-methylombeliferone has shown that this compound inhibits hyaluronic acid production in multiple cell lines and tissue types in vitro and in *vivo*(**39**)

I.8.1.f.Practical applications:

Recent studies on the activity of 7-hydroxy-4-methylcoumarin have demonstrated that the substitution of a hydroxyl group at C7 gives the molecule considerable cytotoxic character against selected cells(**39**).

The tumour-related isoforms IX and XII of the hCA (human carbonic anhydrase) enzyme were were moderately inhibited by hymecromone with a Ki value (Ki : Inhibitory concentration at which 50% inhibition is observed) between 754-8100 nm. However, it weakly inhibits isoform I of the cytosolic hCA enzyme()

I.8.2. 8-nitro-7-hydroxy-4-methylcoumarin

I.8.2.a. Generalities:

It is a heterocycle derived from coumarin obtained by the nitration of 7-hydroxy-4-methylcoumarin.

- **Brute formula:** C10H7NO5
- **Mr:** 221.17 g/mol
- **Chemical structure:**

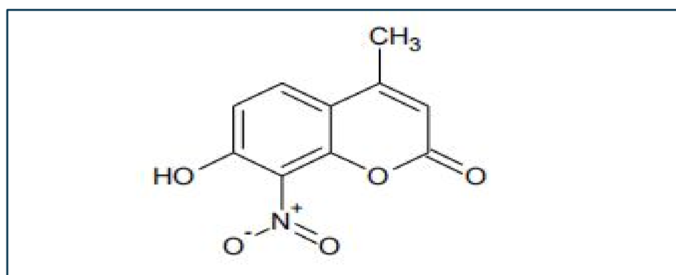


Figure13: Chemical structure of 8-nitro-7-hydroxy-4-methylcoumarin

(**https://pubchem.ncbi.nlm.nih.gov/#query=8-nitro-7-hydroxy-4-methylcoumarin)**

I.8.2.b. Reaction:

This is an ionic nitration, most often used with aromatic compounds. Mixtures of acids containing nitric acid and a strong acid, such as sulphuric acid, perchloric acid or ion exchange resins carrying sulphonic acid groups, are used as nitration agents. They catalyze the formation of nitronium ion NO2+(**17**).

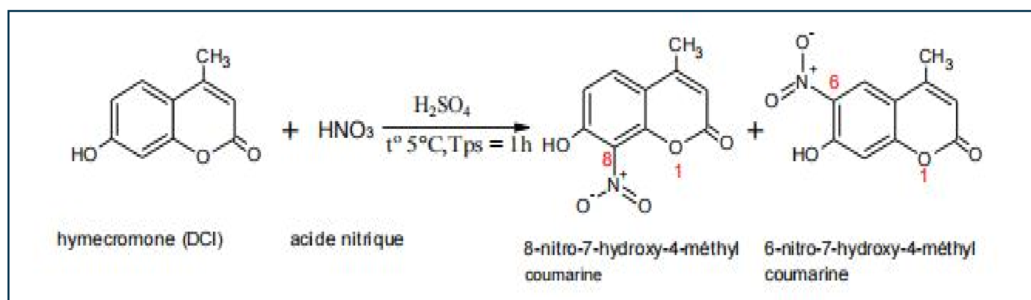


Figure 14: Synthesis reaction of 8-nitro-7-hydroxy-4-methyl coumarin

I.8.2.c. Practical applications of synthesis:

The work of **Swayam Sourav Sahoo et al. (2012)** proceeded to use sulfuric acid as a catalyst and leaving the mixture at a temperature room temperature for one hour. **(18)**

They used ethanol as the recrystallization solvent and resulted in a yield is equal to 60 % **(18)**

I.8.3. 8-amino-7-hydroxy-4-methylcoumarin

I.8.3.a. Generalities:

It is an amino coumarin derivative

- **Chemical formula:** C10H9O3
- **Mr:** 191.186 g/mol
- **Chemical structure:**

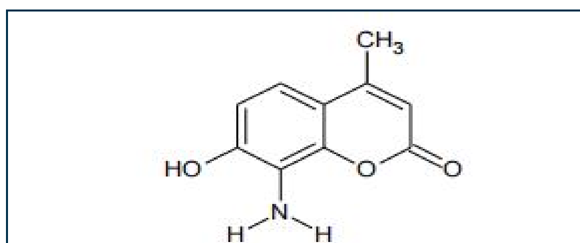


Figure 15: Chemical structure of 8-amino-7-hydroxy-4-methylcoumarin.

I.8.3.b. Reaction:

The reduction of nitro derivatives R-NO2 represents an important method of synthesis of primary amines R-NH2, particularly in aromatic series. Several methods can be used: the action of a metal in acid medium, that of a hydride, catalytic hydrogenation, or hydrogen transfer from a donor compound**(40)**.

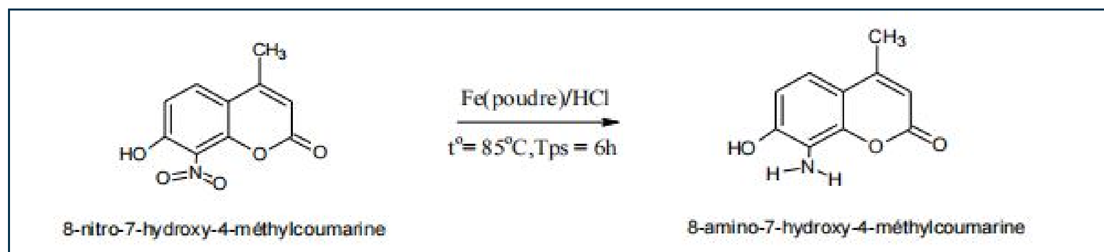


Figure 16: Reaction of the synthesis of 8-amino-7-hydroxy-4- methylcoumarin **1** (**https://pubchem.ncbi.nlm.nih.gov/#query=8-amino-7-hydroxy-4-methylcoumarin)**

I.8.3.c. Therapeutic properties of 8-nitro- and 8-amino-7-hydroxy-4- methylcoumarin

<u>A. Antibacterial activity:</u>

This work is carried out by using the following technique:

The antibacterial activity of the new coumarins was tested against a gram positive bacterium Gram positive bacteria which is Staphylococcus aureus and Gram negative bacteria which is Escherichia coli using api gallery method**(43-45)**.

And to compare the results, the researchers used amoxicillin as a reference. Each of these two compounds (8-nitro-7-hydroxy-4-methylcoumarin and 8-amino-7-hydroxy-4-methylcoumarin) was dissolved in a solution of DMSO [5 mL at a concentration of 1000µg/mL]**(43-45).**

They also prepared a solution of amoxicillin at a concentration of 1000µ/mL in distilled water. After incubation, the diameter of the zone surrounding each of these coumarin compounds was measured using an antibiotic zone reader**(43-45)**.

B. Results and discussion:

Compound	Inhibition zone (in mm)	
	Staphylococcus aureus	*Escherichia coli*
8-nitro-7-hydroxy-4-methylcoumarin	17 ±1.93	20 ± 1.43
8-amino-7-hydroxy-4-methylcoumarin	17 ± 1.74	18 ± 1.21
Amoxicillin	29 ± 1.32	36 ± 1.43

Table II: Evaluation of the antibacterial activity of 8-nitro-7-hydroxy-4-methylcoumarin and 8-amino-7-hydroxy-4-methylcoumarin

The above table shows that both compounds (8-nitro-7-hydroxy-4- methylcoumarin and 8-amino-7-hydroxy-4-methylcoumarin) show considerable antibacterial activity for anti-bacterial activity for both species (Staphylococcus aureus and Escherichia coli)

I.8.4.Umbelliprenin

I.8.4.1. Generalities:

Is a naturally occuring prenylated coumarin which is synthesized by various *Ferula* species

- Belonging to the sesquiterpene coumarin family

- Found in various plant species such as in celery, *Angelica archangelica*, *Coriandrum sativum* and *Citrus limon*

- **Chemical formula:**C24H30O3
- **Mr:**366.5 g/mol
- **Chemical structure:**

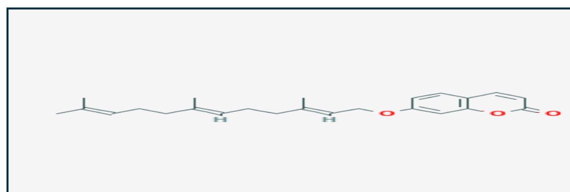


Figure 17: Chemical structure of Umbelliprenin.

I.8.4.2. Chemical names:

7-[(2*E*, 6E)-3, 7, 11-trimethyldodeca-2, 6, 10-trienoxy] chromen-2-one

I.8.4.3. Reaction:

Umbelliprenin (7-farnesyloxycoumarin) was synthesized in 71% yield by reaction between 7-hydroxycoumarin and*trans-trans*farnesyl bromide in acetone with the presence of DBU (1, 8-diazabicyclo undec-7-ene) **(46)**

I.8.4.4. Therapeutic properties:

1-Inhibits the red pigment production in *Serratia marcescens***(46)**

2-Inhibits squalene-hopene cyclase (SHC) (an enzyme taking part in sterol synthesis)**(46)**

3- Decreases matrix metaloproteinase (MMP) activity

4- Exhibits antileishmanial activity against promastigotes **(46)**

5- Induces apoptosis in human M4Beu **(47)**

6- Metastatic pigmented melanoma cells **(47)**

7- Exerts cancer chemopreventive activity **(47)**

I.8.5. Isopentenyloxycoumarin

I.8.5.a. Generalities:

It is among the most widespread naturally occurring prenyloxy umbelliferone derivatives has also been found in a very restricted number of plant families, namely Apiaceae, Asteraceae, and Rutaceae.

- **Brute formula:**C14H14O3
- **Mr:**230.26 g/mol
- **Chemical structure:**

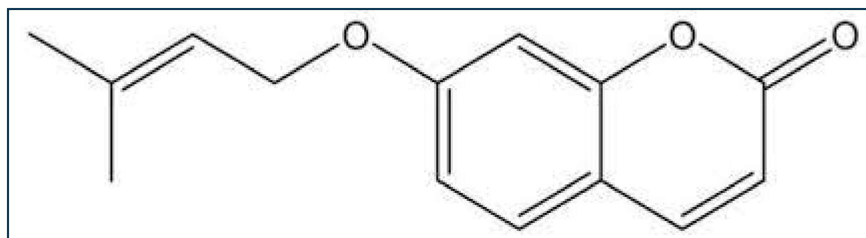


Figure 18: Chemical structure ofIsopentenyloxycoumarin

I.8.5.b. Chemical name:

3-(3-methylbut-3-enoxy) chromen-2-one.

I.8.5.c. Reaction:

Its biosynthetic pathway, one of the very few for oxyprenylated phenylpropanoids characterized until now, was discovered in cell suspension cultures of *Ammi majus* L. (Apiaceae) by **Hamerski and coworkers in 1990**(48).

Thus, 7-isopentenyloxycoumarin is obtained by the coupling reaction between umbelliferone and dimethyallyl diphosphate catalyzed by an enzyme located in the endoplasmic reticulum of plant cells, named dimethylallyl diphosphate umbelliferone transferase (DDU-7) transferase E.C. 2.5.1.

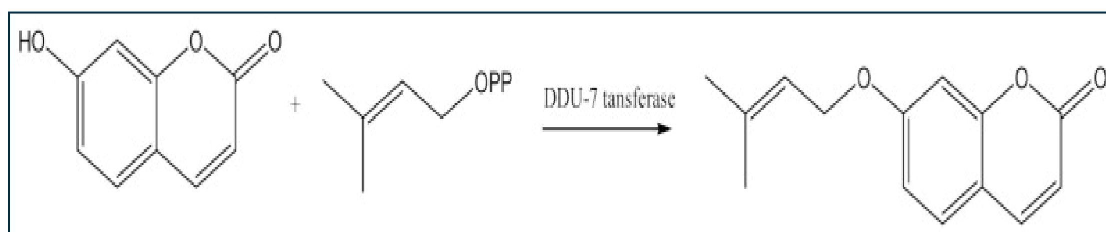


Figure 19: Reaction report of Isopentenyloxycoumarin

I.8.5.d. Therapeutic properties:

The 7-isopentenyloxycoumarin has an antifungal activityand neuroprotective activity**(48).**

Is used like Neuroprotective Activity& cytotoxic activity against three human cancer cell lines, namely ovarian carcinoma (CH1), lung cancer (A549), and melanoma (SK-MEL-28)**(48)**

It has an effect on glucose metabolism, and its modulatory activity on melanogenesis**(49)**.

I.8.6. Skimmin

I.8.6.a. Generalities:

Is a glucoside of umbelliferone.

Occur naturally and are used for the fluorometric determination of glycoside hydrolase enzymes**(50)**

- **Brute formula:**C15H16O8
- **Mr:**324.28 g/mol
- **Chemical structure:**

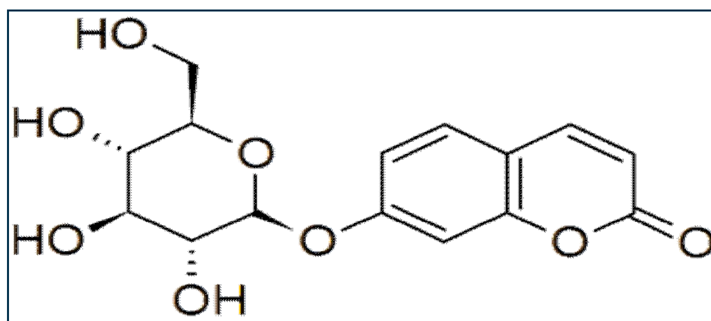


Figure20: Chemical structure of skimmin

I.8.6.b. Chemical names:

7-*O*-β-D-glucopyranosylumbelliferone

7-Hydroxycoumarin glucoside

I.8.6.c.Therapeutic properties:

Anti-diabetic effect:

- Preventive effect on the progression of diabetic nephropathy by the suppression of streptozotocin (decrease the glucose level in blood)

- Slow down the renal fibrosis by regulating TGF-β1 signal pathway.

- Have inhibitory effects on the human monoamine oxidase (hMAO) A and B enzymes, whereas they did not exhibit any anticholinesterase activities with (IC50 =73.47 µM) and hMAO B (IC50 = 1.63 mM)(**51**).

- Improves Insulin Resistance *via* Regulating the Metabolism of Glucose (skimmin can improve the insulin resistance by increasing glucose uptake and decreasing glucose output)**(51).**

- Skimmin enhanced the glucose uptake *via* inhibiting reactive oxygen species (ROS) and reducing the level of inflammatory correlation factor. Meanwhile, skimmin reduced the glucose output by promoting PI3K/Akt signaling pathway and down-regulating the expression of glycogen synthase kinase-3β (GSK3β) and glucose-6-phosphatase (G6Pase(**51**)).

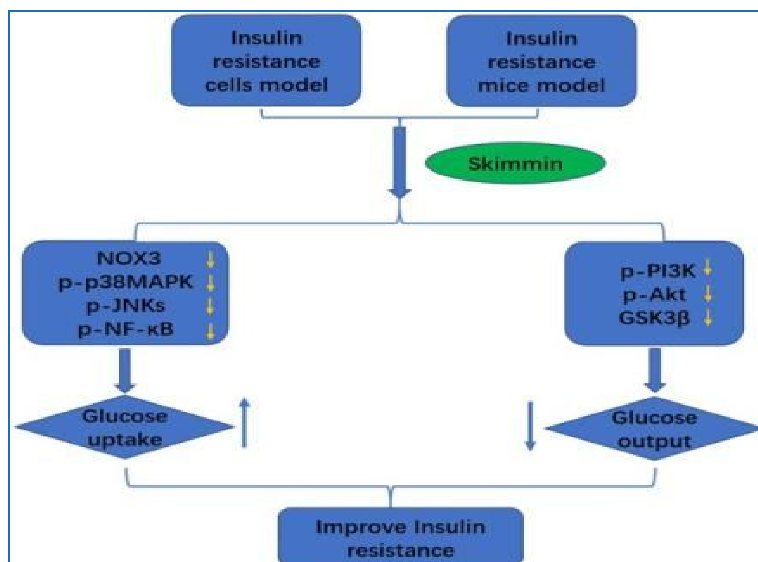


Figure 21: Skimmin improve the insulin resistance by promoting the glucose intake, and inhibiting the glucose output**(51)**

- Also it has a renoprotective activity, and antiamoebic properties.

I.8.7. Psoralen

I.8.7.a. Generalities:

Is the parent compound in a family of naturally occurring organic compounds known as the linear *furanocoumarins*

Occurs naturally in the seeds of *prosalea*, *corylifolia* as well as in the common fig, *Celerey*, *parsley*, west Indian satinwood, and in all citrus fruits.

- **Brute formula:** $C_{11}H_6O_3$
- **Mr:** 186.16 g/mol
- **Chemical Structure:**

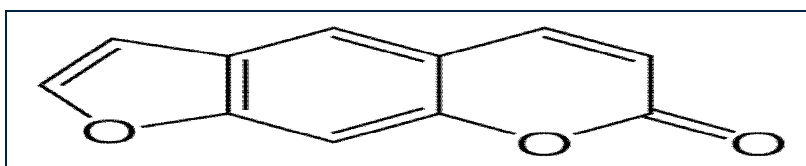


Figure22: Chemical structure of Psoralen**(https://pubchem.ncbi.nlm.nih.gov/#query=Psoralen)**

I.8.7.b. Chemical names:

- Ficusin
- 7H-Furo [3, 2-g] chromen-7-one
- ND methoxsalen (treatment for skin diseases)

I.8.7.c. Reaction:

The first step to enter the furocoumarin biosynthetic pathway is the prenylation of umbelliferone. It corresponds to the addition of a dimethylallyl diphosphate (DMAPP) group in position 6 or 8 of umbelliferone to give demethylsuberosin, precursor of linear furocoumarins.A prenyltransferase catalysing the reaction and localised to the plastids. DMAPP was predominantly derived from the 1-deoxy-D-xylulose (DOX) pathway that occurs in the plastids and not from the mevalonate-dependent pathway **(52)**.

Cyclisation of the prenyl groups on the hydroxyl groups of carbons 7 of demethylsuberosin leads to the formation of marmesin .marmesin synthase (MS) has been identified in Ammi majus and is thought to be a cytochrome P450 **(52)**

The carbon-carbon bonds of the marmesin side chains can be cleaved to lead to the formation of psoralen .This reaction involves psoralen synthase (PS: cytochrome P450 of the CYP71AJ)**(52)**

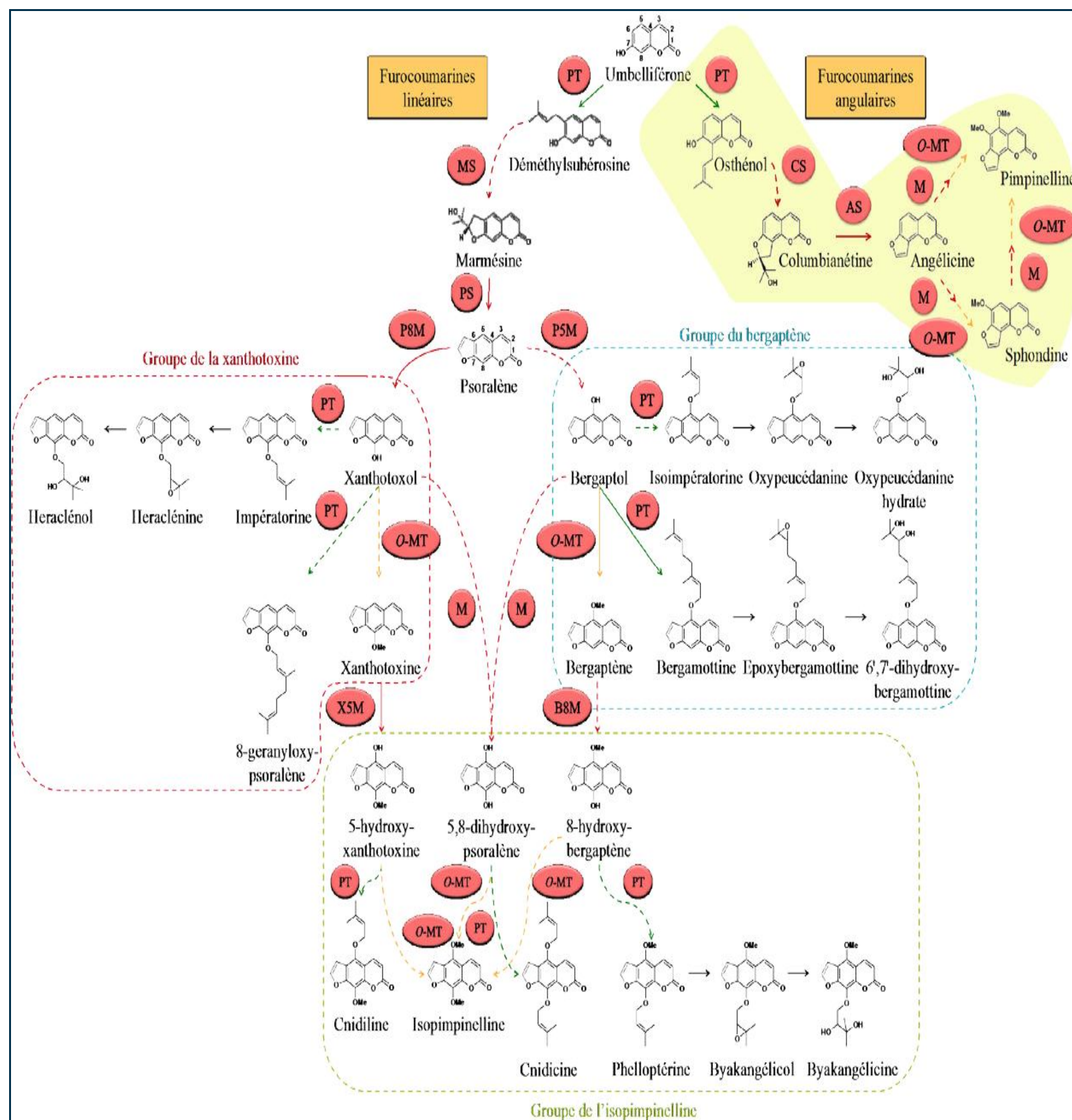


Figure 23: Simplified biosynthetic pathway of furocoumarins

PT: Prenyltransferases **MS:** Marmesin synthase **CS:** Columbianetin synthase

PS: Psoralen synthase **AS:** Angelicin synthase **M:** Monooxygenases

O-MT: O-methyltransferases **P8M:** Psoralen-8- monooxygenase

P5M: Psoralen-5-monooxygenase **X5M:** Xanthotoxin-5-monooxygenase

B8M: Bergapten -8-monooxygenase

I.8.7.d. Therapeutic properties:

- Psoralen is a mutagen, and is used for this purpose in molecular biology research. Psoralen intercalates into DNA and on exposure to ultraviolet (UVA) radiation can form monoadducts and covalent interstrand cross-links (ICL) with thymines, preferentially at 5'-TpA sites in the genome, inducing apoptosis. Psoralen plus UVA (PUVA) therapy can be

used to treat hyperproliferative skin disorders like psoriasis and certain kinds of skin cancer. Unfortunately, PUVA treatment itself leads to a higher risk of skin cancer(**53**).

- An important use of psoralen is in PUVA treatment for skin problems such as psoriasis and, to a lesser extent, eczema and vitiligo. This takes advantage of the high UV absorbance of psoralen. The psoralen is applied first to sensitise the skin, then UVA light is applied to clean up the skin problem(**53**).

- Psoralen has also been recommended for treating alopecia. Psoralens are also used in photopheresis, where they are mixed with the extracted leukocytes before UV radiation is applied(**53)**

I.8.8. Auraptene

I.8.8.a. Generalities:

Auraptene is a natural bioactive monoterpene coumarin ether

7- Extracted from plants belonging to the genus *Citrus.*

❖ **Brute formula:**C19H22O3

❖ **Mr:**298.4 g/mol

❖ **Chemical structure:**

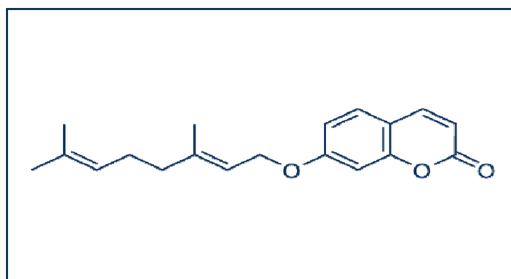


Figure 24: Chemical structure of Auraptene

I.8.8.b. Chemical names:

7-Geranyloxycoumarin

7-[(2*E*)-3, 7-dimethylocta-2, 6-dienoxy] chromen-2-one

I.8.8.c. Reaction:

Auraptene was synthesized from umbelliferone by prenylation with NaH and geranyl bromide in DMF(**54**).

I.8.8.d. Therapeutic properties:

Known such as potent cancer chemo preventive and anti-tumor agent against many types of cancers.A Study shown that auraptene efficiently suppresses the migration and invasion capacity of human cervical (Hela) and ovarian (A2780) cancer cells by reduction of MMP-2 and MMP-9 activity**(54)**.

-It exerts anti-inflammatory activity, it is capable of suppressing the release of tumor necrosis factor alpha (TNF-α), superoxide anion generation by inflammatory leukocytes, and IKB (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor) degradation**(54)**.

-Auraptene also causes complete inhibition of platelet aggregation, induced by arachidonic acid and platelet activated factor *in vitro***(54)**.

-Exhibit spasmolytic activity, and neuroprotective activity**(54)**.

I.8.9 Herniarin

I.8.9.a. Generalities:

Is a natural chemical compound

It is found in *Herniaaria glabra*, Ayapana triplinervis and in species of the genus *Prunus* (*P.mahaleb*, *P.pensylvanica*, and *P.maximowiczii*).

- **Brute formula:** $C_{10}H_8O_3$
- **Mr:** 176.171 g·mol−1
- **Chemical Structure:**

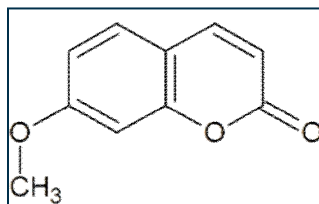


Figure 25: Chemical structure of Herniarin
(https://pubchem.ncbi.nlm.nih.gov/#query=Herniarin)

I.8.9.b. Chemical names:

7-Methoxycoumarin.

7-*O*-methylumbelliferone.

I.8.9.c. Reaction:

Herniarin was also prepared via a reaction between ortho-methoxy phenol and alkynoates in the presence of a palladium catalyst**(55)**

I.8.9.d. Antispasmodic effect:

Study showed that herniarininduced an antispasmodic effect on the guinea pig ileum precontracted with KCl from 31.6 to 316.2 μg/mL with an *E*max of 95.2 ± 3.0% and 55.7 ± 10.1% and an EC50 of 75.2 ± 1.1 and 160.1 ± 1.2 μg/mL From BIM-1157171**(55)**

Herniarin was introduced to have antidermatophytic activity**(55)**.

I.8.9.e. other effect:

Herniarin is used as a scent or aroma agent in perfumes, lotions, facial moisturizers, hand creams, hair conditioners, household aerosols, lip balms and baby oils.

1- 7-methoxycoumarin showed anti-fungal and antibacterial activities**(56)**

2- the antibacterial and anticancer activities**(56)**

Herniarin exhibited antibacterial activity against Gram-positive bacteria Staphylococcus aureus sub sp. aureus KCTC 1916 (S. aureus) and Bacillus subtilis KACC 17047 (B. subtilis) with a zone of inhibition values of 9 ± 0.12 mm and 8.5 ± 0.18 mm.

A study shown Herniarin is cytotoxic to breast carcinoma cell line MCF-7 with an IC_{50} of 207.6 µM. Herniarin (100 µM) also induces apoptosis in MCF-7 cells. Herniarin alone has no obvious cytotoxicity on transitional cell carcinoma (TCC) cells, but when in combination with 5 µg/mL cisplatin, Herniarin (80 µg/mL) potently enhances the antitumor effect of cisplatin, and increases chromatin condensation **(56).**

Chapter 2:
Structure activity relationship

II.1. Structural analysis:

II.1.a. Fluorescence under UV light:

Coumarins are characterized by UV fluorescence at 366 nm.

This fluorescence is usually

- ❖ Blue for 7-hydroxylated coumarins (Umbelliferone).
- ❖ Purple for prenylated coumarins.
- ❖ Yellow for furocoumarins**(57)**

This fluorescence is intensified on the chromatogram, observed under UV light.

- ❖ By NH3 vapors.
- ❖ By spraying the chromatogram.
- ❖ By Bornträger's reactif.
- ❖ Lead acetate.

The presence of aromatic rings in furocoumarins confers a strong absorbance for wavelengths between 270 and 300 nm**(57)**

II.1.b. IR infrared spectroscopy:

This technique provides information on the functional groupings of amolecule.

For the Umbelliferone:

- ❖ The C = O absorption of the conjugated lactone appears almost in the region [1600-1780] cm -1**(33)**
- ❖ The OH absorption of phenol is observed at 3550 cm -1(**57**).

II.1.c. UV ultraviolet spectroscopy:

Umbelliferone have a characteristic UV spectrum, strongly influenced by nature and position of the substituents, deeply modified in an alkaline medium: KOH, NaOH, NaOCH3 and in the presence of AlCl3(**57**).

AlCl3 forms a complex with the hydroxyls carried by adjacent carbons, which induces a bathmochrome displacement(**57**).

For umbelliferone, the maximum absorptions are presented in two bands at 276nm and 311 nm.The presence of the alkyl substituents induced by changes in the values of the band in 311nm to 325nm. Depending on the OH position, the displacement is more or less strong**(57)**.

II.1.d. NMR nuclear magnetic resonance spectroscopy:

The appearance of the 1H NMR spectra of coumarins is characteristic, the olefinic protons H-3 and H-4 appear respectively as two doublets at: δ (ppm) [6.10 - 6.40], [7.50 - 7.90] for simple coumarins and furocoumarins**(57)**.

II.1.e.Umbelliferone mass spectrometry:

Strongly unsaturated cyclic compounds such as "umbelliferone", and evenions which formed by α fragmentation at the level of a carbonyl group, have the property to be able to lose a CO group (28 amu). If there are several CO groups in amolecule, they can be eliminated one after the other.

- hepatotropic (increases the production of bile and fights against spasms of the bile ducts).

- Listed in several pharmacopoeias, such as the European Pharmacopoeia 7th edition, the Japanese pharmacopoeia 16th edition**(57)**.

II.2.a.What is SAR?

The Structure Activity Relationship (SAR) is an approach designed to study the interactions between the chemical structure of a ligand (or the compound under study) and the biological target. A higher level of information regarding the molecular level of a drug and its mechanism of biological activity leads to a better understanding in order to develop the drug with optimal efficacy. Similarly, for all molecules it is also

assumed that similar molecules have similar activities. This assumption is well considered as the guiding principle of the pharmacophore in the concept of SAR. These link-structural properties of a drug can be solved either by computational method (in silico) or by experimental method in vivo and in vitro. **(58)**.

II.2.b.Why SAR?

SAR is used to determine the parts of the structure of a compound that are responsible for its beneficial biological activity, i.e. its pharmacophore, and its adverse effects. Therefore, SAR is necessary to determine the physico-chemical behavior and biological activity in a

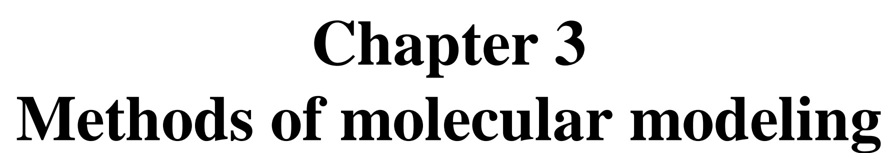
consistent manner. SAR allows researchers to identify changes in pharmacological properties by making minor changes in the drug molecule. SAR allows researchers to understand and explain the mechanisms of activity in a set of ligands**(58)**.

II.2.c.SAR of Umbelliferone in diseases:

Umbelliferone has a remarkable range of biochemical and pharmacological activities. Parts of this group of compounds can significantly affect the function of various mammalian cell systems. Chemically, umbelliferone is an aromatic compound consisting of a heterocycle (a bi-cyclic structure) of which the 1st ring is benzene linked to a hydroxyl group (phenol) and the 2nd ring is a lactone consisting of a ring linked to a carbonyl group.

In general, the substitution of carbons by an electronegative atom is effective for the formation of hydrogen bonding and thus for water solubility, on the other hand, the presence of an aromatic ring and possible heterocycles as substituents is responsible for hydrophobicity**(59)**

These properties are at the origin of a better interaction of the molecule with a receptor site. The biological activity of coumarins is more significant by the substitution of the various carbons of the bi-cyclic system. The pharmacological and biochemical properties and therapeutic applications of single coumarins depend on the substitution motif. Therefore, it is necessary to pay special attention to the study of SAR of umbelliferone. The biological activity can be anticancer activity, Antibiotic activity, Antioxidant activity, Anti-tumor activity**(59)**



Chapter 3
Methods of molecular modeling

III.1. Introduction:

Molecular modeling is defined as the application of theoretical and computational methods to solve problems involving molecular structure and chemical reactivity. These methods can be relatively simple and quickly usable or, on the contrary, they can be extremely complex and require hundreds of hours of computation on a computer, even on a supercomputer**(60)**

Molecular modeling is based on the development of theoretical and computational methodologies, to model and study the behavior of molecules, from small chemical systems to large biological molecules and material assemblies. The application fields of molecular modeling regard computational chemistry, drug design, computational biology and materials science. The basic computational technique to perform molecular modeling is simulation. Molecular simulation techniques require specific additional computational and software requirements **(60)**.

III.2. Molecular docking:

III.2.1. Theoretical principles:

Molecular docking aims at predicting the structure of a molecular complex from isolated molecules, in which different approaches are combined to study the modes of interaction between two molecules. Docking software is therefore a very useful tool in biology, pharmacy and medicine, as most active ingredients are small molecules (ligand) that interact with a biological target of therapeutic interest. As the macromolecular receptor is most often a protein, the term docking alone is commonly used to designate "protein-ligand docking".

The aim of molecular docking is to determine the interaction mode of a complex formed by two or more molecules, by looking for orientations in space and favorable conformations for the binding of a ligand to a receptor**(61).**

A docking simulation essentially consists of two steps: docking itself and scoring.

- Docking (the first step) is the selection step, consisting of placing the ligand in the active site of the protein and sampling the possible conformations, positions and orientations (poses), retaining only those that represent the most favorable interaction modes

- Scoring (the second) is the ranking step, which consists of evaluating the affinity between the ligand and the protein and giving a score to the poses obtained during the

docking phase. This score will make it possible to select the best pose among all those proposed.

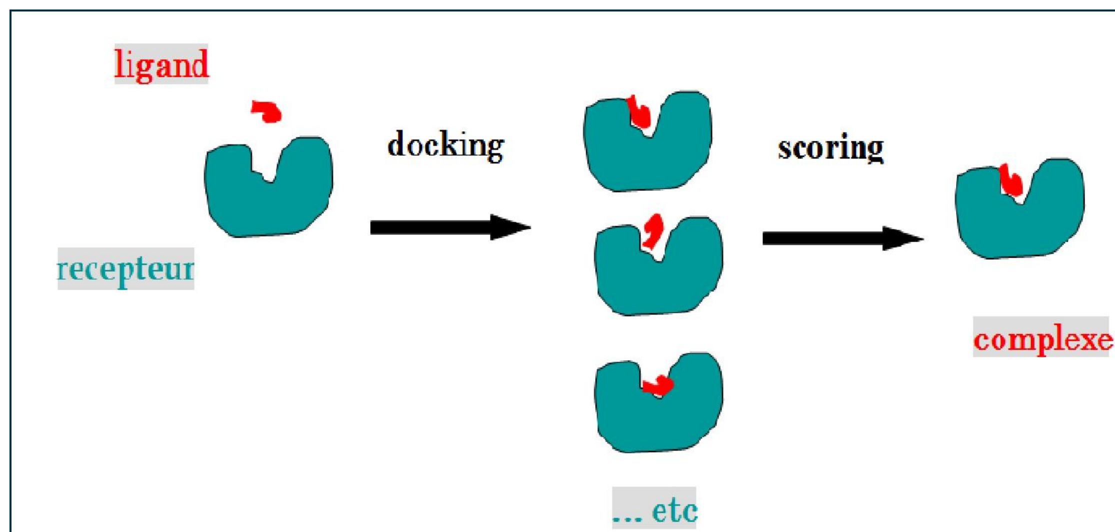


Figure 26: Docking steps

III.2.2. Docking algorithms:

In principle, a docking can be done "manually" directly by the modeler, by placing the ligand in the active site of the protein using a graphical interface. Then, the geometry of the assembly is optimized in order to correct steric problems and obtain an energetically stable complex. This approach is applied when one has a precise idea of the actual interaction mode of the ligand**(62)**

However, in most cases, the actual interaction mode is not known. In this case, manually testing all ligand conformations and orientations is practically impossible, even when considering the protein as a rigid body. To overcome this difficulty, docking algorithms have been designed to search objectively, quickly and efficiently for the most favorable protein-ligand association modes

Docking algorithms can be divided into two main classes: those that do not take into account the flexibility of the protein, treating it as a rigid body, and those that are able to take into account, partially, the flexibility of the receptor.

In docking procedures that treat the protein as a rigid body, the flexibility of the ligand alone is taken into account in obtaining complexes. Depending on the method used to generate the ligand conformers and place them in the catalytic cavity of the receptor, the

algorithms can be subdivided into molecular mechanics simulation**(63)** algorithms and systematic and stochastic molecular dynamics algorithms. Several review articles describe the principle, strengths and limitations of these methods**(64)**. However, this classification should be viewed with caution, as many algorithms combine more than one method for ligand conformation generation and sampling. In most cases, the use of algorithms that consider the protein as a rigid body leads to good results, mainly when the protein has flexibilitylimited. Indeed, in such cases, the crystallographic structure can be considered more representative of the state of the protein in its natural environment, thus increasing the chances of correctly simulating ligand complexation**(65)**

Some proteins naturally exhibit regions of high flexibility, undergoing considerable rearrangement in the presence of a ligand. In this case, neglecting the flexibility of the protein can jeopardize the reliability of the docking results, and makes it necessary to use approaches that are able to take into account the flexibility of the whole system**(66)**. Indirect or direct methods, where the flexibility of the protein is partially or fully taken into account, are described in the literature. However, these methods are not often used because the gain in accuracy compared to traditional algorithms is generally too small compared to the increase in simulation time **(67)**

III.5. Score functions:

The docking procedure generates a list of complexes representing the favorable association modes between the ligand and the macromolecular receptor. The next step is to evaluate these complexes, in order to find the one(s) most likely to reproduce the real association mode. The association between proteins and ligands is governed by several thermodynamic parameters:

- Hydrophobic interactions,
- Electrostatic interactions,
- Hydrogen bonds,
- Solvation effects and entropy effects.

Theoretically, the complex is favorable if the overall free energy change of complexation is negative (ΔG complexation<0).

In practice, the evaluation of the free energy of complexes is often a computationally expensive task, which limits its routine use. Therefore, approximate methods have been

developed to distinguish (evaluate and classify) the best complexes among those generated by a docking procedure: score functions. According to the principles used in their design, score functions are classified into: force field-based score functions, knowledge-based score functions and empirical score functions. These score functions are constructed from rules based on statistical analysis of experimentally resolved protein-ligand complexes

The PMF (Potential of Mean Force) function belongs to this class of functions**(68)**

It is a complex ranking technique that combines the individual results of several score functions in order to compensate the weaknesses of some with the strengths of others, thus increasing the reliability of the final result.

The principle is to check the frequency with which each complex appears among the X best ranked complexes according to different score functions (X = 5 or 10 most often). From a statistical point of view, the higher this frequency for a certain complex, the higher the probability that this complex represents the real mode of protein-ligand interaction, since it is recognized as such by several distinct score functions**(69)**.

A large number of (commercial and non-commercial) molecular docking programs are available. These include AUTODOCK, FLEXX, GOLD, DOCK, SURFLEX, MOLEGRO VIRTUAL DOCKER, UCSF CHIMERA and Schrödinger (Glide) etc.

They differ from each other in the way the molecular system is represented and the way the docking score (score function) is determined.

III.6. Molecular Docking Steps:

Two approaches are mainly used for modeling the protein-ligand system.

The first step consists of downloading the chemical structures of the targets to be processed (Enzyme in our case), for this it is necessary to go directly to the Bank PDB (http://www.pdb.org) and determine where the structures of these targets are deposited.

The PDB contains several thousand protein structures obtained either by crystallography (X-rays) or by NMR. If the target is not yet deposited in the Bank, and the Bank contains a protein with similar sequences, homology modeling is used to build the 3D structure of the desired target.

After downloading the target (PDB), we use visualization software to see with which ligands the enzyme is co-crystallized (water, ligands, ion,).

The second step concerns the structures of the ligand(s) used in the molecular docking. There are two main databases of chemical structures of ligands. The first represents these structures by molecular modeling computer programs, where the different structures are generated by geometry optimization. These structures are governed by the laws of quantum chemistry. In the second case, they are obtained from databases such as PubChem Project or other structure databases. The latter have different extensions such as PDB (Protein Data Bank), SDF ...etc.

III.7. Choice of UCSF CHIMERA software:

In the field of molecular docking, several software packages have been used to study the different interactions between two molecular entities (Enzyme-ligands). Chimera is a recently developed software that has given good results (genetic algorithm). The score function of Chimera is an empirical function. This type of function allows us to choose the compounds with the highest probability of interacting with the target, does not require a lot of computing power, which allows to screen the target more quickly. It also stands out for its ability to better assess H-bridges as well as electrostatic interactions. The hosting procedure consists of three elements:

a) Identification of the binding site.

b) Search for the best pose among the possible poses.

c) The score function.

III.8. General Docking Protocol:

The approaches currently used are exclusively computational and evaluated by visualization tools. These approaches can be broken down into four to five successive phases (Figure 26):

Choice of the protein representation mode (all-atom, pseudo-atom, grid, etc.),

Conformational exploration (rigid-body ligand position/orientation and/or flexible ligand position/orientation/form),

Minimization of the interaction energy evaluation function (or score function) of the conformations resulting from the exploration,

Grouping by similarity and classification by finer evaluation of the score, accompanied by a non-automatic step of visual evaluation of the results when the score does not allow the native conformation to be discriminated from the various conformations generated.

An optional step of refining the selected complexes by minimization or molecular dynamics.

A search algorithm to explore the possibilities of binding modes, a mechanism to place the ligand in the binding site and a score function to rank the different binding modes.

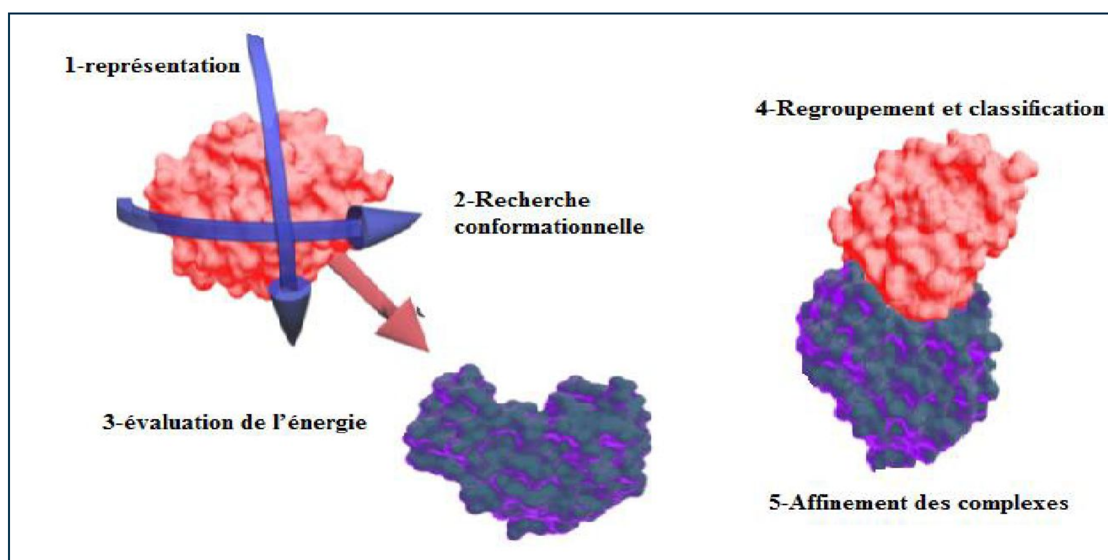
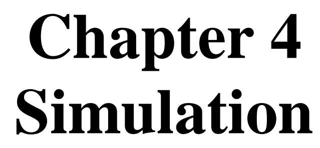


Figure 27: General docking protocol **(69)**

PRACTICAL PART



Chapter 4
Simulation

IV.1. Introduction:

Alzheimer's disease results from a slow degeneration of neurons, starting in the hippocampus (a brain structure essential for memory) and spreading to the rest of the brain. It is characterized by problems with recent memory, executive functions, and orientation in time and space. The patient progressively loses his or her cognitive faculties and autonomy.

Many studies have been carried out to discover the main causes of AD, among the causes, have decreased neurotransmitter acetylcholine (ACh), formation of amyloid b-protein (Ab) plaques, and abnormal post-translational modifications of Tau protein to yield neurofibrillary tangles (Cummings 2004). to treat this disease should use drugs that have shown activity against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1)

Acetylcholinesterase is an enzyme naturally present in humans that hydrolysis of acetylcholine, thus preventing excessive action of the latter (regulation).

It belongs to the hydrolase family and is expressed in the central nervous system and muscles. central nervous system and muscles, its role is to hydrolyze the neurotransmitter acetylcholine in order to terminate the transmission of nerve impulses and thus restore the excitability of cholinergic synapses.

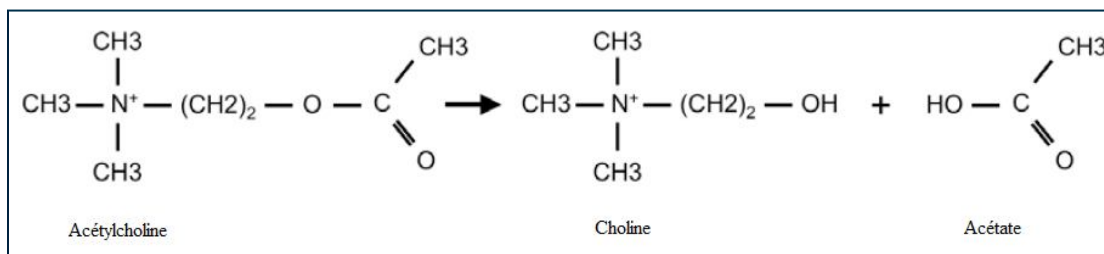


Figure 28: catalytic mechanism of acetylcholinesterase

Acetylcholinesterase is a complex protein with an active center, a multitude of peripheral sites, and numerous hydrophobic domains

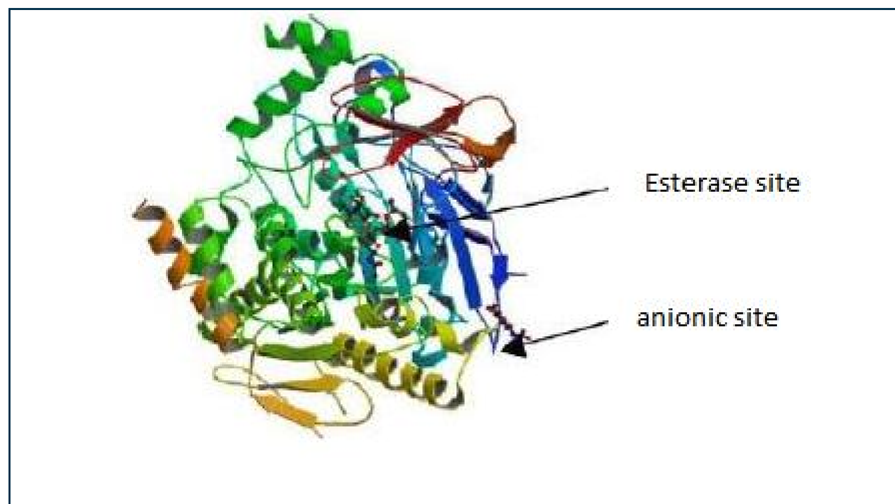


Figure29: Schematic representation of acetylcholinesterase

The catalytic machinery of AChE is located at the bottom of a deep (about 20 Å) and narrow (about 5 Å) groove and includes the hydroxyl group of the serine that reacts with ACh, two subsites are distinguished: the anionic site and the esterase site. The esterase site corresponds to the locus at which the acetyl portion of the ACh molecule binds and forms the tetrahedral intermediate. ACh is then cleaved, releasing the choline and the acylated enzyme intermediate.

This is followed by deacetylation, which regenerates the free enzyme by releasing the acetate (46).

The interaction between a protein and its substrate is the first step in most biological reactions. Understanding how it works means defining which residues are involved:

- The affinity between two molecules.

- The distances between the amino acids of the enzyme's active site and those of the inhibitors.

- The interaction energy.

Similarly, the discovery of new substances that activate or inhibit the biological activity of a protein can only be done by predicting their respective affinity. For this purpose, molecular modeling techniques, grouped under the name of "Molecular Docking", have been developed.

The flexibility of biological molecules results from the high dimensionality of the systems and the intervention of a large number of weak interactions (Van der Waals, hydrogen bonding, hydrophobic effect, etc.). It is known that it is an integral part of the dynamics of macromolecules and their proper functioning.

The neglect of this parameter in calculations introduces errors, it is, therefore, necessary to determine the intrinsic flexibility of a molecule but also to be able to introduce it, at least partially, in a docking procedure.

Docking software is, therefore, a very useful tool in biology, pharmacy, and medicine, as most active ingredients are small molecules (ligands) that interact with a biological target of therapeutic interest, generally, a protein (receptor), to influence the mechanism in which this protein is involved.

Docking approaches are based on the "key-lock" concept.

This work consists of studying the interactions between the three inhibitors.

and acetylcholinesterase , alsoBCHE by molecular modeling methods.

This chapter has included all the computational results from these studies and the discussion is based on the interaction energies and the responsible groups.

IV.2. Material:

IV.2.1. Microcomputers:

Two microcomputers were used for the execution and analysis of our work:

	Microcomputer 1	**Microcomputer 2**
Memory (RAM)	4.00 GO	4.00 GO
System type	64-bit operating system	64-bit operating system
Processor	Intel(R) Core (TM) i5-CPU M 480 @2.67 GHz	Intel(R) Core(TM) i3-3240 CPU @ 3.40GHz 3.40 GHz
OS	Microsoft Windows 7 Ultimate	Windows 10 Professionnel

Table 03: Microcomputers used and their characteristics.

The use of several programs and the consultation of several databases were necessary to carry out our practical part.

V.2.2. Databases and websites:

- ✓ **PDB (Protein Data Bank):**

The PDB is a worldwide collection of data on the three-dimensional structure (or 3D structure) of biological macromolecules: mainly proteins and nucleic acids. These structures are mainly determined by X-ray crystallography or NMR spectroscopy.

- ✓ **PubChem**

It is an American database of chemical molecules managed by the National Center for Biotechnology Information (NCBI), a branch of the US National Library of Medicine under the authority of the National Institute of Health (NIH)**.**

PubChem includes several million compounds available online, free of charge,

for each substance a large amount of data of various chemical, biochemical, pharmacological, production, toxicology.**(70)**

IV.2.3. programs:

- ✓ **OpenBabel:**

In its version 2.4.1. We used it to convert the files, containing the chemical data, from one format to another (pdb, mol2, sdf,......).(71)

AutoDock 4.2

is a Docking software that used a sophisticated gradient optimization method in its local optimization procedure. The gradient actually gives the algorithm for optimizing a "direction of travel" from a single evaluation

- ✓ **PyMO:**

is a free software for the visualization of chemical structures in 3D. We were able to prepare and visualize both the enzyme and the ligands to study the protein and ligand interactions.

- ✓ **ChemSketch:**

Is a molecule editor software, we used to build and minimize molecules structures.

UCSF chimera:

Is an extensible program for interactive visualization and analysis of molecular structures and related data, we used for molecular construction of ligands from the literature and minimization of their intramolecular energy

IV.3.The procedure to be followed:

IV.3.1. Preparation of the enzyme:

* Download the enzymes from the Protein Data Bank (PDB)

* Simplification of one of the two chains.

* Removal of water molecules.

* Removal of co-crystallization inhibitors.

* Addition of hydrogen atoms (only polar)

IV.3.2. Preparation of the ligand:

UMB (inhibitor) is buildby chimera

The other ligands (inhibitors) are drawn using ChemSketch

IV.3.3. Molecular docking:

The Pymol software AutoDock Vina interface is used to better understand the molecular mechanism of inhibition. We analyzed the energies of the complex (inhibitors_ACHE) and (inhibitors_BCHE)the parameters of the AutoDock Vina calculation are:

-The initial state of the ligand (position, orientation, and random or precise conformation)

-Dimensions of the grid to be used

-Finally, launch the Docking ; At the end of each treatment, AutoDock Vina will record the best interaction with the ligand. To enable the ligand-receptor calculations to be performed using the Pymol software with the AutoDock Vina interface, which is a molecular docking program, the following steps must be taken:

-The names of the files containing the receptor and ligand.

-We have simplified the enzyme model by removing water molecules and hetero-atoms from the co-crystallization; the enzyme model retained is a monomer.

Acetylcholinesterase enzyme (5FOQ) crystallizes as a dimer (A&B chain). Represent the acetylcholinesterase/Butyrylcholinestérasemacromolecule with its polar hydrogens and the partial charges of all its atoms

- The prepared proteinsare saved in a file in pdbqt format (Figure 29a, b).

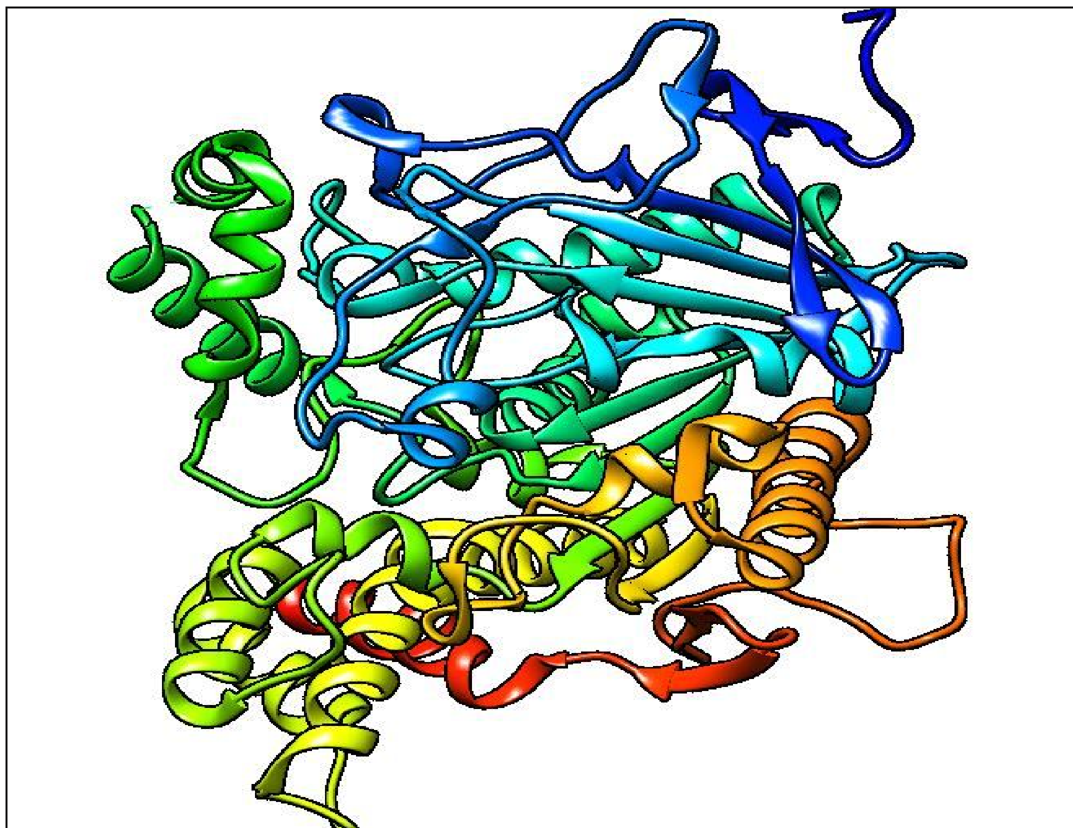


Figure 29a: Simplified acetylcholinesterase

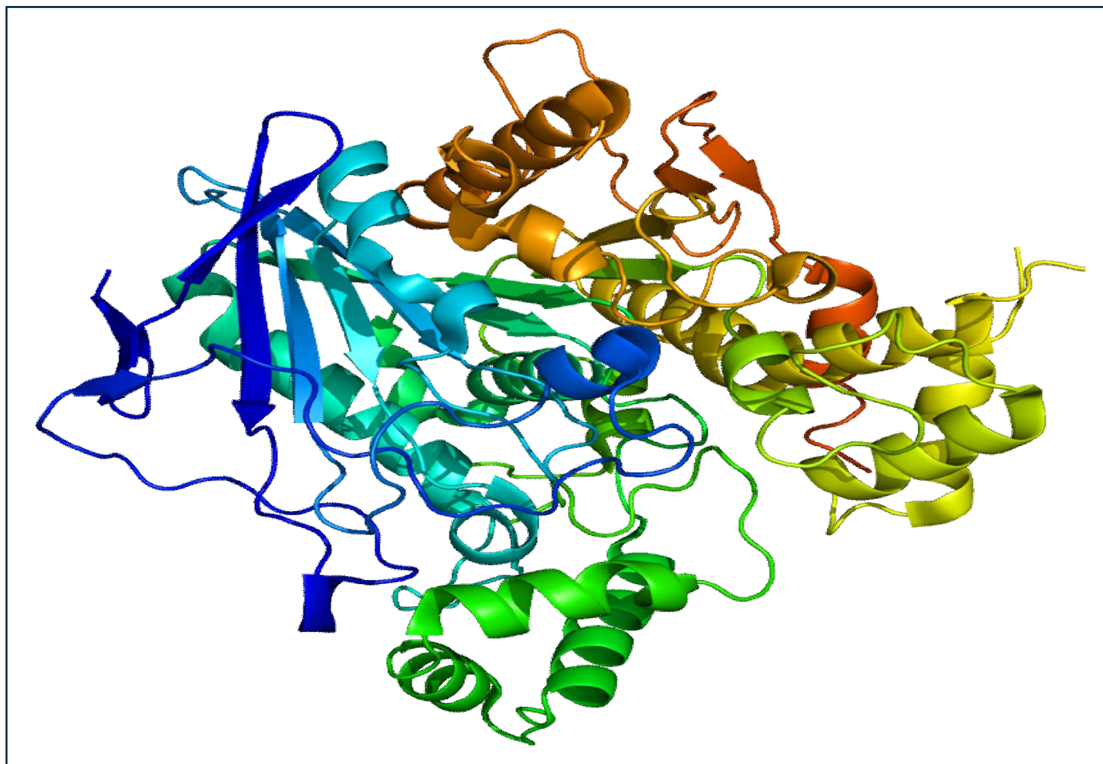


Figure29.b: Simplified Butyrylcholinesterase

IV.4. Method of calculation

IV.4.1. Preparation of the enzyme:

The acetylcholinesterase/Butyrylcholinesterase enzyme was downloaded from the Protein Data Bank (www.rcsb.org/pdb)

The acetylcholinesterase enzyme has two strings (chains), are similar, with 614 amino acids, in The other side the Butyrylcholinesterase enzyme has only one chain with 609 amino acids

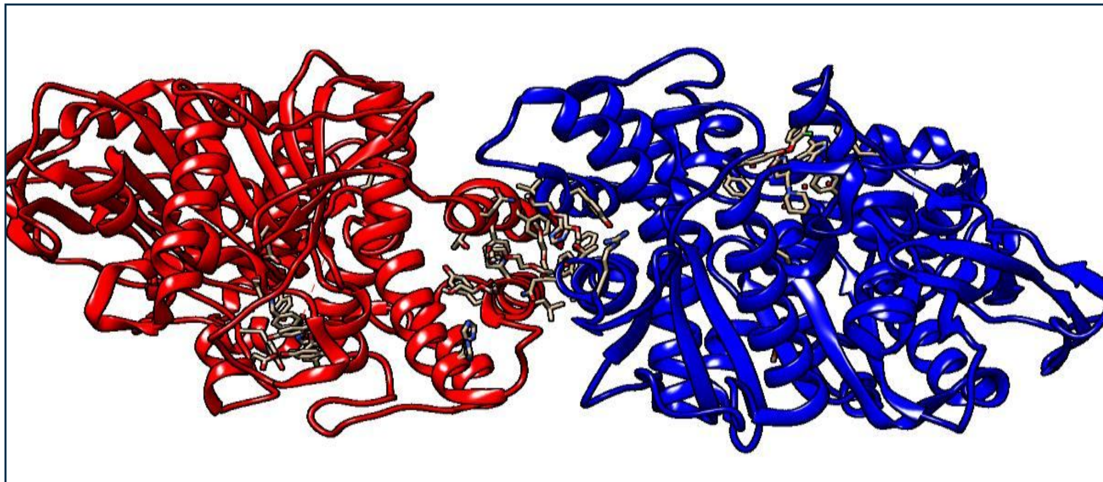


Figure 30: The enzyme acetylcholinesterase unsimplified

-The removal of the water molecules and the co-crystallization inhibitors, allows us to obtain a simplified model of the enzyme

-Add non-polar hydrogen, and Kollman charges

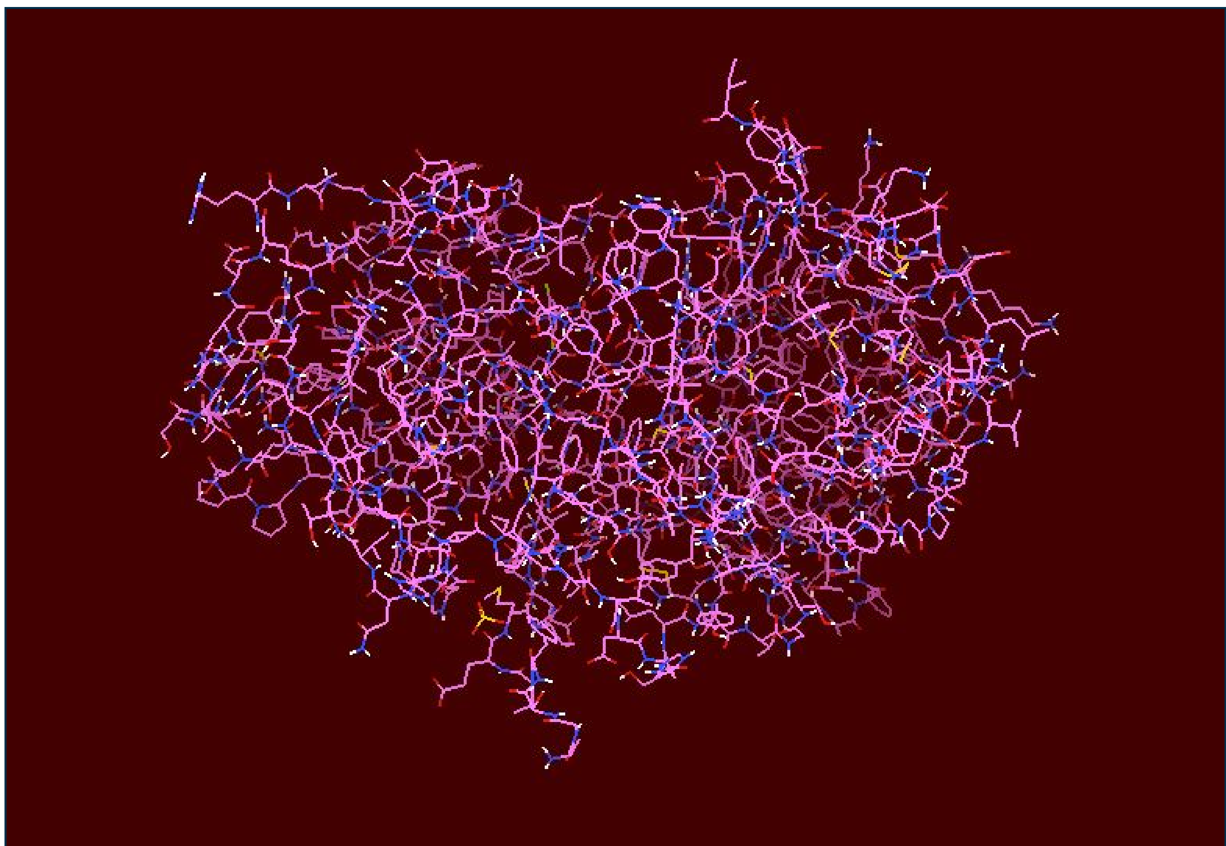


Figure31: stucture of Butyrylcholinesterase after modification

Our study is based on the simplification of the enzyme as a key and essential step to speed up and simplify the calculations. So we eliminate one of the two chains (figure 32) with the co-crystallization molecules and the water molecules (figure33)

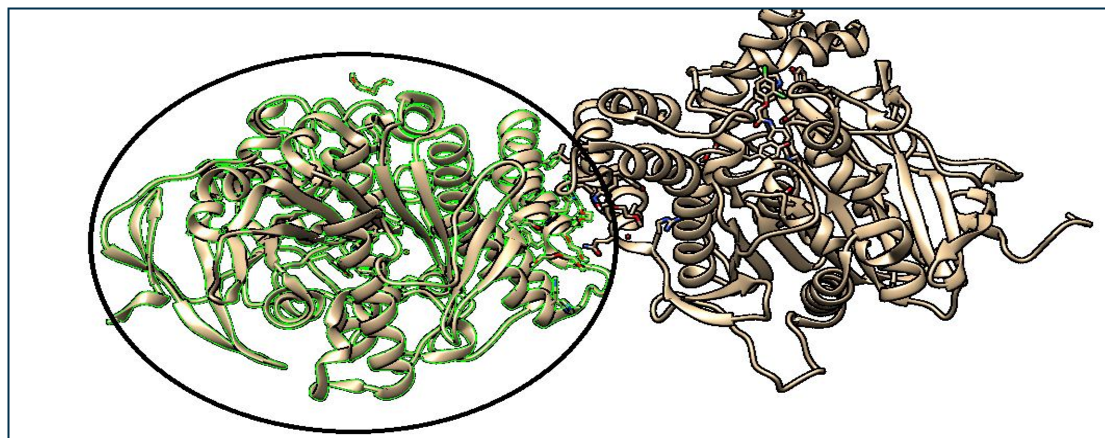


Figure 32: The two chains (A, B) of acetylcholinesterase

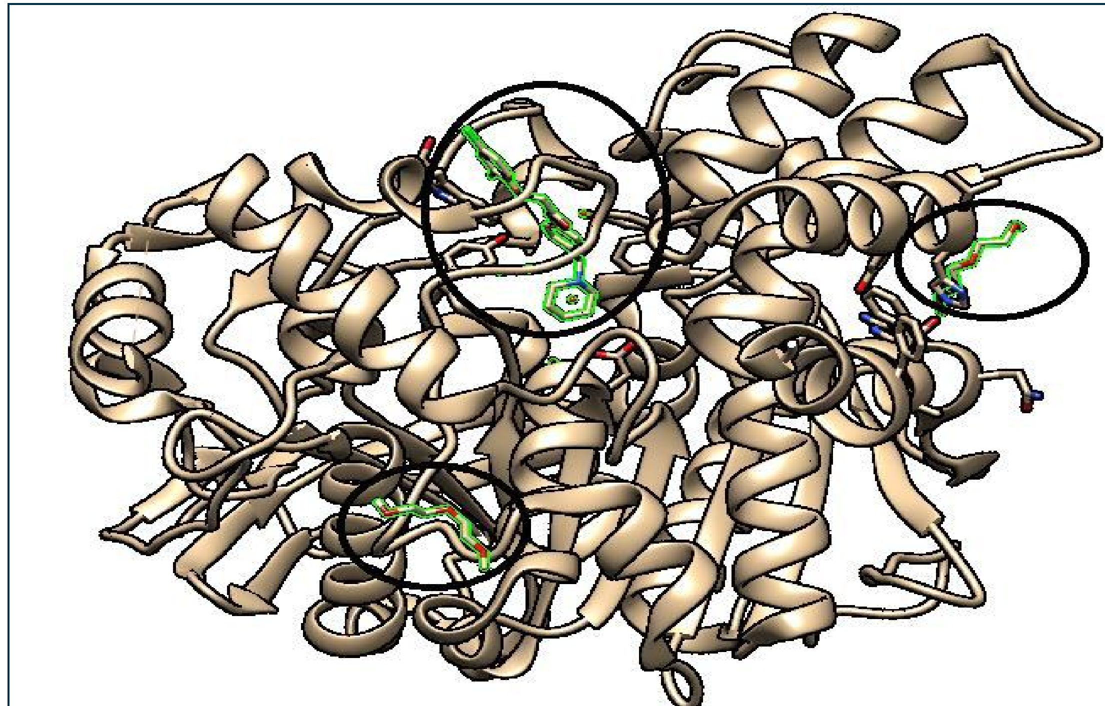


Figure 33: The acetylcholinesterase enzyme with the residues

IV.4.2. Preparation of inhibitors:

The inhibitors used in our work are umbelliferone and its derivatives.

They have been used as drugs against neurodegenerative diseases (progressive loss of neurons) such as Alzheimer's disease and Parkinson's disease...etc

The derivatives (Figure 34) are optimized by ChemSketch and UCSF chimera , they are stored in mol2 format

The three inhibitors (Figure 34) are converted from SDF/mol2 to PDB format, through open babel .

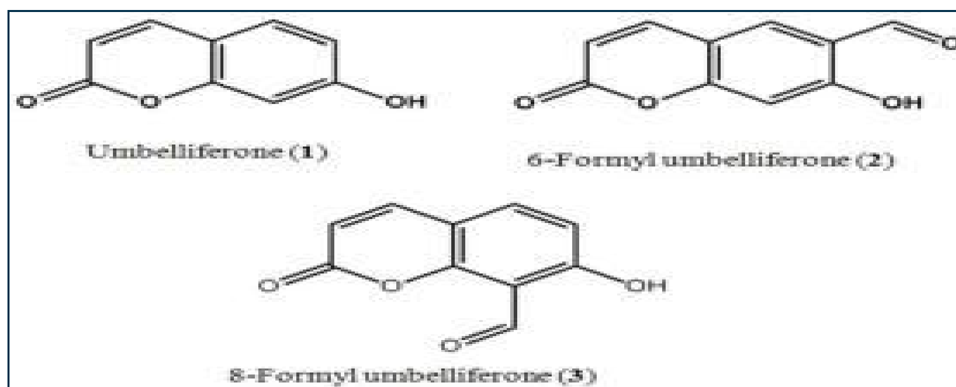


Figure 34: The three inhibitors used

The inhibitory activity of the ligands against the enzyme depends on the position of the (additional) chain linkage

IV.5.Molecular docking study:

Molecular docking aims to determine the interaction mode of a complex formed by two or more molecules, by looking for orientations in space and favorable conformations for the binding of a ligand to a receptor.

A docking simulation essentially consists of two steps: docking and scoring (Figure 35).

- The first step (docking) is the selection step, consisting of placing the ligand in the active site of the protein and sampling the possible conformations, positions, and orientations (poses), retaining only those that represent the most favorable interaction modes

- The second step (scoring) is the ranking step, which consists of evaluating the affinity between the ligand and the protein and giving a score to the poses obtained during the docking phase. This score will make it possible to select the best pose among all those proposed.

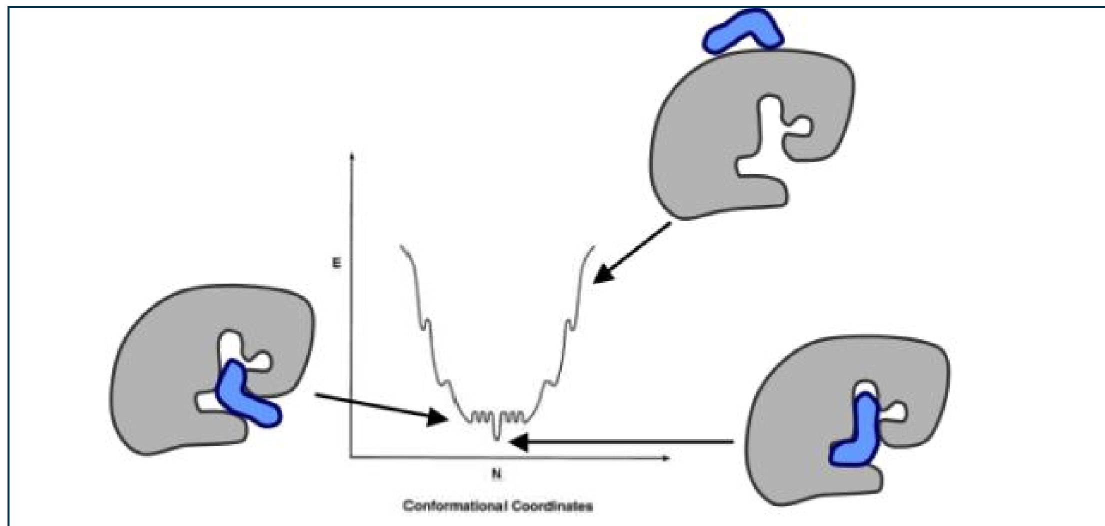


Figure35: General principle of a docking program

V. Results and discussion:

The study of the interaction between the amino acids of the active site of the AchE enzyme and the inhibitors to form a stable complex was carried out using the Pymol software and in a box with known parameters to specify the surface of the calculations.

V.1.Molecular-Docking Study of the Inhibition of BACE1:

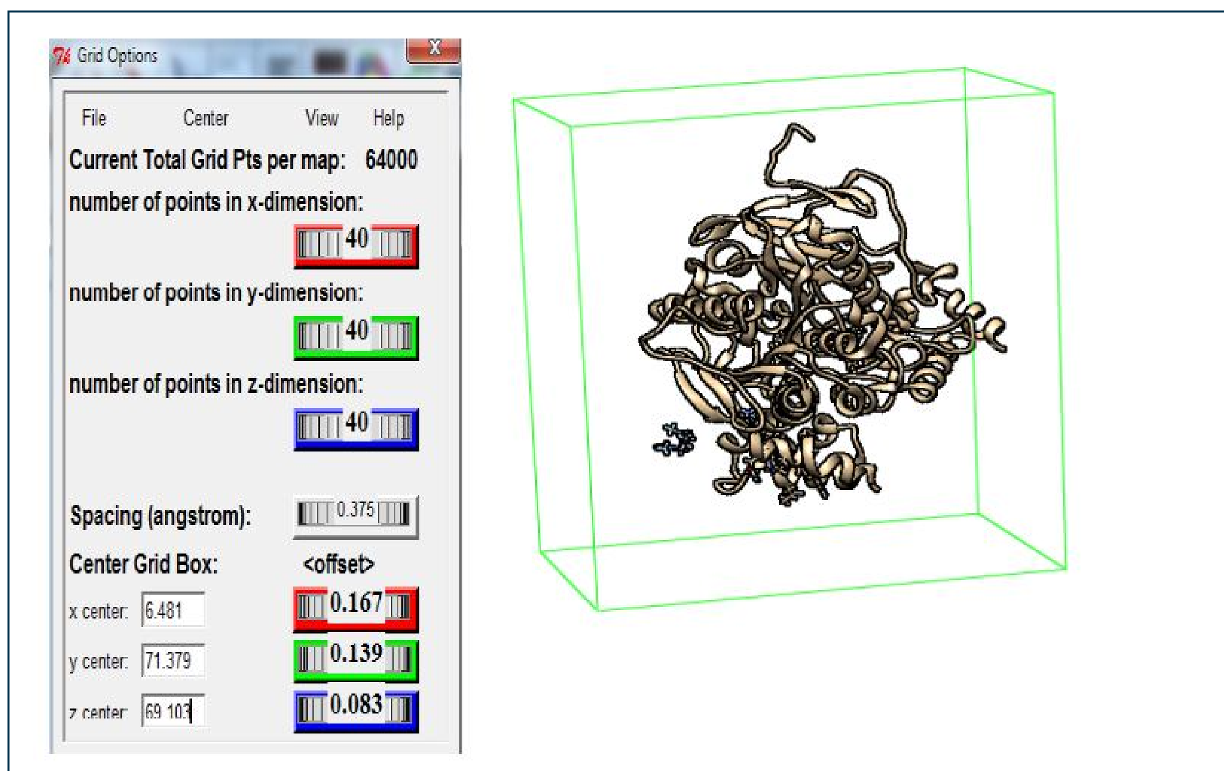


Figure 36: The calculation box

The interactions between the active site of AchE and different inhibitors are calculated during the molecular docking, they are presented in the following table 1:

Compound	Binding Energy (kcal/mol)	No. of H-Bond	van der Waals Interacting Residues	H-Bonds Interacting Residues
1	-6.3	3	Trp279, Ile287, Phe290, Phe330, Tyr334	Tyr121, Phe288, Arg289
2	-6.76	3	Ser81, Trp84, Gly118, Glu199, Phe330, Trp432	Ser200, Tyr334, His440
3	-6.7	1	Trp84, Gly117, Gly118, Phe330, His440, Gly441	Glu199

Table 04: Molecular docking results of the three inhibitors with acetyl cholinesterasegenerated using Autodock 4.2

This study shows that the energies of the Enzyme-Ligand 2 and Enzyme-Ligand 3 complexes are -6.76 Kcal/mol and -6.7 Kcal/mol respectively; whereas that of the Enzyme-Ligand 1 complex is lower and is -6.3 Kcal/mol. Ligands L2 and L3 are therefore better inhibitors than ligand 1. Ligands L2 and L3 have an additional oxygen atom to the ligand, and therefore can form, contrary to ligand L1, hydrogen bonds, strong bonds, with the amino acids of the active site of acetylcholinesterase

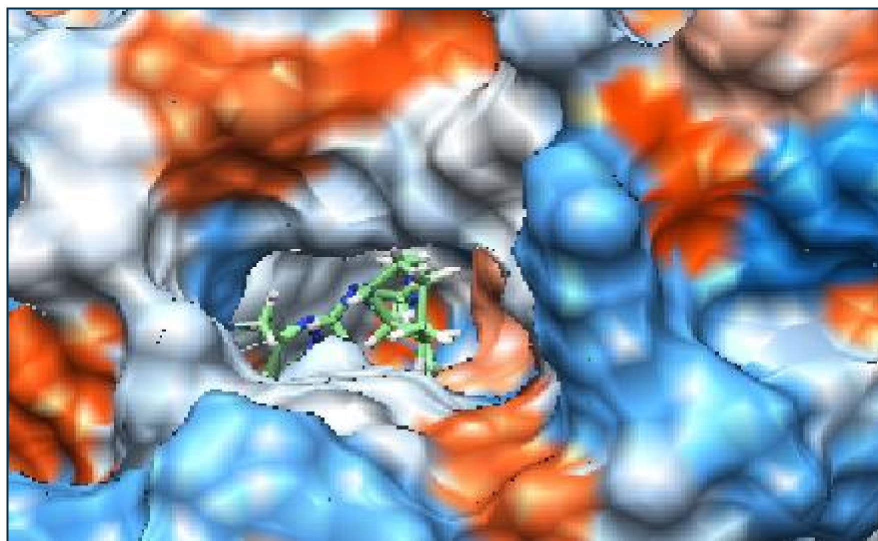


Figure 37: Inhibitor-enzyme complex

Umbelliferone formed three hydrogen bonds with the interacting residues Tyr121, Phe288, and Arg289, the last two were involved in strong hydrogen bonding interactions with

the hydroxyl group at the C-7 position and Tyr121 was involved in hydrogen bonding interactions with the ketone group at C-2.

The important residues of the peripheral anion site (PAS) (Trp279 and Tyr334) of AChE were involved in hydrophobic interactions with 1. (Figure 38)6Formyl-UMB docked in both the anionic catalytic site (CAS) and PAS of AChE (Figure 38). The linkage was formed by residues Ser200, Tyr334, His440, Ser81, Trp84, Gly118, Glu199, Phe330, and Trp432 (Figure 38). An aldehyde group at the C-6 position formed two strong hydrogen bonds with the catalytic triad, Ser200, and His400; Tyr334 interacted with the c-2 ketone group, which is involved in PAS, via a hydrogen bonding interaction.

Docking analysis predicted that the hydroxyl group of 8Formyl-UMB at position C-7 formed a hydrogen bond with residue Glu199.And hydrophobic interactions with residues Trp84, Gly117, Gly118, Phe330, His440, and Gly441 (Figure 38).

These results indicate that inhibitor 2 is active, which could be due to an interaction with important catalytic and peripheral residues of AChE.

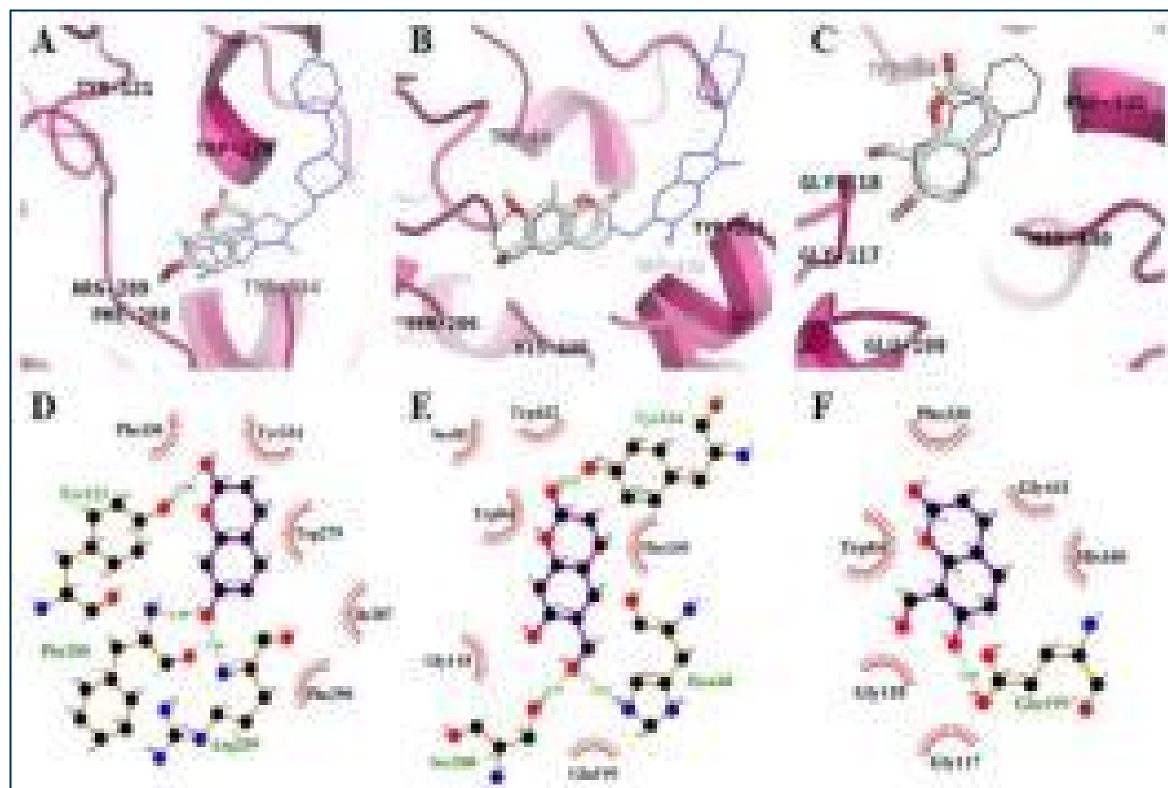


Figure 38: Ligand interaction diagram of AChE inhibition of 1 (D), 2 (E), 3 (F). Dashed lines indicate hydrogen bonds. Carbons are in black, nitrogens in blue, and oxygens in redgenerated using PyMOL **.**

V.2.Molecular-Docking Study of the Inhibition of BACE1:

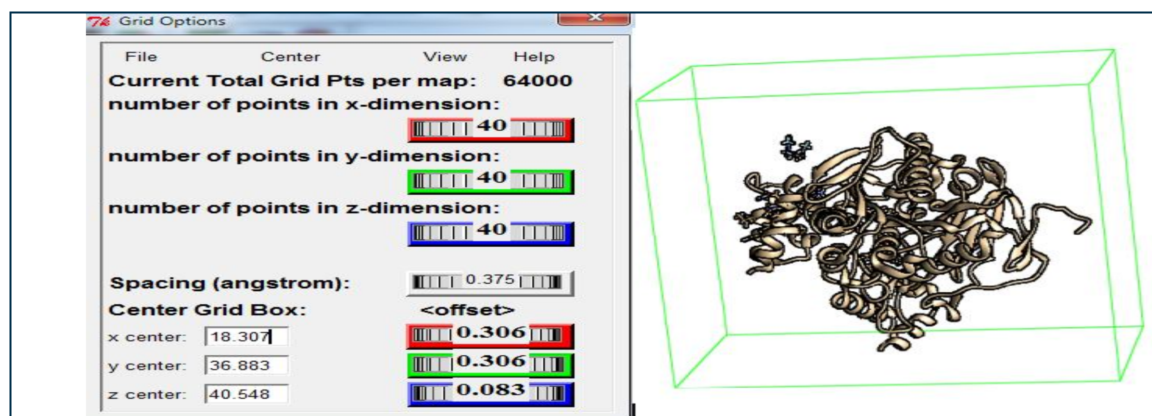


Figure 39: The calculation box

The study of binding interactions in the active site indicated that the hydroxyl group of 1 interacts with the catalytic residue Asp32 of the enzyme using hydrogen bonding. In addition, hydrophobic interactions between umbelliferone and Lys75, Trp76, Val69, Phe108, and Ile118 were observed with a binding energy of -5.4 kcal/mol. In contrast to 1, the top binding sites of 2 and 3 were distant from the catalytic site. (Figure 40B, E), Gly13 of BACE1 formed two hydrogen bonds with the hydroxyl and formyl groups of 2 with an energy of -7.2 kcal/mol.

The hydrophobic interactions between 2 and Ser10, Gly11, Tyr14, Val170, Thr232, Arg307, Pro308, Ala335, and Glu339 of BACE1 also appeared to be important for binding to the allosteric site, according to our Auto Dock 4.2 model study. The molecular docking model of 3 is shown in Figure 40C, F. In the 3-BACE1 complex, Ser10, Gly11, Gly13, Tyr14, Val170, Thr232, Arg307, Ala335, Val336, and Glu339 participated in hydrophobic interactions with 3 without any hydrogen bonding interactions.

Compounds	Binding Energy (kcal/mol)	H-bonds Interacting Residues	No. of H-Bond	van der Waals Interacting Residues
1	-5.4	1	Asp32	Lys75, Trp76, Val69, Phe108, Ile118
2	-7.2	2	Gly13	Ser10, Gly11, Tyr14, Val170, Thr232, Arg307, Pro308, Ala335, Glu339
3	-7	-	-	Ser10, Gly11, Gly13, Tyr14, Val170, Thr232, Arg307, Ala335, Val336, Glu339

Table05: binding energy &The number of hydrogen bonds and all amino acid residues involved in the BACE1 enzyme using AutoDock 4.2.

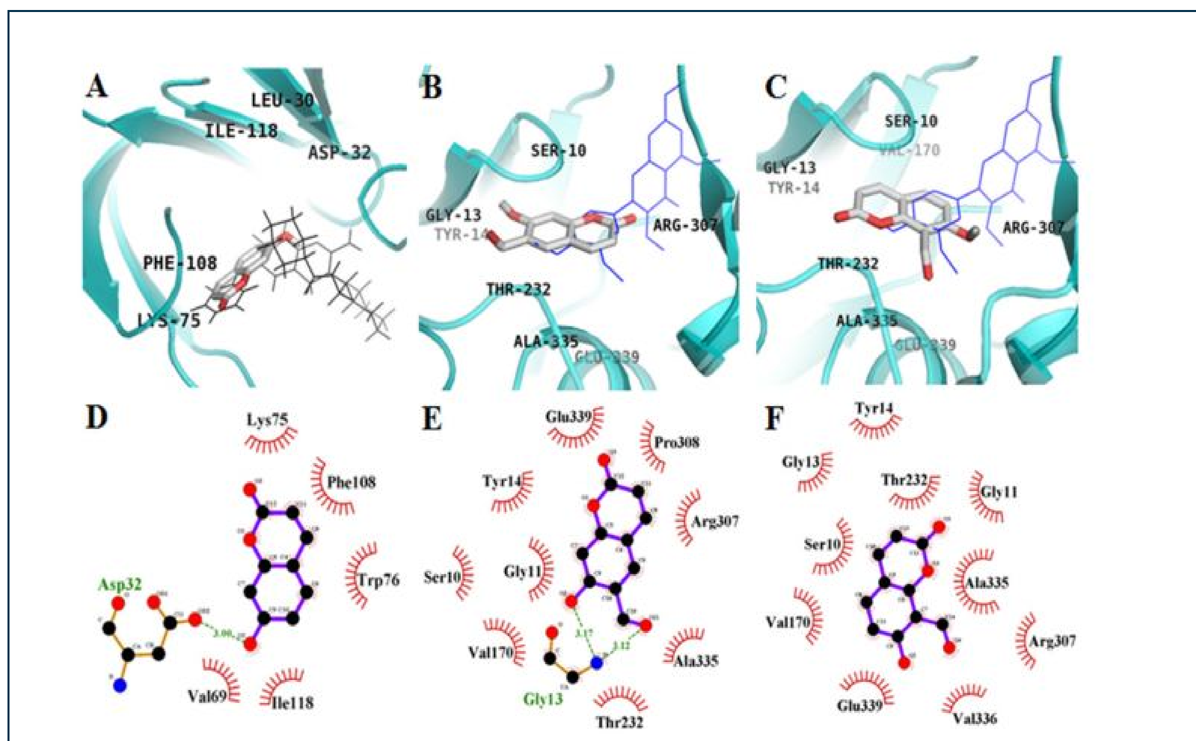


Figure 40: Ligand interaction diagram of BACE1 inhibition of 1(D), 2(E), 3(F). Dashed lines indicate hydrogen bonds. Carbons are in black, nitrogens in blue, and oxygens in red. Figures were generated using PyMOL.

Computational molecular docking analysis can provide insight into the mechanism underlying active site binding interactions. 2 might be related to both CAS (His440 and Ser200) and PAS (Tyr334) of AChE, while 3 only bound CAS (His440). within the study of BACE1, we observed several hydrogen bonds within the BACE1-2 complex. However, 3 didn't form any hydrogen bond with BACE1. These results showed that the hydrogen bonds between the compounds and also the main active residues of the target enzyme play an important role in the release of enzyme inhibition.

Conclusion

Acetylcholinesterase may be a present enzyme in humans that leads to the destruction of acetylcholine. Acetylcholine is a neurotransmitter within the brain involved in the transmission of messages to memory, reasoning, and other thought processes. Low levels of acetylcholine are related to memory disorders, like Alzheimer's disease and other sorts of mental diseases.

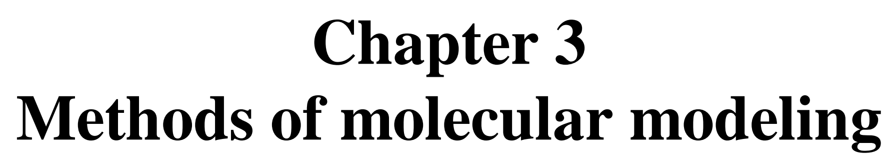
Cholinesterase inhibitors are drugs taken daily to switch the symptoms of Alzheimer's disease.

Alzheimer's disease. They're substances that inhibit the breakdown of acetylcholine, a crucial neurotransmitter related to memory, by blocking the enzyme acetylcholinesterase.

The present study revealed that umbelliferone can have significant anti-AD activity through inhibition of AChE and BACE1 within the Aβ pathway. Especially, 2, which has an aldehyde radical at position 6, inhibited BACE1 during a non-competitive manner compared to 1 and three. Furthermore, the 3D molecular docking study showed that 2 docked to the allosteric site of BACE1.

From this study results, we will conclude that the introduction of the formyl moiety at 1 increases the inhibitory activity against AChEs and BACE1 which its localization at position 6 (as in 2) is simpler than at position 8 (as in 3). To our knowledge, this is often the primary report of the potential anti-MA activity of two and three.

conclude that drugs for neurodegenerative diseases (ND) are umbelliferone-based drugs.



Chapter 3
Methods of molecular modeling

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