Natural and Synthetic Flavonoid Derivatives with Potential Antioxidant and Anticancer Activities

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Dedicated to my loving Mother

I would like to dedicate my thesis to my mother and brothers.

I also want to dedicate my thesis to my loving wife and daughters.

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Abstract

Flavonoids form a family of well known natural products present in most of the plant families. More than 8000 different flavonoids have been isolated from their natural source to date. The structural variations of these flavonoids are associated with many different biological and pharmacological activities, including anticancer activity, protection against cancer formation (chemo-protection), antioxidant activity, cardiovascular and hepatic protection, antibacterial, antifungal and antiviral activity. Flavonoids have also been reported to play an important role in hormone-related female diseases, such as breast cancer and menopausal syndrome. Natural flavonoids have therefore been subjected to many chemical modifications in order to improve their activity. As part of this thesis, I have added moieties such as an amino alkyl chain, selenium and tellurium containing moieties. As part of an approach to generate flavonoid derivatives more active and specific toward cancer cells, addition of a chromene ring (ring **D**) to the flavonoid core structure resulted in chromene-flavone derivative. Aromatase is a group of cytochrome P450 enzymes responsible for the biosynthesis of estrogen, the main stimulant of breast cancer cell growth. Some of the flavonoid derivatives tested (natural as well as synthetic) turned out to be good aromatase inhibitors and may be studied further in the treatment of breast cancer. Ten natural products in addition to 38 synthetic flavonoid derivatives were subjected to various *in vivo* and *in vitro* bioassays in order to understand the various antioxidant, cytotoxic and aromatase inhibiting properties associated with these compounds.

Kurzdarstellung

Die Familie der Flavonoide ist als Naturprodukte sehr gut bekannt und findet sich in einem Großteil der Pflanzenfamilie wieder. Mehr als 8000 verschiedene Flavonoide wurden zum jetzigen Zeitpunkt aus Quellen natürlichen Ursprungs isoliert. Die strukturellen Unterschiede dieser Flavonoide, sowie deren Wirkung gegen Krebs, Schutz vor Krebsentstehung, Antioxidantien-Aktivität, kardiovaskulärer und hepatischer Schutz sowie antibakterielle, antifungizide als auch antivirale Aktivität, spiegeln sich in verschiedenen biologischen und pharmakologischen Aktivitäten wider. Flavonoide spielen zudem bei hormonell bedingten Erkrankungen, die überwiegend bei Frauen auftreten, wie z.B. Brustkrebs oder das menopausale Syndrom, eine wichtige Rolle. Aus diesem Grund wurden natürliche Flavonoide

für diese Studien ausgesucht, die chemisch modifiziert worden sind, um deren Aktivität erhöhen zu können. Als Teil dieser Doktorarbeit wurden verschiedene Reste, wie z.B. Aminoalkylketten, Selen sowie auch Tellur hinzugefügt.

Um eine höhere Aktivität sowie Spezifität der Flavonoid-Derivate gegenüber Krebszellen erzielen zu können, wurde das Flavonoid-Grundgerüst mit einem Chromen-Ring (Ring **D**) ergänzt.

Aromatase gehört zur Gruppe der Cytochrom P450 Enzyme, die für die Biosynthese von Östrogen verantwortlich sind, einem wichtigen Förderer des Wachstums von Brustkrebs-Zellen.

Sowohl natürliche als auch synthetische Flavonoid-Derivate stellten sich als gute Aromatase-Inhibitoren heraus and könnten später vielleicht in der Brustkrebs-Therapie eingesetzt werden.

Zehn natürliche Produkte sowie 38 auf synthetischem Wege hergestellte Derivate wurden in verschiedenen *in vivo* und *in vitro* Bioassays eingesetzt, um die antioxidativen, cytotoxischen, als auch die Aromatase inhibierenden Eigenschaften besser verstehen zu können.

Abreviations

A	Unlabeled androstenedion
ANS	Anthocyanin synthase
ATP	Adenosine triphosphate
br s	Broad singlet
Brine	Saturated solution of sodium chloride
calcd	Calculated
cAMP	Cyclic adenosine monophosphate
CC	Column chromatography
$CDCl_3$	Chloroform
CH_2CL_2	Dichloromethane
CHI	Chalcone isomerase
CHS	Chalcone synthase
^{13}C NMR	Carbon Nuclear magnetic resonance
COSY	Correlation spectroscopy
d	Duplet
dd	Duplet of duplet
DFR	Dihydroflavone-4-reductase
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ER	Estrogen receptor
EtOAc	Ethyl acetate
EtOH	Ethanol
FCS	Fetal calf serum

Fig	Figure
FLS	Flavone synthase
FSH	Follicle stimulating hormone
g	Gram
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
^{3}HA	Labeled androstenedione
HDL	High density lipoprotein
HMBC	Heteronuclear multiple bond correlation
^{1}H NMR	Proton Nuclear magnetic resonance
HR-MS	High resolution-mass spectroscopy
hrs	Hours
HSQC	Heteronuclear single quantum coherence
IC_{50}	50 % inhibition concentration
IFS	Isoflavone synthase
Kg	Kilograms
L	Litre
LC-MS	Liquid chromatography-mass spectroscopy
LDL	Low density lipoprotein
m/z	Mass to charge ratio
m	Multiplet
mCi	Millicurie
MeOH	Methanol
mg	Milligrams
min	Minutes
mL	Millilitres
MMP	Matrix metallo protein

mp	Melting point
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
n.d.	Not determined
NMR	Nuclear magnetic resonance
OS	Oxidative stress
PBS	Phosphate buffered saline
PgR	Progesterone receptor
PLA	Phenylalanine amminolyase
ppm	Part per million
q	Quartet
RNO	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640-medium
r.t.	Room temperature
s	Singlet
SERM	Selective estrogen receptor modulator
t	Triplet
TLC	Thin layer chromatography
TNFα	Tumor necrosis factor α
δ	Chemical shift
%	Percent
μCi	Microcurie
μM	Micro molar
μL	Micro litre

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Chapter I: Introduction

Natural, mainly plant-derived constituents have long been sources of drugs, and a large proportion (30–40%) of the pharmaceuticals available in modern medicine is directly or indirectly derived from natural sources. Natural products are also of great interest in the process of drug discovery. Due to their large diversity in nature, they permit the identification of leading molecules of interest for the development of new therapeutic agents. Furthermore they provide biochemical and molecular tools needed to clarify complex cellular and molecular mechanisms of action involved in most physiological and pathological processes. Ultimately, a growing world-wide interest in the use of phytopharmaceuticals as complementary or alternative medicine, either for the prevention or treatment of many diseases, has been noted in recent years. It is believed that about 80% of world's population uses plants as their primary source of medicinal agents. Flavonoids in particular are considered to be one of the most important natural products and one of the most commonly used compounds around the world.

1.1. Flavonoids

1.1.1. Flavonoids in nature

Flavonoids are a group of naturally occurring polyphenolic compounds ubiquitously found in plants [1]. Considering the means of defense and communication that plants acquired through evolution, flavonoids form one of the most important chemical classes of natural products. Flavonoids first appeared in green algae 500 million years ago, resulting from the fusion of two biogenetic pathways, namely the cinnamate and the ancient polyketide route. They have then become more and more complex with plant evolution [2].

We can attribute a large biological importance to flavonoids, not only for the vegetal kingdom itself, but also for humans and animals. Flavonoids are increasingly thought to be responsible for the longer life expectancy of populations with well-balanced and healthy diets. Such a diet consist of a high amount of fruit and vegetables as well as beverages from vegetable origin, particularly tea and juice, which make it rich in flavonoids and other

polyphenols [3, 4]. This nutritious phenomenon has often been referred to as the '*French paradox*' [5]. French paradox is the observation that the French suffer a relatively low incidence of coronary heart disease. The phenomenon was first noted by Irish physician Samuel Black in 1819. The term *French paradox* was coined by Dr. Serge Renaud, a scientist from Bordeaux University in France in 1992 [6].

Flavonoids generally occur in plants as glycosylated derivatives, and they contribute to the brilliant shades of blue, scarlet, yellow and orange, in leaves, flowers, and fruits [7]. Apart from various vegetables and fruits, flavonoids are found in seeds, nuts, grains, spices, and different medicinal plants as well as in beverages, such as wine (particularly red wine), tea, and at lower levels also in beer [8].

Over 8000 different naturally occurring flavonoids have been discovered [9] and the list is still growing. Most of these structurally different flavonoids can be arranged in various classes, and differ in the level of oxidation of the **C**-ring of the basic benzo-γ-pyrone structure. Common family members of flavonoids include flavones, flavanes, flavonols, catechins, and anthocyanidins (***Fig 1***). The structural difference in each flavonoid family results from the variation in the number and substitution pattern of the hydroxyl groups as well as the extent of glycosylation of these groups [10].

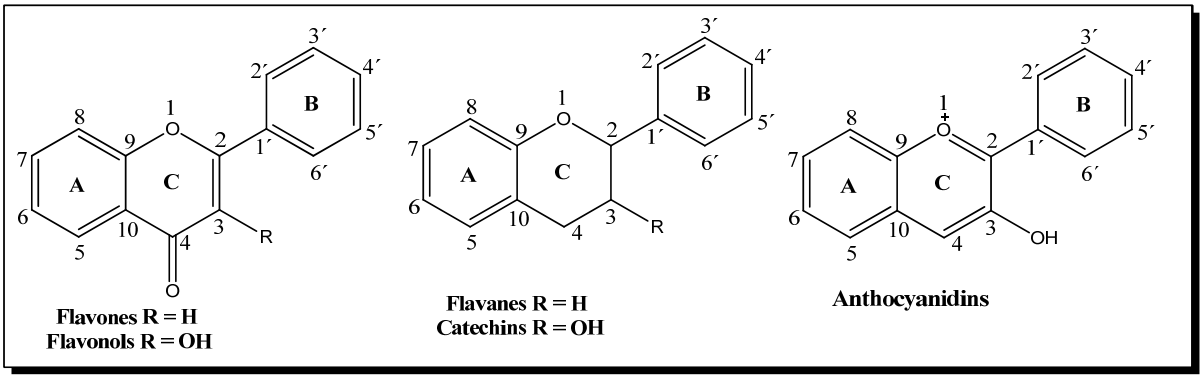


Fig 1: *Chemical structure of some flavonoid members.*

1.1.2. **Biosynthesis of flavonoids** [10, 11]

The **B**-ring and part of the heterocyclic ring of the flavonoid skeleton are provided by a suitable hydroxy-cinnamic acid-CoA ester, usually 4-coumaroyl-CoA, whereas the **A**-ring

originates from three acetate units via malonyl-CoA (***Fig 2***). Both precursors are derived from carbohydrates. Malonyl-CoA is formed from acetyl-CoA and CO_2 catalysed by acetyl CoA carboxylase. 4-Coumaroyl-CoA and related hydroxycinnamic acid esters are supplied by the first steps of the general phenylpropanoid pathway. This pathway starts from the aromatic amino acid phenylalanine, which is synthesized via the shikimate arogenate pathway. The key reaction is the deamination of phenylalanine catalysed by phenylalanine ammonialyase (PAL). This enzyme links the primary metabolism with the phenylpropanoid pathway. The product of the reaction, *trans*-cinnamate, is hydroxylated to 4-coumarate by cinnamate 4-hydroxylase, a cytochrome P450 mixed-function monooxygenase. Activation of 4-coumarate by formation of the CoA ester is catalysed by 4-coumarate-CoA ligase. 4-Coumaroyl-CoA can be hydroxylated in position 3 to caffeoyl-CoA, which may serve as a substrate for chalcone formation besides 4-coumaroyl-CoA in some plant species. Three different enzyme activities have been demonstrated for caffeoyl-CoA formation from 4-coumaroyl-CoA.

The key enzyme for the formation of the flavonoid skeleton is chalcone synthase (CHS), which catalyses the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaroyl-CoA to the fifteen carbon intermediate 2′,4′,6′,4-tetrahydroxychalcone (***Fig 2***). The respective 6'-deoxychalcone, isoliquiritigenin, is likewise synthesized from malonyl-CoA and 4-coumaroyl-CoA by chalcone synthase but in coaction with a (reduced nicotinamide adenine dinucleotide phosphate) (NADPH)-dependent reductase. Both chalcone types may be the direct precursors for aurones and other diphenylpropanoids. The enzymes involved in these reactions are still unknown. But, in particular, the 6′-hydroxy- and 6′-deoxychalcones are the immediate precursors for all flavonoid compounds. The stereospecific cyclization of the chalcone, catalysed by chalcone isomerase, provides 25-flavanones with the typical flavonoid skeleton (***Fig 2***). Two types of chalcone isomerases are known: one catalysing cyclization of 6′-hydroxy-chalcone to 5-hydroxyflavanone and another isomerizing both 6′-hydroxy- and 6′-deoxychalcone to 5-hydroxy-and 5-deoxyflavan-one, respectively.

Flavanones are the direct precursors for other natural products, such as the large class of flavones, isoflavones that are involved in phytoalexin synthesis, and the two flavonoid intermediates, the flavan-4-ols and the dihydroflavonols. Flavones are synthesized from flavanones by introduction of a double bond between C-2 and C-3. Two types of enzymes, flavone synthase I, (a 2-oxoglutarate-dependent dioxygenase), and flavone synthase II, (a cytochrome P450 mixed-function monooxygenase), were found to catalyse this reaction. Formation of isoflavones from flavanones is catalysed by 2-hydroxy-isoflavanone synthase,

another cytochrome P450 mixed-function monooxygenase, coacting with a dehydratase protein. The enzyme accepts both 5-hydroxy- and 5-deoxyflavanones as substrates. The reaction involves an oxidative rearrangement of the flavanone, including a shift of the aryl ring from position 2 to 3.

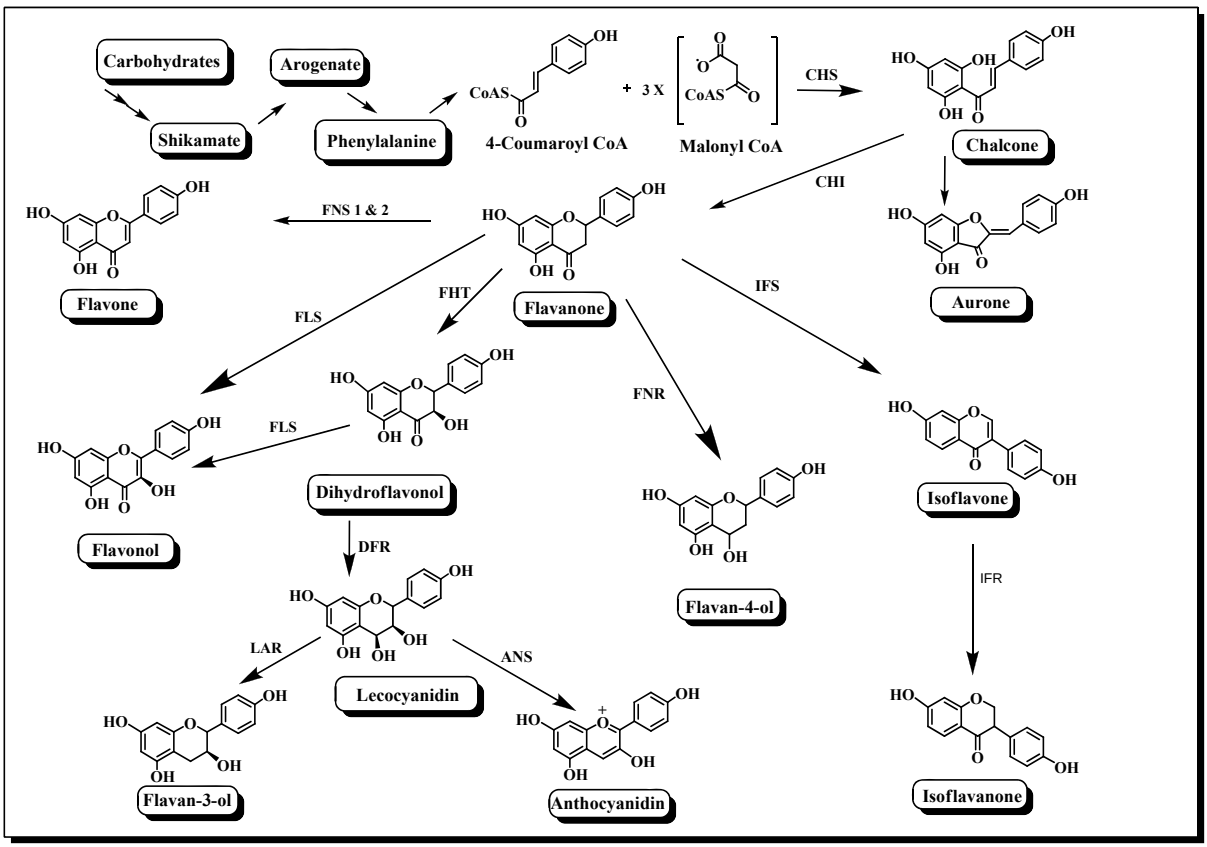


Fig 2: *Schematic overview of the major branch pathways of flavonoid biosynthesis, starting from carbohydrates and leading to twelve flavonoid groups adapted according to [10].*

The reduction of the carbonyl group of flavanones gives rise to flavan-4-ols. The reaction is catalysed by flavanone-4-reductase and provides the immediate precursors for the formation of 3-deoxy-anthocyanins (***Fig 2***).

Finally, flavanones can be hydroxylated in position 3 to dihydroflavonols, which are biosynthetic intermediates in the formation of flavonols, catechins, proanthocyanidins and anthocyanidins. This reaction is catalysed by flavanone 3-hydroxylase, a 2-oxoglutarate-dependent dioxygenase.

Dihydroflavonols are the direct substrates for most of the flavonols and flavan-3,4-diols, which are also known as leucoantho-cyanidins. Flavonols are formed from dihydroflavonols

by introduction of a double bond between C-2 and C-3. The reaction is catalysed by flavonol synthase, another 2-oxoglutarate-dependent dioxygenase. Reduction of dihydroflavonols in position 4, catalysed by dihydroflavonol-4-reductase, leads to flavan-2,3-*trans*-3,4-*cis*-diols, which are intermediates in catechin, proanthocyanidin and anthocyanidin formation. Catechins are synthesized from leucoanthocyanidins by further reduction in position 4. This reaction is catalysed by flavan-3,4-*cis*-diol-reductase. Proanthocyanidins probably originate from leucoanthocyanidins and catechins by a condensation reaction. The enzyme catalysing this reaction is not yet known (***Fig 2***).

The formation of the glycosidic linkage(s) is catalyzed by transferases which are also highly specific, as far as the substrate and the glycosylation positions are concerned. These enzymes require the presence of uridine diphospho-saccharides (UDP-saccharides). Acyl-transferases are also specific enzymes for the acylation of some glycosides such as anthocyanins.

1.1.3. Metabolism of flavonoids

During metabolism of flavonoids, hydroxyl groups are added (Phase I biotransformation), and then methylated, sulfated or glucuronidated (Phase II biotransformation). In food, flavonoids exist primarily as 3-*O*-glycosides and polymers [12]. Several types of higher structures exist, and polymers comprise a substantial fraction of dietary flavonoid intake [13]. Enzymatic oxidation of green tea leaves during fermentation to black tea results in polymerization of flavanols to tannins and other complex compounds [14].

The most common glycosidic unit is glucose, but other examples include glucorhamnose, galactose, arabinose, and rhamnose [15]. Not surprisingly, the *β*-linkage of these sugars resists hydrolysis by pancreatic enzymes, so it had long been assumed that intestinal microbiota were responsible for beta-hydrolysis of sugar moieties. However, two β-endoglucosidases capable of flavonoid glycoside hydrolysis have since been characterized in the human small intestine, including lactase phlorizin hydrolase [16, 17] and a nonspecific cytosolic enzyme believed to deglycosylate flavonoids to allow a site for conjugation [18, 19]. Luteolin-7-glucoside, kaempferol-3-glucoside and quercetin-3-glucoside are hydrolyzed and absorbed by the small intestine, supporting *β*-glucosidase activity [20], but it's also known for some flavonoid glycosides to be absorbed intact, *e.g*. anthocyanin glycosides [21].

Absorption may also depend on dosage, vehicle of administration, antecedent diet, sex differences [22], and microbial population of the colon. For hydrolysis and absorption of some flavonoid glycosides, enteric bacteria are necessary. The requirement of colonic microflora for hydrolysis of rutinosides, for instance may explain the low bioavailability of rutin (quercetin-3-rutinoside) compared to quercetin-3-glucoside in human studies.

Researches have shown that a glucose moiety increases absorption of quercetin in the small intestine to 52 %, compared to 24 % for the aglycone and 17 % for rutin. Quercetin glucoside, but not rutin, has been reported to interact with epithelial glucose transporters, offering a possible explanation for the rapid uptake and bioavailability of glucosides [23].

1.1.4. Distribution of flavonoids

Flavonoids constitute one of the most important classes of compounds in higher plants. Many flavonoids are easily recognized as flower pigments in most angiosperm families (flowering plants). Their occurence is not restricted only to the flowers, but can be found in all parts of the plant.

Flavonoids are present in all vascular plants but some classes are more widely distributed than others. While flavones and flavonols are universal, isoflavones and biflavonoids are found only in few plant families [24].

Bryophyte plants as well as certain types of green algae, particularly *Nitella hookeri* [24], fungi, such as *Aspergillus candidus* [25], and marine coral *Echinophora labellosa* [26], also have a biosynthetic ability to produce flavonoids.

Out of all the plant families, the family *Leguminosae* is perhaps most endowed with flavonoid constituents, and many of such flavonoids are only found in *Leguminosae* plants [27]. Anthocynin pigments are widely distributed in the floral tissue of this family and many flavones and flavonol glycosides have been isolated from different leaves, bark, stems and roots of *Leguminosae* plants. A typical flavonol glycoside found in *Leguminosae*, for instance, is Robinin, which has first been recorded in the leaves of *Robinia pseudoacacia* [27]. Isoflavonoids are almost unique to the *Leguminosae*, and most of these compounds are restricted to the sub family *Papilionoideae* [27].

1.2. Botanical general characters of family *Leguminosae*

The family *Leguminosae* includes trees, shrubs, woody vines, and annual or perennial herbs. Leaves are usually alternate and compound–bipinnate, simple pinnate, or palmate, but rarely simple. Inflorescence is variously racemose, in simple racemes, panicles, spikes, or heads. Flower structure varies to the extent that 3 subfamilies (*Mimosoideae*, *Caesalpinioideae*, *Papilionoideae*) are recognized; corolla typically 5-parted; stamens 3-many, mostly 10, free, or united by their filaments in various ways, pistil simple, free. The Fruit is characteristically a legume, dehiscent or indehiscent [28, 29].

Among the various leguminal plants we did select *Delonix regia* and *Robinia pseudoacacia* in this study in order to isolate the flavonoid constituents and so investigate their biological activities.

1.2.1. *Delonix regia*

Delonix regia is a species of flowering plant from the *Fabaceae* family (the new name of the family *Leguminosae*). This tree is native to Madagascar and is often grown as an ornamental tree in many countries around the world, consistently voted among the Top Five most beautiful flowering trees in the world. *Delonix regia* is also known as the **Royal Poinciana** or **Flamboyant** [32]. This plant was previously placed in the genus *Poinciana*, named after Phillippe de Longvilliers de Poincy (1583-1660), who is credited with introducing the plant to the Americas.

Taxonomy

Delonix regia is classified under class *Dicotyledons*, subclass *Rosidae*, order *Fabales*, family *Fabaceae*, genus *Delonix* and species *regia*.

1.2.2. General botanical character of *Delonix regia*

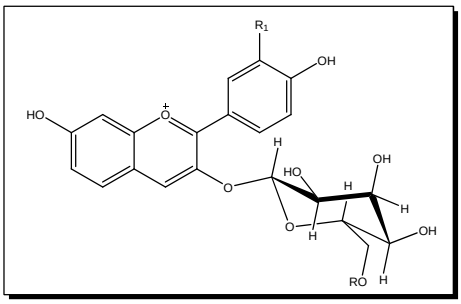
The Royal Poinciana grows to a height of 9-15 meters with an elegant, wide-spreading, umbrella-like canopy which sometimes exceeds the height of the tree in diameter. Royal Poinciana is deciduous in climates that have a marked dry season, but in tropical areas (where the winter is not that much dryer than the summer), it is a semi-evergreen tree.

The leaves are even-pinnate, alternate, and can grow up to 66 cm long, 10-25 pairs of opposite pinnate, and 12-40 pairs of small oblong leaflets. The flowers are Caesalpinaceous, can grow up to 12 cm wide, consisting of 5 red or yellow petals, or standard of red splashed with burgundy spots. They are arranged on racemes that can reach up to 25 cm long. The fruits are Pods, long, hard, flat, slightly curved and dehiscent [30, 31].

1.2.3. Destribution of flavonoids in *Delonix regia*

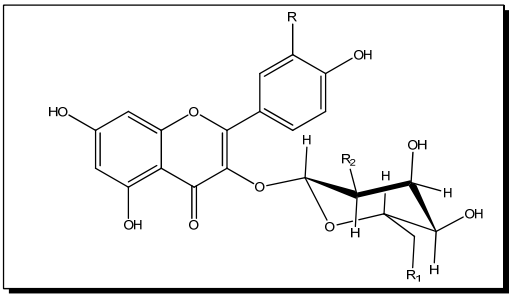
Many publications take in consideration the *Delonix regia* fatty acids and sterols (Stigmasterol, γ-Sitosterol, β-Amyrin) as well as protein and lectin content. In contrast only two of these publications deal with the isolation and characterization of *Delonix regia* flavonoids [32, 34]. The flavonoids isolated from this plant are summaried in ***Table 1*** and ***Table 2***.

Table 1: *Cyanidin glycosides isolated from Delonix regia* [32, 33].



Compound	R	R_1
cyanidin 3-*O*-glucoside	H	OH
cyanidin 3-*O*-rutinoside	Rhamnoside	OH
pelargonidin 3-*O*-rutinoside	Rhamnoside	H
cyanidin 3-*O*-gentiobioside,	Glucoside	OH

Table 2: *Flavone glycosides isolated from Delonix regia* [34].



Compound	R	R_1	R_2
Kaempferol-3-O-glucoside	H	H	H
Kaempferol-3-O-rutinoside	H	Rhamnoside	H
kaempferol -3-O- rhamnosyl neohesperidoside	H	Rhamnoside	Rhamnoside
Kaempferol-3-O-neohesperidoside	H	H	Rhamnoside
Quercetin-3-O-rutinoside	OH	Rhamnoside	H

1.2.4. Traditional uses of *Delonix regia*

The extract of *Delonix regia* is known to have medicinal properties [35, 36]. This plant is used in several countries to prepare extracts with antimicrobial and antifungal activities [37]

and can be used as an antibiotic [38]. In Ivory Coast, for instance, traditional medicines are prepared from several parts of the tree, including the flowers. In rural areas, water extracts are generally homemade from *Delonix regia* flowers.

1.2.5. *Robinia pseudoacacia*

Robinia pseudoacacia, the black locust, is named after Jean Robin (1550-1629), and his son, Vespasian (1579–1662), the Royal Gardeners in Paris during the reign of Henry IV. According to one historical record, seeds of *R. pseudoacacia* were sent to Jean from Canada and cultivated by him on a large scale in 1601. Another record cites the planting of the seed by Vespasian in 1635, who was at that time the arborist to Louis XIII in the Jardin des Plantes, Paris [39].

The genus *Robinia* has 40 species native to temperate regions of north-east America and is noteworthy for its ability to tolerate frost. *Robinia* species have fragrant flowers and are a good source of pollens necessary to bee honey production. In the Danube Basin, this so-called acacia honey commands a higher price than other kinds of honey.

Taxonomy

Robinia pseudoacacia is classified under class *Dicotyledons*, subclass *Rosidae*, order *Fabales*, family *Fabaceae*, genus *Robinia* and species *pseudoacacia*.

1.2.6. General botanical character of *Robinia pseudoacacia*

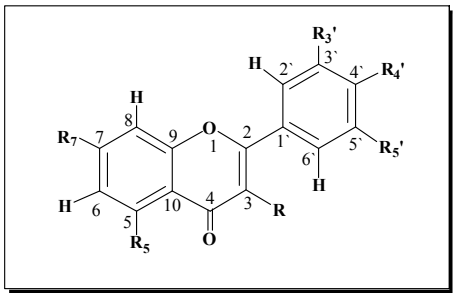
The black locust is a medium-sized, native, deciduous tree belonging to Family *Leguminosae*. The height of a mature plant can range anywhere between 12-18 m. This deciduous plant can take the form of a shrubs or a tree, and can vary in size (from small to large). The trunks of the black locust tend to be long, straight and slender. Their branches are known to be slender, terete, angled, and zigzagged, and their bark is furrowed. The stipular spines grow on twigs and young branchlets. The leaves are imparipinnate and petioled while the leaflets consist of 6-20 pairs and are small, oblong, penniveined and petiolated. The stipules present are stipules setaceous, spinescent at maturity and are persistent. The flowers are white or pink, and present on long slender pedicels. The bracteoles are caduceus or absent. The calyx is campanulate and divided into 5 lobes, the upper 2 lobes are shorter than others.

The stamens are didelphous and around 10 in number. The ovary is linear-oblong and inserted at the base of the calyx [40].

1.2.7. Distribution of flavonoids in *Robinia pseudoacacia*

As with *Delonix regia*, *Robinia pseudoacacia* contain many fatty acids, sterols, protein and lectins, which have reported, together with a large number of triterpenoidal saponins and carbohydrates [41-43]. Importantly *Robinia pseudoacacia* also contain several types of flavonoids which may exert significant health benefits. ***Tables 3-7*** and ***Fig. 3*** and ***Fig 4*** provide an overview of some of the flavonoids found in *Robinia pseudoacacia.*

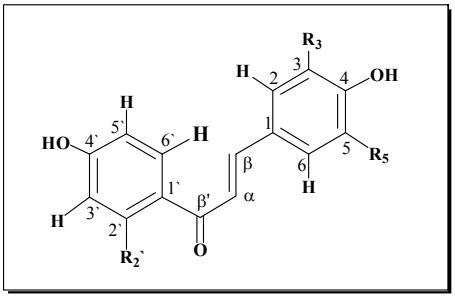
Table 3: *Flavones and Flavonols isolated from Robinia pseudoacacia* [44-50].



Compound	R	R_5	R_7	R_3`	R_4`	R_5`
4`,7-dihydroxyflavone	H	H	OH	H	OH	H
Apigenin	H	OH	OH	H	OH	H
Chrysoeriol	H	OH	OH	H	OH	OCH_3
Acacetin	H	OH	OH	H	OCH_3	H
Robinetin	OH	H	OH	OH	OH	OH
Fisetin	OH	H	OH	OH	OH	H
Quercetin	OH	OH	OH	OH	OH	H
Robinin	A	H	O-Rha	H	H	H
Robtin	Rha	H	Rha	H	H	H

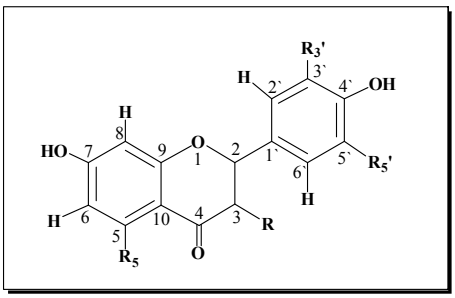
A= Robinobioside (rhamnosyl(1to 6)galactose.

Table 4: *Chalcones isolated from Robinia pseudoacacia* [51-53].



Compound	R_3	R_5	R_2`
isoliquiritigenin	H	H	H
Robtein	OH	OH	OH
Butein	OH	H	OH

Table 5: *Dihydroflavones and dihydroflavonols isolated from Robinia pesudoacacia* [46, 49, 54, 55].



Compound	R	R_5	R_3`	R_5`
Chrysoeriol	H	OH	H	OCH_3
Dihydrofisetin	OH	H	OH	H
Dihydrorobinetin	OH	H	OH	OH
Naringenin	H	OH	H	H

Fig 3: *Flavanes isolated from Robinia pesudoacacia* [56].

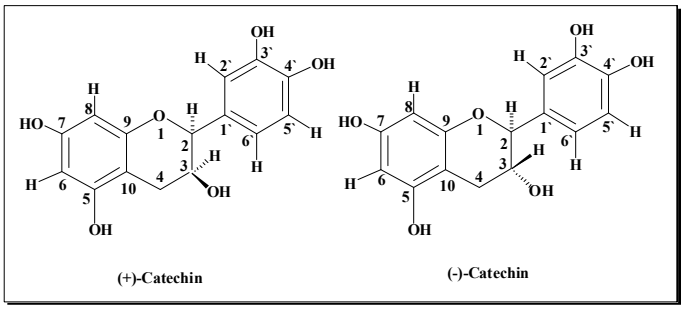
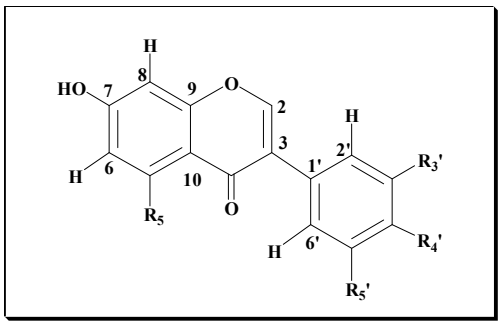
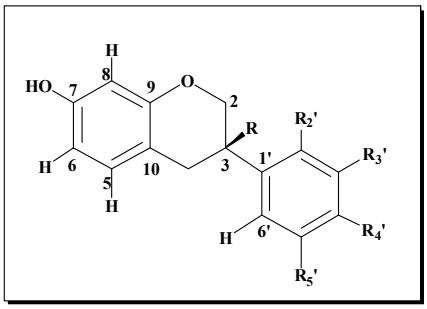


Table 6: *Isoflavones isolated from Robinia pseudoacacia* [57].



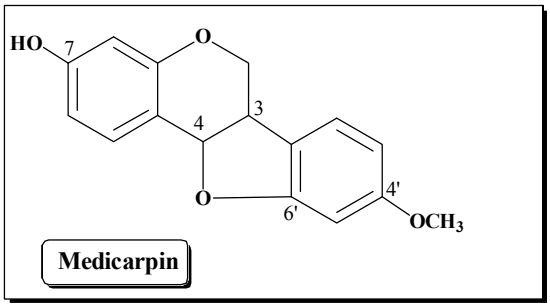
Compound	**R_5**	**$R_{3'}$**	**$R_{4'}$**	**$R_{5'}$**
3`-Methoxydaidzein	H	OCH_3	OH	H
Formonetin	H	H	OCH_3	H
Biochanin A	OH	OH	OCH_3	H
Mucronulatol	H	OCH_3	OH	OCH_3

Table 7: *Isoflavanes isolated from Robinia pseudoacacia* [58].



Compound	R	R_2`	R_3`	R_4`	R_5`
Isovestitol	H	OCH_3	H	OH	H
Isomucronulatol	H	OH	OCH_3	OCH_3	H
Secondifloran	OH	OH	OH	OCH_3	$-C(CH_3)_2$ $CH=CH_2$

Fig 4: *Pterocarpan isolated from Robinia pesudoacacia* [59].



1.2.8. Traditional uses of *Robinia pseudoacacia*

Black locust flowers have a long tradition in the preparation of tea. Tonic, purgative, and emetic properties have been reported for the inner bark and roots of this tree [47].

1.3. Biological activities of flavonoids

Flavonoids may have existed in nature for over 500 million years and thus have interacted with evolving organisms over the eons. Clearly, flavonoids serve important purposes in nature, having survived in vascular plants throughout evolution [2]. Flavonoids not only equip the plants themselves with unique properties (such as colors), but also exert an influence on animals living with plants. The long association of plant flavonoids with various

animal species and other organisms throughout evolution may account for the extraordinary range of biochemical and pharmacological activities of these chemicals in mammalian and other biological systems.

First of all, we will consider the effects on the plant itself. Flavonoids have important roles in plant biochemistry and physiology, they act as antioxidants, enzyme inhibitors, and precursors of toxic substances, while they also take part in nitrogen fixation, and act as pigments and light screens [60]. In addition, these compounds are involved in photosensitization and energy transfer. Certain flavonoids also function as plant growth hormones and growth regulators. They are involved in the control of respiration, photosynthesis, morphogenesis, and sex determination, as well as defence against infections [61].

On the other hand, in humans and higher animals, flavonoids have long been recognized to possess anti-inflammatory, antioxidant, ant-allergic and hepato-protective properties. They are also believed to be antithrombotic, antibacterial, antifungal, antiviral, and cancer protective, and also to protect against cardiovascular disease [62-64].

Small alternations in the chemical structure of flavonoids may lead to significant changes in biological activities, *e.g*. chrysin is a poor antioxidant compared to quercetin (***Fig 5***) -the latter has increased antioxidant activity due to the presence of three additional hydroxyl groups, yet chrysin is 20 times more effective in the inhibition of the human aromatase enzyme (see below) [65].

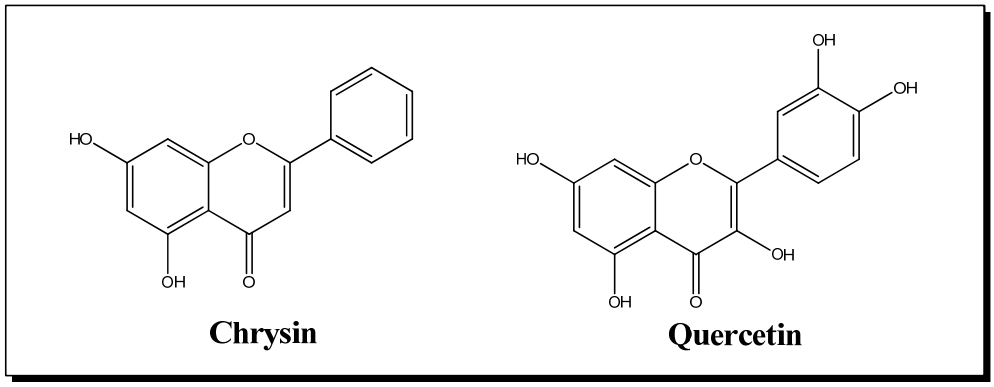


Fig 5: *Chemical structure of chrysin and quercetin.*

1.3.1. Antioxidant activities associated with flavonoids

Numerous publications have investigated the antioxidant activities of flavonoids and how they can contribute to the treatment of several diseases. Considering these publications, they indicate that biological and pharmacological effects of flavonoids may depend upon their behavior as either antioxidants or as prooxidants. Some flavonoids can behave as both antioxidants and prooxidants, depending on concentration and the redox environment present. For instance, certain flavonoids act as antioxidants against free radicals, yet demonstrate prooxidant activity when a transition metal such as $Cu^{2+}$is present [66].

Three important points must be illustrated in order to better understand antioxidant activity of flavonoids and the important role of flavonoids compounds in human biology:

a. The role of oxidative stress in human diseases.
b. The antioxidant structure-activity relationship of flavonoids.
c. The structure characteristics of an effective flavonoid antioxidant.

a. The role of oxidative stress in human diseases

Oxidation is the transfer of electrons from one atom to another. It represents an essential part of our metabolism and aerobic life in general, since oxygen is the ultimate electron acceptor in the electron flow systems that transport energy in the form of ATP [67]. Problems may arise however when the electron flow generates free radicals, such as O_2-centred free radicals, known as reactive oxygen species (ROS), and including superoxide ($O_2^{\cdot -}$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$), hydroxyl ($HO^{\cdot}$) and nitric oxide ($NO^{\cdot}$) radicals.

The contribution of free radical-mediated processes to the pathogenesis of human disease is indicated by biomarkers of oxidative damage to lipids, proteins, and DNA (***Fig 6***). Such markers have been identified in patients with atherosclerosis, certain cancers, neurodegenerative diseases, and lung disorders, especially those with an inflammatory component to their etiology. A range of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the mechanisms of damage associated with disease development, including superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide ($HOO^{\cdot}$), hypochlorite radical ($ClO^{\cdot}$), ferryl heme protein species, lipid alkoxyl ($RO^{\cdot}$) and peroxyl radicals ($ROO^{\cdot}$), peroxynitrite ($ONOO^{-}$), nitric oxide ($NO^{\cdot}$), and nitrogen dioxide radicals ($NOO^{\cdot}$).

Reactive oxygen species (ROS) obey different rules *in vivo*. Some are involved in energy production, phagocytosis, regulation of cell growth and intracellular signaling, as well as recognizing of biologically important compounds [68].

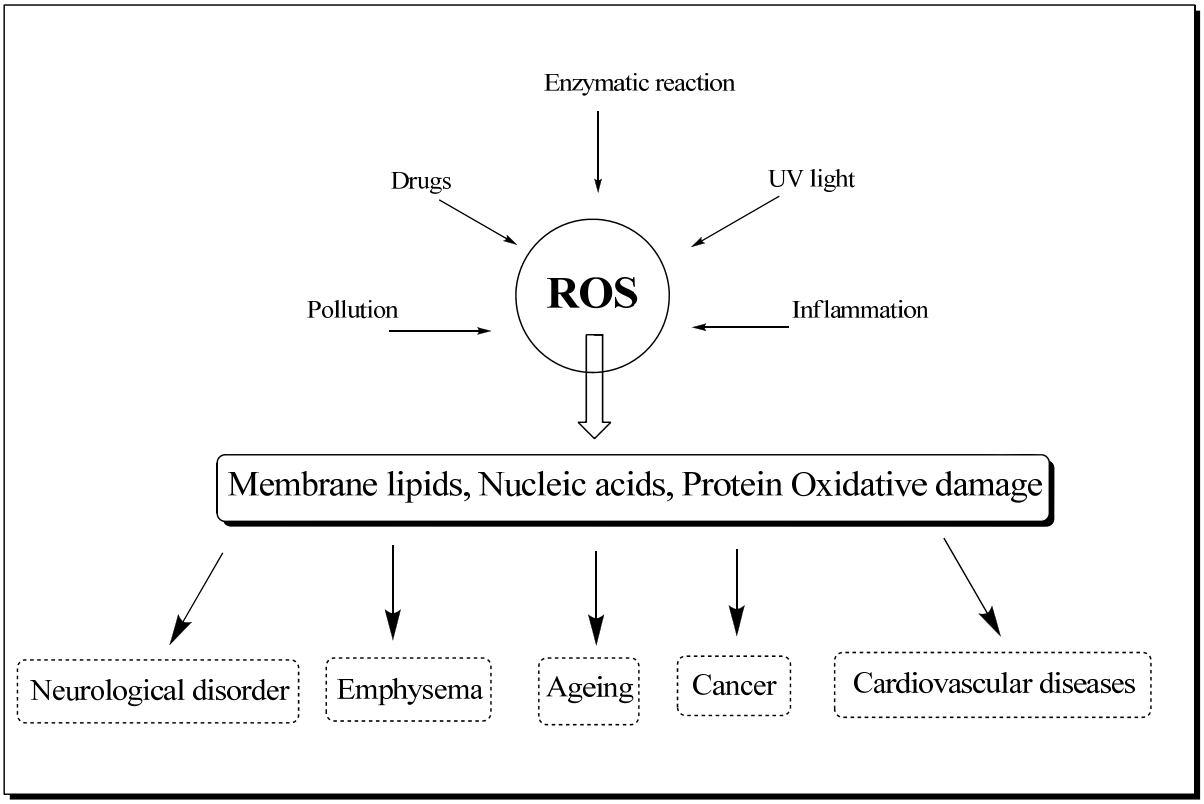


Fig 6: *Formation and impact of ROS on the human body.*

ROS may be very damaging, since they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA to induce oxidative modifications, which cause membrane damage, loss of protein function and DNA damage. This oxidative damage is considered to play a causative role in ageing and several degenerative diseases associated with it, such as heart disease, congestive dysfunction and cancer. Humans have evolved antioxidant systems to protect against free radicals. These systems include some antioxidants produced in the body (endogenous antioxidants) and others obtained from the diet (exogenous antioxidants).

Defense systems against damage induced by ROS fall into three categories:

- Preventative antioxidants that suppress free radical formation.
- Radical-scavenging antioxidants that inhibit initiation of chain reactions and intercept chain propagation, including catalytic antioxidants.

- Antioxidants involved in repair processes.

b. The antioxidant structure-activity relationship of flavonoids

Flavonoids are polyphenolic substances that are based on the flavan nucleus which consists of 15 carbon atoms arranged in three rings (C_6–C_3–C_6) (***Fig 7***). The rings are labeled **A**, **B** and **C**.

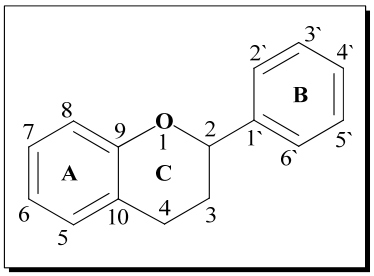


Fig 7: *Flavan skeleton.*

The major flavonoid classes include flavonols, flavones, flavanones, isoflavones flavanols, chalcones and aurones. Differences between the flavonoid classes are mostly limited to the pyrone ring (absence or presence of double bond, presence of 3-hydroxy and/or 4-oxy groups) and in the number of hydroxyl groups in rings **A** and **B** (***Fig 8***).

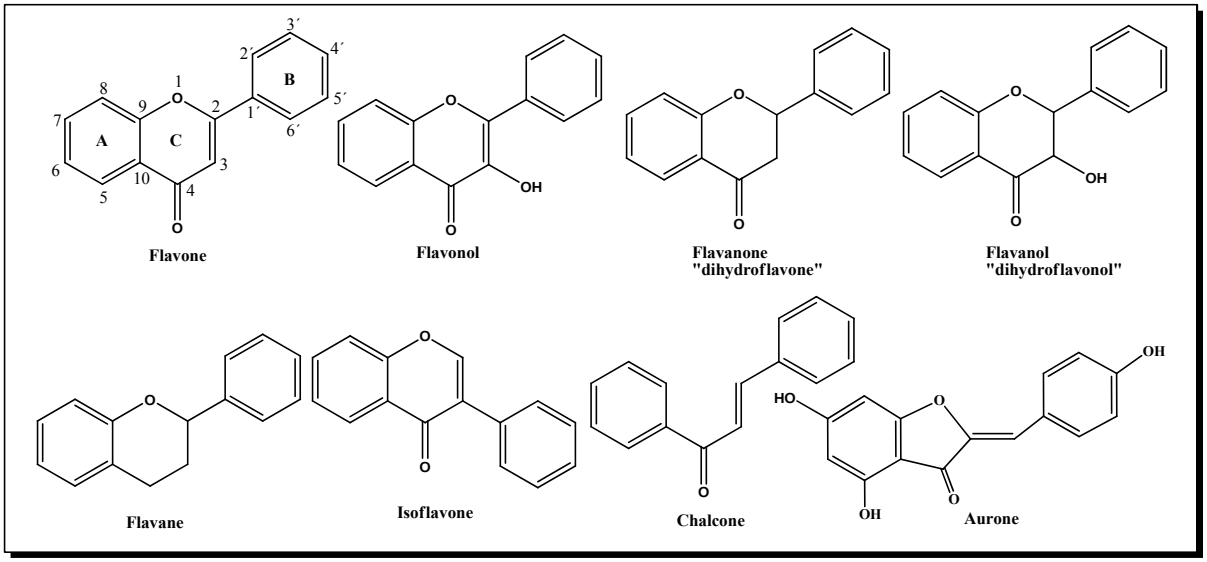


Fig 8: *Various classes of flavonoids which differ in the degree of oxidation and substitution.*

Flavonoids may be monomeric, dimeric, or oligomeric and vary greatly in molecular weight. Polymeric derivatives, called tannins, are divided into two groups relating to their

structure, *i.e.* condensed and hydrolysable. Condensed tannins are polymers of flavonoids and hydrolysable tannins contain gallic acid.

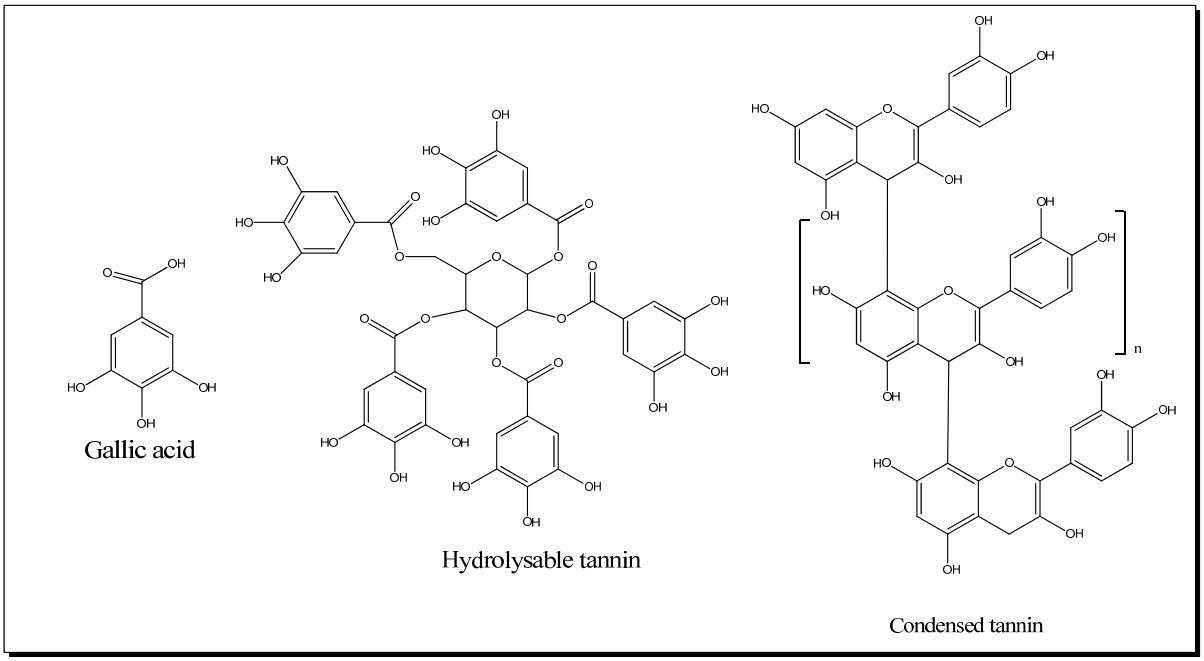


Fig 9: *Chemical structure of condensed and hydrolysable tannins.*

In order for a phenol to be classified as an antioxidant it must possess two properties: firstly, it should be oxidized properly when present in low concentrations compared to the substrate, hence delaying or preventing the autoxidation or free radical-mediated oxidation; secondly, the phenol free radical formed after scavenging must be stable (through intramolecular hydrogen bonding) to further oxidation and can not act as oxidant in its own right [67, 69]. Oxidisable substrates include almost all organic molecules found in food and living tissues including lipids, carbohydrates, proteins and DNA.

The chemistry of the flavonoids is predictive of their free radical scavenging activity as the reduction potentials of flavonoids and the consequently radical form, are lower than those of alkyl peroxyl radicals and the superoxide radical, which therefore means the flavonoids may inactivate these radical species and prevent the deleterious consequences of their reactions [70-73].

The electron/H-donating properties of flavonoids are considered to be the basis of their antioxidant action. Their free radical scavenging properties are best approached through structure-antioxidant activity relationships. The ability of flavonoids to act as antioxidants by electron donation depends directly on the reduction potentials, and inversely on the reactivity

of the flavonoid molecules with dioxygen, as the generation of peroxyl radicals will propagate oxidative reactions. These concepts have been reviewed [74].

In essence, three types of structural properties have been recently known to appear to increase the antioxidant activity of the flavonoids;

1- The *ortho*-dihydroxy structure in the **B** ring [75], which confers higher stability to the radical form and participates in electron delocalization.
2- The 2,3 double bond in conjugation with a 4-oxo function in the **C** ring is responsible for electron delocalization from the **B** ring [69]. The antioxidant potency is related to structure in terms of electron delocalization of the aromatic nucleus. When these compounds react with free radicals, the phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus.
3- The 3,5-OH and 4-oxo functional groups in the **A** and **C** rings are required for maximum radical scavenging potential.

c. The structure characteristics of an effective flavonoid antioxidant.

Quercetin is perhaps the best example of a flavonoid that displays these three structural properties, and so efficiently captures free radicals.

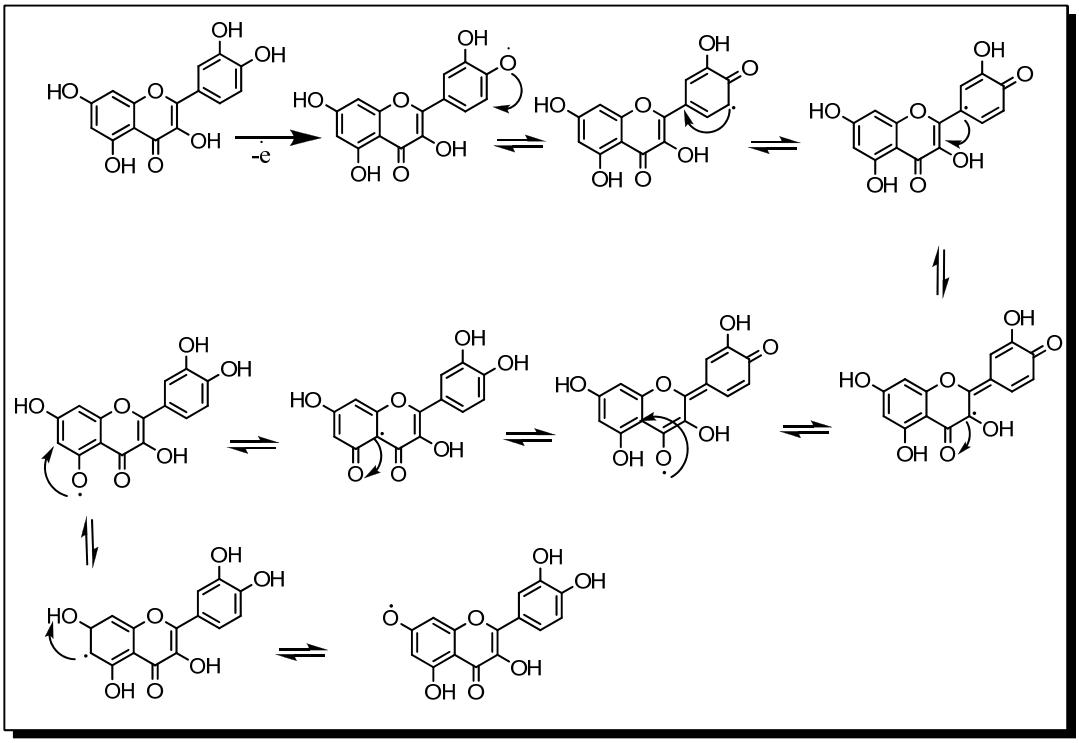


Fig 10: *Delocalization and resonance of quercein radical.*

Quercetin has five free hydroxyl groups which can donate electrons and a complete resonance system which can stabilize the quercetin radical (***Fig 10***). Furthermore the hydroxyl groups at position 3, 5, 3´ and 4´ have a specific configuration that enables the quercetin molecule to bind with up to three metal ions (***Fig 11***) such as Cu^{2+} or Fe^{3+}. These redox active metal ions may contribute to ROS production [76], *via* a Fenton-type radical generating chemistry. Sequestration of such adventitious metal isons may therefore also be considered as an antioxidant activity, as previously shown for the chelator desferoxamin [77]

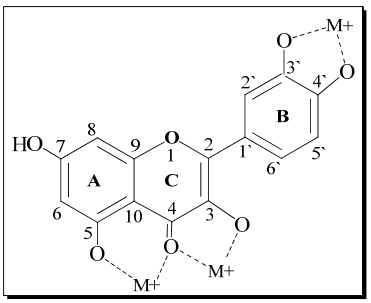


Fig 11: *Metal binding site of quercetin.*

1.3.2. Flavonoids and cancer therapy

Cancer is the second most abundant cause of death in the United States and in many other western countries. According to the annual report of German cancer research center, over 450,000 new cases are diagnosed in Germany with 270,000 cancer-related deaths each year in females alone. The prognosis for a patient with metastatic carcinoma of the lung, colon, breast, or prostate remains a concerning issue and accounts for more than half of all cancer deaths [78].

Cancer may be controlled by a variety of means, including suppression, blockage, and transformation. Suppressing agents prevent the formation of new cancers from procarcinogens; blocking agents prevent carcinogenic compounds from reaching critical initiation sites; and transformation agents act to facilitate the metabolism of carcinogenic components into less toxic materials or prevent their biological actions. Flavonoids can act in all three ways [79].

Flavonoids may act at the different development stages of malignant tumors by protecting DNA against oxidative damage, inactivating carcinogens, inhibiting the expression of the mutagenic genes and enzymes responsible for activating procarcinogenic substances, and activating the systems responsible for xenobiotic detoxification [80].

Although most flavonoids appear to be nontoxic to humans and animals, they have been demonstrated to inhibit proliferation in many kinds of cancerous cell lines. For instance, it has been reported that flavonoids (quercetin and taxifolin) have antiproliferative effects on squamous cell carcinoma HTB43 [81]. Quercetin at 10 μM shows an antiproliferative activity against meningioma cells [82] and against colon cancer cells (Caco-2 and HT-29), with a dose-dependent effect [83]. Diosmin, another important *Citrus* flavonoid, which is on the market as a venotonic, has shown antiproliferative activity in Caco-2 and HT-29 colon cancer cell lines (IC_{50} 203 μM), although with less efficacy than quercetin [84].

Comparison to the structure of the flavonoids shows that the presence of a double C2-C3 bond in polyhydroxylated flavonoids increases the antiproliferative activity against certain cancer cell lines [85, 86]. Another structural element that may influence antiproliferative activity is the number and position of the substituents in the flavonoid base structure. Taking quercetin and myricetin as an example, the presence of one additional hydroxyl group in the

B-ring of myricetin leads to greater activity of this flavonol compared to quercetin when tested in B16F10 and Melan-A cell lines [87, 88].

Various mechanisms have been proposed to explain the antiproliferative activity of flavonoids. Flavonoids have been shown to inhibit several kinases involved in signal transduction, such as protein kinase C, tyrosine kinases, PI 3 kinases, or S6 kinase [89]. They can also interact with estrogen type-II binding sites [90, 91]. Flavonoids have been found to arrest cell-cycle progression at either the G1/S or the G2/M transition.

Flavonoids have also been shown to affect formation of the metastases. Metastases form when cancer cells invade beyond the boundaries of the primary site and establish new tumors in distant organs, an bent considered to be a major cause of cancer death [92]. The invasion of surrounding tissues by cancer cells involves several steps, including matrix metalloproteinase (MMP) secretion, migration, invasion, and adhesion. Flavonoids have shown beneficial influences on all of these steps. The flavonoids quercetin and apigenin, for instance have been reported to possess the ability to inhibit lung colonization by the melanoma B16-BL6 cell line in a dose-dependent manner *in vivo* [93].

A particularly interesting influence on cell proliferation is observed in breast cancer. This type of cancer is the most common cancer among women in the Western world, with about 40,000 deaths from breast cancer in the US alone in 2000 [94].

Around 94 % of breast cancer cases are diagnosed at the early stage of the disease. The primary aim of treatment for early breast cancer is to maximize local control and to prevent the progression of the disease to metastatic sites as metastatic breast cancer is currently incurable [95]

Approximately 75 % of breast cancers are positive for the estrogen receptor (ER) and/or progesterone receptor (PgR). As estrogen is the main stimulant in the development and growth of these tumors, the deprivation of estrogenic signaling has been the main form of hormonal therapy for patients with ER-positive and/or PgR-positive disease. Tamoxifen, which works by blocking the tumor's ability to respond to estrogen stimulation, has been the main hormonal therapy used. Furthermore, aromatase inhibitors help to prevent the growth of these tumors by lowering the amount of estrogen in the body. This approach has recently attracted considerable attention.

1.3.3. Aromatase and estrogen biosynthesis

Aromatase is an enzyme responsible for a key step in the biosynthesis of estrogens. It is a cytochrome P450 enzyme and belongs to family 19, subfamily A, polypeptide 1. In humans, aromatase is expressed in both the granulosa and luteal cells of the ovary, and also in various extra-glandular sites including the placenta, brain, bone, testis and adipose tissue [96]. Aromatase is encoded by the CYP19 gene, which maps to chromosome 15q21.2 in humans [97]. The structure and hormonal regulation of CYP19 is complex: the gene spans 123 kb, with a coding region of 30 kb comprising nine translated exons [98-101]. A number of untranslated first exons, each driven by a unique promoter, exist upstream of exon II [102, 103]. These are spliced to a common site in the 5' untranslated region. Tissue-specific regulation of CYP19 expression is achieved through the use of these different promoters, each of which is regulated by distinct hormonal factors. In the ovary, CYP19 expression is regulated by Follicle-stimulating hormone (FSH) which acts (through cAMP) via promoter II [97], whereas in the placenta, promoter I.I regulates CYP19 expression in response to retinoids [104]. In contast, in bone and adipose tissue, a distal promoter (promoter I.4) drives CYP19 expression under the control of glucocorticoids, class 1 cytokines or TNFα [105-107].

Estradiol is the most potent endogenous estrogen. Estradiol is biosynthesized from androgens by the cytochrome P450 enzyme complex known as "aromatase" [108]. This enzyme complex is found in the endoplasmic reticulum of the cell and is comprised of two major proteins [108, 109]. One protein is cytochrome $P450_{arom}$, a hemoprotein that converts C19 steroids (androgens) into C18 steroids (estrogens) that contain a phenolic **A**-ring [108, 110]. The second protein is NADPH-cytochrome P450 reductase, which transfers reducing equivalents to cytochrome $P450_{arom}$.

Three moles of NADPH and three moles of oxygen are used in the conversion of one mole of androgen substrate into one mole of estrogen product (***Fig 12***). Aromatization of androstenedione, the preferred substrate, proceeds via three successive oxidation steps, with the first two being hydroxylation of the angular C-19 methyl group. The final oxidation step proceeds with the aromatization of the **A** ring of the steroid and loss of the C-19 carbon atom as formic acid.

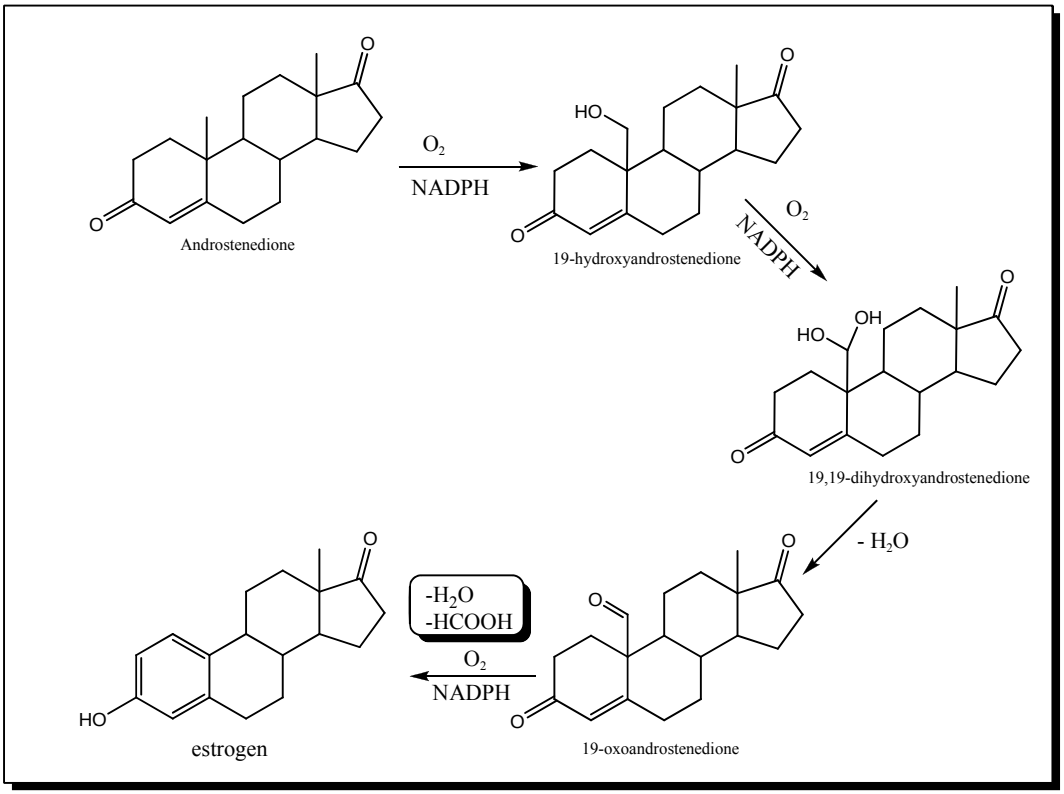


Fig 12: *Estrogen biosynthesis pathway catalysed by aromatase (Figure adaptaed according to [111]).*

1.3.4. Inhibitors of human aromatase enzyme

Two primary approaches have been developed to reduce the growth-stimulatory effects of estrogens in breast cancer: firstly it is possible to interfere with the ability of estrogen to bind to its receptor. (The drug tamoxifen, which is widely used in the treatment of breast cancer acts via this pathway). Secondly, the circulating levels of estrogen may be decreased by inhibiting estrogen synthesis via aromatase inhibitors. This approach has been proven to be rather effective [111], and aromatase inhibitors have been developed as therapeutic agents for controlling estrogen-dependent breast cancer. Investigations on the development of aromatase inhibitors began in the 1970s and have greatly expanded over the past three decades [112-119]. Today, we can distinguish two types of aromatase inhibitors, namely steroidal and nonsteroidal inhibitors.

1- Steroidal inhibitors represent competitive inhibitors which compete with the substrate androstenedione for noncovalent binding to the active site of the enzyme and thereby decrease the amount of estrogen product level. The development of steroidal inhibitors builds upon the basic androstenedione nucleus and incorporates chemical substituents at

varying positions on the steroid. This is illustrated by formestane and 1-methyl-ADD, two estoroidal aromatase inhibitors commonly used to treat breast cancer (***Fig 13***). These inhibitors bind to the aromatase cytochrome P450 enzyme in the same manner as the substrate androstenedione. [111].

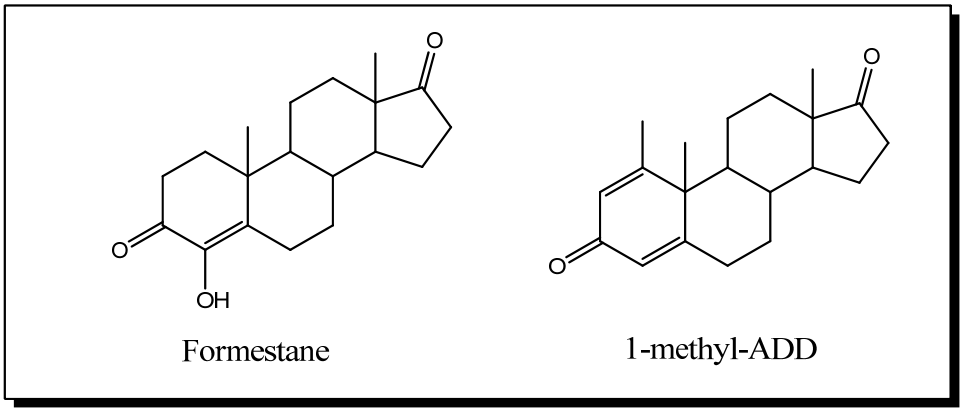


Fig 13: *Formestane and 1-methyl-ADD, two steroidal inhibitors of aromatase commonly used in the treatment of breast cancer.*

2- Nonsteroidal inhibitors

a- First generation aromatase inhibitos: nonsteroidal aromatase inhibitors possess a heteroatom as a common chemical feature and interfere with steroid hydroxylation by the binding of this heteroatom to the heme iron of the cytochrome P450s. Initial nonsteroidal inhibitors were nonspecific to the enzyme and also inhibited other cytochrome P450-mediated hydroxylations of steroidogenesis which resultes in significant toxicity. Aminoglutethimide (***Fig 14***) is the prototype for nonsteroidal aromatase inhibitors [120]. This compound was originally developed as an antiepileptic agent but was removed from the market due to serious side effects. Aminoglutethimide also inhibites cytochrome $P450_{SCC}$ and other enzymes, yet is more selective for cytochrome $P450_{arom.}$

Because aminoglutethimide was the first aromatase inhibitor to be studied in patients, it is referred to as a first generation aromatase inhibitor. Aminoglutethimide has been used in the clinic with some success to treat patients with advanced breast cancer, but must be administered with corticosteroid due to the inhibitory effects on cortisol and aldosterone biosynthesis (see above) [120, 121].

b- Second generation aromatase inhibitors: the second generation inhibitors are represented by fadrazole (***Fig 14***) which is more selective than Aminoglutethimide. It is inhibitory

activity is 700 times more potent. Clinical studies using fadrazole have shown that this nonsteroidal inhibitor is effective in the treatment of some postmenopausal women with advanced breast cancer [111].

c- Third generation aromatase inhibitors: several nonsteroidal aromatase inhibitors containing a triazole ring have been successfully developed. Anastrozole (***Fig 14***) is a potent aromatase inhibitor with an IC_{50} of just 15 nM in human placental microsomes. *In vitro*, anastrozole is very specific and has no effect on other P450 enzymes such as $P450_{SCC}$, 11β-hydroxylase, 18-hydroxylase, 17α-hydroxylase, and lanosterol-14α-demethylase [111].

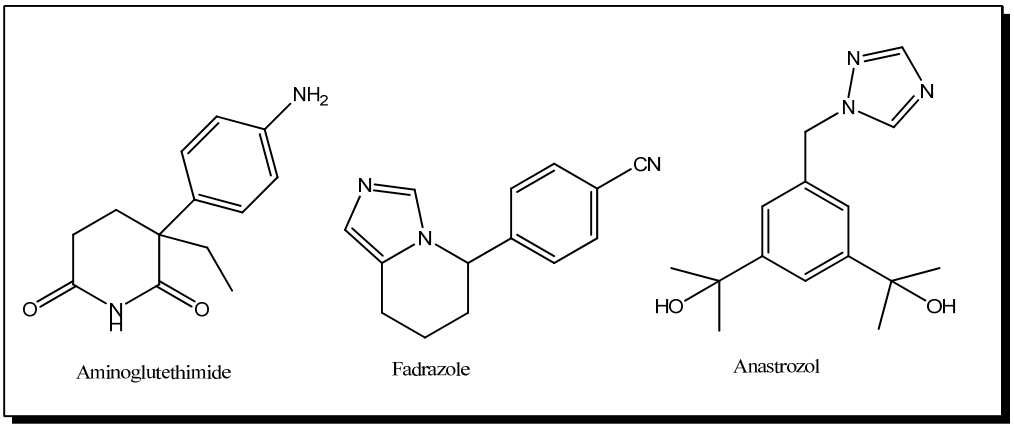


Fig 14: *First, second and third generation aromatase inhibitors.*

d- Flavonoid based aromatase inhibitors: several flavonoids demonstrate inhibitory activities against the aromatase enzyme, thus lowering estrogen biosynthesis and circulating estrogen levels [122]. Strong evidence for the binding of flavones to the active site of aromatase has been obtained by different spectral absorption studies [123], with 7,8-benzoflavone displacing androstenedione from the aromatase active site and inducing a spectrum consistent with the low-spin state of iron. Binding of flavonoids to aromatase requires certain structural features. Reduction of the flavone 4-keto group for instance is detrimental to aromatase inhibition by these compounds [124]. Based on data obtained from site-directed mutagenesis studies and ligand docking into a homology model of the aromatase protein, a binding orientation has been predicted in which the **A** and **C** rings of the flavone mimic the **C** and **D** rings of the steroid substrate, respectively. The 2-phenyl substituent (ring **B**) is orientated in a region similar to that occupied by the **A** ring of the

steroid (***Fig 15***). This analysis places the flavone 4-keto functionality in the same position as the steroid 19-angular methyl group with respect to the heme iron [125].

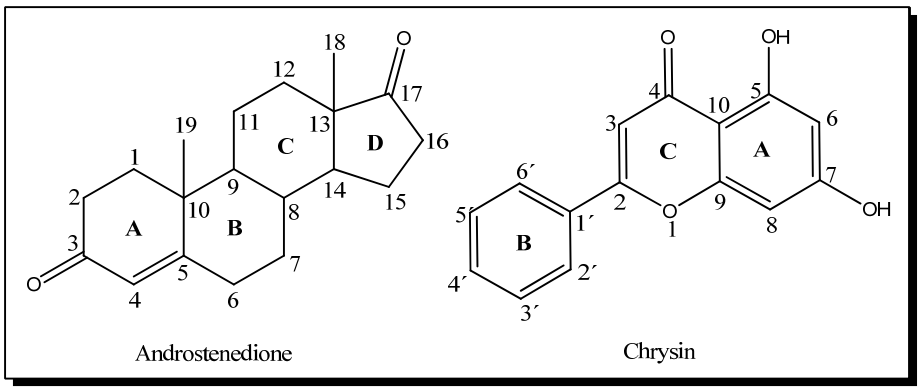


Fig 15: *Structural similarities between chrysin and aromatase substrate androstenedione (Figure adapted according to [125])*

Generally, flavones and flavanones have higher aromatase inhibitory activity than isoflavones [111]. Furthermore medicinal chemistry approaches to develop synthetic flavonoids, chromones, or xanthone analogs with enhanced aromatase inhibitory activity have identified more selective and/or more potent agents for future development [126-128].

1.3.5. Receptor binding activities: Treatment of menopausal syndrome

The estrogen receptor (ER) belongs to the steroid/thyroid nuclear receptor superfamily whose members act as transcriptional activators via a direct interaction with DNA sequences termed response elements [129]. Ligand (*e.g.* estradiol) binding to the ER induces a conformational change in the receptor important for the association of the receptor-DNA complex with transcriptional coactivators and the transcriptional components of the cell [130]. This association then culminates in the synthesis of estrogen-responsive genes and an estrogen-induced cell and/or tissue response.

An apparent consequence of estrogen deficiency is the increase in short-term menopausal symptoms, including vasomotor hot flashes, urogenital atrophy and changes in psychological functioning. The "hot flash" is the classic sign of menopause and the primary clinical symptom experienced by women during this transitional stage [131].

At menopause, an accelerated loss of bone mass takes place during the first five years, with changes in bone structure (3 %/year). Estrogen slows the rate of bone remodeling and protects against bone loss directly, through the estrogen Receptor (ER), and indirectly, through its effects on collagen. It has been suggested that in E2 deficiency, the loss of transcriptional effect on the lifespan of mature osteoclasts may be responsible for the imbalance between formation and resorption and the progressive loss of bone mass and strength [132].

The incidence of heart disease among pre-menopausal women is low compared with males, whereas the incidence among post-menopausal women approaches that of males. The administration of estrogen to post-menopausal women decreases the incidence of heart disease [133]. This protective effect of estrogen may partially be attributed to its influence in decreasing the ratio between LDL (low density lipoprotein) and HDL (high density lipoprotein) [134], on reducing thrombus formation and in improving vascular compliance.

Not surprisingly, there is a great motive for women to start taking hormone replacing therapy (HRT), which among other things alleviates sleep problems, mood change, loss of drive and energy and hence, improves quality of life [135]. Unfortunately women taking estrogen have an increased risk of breast and epithelial ovarian cancers. Estrogen can stimulate malignant growths, and hence contributes to the development of estrogen-dependent tumors, such as breast and uterus cancer (see above) [136].

Phytoestrogens (***Fig 16***) are a group of polyphenolic non-steroidal compounds of plant origin. They function at least in part as selective estrogen receptor modulators (SERMs) and may influence the transcriptional response to estrogens [137, 138]. These phytoestrogens are thought to act as estrogen agonists (or antagonists) and may promote a significant advancement in the prevention of some diseases caused by estrogen deficiency in post menopausal women compared to conventional hormonal therapy [139].

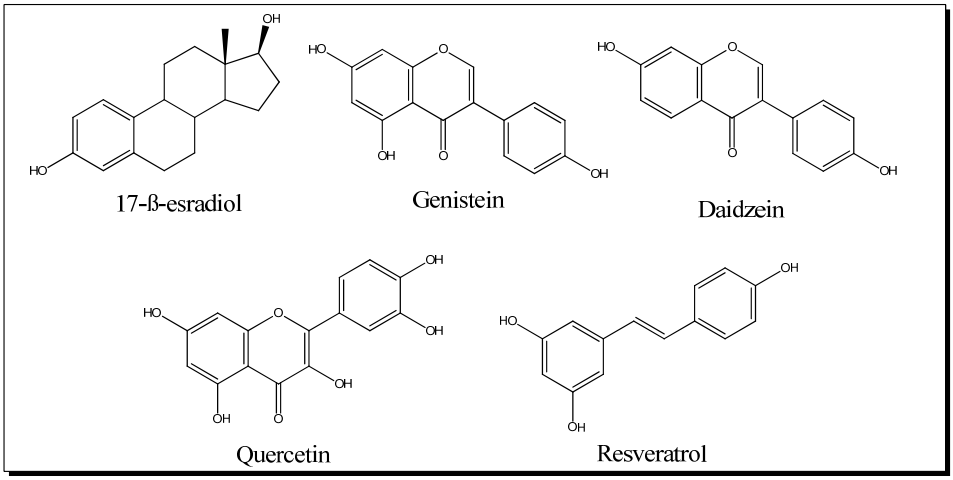


Fig 16: *Chemical structure of selected phytoestrogens.*

Shared structural features of phytoestrogens include a pair of hydroxyl groups and a phenolic ring, which is required for binding to estrogen receptors ER-α and ER-β, with the position of these hydroxyl groups appearing to be an important factor in determining their ability to bind to ERs and activate transcription [140]. Four main classes of compounds are currently recognized as phytoestrogens, *i.e.* the isoflavones, stilbenes, coumestans, and lignans [141, 142]. These phytochemicals are some of the most prevalent compounds found in fruits, vegetables, legumes, and tea and are generally concentrated in the fruit skin, bark, and flowers of plants [141]. Among them, Resveratrol, daidzein, quercetin, and genistein represent four of the most commonly ingested and most intensely studied phytoestrogens.

1.4.Chemistry of flavonoids

Flavonoids are formed in plants from the aromatic amino acids phenylalanine and tyrosine, and malonate. As already mentioned, the basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), labeled **A**, **B**, and **C** (***Fig 7***).

The various classes of flavonoids differ in the degree of oxidation and pattern of substitution of the **C**-ring, while individual compounds within each class differ in the pattern of substitution of the **A** and **B**-rings. The flavonoid classes include flavones, flavanones, isoflavones, flavonols, flavanonols, flavan-3-ols, and anthocyanidins, biflavones, chalcones, aurones (***Fig 8***), and most of these classes are present in plants as glycosides.

Synthesis, redox behavior and electrochemistry

Flavonoids represent a well-known family of compounds and their synthesis has been the object of a great number of studies. In general, procedures for laboratory synthesis of flavonoids are still based on the approaches originally developed by Robinson [143], with other methods including the Baker–Venkataraman rearrangement [144, 145], synthesis via chalcones [146], and synthesis via an intramolecular Wittig reaction [147]. Despite the number of steps often involved in these methods, they constitute the most popular methodologies used nowadays for the preparation of flavonoids. The synthesis of flavonoid derivatives is also an area of significant interest and many synthetic routes have been used to add moieties to the natural flavonoids nucleus. The Mannich reaction, for instance, is used for the addition of alkyl amine derivatives [148]. The Aldol condensation reaction is used either for the synthesis of natural flavonoids or for the addition of alkyl or benzyl moieties [149]. Many puplications covering flavonoid biotransformations also focus on oxidation, methylation, glucosidation [150-152], and halogenation [153].

The biochemical reaction activities of flavonoids and their metabolites depend on their chemical structure and the relative orientation of the various moieties present in the molecule. The shape of the flavonoid is important as flavones and flavonols have a planar benzopyranone skeleton whereas the dihydroflavonols have a less planar dihydrobenzopyranone skeleton. Chrysin has a double bond between C-2 and C-3, and the conjugation within the molecule is causing ring **B** to be a coplanar with the rings **A** and **C**. Saturation of this double bond, as in taxifolin, will destroy conjugation and coplanarity [155].

The antioxidant property of flavonoids may be investigated using electrochemical methods. The ability to act as chemical defence agents by donating an electron to an oxidant frequently depends on the redox potentials of the flavonoid and the corresponding radical [154].

The mechanism of action as antioxidants seems to involve the ability of phenols to scavenge radicals with an H-atom, or by an electron transfer process in which the phenol is converted into a phenoxyl radical resulting in the formation of the semiquinone, which can donate a further electron to form the quinone (***Fig 17***) [156]. Electrochemical measurements lead to physicochemical parameters for antioxidants, which help in evaluating their antioxidative abilities [157–159], and understanding their reaction mechanisms.

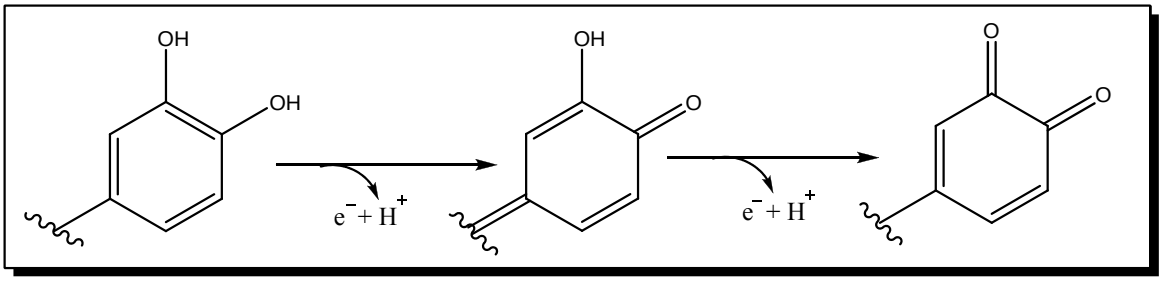


Fig 17: *Quinone formation in ring B by oxidation with free radicals.*

Some information regarding the mechanism of polyphenol oxidation is provided by comparing the redox potentials at different pH. An increase in pH is often associated with deprotonation of the OH groups and a decrease of the oxidation potential [155].

Studies of the electrochemical properties of flavonoids have provided useful information about the redox potential and strongly support the antioxidant structure-activity relationship of different flavonoids which may also be measured by different biological and bioassay methods. Electrochemical analysis of quercetin, (±)-catechin, galangin, rutin, and taxifolin show that quercetin, with a 3′,4′-dihydroxy subistitution in **B**-ring and double bond between C2 and C3 has the lowest redox potential of all [75, 160]; it can therefore be assumed that quercetin has the highest antioxidant activity of the compounds tested.

1.5.The objectives of this project

Flavonoids form a group of natural products present in most plant families. They reportedly play an important role in plant growth and defense mechanisms. More than 8000 different flavonoids have been isolated from natural sources todate, and scientists still endeavor to find new flavonoid structures.

Scientists were also trying to synthesise these flavonoids to make it then available in high amounts or to add some more functional groups that may enhance the biological or pharmacological behavior. Biotransformation of the flavonoids is also a current area of interest to obtain novel biologically and pharmacologically active flavonoid compounds.

Thousands of publications underline this interest in the different biological activities and pharmacological behavior (absorption, metabolism, excretion) of the various flavonoid

classes. There is hardly any flavonoid structure without a biological use of interest, and structural variations between flavonoids may be related to the different activities observed in diseases in the humans and animals body.

Quercetin for instance is a well known flavonoid isolated from many plant families. It has a unique structural feature that enables it to play a significant role as an antioxidant in addition to many other roles in the protection against cancer and coronary heart diseases. Chrysin, another well known flavonoid natural product, is reported to possess interesting biological activities, such as an aromatase inhibitor and certain cytotoxicity toward cancer cell lines.

The synthesis of novel quercetin and chrysin derivatives, based on the Mannich reaction may be a good strategy to introduce changes in the biological behavior of quercetin and chrysin, *e.g*. by addition of alkyl amine moieties. Such changes may enhance the cytotoxic and aromatase inhibiting activity of the quercetin, and, at the same time, may also improve the antioxidant activity of this compound.

Chrysin and most other flavonoids are able to mimic the substrate of aromatase (*i.e.* androgen), due to the similarity between rings **A** and **C** in flavonoids and the **C** and **D** rings of the aromatase substrate. Addition of one more ring to chrysin may enhance this aromatase inhibiting activity. It was therefore decided to synthesise a set of novel chromene-flavone compounds that consist of both a flavone (chrysin) and a chromene nucleus in the same molecule.

Chapter II: Materials and Methods

2.1. Isolation and characterization of natural products

The plant materials used in this work are the roots of *Delonix regia* (growing in Egypt and collected in July 2007, from the garden of Al-Azhar University, Nasr city, Cairo, Egypt), and *Robinia pseudoacacia* (growing in Germany and collected in August 2008 from the forest of Saarbrücken, near to the Waldhaus, Saarbrücken, Germany). *Delonix regia* plants were kindly identified by Dr. Nabil El-hadidy, Professor of Plant Taxonomy, Faculty of Science, Cairo University, and by Engineer Badeia Hassan Aly Dewan, Consultant of Egyptian Flora, Agricultural Museum, Dokki, Giza, Egypt. *Robinia pseudoacacia* were kindly identified by Professor Rüdiger Mues, professor of Molecular Plant Biology and Botany, Saarland University, Saarbrücken, Germany.

2.1.1. Chromatographic studies

Thin layer chromatography (TLC):

Silica gel 60 F_{254} (with fluorescent indicator) pre-coated sheets 20 x 20 cm wide and 0.2 mm thick (Merck, Germany) were used for TLC analysis in the synthesis and isolation of natural products. The solvent systems used for TLC were mixtures of methanol in dichloromethane or ethyl acetate in petroleum ether (40-65 °C) in different concentrations.

Vanillin/H_2SO_4 (TLC spraying reagent) was applied to the TLC, as a means of detection [161]. Vanillin/H_2SO_4 are generally used to visualize hydrocarbon compounds, especially flavonoid glycosides. Vanillin powder (1 g) was dissolved in 100 mL of H_2SO_4 and then the mixture was heated at 100 °C for 5 min.

Column Chromatography (CC):

Silica gel 60 (0.063-0.200 mm) 70-230 mesh (Merck, Germany) and Sephadex LH-20 (Pharmacia, Germany) were used as stationary phases for column chromatography. The solvent system applied for the Silica gel column was a mixture of methanol and dichloromethane or mixture of ethyl acetate and petroleum ether (40-65 °C) in different concentrations. The solvent used for the Sephadex column was pure methanol.

Solvents used for NMR spectroscopy:

DMSO-d_6, $CDCl_3$ and CD_3OD were the solvents used for ^1H and ^{13}CNMR spectroscopy.

2.1.2. Extraction

Delonix regia*:* The extraction of *Delonix regia* is shown in ***Fig 18***. 3000 g of air-dried powdered roots were subjected to exhaustive extraction with 5×10 L ethanol (70 %). The combined ethanol extracts were concentrated under vacuum (Vakuum, PC2001 VARIO, Germany) at 40 °C to dryness (This yields 370.0 g of dried ethanol extract). The dried ethanol extract was then suspended in distilled water (500 mL) and filtered through filter paper.

The water soluble portion (255.0 g) was extracted with petroleum ether (3×500 mL) to remove the fat and nonpolar compounds. The combined petroleum ether extract were concentrated under vacuum at 40 °C to dryness (166.0 g).

The resulting crude extract (defatted water extract) was partitioned several times with ethyl acetate, and then with *n*-butanol. The combined ethyl acetate and *n*-butanol extracts were concentrated under vacuum at 40 °C to produce 12.0 g of ethyl acetate extracts, and 20.4 g of *n*-butanol extracts respectively.

Robinia pseudoacacia: The extraction of *Robinia pseudoacacia* is shown in ***Fig 19.*** 850 g of air-dried powdered roots were subjected to exhaustive extraction with 5×4 L ethanol (70 %). The combined ethanol extracts were concentrated under vacuum at 40 °C to dryness (95.0 g). The concentrated ethanol extract was then suspended in distilled water (200 mL) and filtered through filter paper.

The water soluble portion (62 g) was defatted with petroleum ether (3×200 mL), and the combined petrol extracts were concentrated under vacuum at 40 °C to dryness (35.0 g).

The defatted water crude extract was partitioned several times with ethyl acetate, and then with *n*-butanol. The combined ethyl acetate and *n*-butanol extracts was concentrated under vacuum at 40 °C to produce 5.0 g of ethyl acetate extracts, and 8.5 g of *n*-butanol extracts respectively.

2.1.3. Isolation

Sequential percolation of the powdered roots of *Delonix regia* and *Robinia pseudoacacia* with petroleum ether, ethyl acetate and *n*-butanol yielded the respective crude extracts. Chromatographic purification of the ethyl acetate fraction is shown in ***Fig 18*** and ***Fig 19***.

Delonix regia constituents isolation

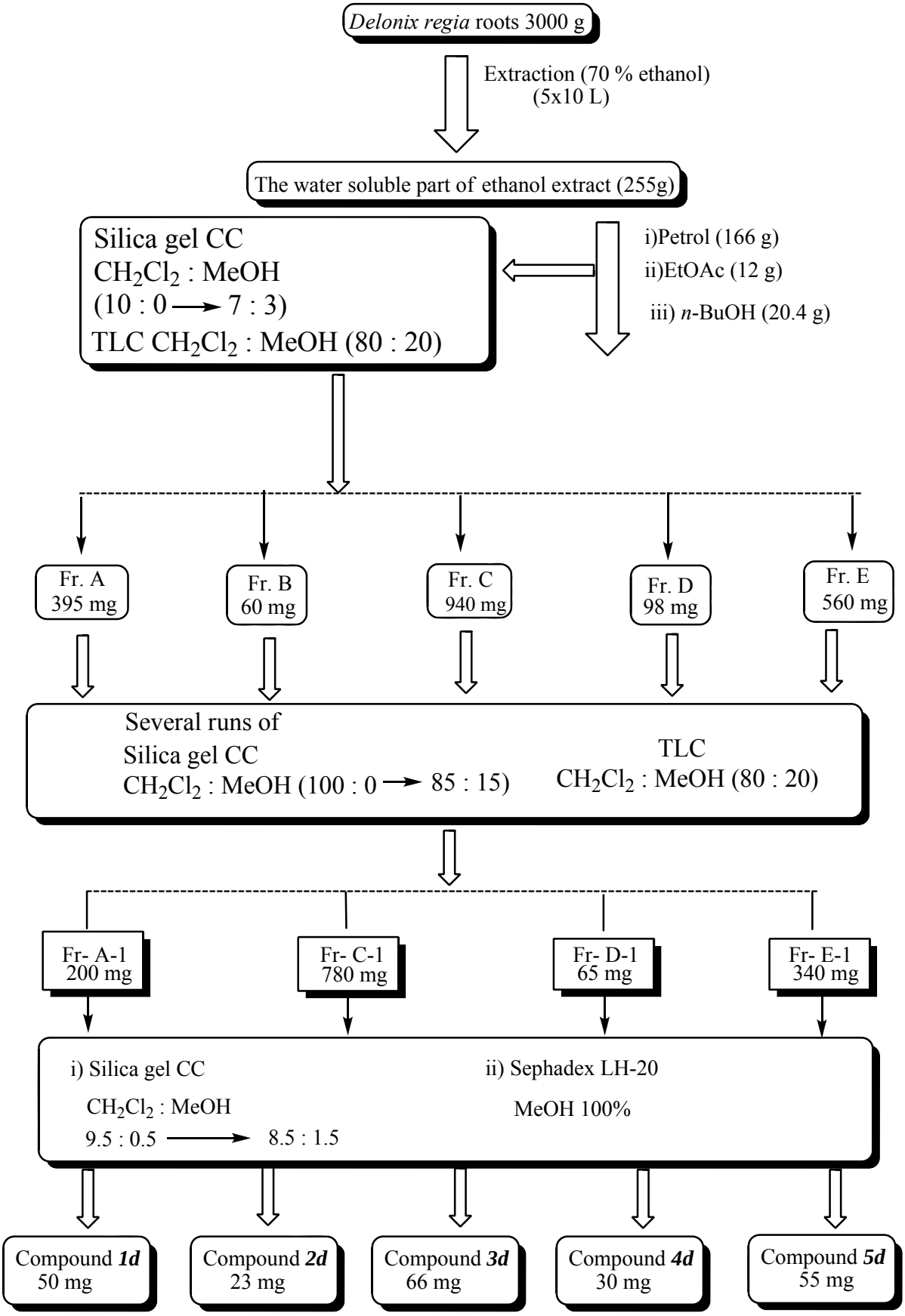


Fig 18: *Separation procedure for Delonix regia root constituents.*

Robinia pesudoacacia constituents isolation

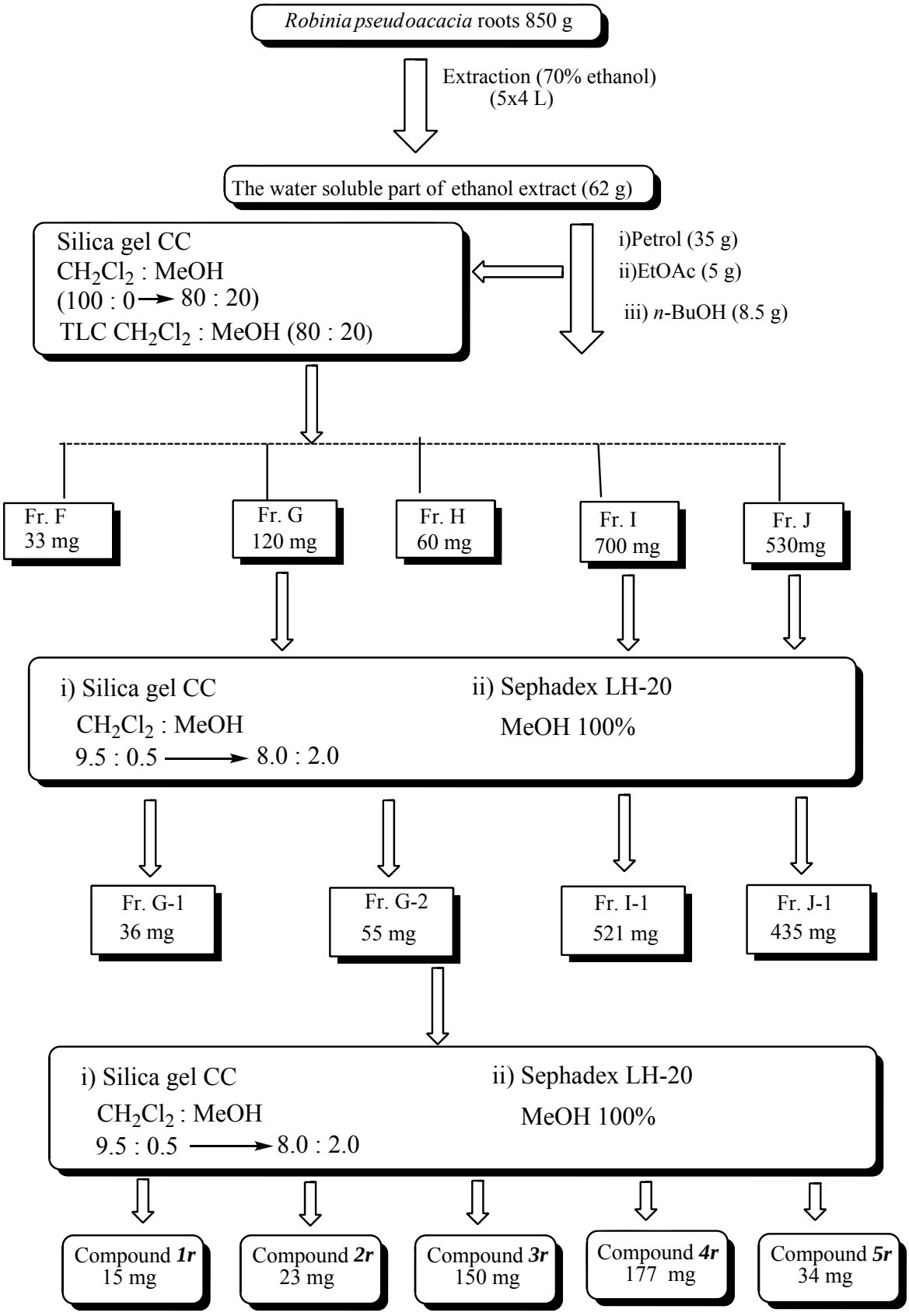


Fig 19: *Separation procedure for Robinia pseudoacacia root constituents.*

2.2.Synthesis of compounds

2.2.1. Mannich reaction

This reaction is named after Carl Mannich [162]. The Mannich Reaction is an important carbon-carbon bond forming reaction that is commonly employed in the synthesis of alkaloid natural products and is involved in a number of biosynthetic pathways [163]. The reaction uses three components: An amine, a non-enolizable aldehyde, and a compound containing an enolizable carbonyl moiety.

In short, compounds with an active hydrogen atom are treated with aldehyde and ammonia or a primary or secondary amine, thereby replacing the active hydrogen with an amino alkyl group (***Fig 20***) [164].

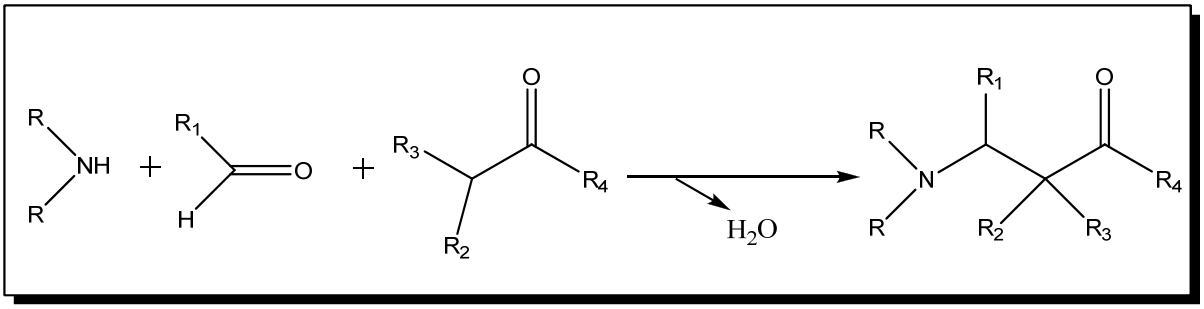


Fig 20: *General scheme of Mannich reaction.*

Materials and analytical methods

Flavonoids used in the reactions, *i.e* 5,7-dihydroxyflavone (chrysin), and 3,5,7,3′,4′-pentahydroxyflavone (quercetin), and all other starting materials used in synthesis were purchased from Aldrich, Acros, Lancaster, Merck, or Fluka and were used without further purification. Reaction progress was monitored by TLC on Alugram SIL G UV254 (Macherey-Nagel). Melting points were measured on a Mettler FP1 melting point apparatus and are provided uncorrected.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 instrument. Chemical shifts are given in parts per million (ppm), and tetramethylsilane (TMS) was used as the internal standard. All coupling constants (*J*) are given in Hertz. Mass spectra (LC-MS) were measured on a TSQ Quantum (Thermo Electron Corporation) instrument with a RP18 100-3

column (Macherey Nagel) and with water/acetonitrile mixtures as eluents. HR-MS was measured with a Finnigan *MAT 95S* mass spectrometer.

General synthetic procedure for the Mannich reaction

The primary or secondary amine (1.5 mmol), was added to a solution of flavones (1 mmol) and aldehyde (1.5 mmol) in EtOH (10 mL) at r.t. The mixture was then heated to 40–65 °C, depending on the flavonoid and secondary amine (***Fig 21*** and ***Fig 22***), and progression of the reaction was monitored by TLC.

After the reactants were consumed, EtOAc (30 mL) and diluted HCl (30 mL, pH 3) were added to the mixture. The aqueous layer was separated and the pH was adjusted to 7-8. The aqueous phase was then extracted with EtOAc (3 × 15 mL). The combined extracts were dried over anhydrous Na_2SO_4, the solvent was removed under vaccum. The crude product was subsequently purified by silica gel column chromatography using a mixture of ethyl acetate and petroleum ether (40-65 °C) as eluent.

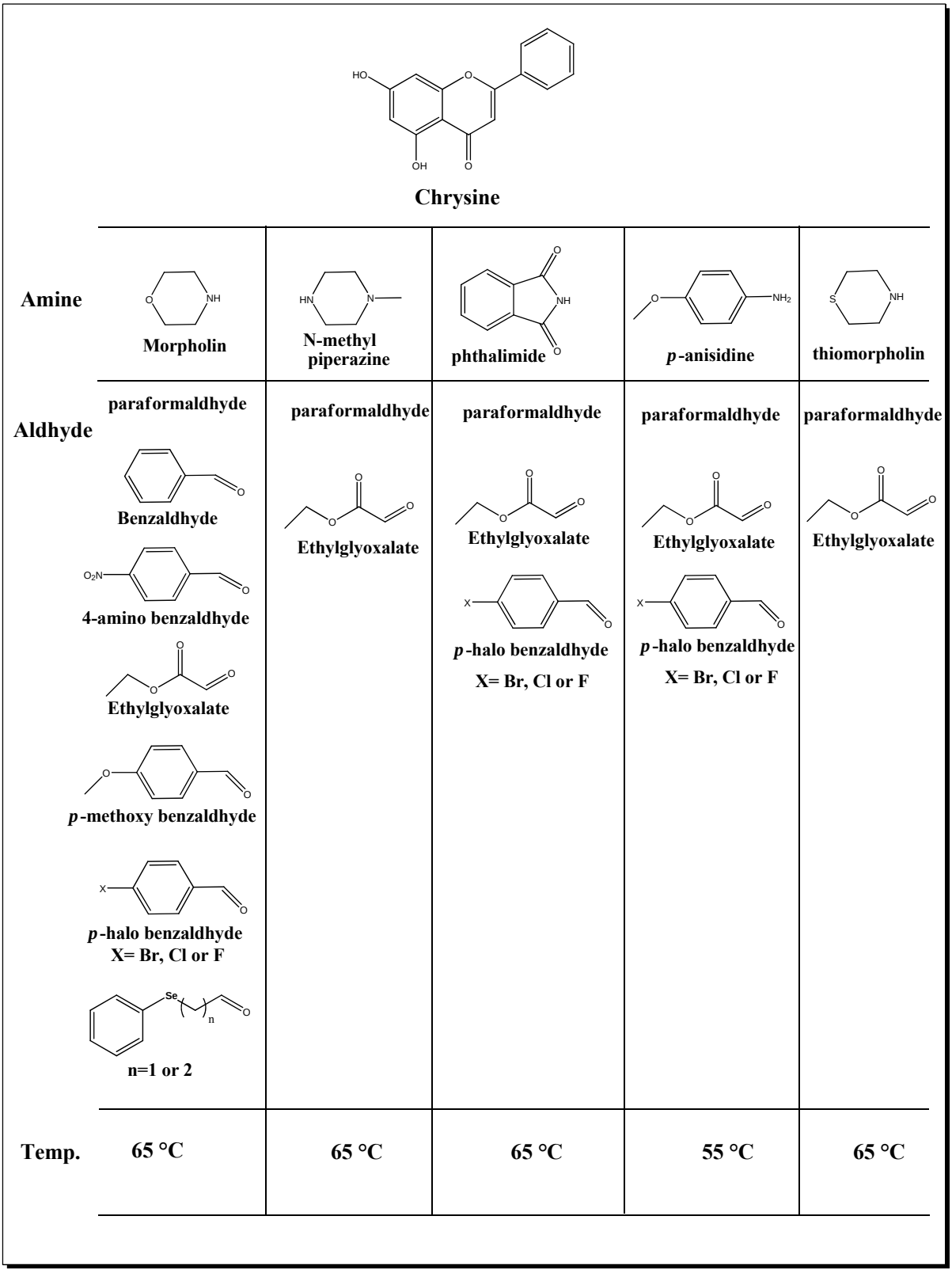


Fig 21: *Amines and aldehydes used in the Mannich reaction employing chrysin. The temperatures needed for the reaction are provided.*

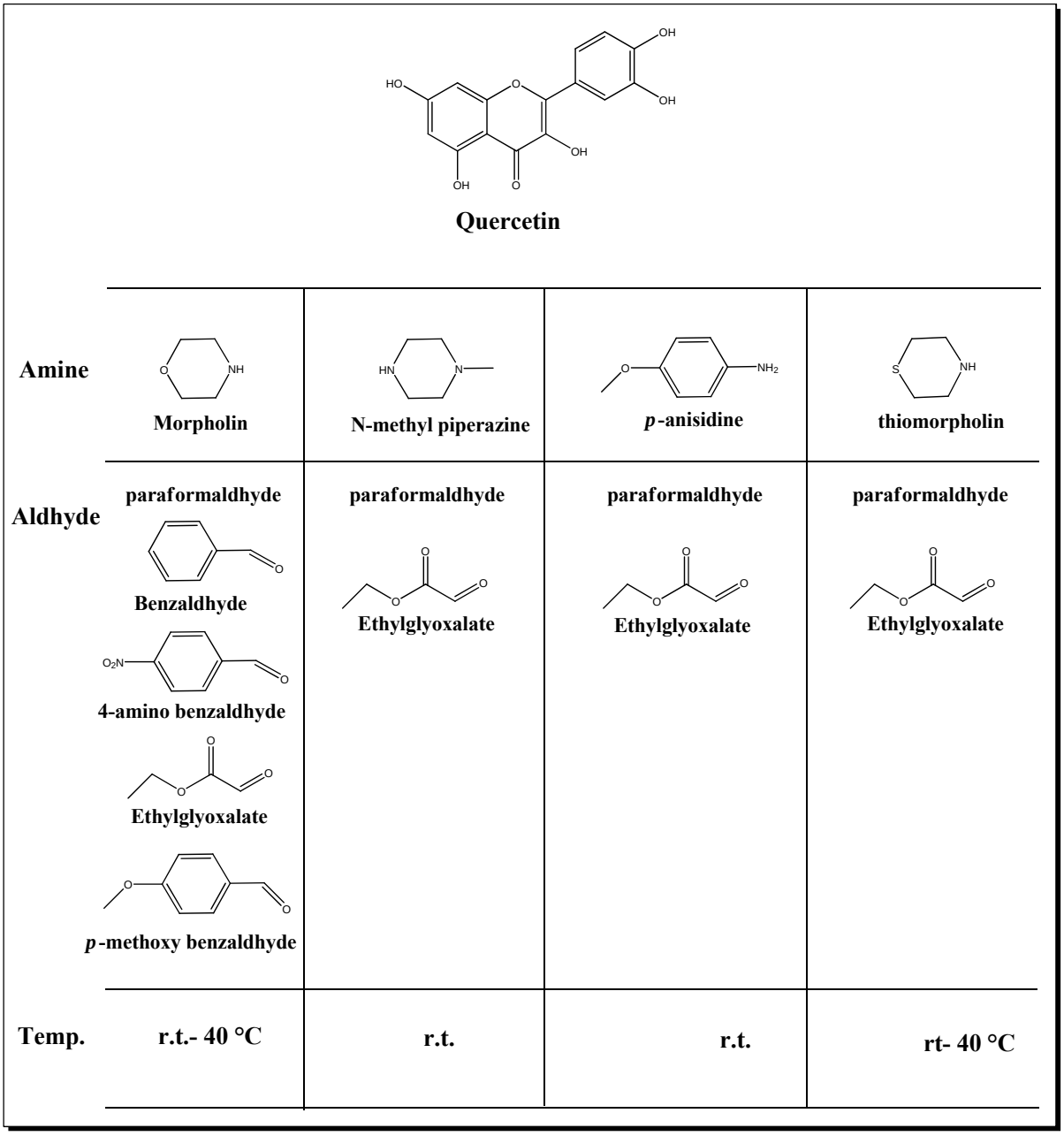


Fig 22: *Amines and aldehydes used in the Mannich reaction employing quercetin. The temperatures needed for the reaction are provided.*

2.2.2. Chromene-flavone reaction

By reacting a flavonoid with alkyl isocyanides and dialkyl acetylenedicarboxylate it is possible to create new tricyclic compounds that contain both, flavone and chromene-like moieties. The new molecules may possess interesting biological and pharmacological activities.

General synthetic procedure for the chromene-flavone reaction

The isocyanide derivative (1.5 mmol) was added drop-wise to a magnetically stirred solution of flavonoids (1.5 mmol) and dialkyl acetylene dicarboxylate (1.5 mmol) in 10 mL CH_2Cl_2 at -20°C over 10 min. The reaction mixture was then allowed to warm up to room temperature and was stirred for another 24-48 hrs, and progress of the reaction was monitored by TLC. The solvent was removed under reduced pressure and the residue was separated by silica gel column chromatography using a mixture of petroleum ether (40-65 °C) and ethyl acetate as eluent.

2.2.3. Synthesis of 7-(3-phenyl selenide propan)-3-hydroxyflavone and 7-(3-phenyl telluride propan)-3-hydroxyflavone

General synthetic procedure

A previously reported synthetic method was used for this synthesis [165]. In brief, diphenyl diselenide or diphenyl ditelluride (1 mmol) was dissolved in 30 mL of EtOH and cooled with an ice-water bath. Sodium borohydride 0.83g (21.9 mmol) and 1,3-dibromopropane (400 mg, 2 mmol) were added to the solution under a nitrogen atmosphere, and the solution was stirred at room temperature for 3 hrs. The reaction mixture was poured into water, extracted with ether, and the organic portion was washed successively with aqueous Na_2CO_3 and brine. The ether fraction was dried over anhydrous Na_2SO_4, and the solvent was removed in *vacuo*. The crude products 1-bromo(3-phenyl selenide)-propane, or 1-bromo(3-phenyl telluride)-propane, were used directly in the next step.

A mixture of 5,7-dihydroxyflavone (1 mmol), 1-bromo(3-phenyl selenide)-propane, or 1-bromo(3-phenyl telluride)-propane (1.5 mmol) and K_2CO_3 (5.0 g), in anhydrous acetone (25.0 mL) was stirred at 40 °C for 24 hrs. The insoluble material was filtered off, and the solvent was removed under reduced pressure. The residue was separated by silica gel column chromatography using a mixture of petroleum ether and ethyl acetate as eluent.

2.3. Cell culture

HL-60 cell line as a model to measure the antioxidant/ cytotoxic activities of compounds

Apparatus

Multi-plate reader	Sunrise Absorption Reader, Tecan (Osterreich)
Autoclave	Autoklavi Spa, Fedegari (Italien)
Incubator	Steri-Cycle CO_2 Incubator Hepa Class 100, Thermo
Cryobox	Nalgene Cryo 1 °C Freezing Container
Digital multichannel Pipette	Finnipette, Thermo
Electric Pipette	Accu-jet pro, Brand
Fine scale balance	Mettler AE 50
Microscope	Axiovert 40 CFL, Zeiss
pH-Meter	HI 8314, Hanna
Pipettes	Pipettman, Gilson
Stepper	Handy-Step, Brand
Sterile Workbench	Biowizard Kojair, Axon
Vortex	Vortex Genie 2, Scientific Industries
Waterbath	Haake W13, Thermo
Centrifuge	Sigma 4-10, B.Braun
Freezer (-80 °C)	C660, New Brunswick scientic

Stock solution and media

1- Phosphate buffered saline (PBS)

8.00 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were dissolved in distilled water (1000 mL) and the pH was adjusted to 7.4. The solution was then autoclaved in a stem autoclave at 120 °C for 20 min. It was opened only in a clean, sterile area.

2- Hydrogen peroxide (H_2O_2) stock solution

Freshly prepared 500 mM of H_2O_2 was used as a stock solution. 5 mM of final concentration was prepared by adding 10 μl from the stock solution to 990 μl of cell medium.

3- Stock solution of the test compounds

Serial dilutions of all compounds at 2.5, 10, 20, 30, 40, and 50 mM were prepared as stock solutions in pure DMSO. 4 μl from this stock solution in 996 μL of medium was used to prepare 1000 μl of 10, 40, 80, 120, 160 and 200 μM, respectively (please note: the DMSO concentration must not exceed 0.4 %).

4- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution

50 mg of MTT solid powder was dissolved in 10 mL of PBS (5 mg/mL) and used directly without dilution. This solution was stored below 4°C, in the dark and was used within two weeks.

HL-60 cell line culture

Human promyelocytic leukemia HL-60 cells were kindly provided by Prof. Dr. Alexandra K. Kiemer (Dept. of Pharmaceutical Biology, Saarland University, Germany). Cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY, USA), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 1 % l-glutamine (Sebac, Germany) in an atmosphere of 5 % CO_2 in humidified air at 37 °C. In all experiments, exponentially growing cells were used.

In order to establish the activity of the test compounds, 2-day experiments were set up. On the first day, one mL of cell suspension was added in a concentration of 9×10^5 cell/mL to Eppendorfs (size 1.5 mL or 2 mL, Greiner Bio-One). To each cell suspension, the compounds were added at different concentrations (with a DMSO concentration equal to 0.4%). DMSO was used as control. For the antioxidant assay, H_2O_2 was added to the mixture of cell

suspension and compounds in 5 mM final concentration. In contrast, samples prepared for the cytotoxicity test will not contain H_2O_2. To a 96 well plate (Greiner Bio-One) 100 μl of cell suspension with compounds were added. This micro plate was then incubated for 24 hours in an atmosphere of 5 % CO_2 in humidified air at 37 °C.

On the second day, 10 μl of MTT stock solution was added to each well (final conc. 0.5 mg /mL), in the dark and under sterile conditions. The plate was then incubated at 37 °C for 2 hrs in the dark. 200 μl of DMSO was then added in each well to dissolve the formazane formed. The plate was gently shaken and the color was immediately measured with a microplate reader at 550 nm (reference 690 nm).

2.4.Nematode assay

These assays were carried out together with Mr. Mohammed Badr Sarakbi, a Diploma student in Prof Jacob´s group who was supervised by me since February 2009. Nematodes, *i.e.* primarily *Steinernema feltiae,* were purchased as soft cake product used in gardening (from Schneckenprofi Hennstedt Ltd., Germany), and stored at 4 °C until the time of use (the viability of nematodes was checked prior to each assay; the viability must be more than 80 %). A suitable suspension of the nematodes was prepared by mixing 2 g of the nematode soft cake with approximately 250 mL distilled water. The suspension was set aside at room temperature for 15-30 minutes before use.

The compounds were dissolved in DMSO to prepare a stock solution of a desired concentration, and were then subjected to serial dilution with DMSO and distilled water. The samples were prepared by transferring 900 μL of the compound solution at each test concentration to an Eppendorf tube (1 mL). 100 μL of the prepared nematode suspension was added, resulting in 1 mL test solution containing 200-400 nematodes with final concentrations of 50, 100, 200, and 400 μM of the tested compounds, respectively (please note that the DMSO concentration in each tube must be 2 %). A blank of 2 % DMSO was used as negative control.

Assay procedure:

Three replicates were prepared for each concentration. For each replicate, four samples of 100 μL were transferred into 4 wells in a 96-well micro plate and immediately examined under a light microscope at 4 fold magnification. Only living (mobile) nematodes in each sample were counted, producing a combined total from the four samples. The micro plate was

then incubated in the dark, and re-examined after 24 and 48 hours. The viability (survival) percentage after each time period was calculated by the formula:

Viability % = (A_t/A_0) X 100

Where A_0 is the number of live nematodes at the beginning of the test, A_t is the number of live nematodes after the time period (24 and 48 hours).

The test was conducted in two independent measurements, and the final result was estimated as the mean ± standard deviation.

2.5.*In vitro* bioassay

2.5.1. Aromatase assay

Preparation of stock solution

Sodium phosphate buffer

6.00 g of Na_2HPO_4 and 8.90 g of NaH_2PO_4 were dissolved in 2000 mL of distilled water and the pH was adjusted to be 7.4.

Labeled androstenedion solution (^{3}HA-stock)

100 μL of androsten-4-ene-3,17-dione,[1β-^{3}H(N)] (NET-926, 1,0 mCi) was diluted to 1000 mL with ethanol (1 μL contain 1 μCi)

Normal androstenedion stock solution (A-stock)

6.945 mg of androstenedion was dissolved in 50 mL of ethanol.

Normal androstenedion diluted solution

250 μl of androstenedion stock solution was diluted in 50 mL of the cooled phosphate buffer.

Mercuric chloride solution

27.25 mg of $HgCl_2$ was dissolved in 100 mL of dist. water (1mM).

Glucose-6-phosphate (G6P)

120.56 mg of glucose-6-phosphate was dissolved in 4 mL of cooled sodium phosphate buffer.

Nicotinamid adenine dinucleotide phosphate (NADPH) solution

18.11 mg of NADP was dissolved in 1mL of cooled sodium phosphate buffer.

Glucose-6-phosphate dehydogenase (G6PDH) solution

4 μl of G6PDH solution (1 mL contain 40 units) was diluted in the cooled sodium phosphate buffer to a final volume of 1 mL.

Aromatase

Human aromatase was obtained from the microsomal fraction of freshly delivered human term placental tissue according to the procedure of Thompson and Siiteri [166]. In brief, freshly delivered human term placentas were washed in cold 0.15 M KC1, and adhering membranes and large blood vessels were removed by dissection. After thorough mincing with scissors, the tissue was weighed and 1 mL of cold 0.25 M sucrose was added per 1 g of tissue. Homogenization was accomplished and mitochondria, nuclei, and cell debris were removed by centrifugation at 20,000 rpm for 30 min. The postmitochondrial supernatant was then subjected to centrifugation at 148,000 rpm for 45 min to yield a microsomal pellet which was resuspended in 0.05 M potassium phosphate buffer, pH 7.4. The volume of this microsomal suspension was adjusted to 400 mL with the above buffer, after which the microsomes were resedimented at 148,000 rpm for 45 min. The microsomal pellet from the second high speed centrifugation was again resuspended in buffer and resedimented under the same conditions. Twice washed microsomes were finally suspended in a minimal volume of buffer and stored frozen at -80 °C in 1 mL Eppendorf tubes. Prepared in this manner, microsomes retained their full aromatase activity for at least 4 months.

Working enzyme solution was prepared by dilution of the microsomes obtained by the above procedure with phosphate buffer (pH 7.4) (1: 50). This diluted enzyme preparation was stored in -80°C for several months.

Charcol suspension

2 g of charcol was suspended in 100 mL of dist. Water.

Assay procedure

Pre test preparation

1- ^{3}HA/A: 20 μl of labeled androstenedione ^{3}HA was dissolved in 1000 μl of cooled, diluted, unlabeled androstenedion and was mixed well for 10 min.
2- Preparation of the regenerating system: G6P solution, NADP solution and G6PDH solution were mixed in 2:1:1 ratio respectively.
3- Preparation of inhibitors (compounds under investigation): at least 3 different concentrations from each compound were used and all compounds were dissolved in DMSO (with the concentration of DMSO not exceeding 2 %), and dilution factor of 50 was used.

Test procedure

To each Eppendorf, 95 μl of phosphate buffer, 50 μl of the regenerating system, 5 μl of DMSO (control) or compound solution, and 50 μl of ^{3}HA/A solution was added (the previous mixtures were added in only two Eppendorfs, except for the presence of DMSO or compounds solution, as they were used as a zero control). To each zero control, 200 μl of $HgCl_2$ was added to stop the reaction. The diluted aromatase solution (1: 50) was removed from the -80 °C freezer and incubated with the prepared Eppendorf samples in 30 °C water bath (Grant, Type VAB18EU, England) for 5 min. The reaction was started by the addition of 50 μl of diluted aromatase solution to each reaction tube; after thorough mixing (IKA-Vibrax-VXR, Germany), these tubes were incubated for 14 min in a water bath at 30 °C. The reaction was stopped directly after the incubation period by the addition of 200 μl of $HgCl_2$, and was then mixed well for 2-3 min. 200 μl of charcoal solution was added after vigorous mixing and the reaction tubes were shaken for at least 30 min before being centrifuged (Thermo scientific, Heraeus Fresco 17 centrifuge, Germany) for 5 min at 12500 rpm at 4 °C. 400 μl of supernatant from each tube was transferred to new tubes and again centrifuged for 5 min at 12500 rpm at 4 °C. 200 μl of supernatant from each tube was then transferred to 2 mL Eppendorf tubes, followed by the addition of 1000 μl of Szintillator 212. All tubes were then mixed well. The $^{3}H_2O$ was measured using a ß-counter (WALLAC, Micro Beta Trilux, Finland).

2.5.2. 2,2-Diphenyl-1-picrylhydrazyl, 95 % (DPPH) assay

Preparation of DPPH solution

DPPH stock solution

Dissolve 125 mg of DPPH in 100 mL of ethanol, sonicate for 10 minutes. The stock solution may be stored for 2 weeks in the refrigerator (–20 °C).

DPPH working solution

Dilute the stock solution 1: 10 (V/V) in ethanol. The concentration of DPPH in the working solution is 316 μM.

Sample preparation

Serial dilution of all compounds at 0.2, 1, 2, 4 and 8 mM was prepared in pure DMSO. 10 μl from these dilutions was necessary to prepare 200 μl of 10, 50, 100, 200 and 400 μM respectively. Controls (D, L–α–tocopherol (vitamin E) and Trolox®) were prepared in the same concentration as test compounds.

Assay procedure

In a 96–well plate, 10 μL of test compounds stock solution sample was added to 190 μL of DPPH working solution. A blank control was prepared; where as 10 μL of DMSO was added to 190 μL of DPPH working solution. The final volume in each is therefore equal to 200 μL. In final solution, the concentration of DPPH is 300 μM. The plate was then incubated in the dark for 30 minutes at room temperature. After incubation, the absorbance was measured using the microplate reader spectrophotometer (Wallac, Victor, 1420 multilabel counter) at 530 nm.

Calculations

Inhibition %= (1- $Sample_{530}/blank_{530}$) X 100

The percentage of inhibition versus compound concentration was plotted on a graph. From the equation of the slope, I was able to calculate the test sample concentration required to reduce the absorbance at 520 nm by 50 % (IC_{50}).

2.5.3. Electrochemistry

The electrochemical behavior of the chromene-flavone compounds was studied by Ms. Elena Gurevich, a Diploma student in Jacob´s group. Studies were performed by using Differential Pulse Polarography (DPP) with a BAS 100W potentiostat. The compounds were at first solved in 5 % DMSO and then diluted in 33 % of methanol/water (v:v) and phosphate buffer pH = 7.4. to a final concentration of approximately 500 μM. The Polarogramms were recorded using a glassy carbon working electrode, a silver/silver chloride reference electrode and platium reference electrode. The potential range was between -1000 to 1500 mV with a scan rate of 10 mV/s, the puls amplitude was 80 mV, with a sample width of 45 ms and pulse width of 100 ms. For Cyclic Voltammetry (CV), the same experimental setup was used. The potential range was between -1500 mV to 1500 mV with scan rates of 100 mV/s and 500 mV/s.

Chapter III: Results

3.1. Characterization of natural product

The water soluble portion of the ethanolic extracts of *Delonix regia* and *Robinia pseudoacacia* roots were fractionated by solvent partition (ethyl acetate and *n*-butanol). A successive silica gel column chromatography followed by further purification via Sephadex (LH-20) column chromatography allowed for the isolation of five compounds from *Delonix regia* root (two 3,4-flavandiols ***1d***, ***2d***, one legnan derivative ***3d***, one flavan-3-ol ***4d***, and one dihydroflavone ***5d*** (***Fig 23***)), and five compounds from *Robinia pseudoacacia* root (one dihydroflavone ***1r***, three chalcones ***2r***, ***3r***, ***4r***, and one indol derivative ***5r*** (***Fig 24***)).

Identification of these compounds was based on extensive ^{1}H, ^{13}C NMR (1D and 2D), and mass spectroscopy data.

3.1.1. Isolated natural products from *Delonix regia* root

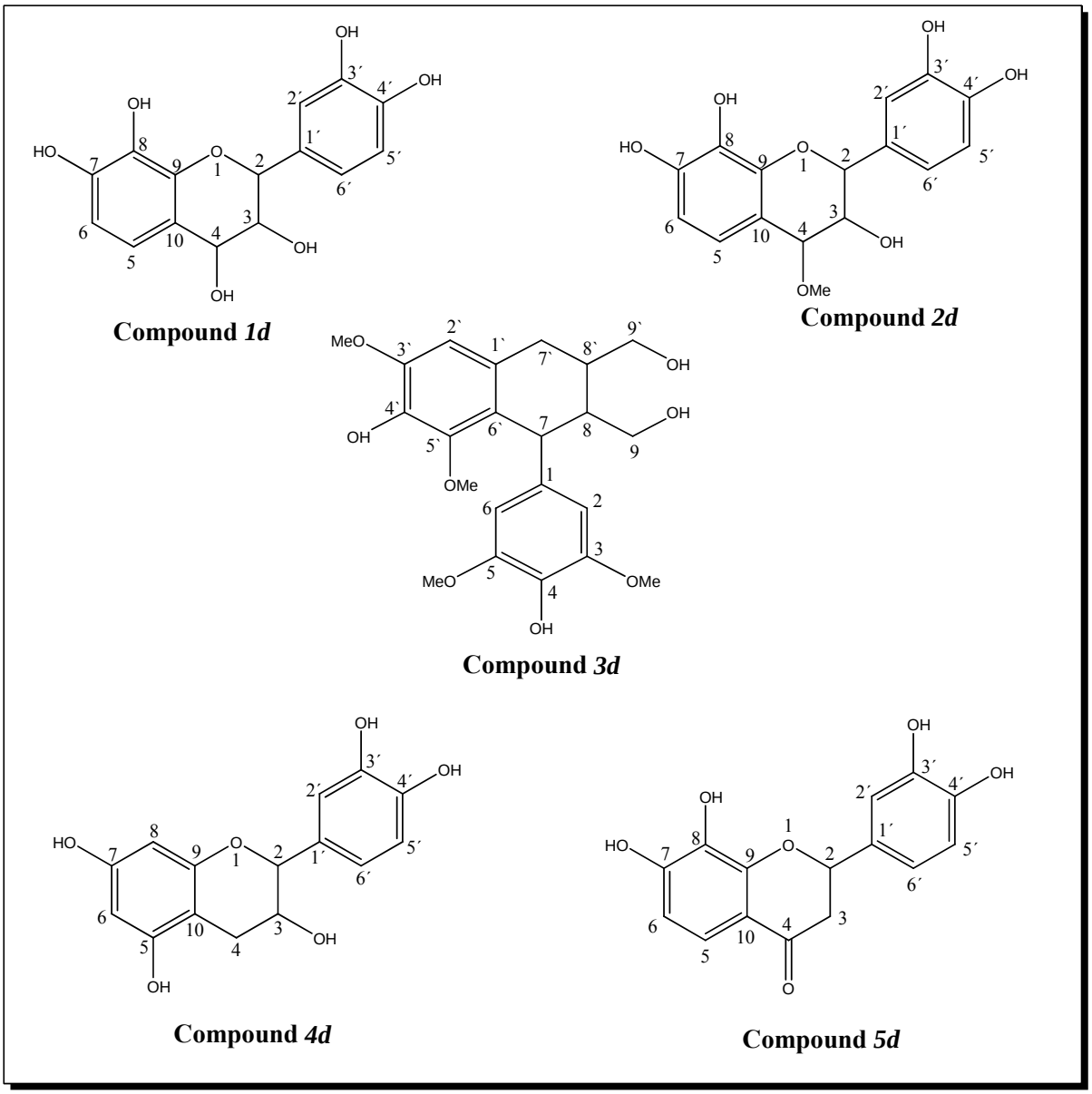


Fig 23: *Chemical structure of natural compounds isolated from Delonix regia roots*

3.1.1.1. Compound *1d*

Compound ***1d*** was isolated as a yellowish brown amorphous powder. On TLC, it resulted in a greenish black spot when treated with vanillin/H_2SO_4. According to IUPAC system, the compound was named as 2-(3,4-dihydroxyphenyl) chromane-3,4,7,8-tetrol (***Fig 23***). Following the normal nomenclature of flavonoids, it can be also referred to as 7,8,3′,4′-

teteahydroxy-3,4-flavandiol. After reviewing the literature, compound ***1d*** has never been isolated from *Delonix regia* before, but was isolated from many other *Leguminosae* plants [167].

Compound ***1d*** has a molecular formula $C_{15}H_{14}O_7$. LC-MS-ESI: t_R = 3.95 min, m/z calcd 306.07, m/z found 290.96 $[M\text{-}OH+H]^+$.

^1H, ^{13}C NMR and mass spectroscopy were all compatible with the structure of ***1d*** (see ***Table 8***).

3.1.1.2. Compound *2d*

Compound ***2d*** was isolated as a yellowish brown amorphous powder. On TLC, it resulted in a greenish black spot when treated with vanillin/H_2SO_4. According to IUPAC system, the compound was named as 2-(3,4-dihydroxyphenyl)-4-methoxychromane-3,7,8-triol (***Fig 23***). By the normal nomenclature of flavonoids, it can also be referred to as 7,8,3´,4´-teteahydroxy-4-methoxy-flavan-3-ol. Compound ***2d*** has been a known compound since July 2008, when its isolation from heartwood of *Acacia confuse* family *Leguminosae* was reported [168]. (NB, I isolated this compound for the first time in September 2007).

The position of methoxy group is confirmed by the correlation of the methoxy protons and the carbons 3, 4 and 10. Whereas the stereochemistry of protons on the chiral carbons 2 and 3 in addition to stereochemistry of the methoxy group is more complicated and still requires more analysis.

Comound ***2d*** has a molecular formula of $C_{16}H_{16}O_7$, LC-MS-ESI: t_R = 2.32 min, m/z calcd 320.09, m/z found 288.93 $[M\text{-}OCH_3+H]^+$.

HRMS [M-H] negative mode calcd= 319.0817, found= 319.0823.

^1H, ^{13}C NMR and mass spectroscopy were all compatible with the structure of ***2d*** (see ***Table 8***).

Table 8: *^{1}H, ^{13}C NMR and DEPT spectral data of compounds* ***1d*** *and* ***2d****.*

Position Nr.	Compound *1d*		Compound *2d*		DEPT
	δ H	δ C	δ H	δ C	
2	4.93, br. s.	69.50	4.97, br. s.	76.80	CH
3	3.87, dd, *J* = 0.63, 3.99	67.48	3.95, dd, *J* = 0.80, 2.70.	69.47	CH
4	4.84, d, *J* = 3.31	79.04	4.07, d, *J* = 3.30.	78.68	CH
5	6.68, d, *J* = 7.95	118.34	6.67, d, *J* = 8.60.	123.32	CH
6	6.36, d, *J* = 8.34	108.09	6.45, d, *J* = 8.60.	109.26	CH
7	-	144.81	-	147.25	C
8	-	131.92	-	133.84	C
9	-	143.05	-	145.35	C
10	-	114.28	-	112.61	C
1´	-	130.34	-	131.44	C
2´	7.01, d, *J* =1.90	114.51	7.04, d, *J* = 1.96.	115.80	CH
3´	-	144.71	-	146.09	C
4´	-	144.63	-	146.08	C
5´	6.75, dd, *J* = 8.30, 0.90	115.73	6.77, d, *J* = 8.30.	116.02	CH
6´	6.81, dd, *J* = 8.34, 2.28	117.42	6.87, dd, *J* = 8.00, 2.20.	119.85	CH
O-CH_3	-	-	3.45, s.	56.57	CH_3

3.1.1.3. Compound *3d*

Compound ***3d*** was isolated as a colorless amorphous powder. On TLC, it resulted in a greenish black spot when treated with vanillin/H_2SO_4. According to IUPAC system, the compound was named as 8-(4-dihydroxy-3,5 dimethoxyphenyl)-6,7-bis(hydroxymethyl)-1,3-dimethoxy-5,6,7,8-tetrahydronaphthalene-2-ol (***Fig 23***). ***3d*** is considered to be a lignin derivative and was isolated for the first time from the genus *Delonix*. It has also been isolated previously from *Aphanamixis polystachya* family *Meliaceae* [169] and given the name Lyoniresinol or (-)-Lyoniresinol, according to the configuration of the chiral center (C7).

LC-MS-ESI: t_R = 6.29 min, m/z calcd 420.18, m/z found 405.02 $[M\text{-}Me]^+$.

HRMS [M-H] negative mode calcd= 419.1705, found= 419.1716.

^1H and ^{13}C NMR as well as HC-HMBC two dimensional correlations (***Table 9***) and mass spectroscopy were all compatible with the structure of ***3d***.

Table 9: *1D and 2D NMR data of compound* ***3d****.*

Position Nr.	δ ^{1}H	δ ^{13}C	DEPT	HMBC
1	-	139.31*	C	-
2	6.28, s	106.91	CH	C-1,3,4,6,7
3	-	149.00	C	-
4	-	134.56	C	-
5	-	149.00	C	-
6	6.28, s	106.91	CH	C-1,2,4,5,7
7	4.20, d, *J* = 4.87	42.31	CH	C-1,2,9,1′,6′,8′
8	1.85, m	48.00#	CH	C-1,7,9,8′,9′
9	3.40, d, *J* = 5.84	64.21	CH_2	C-7,8,8′
1′	-	129.87	C	-
2′	6.49, s	107.79	CH	C-3′,4′,6′,7′
3′	-	148.67	C	-
4′	-	139.31*	C	-
5′	-	147.71	C	-
6′	-	126.26	C	-
7′	2.47, dd, *J* =11.54, 14.50,ax 2.59, dd, *J* = 4.86, 15.05, eq	33.30	CH_2	C-1′,6′,8′ C-8,1′,6′,8′
8′	1.50, m	40.91	CH	-
9′	3.39, dd, *J* = 2.85, 8.50, ax 3.50, dd, *J* = 4.40, 10.82, eq	66.80	CH_2	C-8,7′,8′ C-8,7′,8′
3,5-OCH_3	3.65, s	56.61	CH_3	C-3,5
3`- OCH_3	3.77, s	56.78	CH_3	C-3′
5`- OCH_3	3.28, s	60.16	CH_3	C-5′

* Observed from HMBC correlations [139.31 ppm for C-1 and C-4′].
# Chemical shift obtained from HMQC, due to solvent interference.

3.1.1.4. Compound *4d*

Compound ***4d*** was isolated as a yellowish white amorphous powder. On TLC, it resulted in a blueish spot when treated with vanillin/H_2SO_4. Compound ***4d*** is a well known compound. It has a common name, (±) catechin. According to the IUPAC system, the compound was named as 2-(3,4-dihydroxyphenyl)chromane-3,5,7-triol (***Fig 23***). Following the normal nomenclature of flavonoids, it can be named 5,7,3′,4′-teteahydroxy–flavan-3-ol. After reviewing the literature, Compound ***4d*** has never been isolated from *Delonix regia*, but it has been isolated from many other plants.

Compound ***4d*** has a molecular formula of $C_{15}H_{14}O_6$, LC-MS-ESI: t_R = 5.54 min, m/z calcd 290.08, m/z found 290.96 $[M+H]^+$.

^1H, ^{13}C NMR and mass spectroscopy were compatible with the structure of ***4d*** and with the data published before (see ***Table 10***) [170].

Table 10: *^1H and ^{13}C of compound* ***4d****.*

Position Nr.	δ H	δ C	DEPT
2	4.64, d. *J* = 7.58	81.48	CH
3	3.87, m	67.43	CH
4	2.40, dd, *J* = 8.20, 16.30, ax 2.74, dd, *J* = 6.24, 16.30, eq	27.14	CH_2
5	-	156.45	C
6	5.82, d, *J* = 2.34	94.12	CH
7	-	156.19	C
8	5.75, d, *J* = 2.18	94.89	CH
9	-	155.54	C
10	-	99.45	C
1′	-	130.85	C
2′	6.73, d, *J* = 2.06	113.86	CH
3′	-	144.85	C
4′	-	144.83	C
5′	6.66, d, *J* = 8.24	114.70	CH
6′	6.61, dd, *J* = 2.60, 8.31	118.64	CH

3.1.1.5. Compound *5d*

Compound ***5d*** is a well known compound. It's known as Isookanin. According to the IUPAC system, the compound was named as 2-(3,4-dihydroxyphenyl)-7,8-dihydroxy-2,3-dihydro-4*H*-chromen-4-one (***Fig 23***). Following the normal nomenclature of flavonoids, is referred to 7,8,3′,4′-teteahydroxy–dihydroflavone.

After reviewing the literature, compound ***5d*** has never been isolated from *Delonix regia* before, but it has been isolated from many other *Leguminosae* plants [171].

Compound ***5d*** has a molecular formula of $C_{15}H_{14}O_6$, LC-MS-ESI: t_R = 2.68 min, m/z calcd 288.06, m/z found 289.00 $[M+H]^+$.

^1H, ^{13}C NMR and mass spectroscopy were all compatible with the structure of ***5d*** and with the data published before (see ***Table 11***) [171].

Table 11: *^{1}H and ^{13}C of compounds* ***5d*** *and* ***1r***.

No	Compound *5d*			Compound *1r*		
	δ H	**δ C**	**DEPT**	**δ H**	**δ C**	**DEPT**
2	5.35, dd. *J* = 2.79. 12.31	82.49	CH	5.28, dd. *J* = 2.90. 13.26	81.06	CH
3	3.04, dd, *J* =12.31, 16.72, ax 2.70, dd, *J* =2.79, 16.72, eq	45.96	CH_2	2.95, dd, *J* = 12.93, 16.91, ax 2.59, dd, *J* = 2.99, 16.50, eq	44.96	CH_2
4	-	194.94	C	-	193.52	C
5	7.27, d, *J* = 8.94	120.27	CH	6.25, d, *J* = 8.01	103.83	CH
6	6.50, d, *J* = 8.82	111.85	CH	6.40, dd, *J* = 2.50, 8.60	111.77	CH
7	-	155.04	C	-	155.04	C
8	-	134.95	C	7.63, d, *J* = 8.12	129.84	CH
9	-	153.62	C	-	158.98	C
10	-	116.58	C	-	114.98	C
1´	-	132.86	C	-	131.37	C
2´	6.96, d, *J* = 1.86	115.93	CH	6.72, d, *J* = 9.57	116.31	CH
3´	-	147.41	C	7.22, d, *J* = 8.12	129.00	CH
4´	-	147.85	C	-	166.86	C
5´	6.76, d, *J* = 8.13	117.16	CH	7.22, d, *J* = 8.12	129.00	CH
6´	6.83, dd, *J* = 1.86, 8.13	120.51	CH	6.72, d, *J* = 9.57	116.31	CH

3.1.2. Isolated natural products from *Robinia pseudoacacia* root

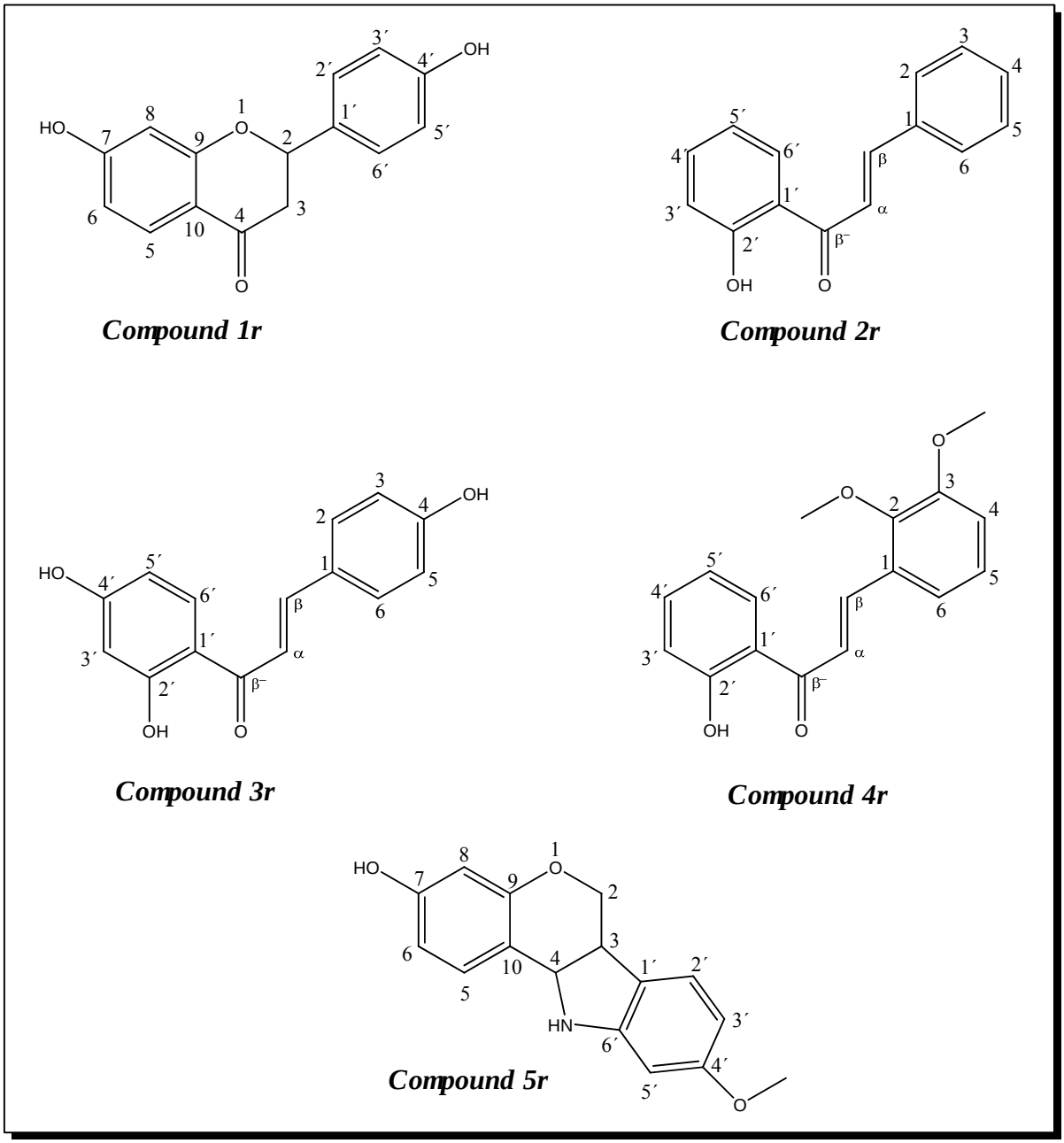


Fig 24: *Chemical structure of natural compounds isolated from Robinia pseudoacacia roots*

3.1.2.1. Compound *1r*

Compound ***1r*** was isolated as a yellowish amorphous powder. On TLC, it resulted in a black spot when treated with vanillin/H_2SO_4. According to IUPAC system, the compound was named as 7-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4*H*-chromen-4-one (***Fig 24***). Following the normal nomenclature of flavonoids, it can be referred to as 7,4′-dihydroxy–

dihydroflavone. After literature reviewing, compound ***1r*** has never been isolated before from the *Robinia pseudoacacia,* but has been isolated from many other plants [172]

Compound ***1r*** has a molecular formula of $C_{15}H_{12}O_4$, LC-MS-ESI: t_R = 7.87 min, m/z calcd 256.07, m/z found 256.95 $[M+H]^+$.

^1H, ^{13}C NMR and mass spectroscopy were all compatible with the structure of ***1r*** (see ***Table 11***).

3.1.2.2. Compound *2r*

Compound ***2r*** was isolated as an intense yellow amorphous powder. On TLC, it resulted in a yellow spot when treated with vanillin/H_2SO_4. The proton and carbon pattern of NMR showed a complete chalcone skeleton [173], with only one OH group at position 2´.

Compound ***2r*** was named according to IUPAC system, as (2*E*)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one. Following the flavonoid nomenclature it can referred to as 2´-hydroxychalcone.

Compound ***2r*** has a molecular formula of $C_{15}H_{12}O_2$, LC-MS-ESI: t_R = 12.72 min, m/z calcd 224.08, m/z found 225.15 $[M+H]^+$.

^1H, ^{13}C NMR and mass spectroscopy were all compatible with the structure of ***2r*** (see ***Table 12*** and ***13***).

3.1.2.3. Compound *3r*

Compound ***3r*** was isolated as an amorphous powder with more intense yellow colouration than ***2r.*** On TLC, it resulted in a yellow spot when treated with vanillin/H_2SO_4. Compound ***3r*** was identified as a chalcone, with NMR data and comparison with compound ***2r*** indicating the presence of two more hydroxyl groups at positions 4 and 4´ [173].

Compound ***3r*** was named according to IUPAC system as (2*E*)-1-(2,4-dihydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one. Following the flavonoid nomenclature, it can be referred to as 4,4´,2´-trihydroxychalcone.

Compound ***3r*** has a molecular formula of $C_{15}H_{12}O_4$, LC-MS-ESI: t_R = 13.85 min, m/z calcd 256.07, m/z found 257.04 $[M+H]^+$.

^1H, ^{13}C NMR and mass spectroscopy were all compatible with the structure of ***3r*** and with the data obtained from the literature (see ***Table 12*** and ***13***) [173].

3.1.2.4. Compound *4r*

Compound ***4r*** was isolated as a yellow amorphous powder. On TLC, it resulted in a yellow spot when treated with vanillin/H_2SO_4. Compound ***4r*** was identified as a chalcone, with NMR data and comparison with compounds ***2r*** and ***3r*** indicating the presence of two more methoxy groups at positions 2 and 3. Two dimensional NMR, HMBC and HMQC spectra also confirm this structure. After reviewing the literature, this compound appears to be a new natural product isolated for only the second time from the plant. I isolated this compound during my master work from leaves of *Robinia pseudoacacia* and recorded this isolation in my MSc. thesis. **N.B**: Compound ***4r*** is known in literature however as a synthetic product [174, 175]. It is used to prepare other chalcons by the process of biotransformation [176].

Compound ***4r*** can be named according to the IUPAC system as (2*E*)-3-(2,3-dimethoxyphenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one. Following the flavonoid nomenclature, it can also be referred to as 2´-hydroxy-2,3-dimethoxy chalcone.

Compound ***4r*** has a molecular formula of $C_{17}H_{16}O_5$, HRMS calcd = 284.1048, found = 284.1055$[M]^+$.

^1H, ^{13}C NMR (***Table 12*** and ***13***) in addition to COSY, HMQC and HMBC as well as mass spectroscopy were all compatible with the structure of ***4r***.

Table 12: *^{1}H NMR spectral data of compounds **2r**, **3r**, **4r**.*

Position Nr.	δ H *compound 2r*	δ H *compound 3r*	δ H *compound 4r*
1	-	-	-
2	7.90, m	6.74, d, *J* = 8.90	-
3	7.48, m	7.51, d, *J* = 8.90	-
4	7.48, m	-	7.00, m
5	7.48, m	7.51, d, *J* = 8.90	7.12, dd, *J* = 2.68, 6.65
6	7.90, m	6.74, d, *J* = 8.90	7.00, m
A	8.04, d, *J* = 15.57	7.56, d, *J* = 15.95	8.07, d, *J* = 15.83
B	7.84, d, *J* = 15.57	7.51, d, *J* = 15.95	7.98, d, *J* = 15.59
B´	-	-	-
1´	-	-	-
2´	-	-	-
3´	8.24, dd, *J* = 1.16, 7.95	6.18, d, *J* = 2.00	8.19, dd, *J* = 1.50, 8.22
4´	7.86, m	-	7.17, m
5´	7.01, m	6.31, dd, *J* = 2.60, 8,82	7.56,ddd, *J* = 1.62, 1.62, 8.43
6´	7.01, m	7.86, d, *J* = 8.29	7.17, m
2´-OH	12.55, s	12.89, s	12.39, s
4´-OH	-	10.12, s	-
4 -OH	-	10.35, s	-
2-OCH_3	-	-	3.81, s
3-OCH_3	-	-	3.84, s

Table 13: *^{13}C NMR spectral data of compounds **2r**, **3r**, **4r**.*

Position Nr.	*compound 2r*		*compound 3r*		*compound 4r*	
	δ C	**DEPT**	**δ C**	**DEPT**	**δ C**	**DEPT**
1	134.42	C	127.87	C	127.99	C
2	128.92	CH	131.80	CH	148.51	C
3	128.50	CH	116.94	CH	153.77	C
4	130.96	CH	161.60	C	115.39	CH
5	129.16	CH	116.94	CH	122.86	CH
6	129.92	CH	131.80	CH	124.42	CH
α	117.68	CH	118.40	CH	117.70	CH
β	144.70	CH	145.58	CH	138.68	CH
β$^-$	193.62	C	193.48	C	193.55	C
1´	120.80	C	114.62	C	120.94	C
2´	161.84	C	166.76	C	161.72	C
3´	119.15	CH	103.88	CH	119.36	CH
4´	136.31	CH	167.56	C	136.25	CH
5´	121.86	CH	109.31	CH	119.17	CH
6´	130.84	CH	133.35	CH	130.77	CH
2-OCH$_3$	-	-	-	-	61.00	CH$_3$
3-OCH$_3$	-	-	-	-	56.00	CH$_3$

3.1.2.5. Compound *5r*

Compound ***5r*** was isolated as a white amorphous powder. On TLC, it resulted in a black spot when treated with vanillin/H_2SO_4.

Compound ***5r*** was identified as a new indol derivative, with ^{1}H, ^{13}C and two dimensional NMR HMBC comparison used to confirm the indol derivative structure. This compound is a new natural product which has not been reported before, and is isolated the first time from the root of *Robinia pseudoacacia*.

The chemical structure of compound ***5r*** gave the typical NMR signals of a pterocarpan (Medicarpin) [177, 178], with one additional broad singlet proton at δ_H 4.99, indicating the presence of an NH group instead of oxygen at the bridge of the three member ring [179, 180]. Compound ***5r*** was named according to IUPAC system as 9-methoxy-6,6a,11,11a-tetrahydrochromeno[4,3-b]indol-3-ol

Compound 5r has a molecular formula of $C_{16}H_{15}NO_3$, LC-MS-ESI: t_R = 12.55 min, m/z calcd 269.11, m/z found 269.1 $[M]^+$.

^{1}H and ^{13}C NMR and HMBC in addition to COSY, HMQC as well as mass spectroscopy were all compatible with the structure of ***5r*** (see ***Table 14***).

Table 14: *^{1}H, ^{13}C as well as HMBC spectral data of compound* ***5r****.*

Position Nr.	δ H	H-C HMBC correlation	δ C	DEPT
2	3.51, m, ax/ 4.21, m, eq	C-2, 1′, 9	66.58	CH_2
3	3.60, t, *J* = 10.95	C-3, 4, 1′, 6′	39.52	CH
4	5.47, d, *J* = 6.85	C-2, 10, 8, 9	78.55	CH
5	7.36, d, *J* = 8.50	C-9, 4	132.22	CH
6	6.43,dd, *J* = 2.50, 8.50	C-5, 10	106.45	CH
7	-	-	161.15	C
8	6.38, d, *J* = 2.50	C-9, 10	96.93	CH
9	-	-	157.01	C
10	-	-	112.70	C
1′	-	-	119.11	C
2′	7.11, d, *J* = 8.45	C-3′, 4′, 5′, 3	124.78	CH
3′	6.53,dd, *J* = 2.20, 8.45	C-1′, 3′, 4′, 5′	109.75	CH
4′	-	-	160.71	C
5′	6.43, d, *J* = 2.20	C-1′, 3′, 4′, 5′	103.69	CH
6′	-	-	156.67	C
N-H	4.99, br.s	C-10, 5′, 6′	-	-
O-CH_3	3.74, s	C-4′	55.45	CH_3

3.2.Mannich reaction

Reaction of aldehydes and primary or secondary amines with the flavones (chrysin or quercetin) produced twenty-one compounds (***1m-21m***), all with different chemical, physical and biological characteristics. The resulting diversity may provide a better understanding of the structure-activity relationships of these compounds (***Fig 26***).

Table 15 provides details of the compounds obtained from the Mannich reaction as well as the reaction conditions and yields associated with them.

It should be noted that carbon number **6** in the flavone nucleus was the most reactive and usually involved in the reaction. In some instances carbon number **8** was also involved, thus creating **6,8**-disubstituted flavones (***Fig 25***).

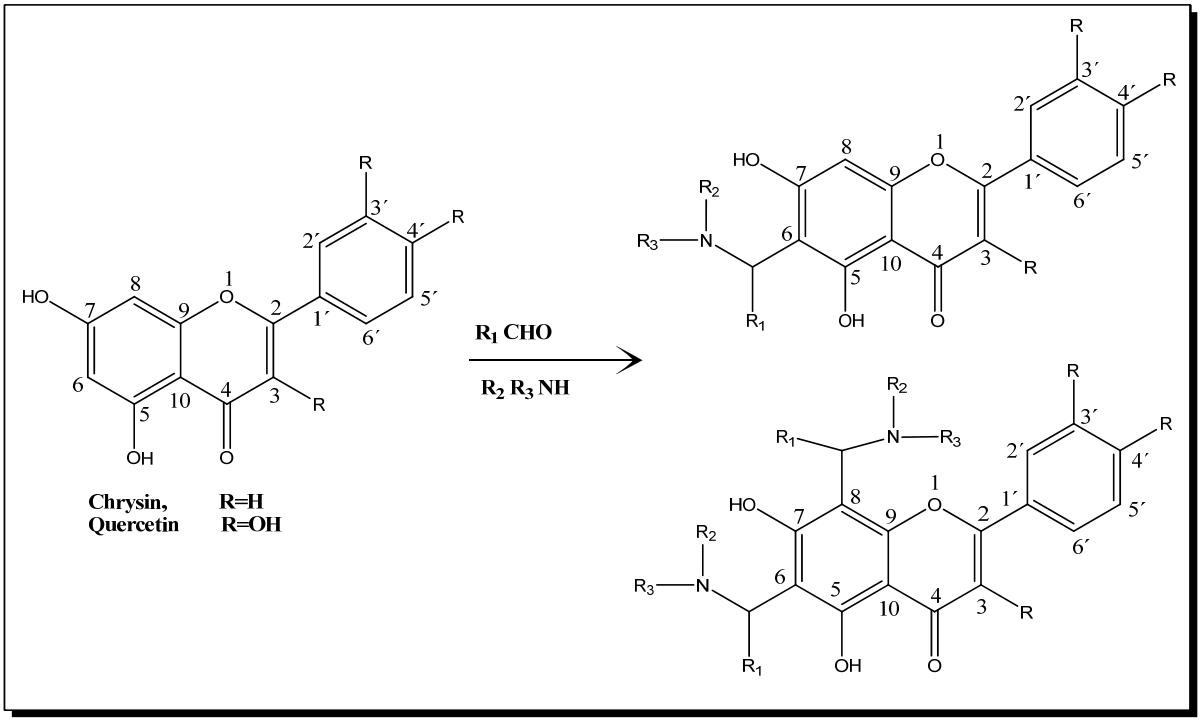


Fig 25: *Schematic overview of the Mannich reaction used to modify chrysin and quercetin.*

Table 15: *Components of the Mannich reaction, including reaction conditions and yields.*

No	Aldehyde	Amine	Flavone	Condition	Reaction site	Yield%
1m	Paraformaldehyde	morpholin	chrysin	24 h, 65 °C	C6	42
2m	Paraformaldehyde	morpholin	chrysin	24 h, 65 °C	C6, C8	22
3m	Paraformaldehyde	thiomorpholin	chrysin	8 h, 65 °C	C6	21
4m	Paraformaldehyde	thiomorpholin	chrysin	8 h, 65 °C	C6, C8	86
5m	Paraformaldehyde	*N*-methyl piperazine	chrysin	4 h, 65 °C	C6	32
6m	Ethylglyoxalate	morpholin	chrysin	8 h, 65 °C	C6	80
7m	Ethylglyoxalate	morpholin	chrysin	8 h, 65 °C	C6, C8	12
8m	Ethylglyoxalate	thiomorpholin	chrysin	4 h, 65 °C	C6	18
9m	Ethylglyoxalate	*N*-methyl piperazine	chrysin	4 h, 65 °C	C6	80
10m	Ethylglyoxalate	morpholin	quercetin	4 h, 40 °C	C6	72
11m	Ethylglyoxalate	thiomorpholin	quercetin	4 h, 35 °C	C6	36
12m	Paraformaldehyde	Phthalimide	chrysin	4 h, 65 °C	C6	10
13m	Ethylglyoxalate	*p*-anisidine	chrysin	4 h, 55°C	C6	10
14m	Ethylglyoxalate	*p*-anisidine	quercetin	8 h, r.t.	C6	39
15m	*p*-Methoxy penzaldehyde	morpholin	chrysin	24 h, 65 °C	C8	42
16m	*p*-Methoxy penzaldehyde	morpholin	chrysin	24 h, 65 °C	C6	18
17m	Benzaldehyde	morpholin	chrysin	24 h, 65 °C	C6	47
18m	*p*-Nitro penzaldehyde	morpholin	chrysin	24 h, 65 °C	C6	22
19m	*p*-Bromo penzaldehyde	morpholin	chrysin	24 h, 65 °C	C6	15
20m	*p*-Chloro penzaldhyde	morpholin	chrysin	24 h, 65 °C	C6	60
21m	*p*-Floro penzaldehyde	morpholin	chrysin	24 h, 65 °C	C6	55

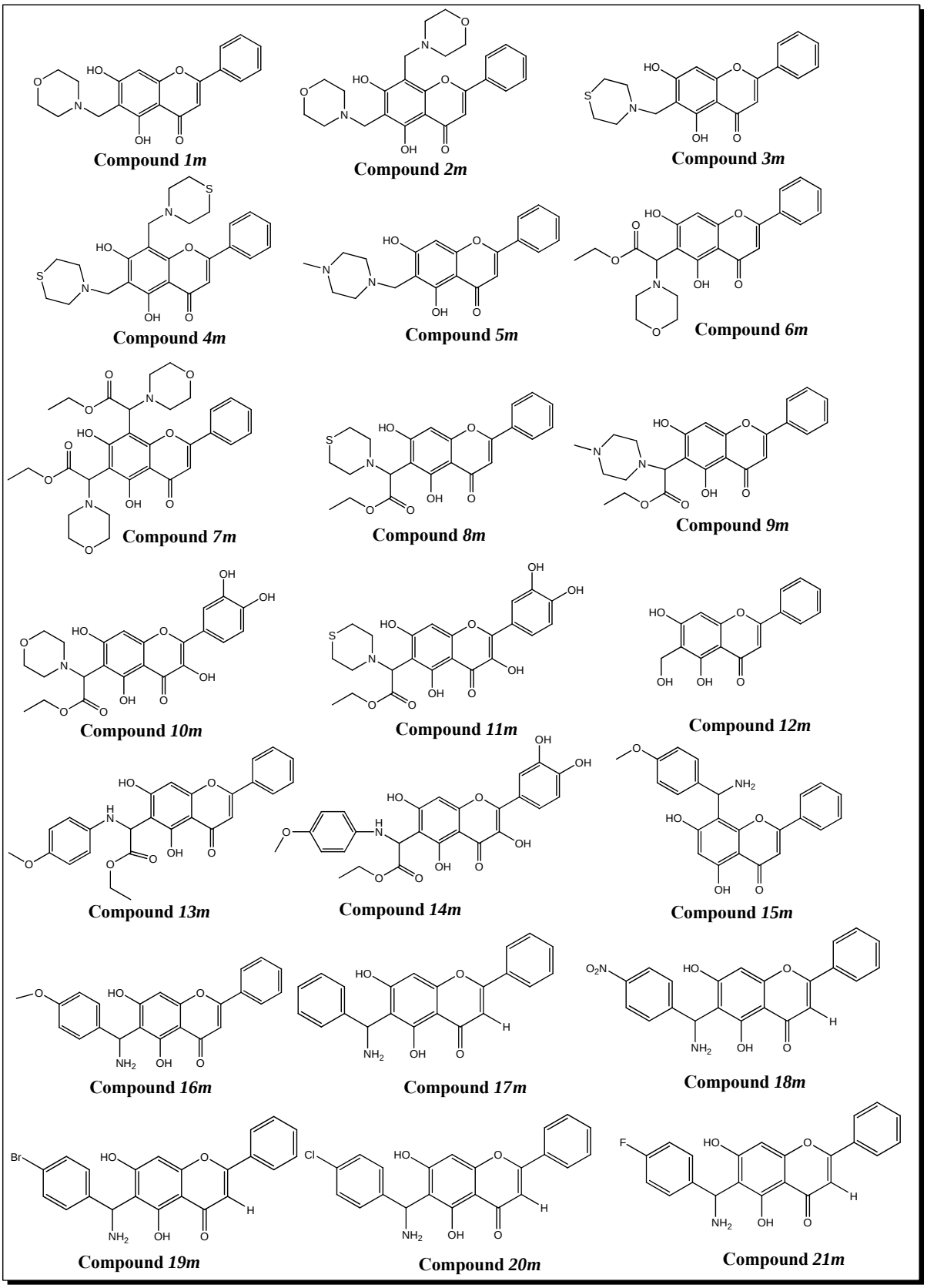


Fig 26: *Chemical structure of synthetic compounds obtained via Mannich reaction.*

Compound 1m: 5,7-Dihydroxy-6-(morpholinomethyl)-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellow powder. Yield = 42 %.

TLC (petrol ether: ethyl acetate = 7: 3): R_f = 0.61, melting point: 209 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.70 (s, 1H), 7.83-7.71 (dd, 2H, *J*=1.49, 7.50 Hz,), 7.57-7.48 (m, 3H), 6.61 (s, 1H), 6.27 (s, 1H), 3.98 (s, 2H), 3.86- 3.63 (br s, 4H), 2.83- 2.47 (br s, 4H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.35 (s), 165.31 (s), 163.13 (s), 161.74 (s), 154.84 (s), 131.79 (d), 131.53 (s), 129.19 (d, 2C), 126.07 (d, 2C), 105.91 (d), 104.84 (s), 100.25 (d), 97.97 (s), 66.63 (t), 54.13 (t), 52.98 (t) ppm.

LC-MS-ESI: t_R = 7.07 min, m/z calcd 353.13, m/z found 354.61 $[M+H]^+$.

Compound 2m: 5,7-Dihydroxy-6,8-bis(morpholinomethyl)-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellow powder. Yield = 22 %.

TLC (petrol ether: ethyl acetate = 7: 3): R_f = 0.77, melting point: 172 ^{o}C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.25 (s, 1H), 9.80 (br s, 1H), 7.80-7.95 (dd, 2H, *J*=2.00, 8.09 Hz,), 7.54-7.47 (m, 3H), 6.63 (s, 1H), 3.82 (s, 2H), 3.81 (s, 2H), 3.75- 3.71 (t, 4H, *J*=4.40), 3.71- 3.68 (t, 4H, *J*=4.40), 2.64- 2.55 (m, 4H)1.49 ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.58 (s), 164.63 (s), 163.34 (s), 159.08 (s), 155.32 (s), 131.72 (d), 131.58 (s), 129.11 (d, 2C), 126.15 (d, 2C), 105.48 (d), 104.03 (s), 103.92 (s), 101.31 (s), 66.87 (t), 66.62 (t), 53.73 (t), 52.86 (t), 52.38 (t), 50.96 (t) ppm.

LC-MS-ESI: t_R = 6.09 min, m/z calcd 452.19, m/z found 453.63 $[M+H]^+$.

Compound 3m: 5,7-Dihydroxy-2-phenyl-6-(thiomorpholinomethyl)-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as a dark yellow powder. Yield = 21 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.8, melting point: 226 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.70 (s, 1H), 7.81-7.78 (dd, 2H, *J*= 1.54, 8.30 Hz,), 7.56-7.50 (m, 3H), 6.61 (s, 1H), 6.27 (s, 1H), 3.99 (s, 2H), 3.00-2.83 (br s, 4H), 2.81-2.71 (br s, 4H), ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.0 (s), 165.46 (s), 163.11 (s), 161.72 (s), 154.82 (s), 131.80 (d), 131.53 (s), 129.20 (d, 2C), 126.06 (d, 2C), 105.91 (d), 104.82 (s), 100.31 (d), 98.05 (s), 54.62 (t), 54.45 (t, 2C), 27.86 (t, 2C) ppm.

LC-MS-ESI: t_R = 7.84 min, m/z calcd 369.10, m/z found 370.49 $[M+H]^+$.

HRMS: $[M]^+$, calcd 369.1034, found 369.1073.

Compound 4m: 5,7-Dihydroxy-2-phenyl-6,8-bis(thiomorpholinomethyl)-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as a dark yellow powder. Yield = 86 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.64, melting point: 226 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.34-12.95(br s, 1H), 10.58-948 (br s, 1H) 7.87-7.83 (dd, 2H, *J*=2.22, 7.67 Hz,), 7.52-7.47 (m, 3H), 6.61 (s, 1H), 3.79 (s, 4H), 2.88-2.81(m, 8H), 2.72-2.67 (t, 4H, *J*=4.52 Hz), 2.67-2.63 (t, 4H, *J*=4.94Hz) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.49 (s), 164.63 (s), 163.25 (s), 158.99 (s), 155.23 (s), 131.67 (d), 131.52 (s), 129.06 (d,2C), 126.09 (d, 2C), 105.42 (d), 104.01 (s), 103.95 (s), 101.45 (s), 54.62 (t), 54.18 (t), 52.62 (t, 2C), 51.35 (t,2C), 27.82 (t, 2C), 27.54 (t, 2C) ppm

LC-MS-ESI: t_R = 7.57 min, m/z calcd 484.15, m/z found 485.55 $[M+H]^+$.

Compound 5m: 5,7-Dihydroxy-6-((4-methylpiperazin-1-yl)methyl)-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as a dark yellow powder. Yield = 32 %.

TLC (petrol ether: ethyl acetate = 7: 4): R_f = 0.64, melting point: 186 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.69 (s, 1H), 7.80-7.77 (dd, 2H, *J*= 1.55, 7.75 Hz), 7.54-7.48 (m, 3H), 6.59 (s, 1H), 6.25 (s, 1H), 3.97 (s, 2H), 2.97-2.32 (m, 8H), 2.30 (s, 3H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.32 (s), 165.64 (s), 163.04 (s), 161.58 (s), 131.73 (d), 131.53 (s), 129.14 (d, 2C), 126.03 (d, 2C), 105.80 (d), 104.68 (s), 100.20 (d), 98.36 (s), 54.69 (t), 53.68 (t), 52.60 (t), 45.79 (q) ppm.

LC-MS-ESI: t_R = 6.18 min, m/z calcd 366.16, m/z found 367.59 $[M+H]^+$.

HRMS: $[M]^+$, calcd 366.1579, found 366.1577.

Compound 6m: Ethyl 2-(5,7-dihydroxy-4-oxo-2-phenyl-4H-chromen-6-yl)-2-morpholinoacetate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as a dark yellow powder. Yield = 80 %.

TLC (petrol ether: ethyl acetate = 7: 3): R_f = 0.61, melting point: 199 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.75 (s, 1H), 7.94-7.91 (dd, 2H, *J*= 1.50, 8.76 Hz), 7.55-7.50 (m, 3H), 6.63 (s, 1H), 6.26 (s, 1H), 4.27 (s, 1H), 4.21-4.12 (m, 2H), 3.81-3.74 (t, 4H, *J*= 4.50), 2.73-2.57 (m, 4H), 1.19-1.14 (t, 3H, *J*= 7.50) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.10 (s), 168.67 (s), 164.60 (s), 163.37 (s), 162.47 (s), 155.21 (s), 131.84 (d), 131.30 (s), 129.14 (d, 2C), 126.23 (d, 2C), 105.91 (d), 105.00 (s), 100.47 (d), 96.81 (s), 66.47 (d), 66.42 (t), 61.78 (t), 51.40 (t), 13.88 (q) ppm.

LC-MS-ESI: t_R = 7.67 min, m/z calcd 425.15, m/z found 426.01 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 426.1552, found 426.1275.

Compound 7m: Diethyl 2,2'-(5,7-dihydroxy-4-oxo-2-phenyl-4H-chromene-6,8-diyl)bis(2-morpholinoacetate)

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as a dark yellow powder. Yield = 12 %.

TLC (petrol ether: ethyl acetate = 7: 3): R_f = 0.64, melting point: 124 ^{o}C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.26 (s, 1H), 7.67-7.62 (dd, 2H, *J*= 2.2, 8.21 Hz), 7.29-7.22 (m, 3H), 6.41 (s, 1H), 4.60 (s, 1H), 4.56 (s, 1H), 3.93-3.85 (m, 2H), 3.84-3.77 (m, 2H), 3.50-3.40 (t, 8H, *J*= 4.50), 2.50-2.40 (m, 4H), 2.40-2.27 (m, 4H), 0.97-0.92 (t, 3H, *J*= 6.90), 0.90-0.86 (t, 3H, *J*= 6.90) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.13 (s), 170.77 (s), 170.06 (s), 170.04 (s), 169.37 (s), 163.44 (s), 163.07 (s), 162.93 (s), 160.51 (s), 154.97 (s), 131.82 (d), 131.94 (s), 129.04 (d, 2C), 126.15 (d, 2C), 105.62 (d), 98.31 (s), 66.65 (t), 66.49 (t), 63.83 (d), 62.07 (d), 61.20 (t), 60.93 (t), 50.70 (t), 50.53 (t), 13.90 (q), 13.79 (q) ppm.

LC-MS-ESI: t_R = 8.69 min, m/z calcd 596.24, m/z found 597.10 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd= 597.2448, found= 597.2416.

Compound 8m: 6-(2-Ethoxy-1-morpholinoallyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a dark yellow powder. Yield = 18 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.61, melting point: 190 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.77 (s, 1H), 7.94-7.91 (dd, 2H, *J*= 2.50, 7.14 Hz), 7.57-7.53 (m, 3H), 6.64 (s, 1H), 6.29 (s, 1H), 4.79 (s, 1H), 4.19-4.14 (m, 2H), 2.96-2.86 (m, 4H), 2.80-2.75 (m, 4H), 1.19-1.15 (t, 3H, *J*= 7.14) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.18 (s), 168.98 (s), 164.80 (s), 163.43 (s), 162.56 (s), 155.25 (s), 131.92 (d), 131.89 (s), 129.23 (d, 2C), 126.30 (d, 2C), 106.03 (d), 105.08 (s), 100.67 (d), 97.04 (s), 66.60 (d), 61.87 (t), 53.05 (t), 27.72 (t), 13.94 (q) ppm.

LC-MS-ESI: t_R = 8.74 min, m/z calcd 441.16, m/z found 442.66 $[M+H]^+$.

Compound 9m: Ethyl 2-(5,7-dihydroxy-4-oxo-2-phenyl-4H-chromen-6-yl)-2-(4-methylpiperazin-1-yl)acetate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as a dark yellow powder. Yield = 80 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.52, melting point: 166 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.75 (s, 1H), 7.94-7.91 (dd, 2H, *J*= 1.75, 7.76 Hz), 7.55-7.50 (m, 3H), 6.64 (s, 1H), 6.28 (s, 1H), 4.71 (s, 1H), 4.19-4.14 (m, 2H), 2.81-2.42 (m, 8H), 2.29 (s, 3H), 1.19-1.14 (t, 3H, *J*= 7.15) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.19 (s), 168.99 (s), 165.00 (s), 163.37 (s), 162.24 (s), 155.15 (s), 131.86 (d), 131.39 (s), 129.19 (d, 2C), 126.27 (d, 2C), 105.91 (d), 104.96 (s), 100.85 (d), 97.36 (s), 66.35 (d), 61.73 (t), 54.63 (t), 51.02 (t), 45.69 (q), 13.93 (q) ppm.

LC-MS-ESI: t_R = 8.06 min, m/z calcd 438.18, m/z found 439.10 $[M+H]^+$.

Compound 10m: Ethyl 2-(2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4-oxo-4H-chromen-6-yl)-2-morpholinoacetate

The compound was purified by column chromatography on silica gel with dichloromethane: methanol = 95: 5. It was obtained as a brownish powder. Yield = 72 %.

TLC (dichloromethane: methanol = 9: 1): R_f = 0.63, melting point: 250 °C.

^{1}H NMR (CH_3OD, 500 MHz): 7.89-7.84 (br s, 1H), 7.75-771 (br s, 1H), 7.64-7.59 (d, 1H, *J*= 8.67 Hz), 6.86-6.75 (s, 1H), 6.07 (s, 1H), 4.07-4.02 (m, 2H), 3.66-3.62 (t, 4H, *J*= 4.88), 2.60-2.55 (m, 4H), 1.05-1.00 (t, 3H, *J*= 7.04) ppm.

^{13}C NMR (CH_3OD, 125 Hz): 177.31 (s), 171.18 (s), 165.19 (s), 164.87 (d), 162.66 (s), 155.62 (s), 148.79 (s), 148.14 (s) 146.31 (s), 137.42 (s), 124.04 (s), 122.21 (d), 116.32 (d), 104.76 (s), 99.66 (d), 99.12 (s), 67.77 (t), 66.05 (d), 62.66 (t), 52.31 (t), 14.30 (q) ppm.

LC-MS-ESI: t_R = 6.74 min, m/z calcd 473.13, m/z found 474.02 $[M+H]^+$.

Compound 11m: 2-(3,4-Dihydroxyphenyl)-6-(2-ethoxy-1-morpholinoallyl)-3,5,7-trihydroxy-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with dichloromethane: methanol = 98: 2. It was obtained as a brownish yellow powder. Yield = 36 %.

TLC (dichloromethane: methanol = 9: 1): R_f = 0.68, melting point: 211 °C.

^{1}H NMR (CH_3OD, 500 MHz): 7.80-7.78 (d, 1H, *J*=2.23 Hz), 7.75-7.72 (dd, 1H, *J*=2.23, 8.36) 7.02-6.98 (d, 1H, *J*=8.38 Hz), 6.28 (s, 1H), 4.81 (s, 1H), 4.21-4.13(m, 2H), 2.97-2.84 (m, 4H), 2.80-2.74 (t, , 4H, *J*=4.47), 1.20-1.16 (t, , 3H, *J*=7.26) ppm.

^{13}C NMR (CH_3OD, 125 Hz): 175.22 (s), 169.57 (s), 165.27 (s), 161.48 (s), 154.42 (s), 146.52 (s), 145.44 (s), 143.80 (s), 135.89 (s), 123.69 (s), 121.81 (d), 115.77 (d), 114.95 (d), 103.50 (s), 100.34 (d), 97.11 (s), 66.94 (d), 62.38 (t), 53.27 (t), 27.96 (t), 14.16 (q) ppm.

LC-MS-ESI: t_R = 7.06 min, m/z calcd 489.11, m/z found 490.63 $[M+H]^+$.

Compound 12m: 5,7-Dihydroxy-6-(hydroxymethyl)-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as a yellow powder. Yield = 10 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.54, melting point: 118 °C.

^{1}H NMR (CH_3OD, 500 MHz): 7.88-7.85 (dd, 2H, *J*= 2.26, 8.28 Hz), 7.49-7.42 (m, 3H), 6.64 (s, 1H), 6.43 (s, 1H), 4.64 (s, 2H) ppm.

^{13}C NMR (CH_3OD, 125 Hz): 184.04 (s), 165.60 (s), 165.03 (s), 161.18 (s), 158.72 (s), 133.05 (d), 132.54 (s), 130.24 (d, 2C), 127.43 (d, 2C), 111.96 (s), 106.14 (d), 105.34 (s), 94.60 (d), 53.68 (t) ppm.

LC-MS-ESI: t_R = 9.64 min, m/z calcd 284.07, m/z found 284.94 $[M+H]^+$.

Compound 13m: Ethyl 2-(5,7-dihydroxy-4-oxo-2-phenyl-4H-chromen-6-yl)-2-(4-methoxyphenylamino)acetate.

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellow powder. Yield = 10 %.

TLC (petrol ether: ethyl acetate = 7: 3): R_f = 0.55, melting point: 152 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.78 (s, 1H), 7.87-7.83 (dd, 2H, *J*= 1.50, 7.80 Hz), 7.54-7.50 (m, 3H), 6.78-6.72 (m, 4H), 6.70 (s, 1H), 6.28 (s, 1H), 5.62 (s, 1H), 4.17-4.07 (m, 2H), 3.68 (s, 3H), 1.13-1.08 (t, 3H, *J*= 6.75) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.37 (s), 170.55 (s), 164.50 (s), 163.43 (s), 162.18 (s), 155.33 (s), 155.03 (s), 138.06 (s), 131.90 (d), 131.44 (s), 129.22 (d, 2C), 126.15 (d, 2C), 118.23 (d), 114.96 (d), 106.10 (d), 105.62 (s), 100.96 (d), 99.11 (s), 62.76 (t), 56.43 (d), 55.49 (q), 13.91 (q) ppm.

LC-MS-ESI: t_R = 10.47 min, m/z calcd 461.15, m/z found 461.95 $[M+H]^+$.

Compound 14m: Ethyl 2-(2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4-oxo-4H-chromen-6-yl)-2-(4-methoxyphenylamino)acetate

The compound was purified by column chromatography on silica gel with dichloromethane: methanol = 95: 5. It was obtained as a brownish black powder. Yield = 39 %.

TLC (dichloromethane: methanol = 9: 1): R_f = 0.63, melting point: 120 °C.

^{1}H NMR (CH_3OD, 500 MHz): 7.73-7.71 (d, 1H, *J*= 2.00), 7.59-7.55 (dd, 1H, *J*= 1.97, 7.87 Hz), 6.81-6.79 (d, 1H, *J*= 8.66), 6.64-6.61 (dd, 2H, *J*= 1.97, 6.69 Hz), 6.59-6.55 (dd, 2H, *J*= 1.97, 7.00 Hz), 6.12 (s, 1H), 5.64 (s, 1H), 4.11-4.01 (m, 2H), 3.54 (s, 3H), 1.04-1.00 (t, 3H, *J*= 7.09) ppm.

^{13}C NMR (CH_3OD, 125 Hz): 177.47 (s), 173.82 (s), 163.43 (s), 162.04 (s), 155.51 (s), 154.83 (s), 148.96 (s), 148.30 (s) 146.33 (s), 142.08 (s), 137.29 (s), 124.06 (s), 121.80 (d), 117.70 (d), 116.36 (d), 116.24 (d), 115.69 (d), 104.83 (s), 99.94 (d), 62.70 (t), 56.06 (q), 54.53 (d), 14.39 (q) ppm.

LC-MS-ESI: t_R = 8.17 min, m/z calcd 509.13, m/z found 509.98 $[M+H]^+$.

Compound 15m: 8-(Amino(4-methoxyphenyl)methyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as yellow powder. Yield = 42 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.54, melting point: 225 ^{o}C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.79 (s, 1H), 12.68 (s, 1H), 7.79-7.75 (dd, 2H, *J*= 1.56, 8.24 Hz), 7.56-7.52 (m, 3H), 7.39-7.35 (d, 2H, *J*= 8.20 Hz) 6.84-6.81 (d, 2H, *J*= 1.56, 8.24 Hz), 6.54 (s, 1H), 6.34 (s, 1H), 4.49 (s, 1H), 3.73 (s, 3H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.22 (s), 164.17 (s), 163.38 (s), 161.54 (s), 159.73 (s), 154.81 (s), 131.75 (d), 130.00 (s), 129.12 (d, 2C), 126.18 (d, 2C), 106.24 (d), 105.10 (s), 103.96 (s), 100.73 (d), 68.31 (d), 55.23 (q) ppm.

LC-MS-ESI: t_R = 9.64 min, m/z calcd 389.13, m/z found 374.09 $[M\text{-}NH_2+H]^+$.

HRMS: $[M\text{-}CH_3+H]$, calcd 375.1106, found 375.1076.

Compound 16m: 6-(Amino(4-methoxyphenyl)methyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 7: 3. It was obtained as yellow powder. Yield = 18 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.49, melting point: 204 ^{o}C.

^{1}H NMR ($CDCl_3$, 500 MHz): 14.00 (s, 1H), 13.08 (s, 1H), 7.85-7.81 (dd, 2H, *J*= 2.30, 7.60 Hz), 7.51-7.45 (m, 3H), 7.42-7.38 (d, 2H, *J*= 8.56 Hz) 6.84-6.80 (d, 2H, *J*= 1.50, 8.20 Hz), 6.56 (s, 1H), 6.45 (s, 1H), 4.87 (s, 1H), 3.74 (s, 3H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.28 (s), 163.99 (s), 163.66 (s), 159.42 (s), 159.25 (s), 157.02 (s), 131.67 (d), 131.14 (s), 130.46 (s), 129.02 (d, 2C), 126.22 (d, 2C), 108.91 (s), 105.48 (d), 104.56 (s), 94.95 (d), 67.74 (d), 55.20 (q) ppm.

LC-MS-ESI: t_R = 9.15 min, m/z calcd 389.13, m/z found 373.95 $[M\text{-}NH_2+H]^+$.

HRMS: $[M\text{-}OCH_3+H]$, calcd 375.1106, found 375.1121.

Compound 17m: 6-(Amino(phenyl)methyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellow powder. Yield = 47 %.

TLC (petrol ether: ethyl acetate = 7: 3): R_f = 0.45, melting point: 195 ^{o}C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.75 (s, 1H), 13.15 (s, 1H), 7.85-7.81 (m, 2H), 7.56-7.51 (d, 2H, *J*= 7.41 Hz), 7.50-7.75 (m, 3H),7.35-7.30 (d, 2H, *J*= 7.92 Hz),7.30-7.25 (m, 1H), 6.58 (s, 1H), 6.49 (s, 1H), 4.96 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.18 (s), 163.89 (s), 163.57 (s), 159.31 (s), 155.99 (s), 138.44 (s), 131.60 (d), 131.27 (s), 128.93 (d, 2C), 128.11 (d), 126.11 (d, 2C), 108.55 (s), 105.41 (d), 104.49 (s), 94.89 (d), 68.35 (d) ppm.

LC-MS-ESI: t_R = 8.99 min, m/z calcd 359.12, m/z found 344.07 $[M\text{-}NH_2+H]^+$.

Compound 18m: 6-(Amino(4-nitrophenyl)methyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as yellow powder. Yield = 22 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.55, melting point: 292 ^{o}C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.20 (s, 1H), 8.18-8.13 (d, 2H, *J*= 8.76 Hz), 7.84-7.80 (d, 2H, *J*= 7.76 Hz), 7.73-7.67 (d, 2H, *J*= 8.76 Hz), 7.52-7.45 (m, 3H), 6.58 (s, 1H), 6.47 (s, 1H), 5.04 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.27 (s), 164.00 (s), 163.31 (s), 159.44 (s), 157.34 (s), 147.62 (s), 145.83 (s), 131.85 (d), 131.18 (s), 129.07 (d, 2C), 126.24 (d, 2C), 124.13 (d) 107.26 (s), 105.52 (d), 104.66 (s), 95.82 (d), 67.59 (d) ppm.

LC-MS-ESI: t_R = 16.91 min, m/z calcd 404.10, m/z found 390.05 $[M\text{-}NH_2+H]^+$.

Compound 19m: 6-(Amino(4-bromophenyl)methyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 85: 15. It was obtained as a dark yellow powder. Yield = 15 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.60, melting point: 163 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.96 (s, 1H), 7.66-7.64 (dd, 2H, *J*=1.67, 7.68 Hz), 7.34-7.28 (m, 3H), 7.26-7.23 (d, 2H, *J*=8.34Hz), 7.22-7.18 (d, 2H, *J*=8.68 Hz), 6.39 (s, 1H), 6.28 (s, 1H), 4.71 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.54 (s), 164.05 (s), 163.89 (s), 159.60 (s), 157.41 (s), 137.80 (s), 132.28 (d), 132.00 (d), 131.57 (s), 129.29 (d, 3C), 126.49 (d, 2C), 122.45 (s), 108.38 (s), 105.77 (d), 104.86 (s), 95.33 (d), 67.98 (d) ppm.

LC-MS-ESI: t_R = 9.98 min, m/z calcd 437.01, m/z found 421.47 $[M\text{-}NH_2]^+$.

Compound 20m: 6-(Amino(4-chlorophenyl)methyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 85: 15. It was obtained as a dark yellow powder. Yield = 60 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.60, melting point: 187 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.11 (s, 1H), 7.80-7.77 (dd, 2H, *J*=1.95, 8.27 Hz), 7.47-7.37 (m, 5H), 7.25-7.21 (d, 2H, *J*=8.35Hz), 6.53 (s, 1H), 6.42 (s, 1H), 4.86 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.28 (s), 163.80 (s), 163.66 (s), 159.34 (s), 157.15 (s), 137.03 (s), 134.04 (s), 131.74 (d), 131.31 (s), 129.04 (d, 2C), 126.23 (d, 2C), 108.20 (s), 105.51 (d), 104.60 (s), 95.07 (d), 67.95 (d) ppm.

LC-MS-ESI: t_R = 9.98 min, m/z calcd 393.06, m/z found 379.75 $[M\text{-}NH_2\text{+}H]^+$.

Compound 21m: 6-(Amino(4-fluorophenyl)methyl)-5,7-dihydroxy-2-phenyl-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 85: 15. It was obtained as a dark yellow powder. Yield = 55 %.

TLC (petrol ether: ethyl acetate = 6: 4): R_f = 0.57, melting point: 166 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.15 (s, 1H), 7.87-7.77 (dd, 2H, *J*=1.98, 8.20 Hz), 7.54-7.39 (m, 5H), 7.01-7.94 (dt, 2H, *J*=1.30, 7.14, 8.47 Hz), 6.57 (s, 1H), 6.46 (s, 1H), 4.91 (s, 1H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.31 (s), 163.79 (s), 163.72 (s), 159.32 (s), 157.12 (s), 134.35 (s), 134.32 (s), 131.73 (d), 131.35 (s), 129.04 (d, 2C), 126.23 (d, 2C), 108.49 (s), 105.52 (d), 104.60 (s), 95.05 (d), 67.58 (d) ppm.

LC-MS-ESI: t_R = 9.26 min, m/z calcd 377.11, m/z found 362.53 $[M\text{-}NH_2]^+$.

3.3.New type of synthesis of chromene-flavone compounds

The reaction of chrysin with alkyl isocyanides and dialkyl acetylene dicarboxylate provided the foundation for the synthesis of fifteen new compounds (***1f-15f***). Each of these compounds shows a tricyclic nucleus containing features of flavones as well as chromenes as a common structural element (***Fig 28***). The reaction was performed at -20 °C during the isonitrile addition step. The reaction was completed at r.t. The solvent used was CH_2Cl_2 for all compounds (***Fig 27***). The reaction times varied (see ***Table 16***).

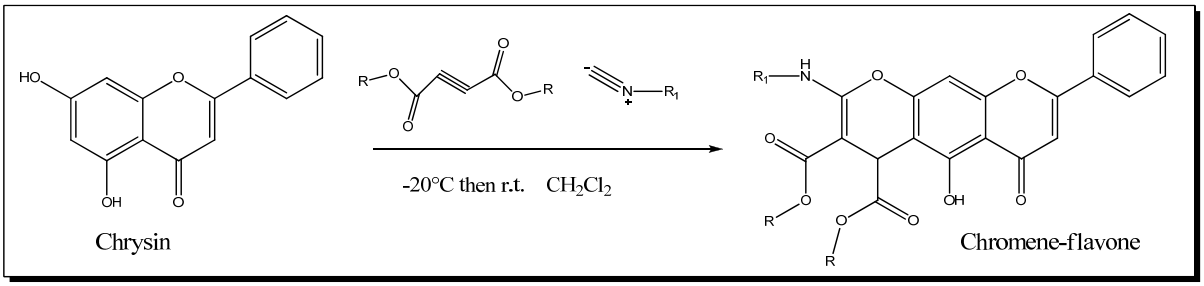


Fig 27: *General scheme employed for the synthesis chromene-flavones. Please note that this is a general scheme only. Details can be found in table 16 and in the text.*

Table 16 lists the isocyanide and acetylene dicarboxylate elements as well as reaction times used and yields obtained.

Table 16: *Individiual building blocks of the chromene-flavone reaction. Reaction times and yields are provided.*

Compound Nr	R	R1	Reaction time	Yield %
1f	methyl	*t*- butyl	24 hrs	63
2f	methyl	*c*-hexyl	36hrs	76
3f	*t*-butyl	*t*-butyl	24hrs	55
4f	*t*-butyl	*c*-hexyl	36hrs	27
5f	ethyl	*t*-butyl	24hrs	55
6f	ethyl	*c*-hexyl	24hrs	65
7f	methyl	*i*-propyl	24hrs	69
8f	ethyl	*i*-propyl	24hrs	70
9f	methyl	1,1,3,3- tetramethyl butyl	36 hrs	85
10f	ethyl	1,1,3,3- tetramethyl butyl	36 hrs	82
11f	*t*-butyl	1,1,3,3- tetramethyl butyl	48 hrs	65
12f	methyl	*n*-pentyl	36 hrs	25
13f	ethyl	*n*-pentyl	36 hrs	33
14f	methy	*n*-butyl	24 hrs	55
15f	ethyl	*n*-butyl	24 hrs	33

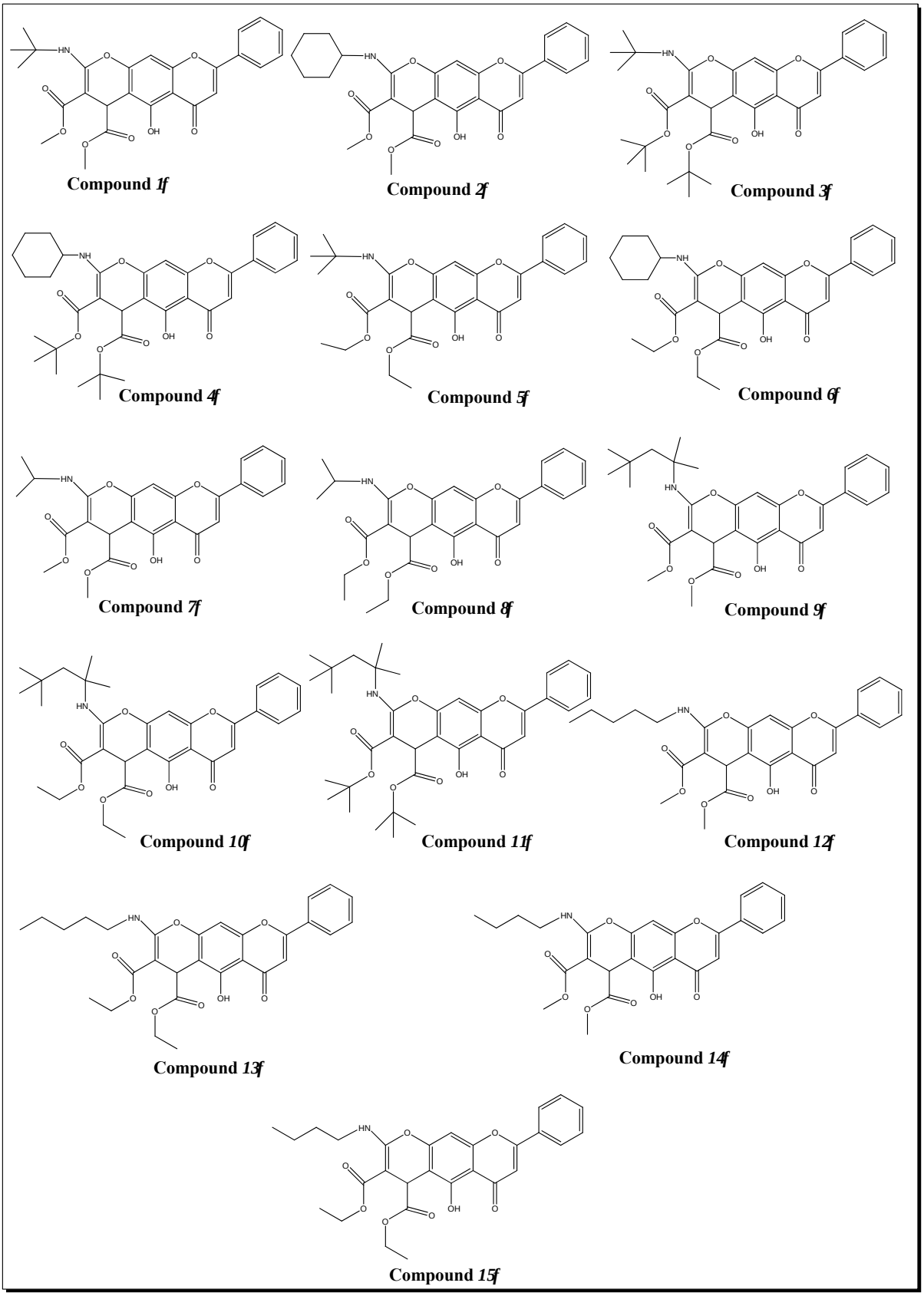


Fig 28: *Chemical structures of chromene-flavones.*

Compound 1f: Dimethyl 2-(tert-butylamino)-5-hydroxy-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellowish powder. Yield = 63 %.

TLC (petrol ether: ethyl acetate = 7: 3): R_f = 0.45, melting point: the compound decomposes at 170 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.81 (s, 1H), 8.91-8.83 (br s, 1H), 8.10-8.05 (dd, 2H, *J*=2.00, 7.69 Hz,), 7.61-7.56 (m, 3H), 6.76 (s, 1H), 6.57 (s, 1H), 5.11 (s, 1H), 3.77 (s, 3H), 3.63 (s,3H), 1.49 (s, 9H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.12 (s), 170.32 (s), 166.91 (s), 162.92 (s), 158.96 (s), 158.65 (s), 151.92 (s), 151.23 (s), 129.64 (d, 2C), 128.43 (s), 126.73 (d, 2C), 124.11(d), 105.70 (s), 103.58 (d), 97.99 (s), 97.16 (d), 69.44 (s), 50.33 (q), 49.92 (s), 48.54 (q), 33.28 (d), 28.07 (q, 3C) ppm.

LC-MS-ESI: t_R = 16.91 min, m/z calcd 479.16, m/z found 480.1 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 480.1658, found 480.1754.

Compound 2f: Dimethyl 2-(cyclohexylamino)-5-hydroxy-6-oxo-8-phenyl-4,6 dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellowish powder. Yield = 76 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.54, melting point: 224-228 °C

^{1}H NMR ($CDCl_3$, 500 MHz): 12.78 (s, 1H), 8.77-8.59 (br s, 1H), 8.09-8.03 (dd, 2H, *J*=2.00, 7.69 Hz), 7.59-7.54 (m, 3H), 6.74 (s, 1H), 6.53 (s, 1H), 5.08 (s, 1H), 3.84-3.77(m, 1H), 3.75 (s, 3H), 3.60 (s, 3H), 2.06-1.99(m, 1H), 1.94-1.88 (m, 1H), 1.82-1.70 (m, 2H), 1.45-1.22 (m, 6H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.65 (s), 172.95 (s), 169.36 (s), 164.60 (s), 161.13 (s), 160.09 (s), 154.70 (s), 153.76 (s), 132.15 (d), 130.95 (s), 129.24 (d, 2C), 126.64 (d, 2C), 108.19 (s),

106.10 (d), 100.45 (s), 99.84 (d), 71.11 (s), 52.41 (q), 51.08 (q), 50.07 (d), 35.92 (d), 33.91 (t), 33.47 (t), 25.46 (t, 2C), 24.50 (t), 24.42 (t) ppm.

LC-MS-ESI: t_R = 13.45 min, m/z calcd 505.17, m/z found 506.1 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 506.1814, found 506.1849.

Compound 3f: Di-tert-butyl 2-(tert-butylamino)-5-hydroxy-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellowish powder. Yield = 55 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.69, melting point: 115 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.66 (s, 1H), 8.66-8.59 (br s, 1H), 8.08-8.05 (dd, 2H, *J*=1.65, 8.24 Hz), 7.57-7.49 (m, 3H), 6.76 (s, 1H), 6.53 (s, 1H), 4.98 (s, 1H), 1.53 (q, 9H), 1.45(q, 9H), 1.27 (q, 9H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.72 (s), 172.02 (s), 168.59 (s), 163.91 (s), 161.21 (s), 160.72 (s), 154.75 (s), 153.35 (s), 132.15 (d), 130.82 (s), 129.08 (d, 2C), 126.56 (d, 2C), 107.96 (s), 105.61 (d), 101.59 (s), 99.55 (d), 80.85 (s), 79.29 (s), 52.54 (s), 37.25 (d), 30.64 (q, 3C), 28.49 (q, 3C), 27.94 (q, 3C) ppm.

LC-MS-ESI: t_R = 23.15 min, m/z calcd 563.25, m/z found 566.3 $[M+3H]^+$.

HRMS: $[M+H]^+$, calcd 564.2597, found 564.2491.

Compound 4f: Di-tert-butyl 2-(cyclohexylamino)-5-hydroxy-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellowish powder. Yield = 27 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.69, melting point: 167 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.64 (s, 1H), 8.56-8.36 (br s, 1H), 8.09-8.03 (dd, 2H, *J*=1.92, 8.19 Hz), 7.58-7.49 (m, 3H), 6.76 (s, 1H), 6.52 (s, 1H), 4.98 (s, 1H), 3.79-3.70 (m, 1H), 2.07-

1.99 (m, 1H), 1.95-1.89 (m, 1H), 1.79-1.70 (m, 2H), 1.64-1.58 (m, 1H), 1.53 (s, 9H), 1.40-1.34 (m, 1H), 1.27 (s, 9H), 1.25-1.22 (m, 4H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 183.02 (s), 172.53 (s), 168.65 (s), 164.19 (s), 160.96 (s), 160.21 (s), 155.34 (s), 153.63 (s), 132.43 (d), 131.12 (s), 129.36 (d, 2C), 126.85 (d, 2C), 108.22 (s), 105.91 (d), 101.86 (s), 100.02 (d), 81.18 (s), 79.50 (s), 50.49 (d), 37.56 (d), 34.37 (t), 33.94 (t), 29.93 (t), 28.80 (q, 3C), 28.23 (q, 3C), 25.79 (t), 25.04 (t), 24.97 (t) ppm.

LC-MS-ESI: t_R = 16.77 min, m/z calcd 589.27, m/z found 590.1 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 590.2753, found 590.2752.

Compound 5f: Diethyl 2-(tert-butylamino)-5-hydroxy-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8.5: 1.5. It was obtained as a yellowish powder. Yield = 55 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.61, melting point: 177 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.73 (s, 1H), 8.84-8.73 (br s, 1H), 8.08-8.04 (dd, 2H, *J*=1.67, 7.95 Hz), 7.58-7.51 (m, 3H), 6.75 (s, 1H), 6.54 (s, 1H), 5.08 (s, 1H), 4.27-4.21 (m, 1H), 4.19-4.12 (m, 1H), 4.10-3.98 (m, 2H), 1.46 (s, 9H), 1.32-1.28 (t, 3H, *J*= 7.14 Hz), 1.12-1.09 (t, 3H, *J*= 7.14 Hz) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.64 (s), 172.72 (s), 168.98 (s), 164.31 (s), 161.39 (s), 161.03 (s), 154.51 (s), 153.68 (s), 132.14 (d), 130.78 (s), 129.13 (d, 2C), 126.60 (d, 2C), 108.13(s), 105.93 (d), 100.65 (s), 99.62 (d), 72.16 (s), 61.07 (t), 59.51 (t), 52.74 (d), 35.88 (d) 30.69 (q, 3C), 14.53 (q), 13.90 (q) ppm.

LC-MS-ESI: t_R = 19.59 min, m/z calcd 507.19, m/z found 508.1 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 508.1971, found 508.1844.

Compound 6f: Diethyl 2-(cyclohexylamino)-5-hydroxy-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 85: 15. It was obtained as a yellowish powder. Yield = 65 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.72, melting point: 39-42 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.74 (s, 1H), 8.71-8.56 (br s, 1H), 8.09-8.04 (dd, 2H, *J*=1.58, 8.05 Hz), 7.57-7.52 (m, 3H), 6.75 (s, 1H), 6.53 (s, 1H), 5.08 (s, 1H), 4.27-4.21 (m, 1H), 4.18-4.13 (m, 1H), 4.10-3.99 (m, 2H), 3.85-3.73 (m, 1H), 2.04-1.98 (m, 1H), 1.94-1.87 (m, 1H), 1.81-1.70 (m, 2H), 1.64-1.55 (m, 2H), 1.44-1.32 (m, 4H), 1.32-1.28 (t, 3H, *J*=7.14 Hz), 1.13-1.07 (t, 3H, *J*=7.14 Hz) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.68 (s), 172.91 (s), 168.97 (s), 164.35 (s), 161.02 (s), 160.03 (s), 154.80 (s), 153.70 (s), 132.16 (d), 130.91 (s), 129.16 (d, 2C), 126.65 (d, 2C), 108.14 (s), 105.91 (d), 100.63 (s), 98.83 (d), 71.34 (s), 61.06 (t), 59.26 (t), 50.04 (d), 36.03 (d), 33.89 (t), 33.51 (t), 25.48 (t), 24.50 (t), 24.49 (t), 14.57 (q), 14.10 (q) ppm.

LC-MS-ESI: t_R = 17.15 min, m/z calcd 533.2, m/z found 534.58 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 534.2127, found 534.2027.

Compound 7f: Dimethyl 5-hydroxy-2-(isopropylamino)-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellowish powder. Yield = 69 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.58, melting point: 117 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.79 (s, 1H), 8.65-8.45 (br s, 1H), 8.09-8.03 (dd, 2H, *J*=1.74, 7.24 Hz), 7.59-7.54 (m, 3H), 6.75 (s, 1H), 6.53 (s, 1H), 5.08 (s, 1H), 4.15-4.08 (m, 1H), 3.75 (s, 3H), 3.60 (s, 3H), 1.33-1.30 (d, 3H, *J*=6.38 Hz), 1.26-1.26 (d, 3H, *J*=6.96 Hz) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.66 (s), 172.94 (s), 169.37 (s), 164.63 (s), 161.16 (s), 160.13 (s), 154.67 (s), 153.78 (s), 132.17 (d), 130.96 (s), 129.25 (d, 2C), 126.65 (d, 2C), 108.23 (s), 106.12 (d), 100.94 (s), 99.84 (d), 71.21 (s), 52.42 (q), 51.09 (q), 43.44 (d), 35.90 (d), 23.82 (q), 23.54 (q) ppm.

LC-MS-ESI: t_R = 14.44 min, m/z calcd 465.14, m/z found 466.58 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 466.1501, found 466.1405.

Compound 8f: Diethyl 5-hydroxy-2-(isopropylamino)-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 8: 2. It was obtained as a yellowish powder. Yield = 70 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f 0.56, melting point: 123 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.73 (s, 1H), 8.60-8.44 (br s, 1H), 8.09-8.05 (dd, 2H, *J*=2.12, 7.30 Hz), 7.56-7.51 (m, 3H), 6.75 (s, 1H), 6.53 (s, 1H), 5.08 (s, 1H), 4.29-4.21 (m, 1H), 4.18-4.00 (m, 4H), 1.32-1.28 (m, 6H), 1.26-1.23 (d, 3H, *J*=6.45 Hz), 1.12-1.08 (t, 3H, *J*=6.83 Hz) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.67 (s), 172.88 (s), 168.96 (s), 164.35 (s), 161.04 (s), 160.07 (s), 154.57 (s), 153.70 (s), 132.16 (d), 130.90 (s), 129.15 (d, 2C), 126.64 (d, 2C), 108.16 (s), 105.97 (d), 100.58 (s), 99.81 (d), 71.43 (s), 61.10 (t), 59.47 (t), 43.39 (d), 36.00 (d), 23.78 (q), 23.57 (q), 14.55 (q), 144.09 (q) ppm.

LC-MS-ESI: t_R = 18.56 min, m/z calcd 493.17, m/z found 494.1 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 494.1814, found 494.1807.

Compound 9f: Dimethyl 5-hydroxy-6-oxo-8-phenyl-2-(2,4,4-trimethylpentan-2-ylamino)-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 85: 15. It was obtained as a yellowish powder. Yield = 85 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.75, melting point: 195-197 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.80 (s, 1H), 8.96-8.77 (br s, 1H), 8.09-7.96 (dd, 2H, *J*=2.58, 6.75 Hz), 7.59-7.49 (m, 3H), 6.73 (s, 1H), 6.54 (s, 1H), 5.08 (s, 1H), 3.74 (s, 3H), 3.58 (s, 3H), 1.86-1.74 (q, 2H, *J*=15.11, 16.75 Hz), 1.51 (s, 3H), 1.49 (s, 3H), 0.98 (s, 9H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.59 (s), 172.70 (s), 169.34 (s), 164.56 (s), 161.29 (s), 161.12 (s), 154.35 (s), 153.69 (s), 132.09 (d), 130.88 (s), 129.19 (d, 2C), 126.58 (d, 2C), 108.16 (s), 106.00 (d), 100.53 (s), 99.57 (d), 71.58 (s), 56.29 (s), 53.33 (t), 52.29 (q), 51.06 (q), 35.78 (d), 31.64 (s), 31.46 (q), 31.73 (q), 31.30 (q) ppm.

LC-MS-ESI: t_R = 16.72 min, m/z calcd 535.22, m/z found 536.66 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 536.2284, found 480.2370 $[M-C(CH_3)_3+H]^+$.

Compound 10f: Diethyl 5-hydroxy-6-oxo-8-phenyl-2-(2,4,4-trimethylpentan-2-ylamino)-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 90: 10. It was obtained as a yellowish powder. Yield = 82 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.75, melting point: 172-177 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.77 (s, 1H), 8.91-8.75 (br s, 1H), 8.09-8.00 (dd, 2H, *J*=2.61, 7.82 Hz), 7.56-7.51 (m, 3H), 6.74 (s, 1H), 6.54 (s, 1H), 5.09 (s, 1H), 4.27-4.20 (m, 1H), 4.19-4.12 (m, 1H), 4.06-4.00 (m, 2H), 1.85-1.75 (q, 2H, *J*=14.99, 16.75 Hz), 1.52 (s, 3H), 1.47 (s, 3H), 1.32-1.27 (t, 3H, *J*=7.17 Hz), 1.12-1.07 (t, 3H, *J*=7.17 Hz), 0.98 (s, 9H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.63 (s), 172.55 (s), 169.00 (s), 164.34 (s), 161.17 (s), 161.02 (s), 154.43 (s), 153.67 (s), 132.11 (d), 130.84 (s), 129.11 (d, 2C), 126.61 (d, 2C), 108.12 (s), 105.87 (d), 100.67 (s), 99.54 (d), 71.86 (s), 60.99 (t), 59.43 (t), 56.23 (s), 53.34 (t), 35.96 (d), 31.64 (q), 31.48 (s), 31.41 (q, 3C), 31.30 (q), 14.49 (q), 14,04 (q) ppm.

LC-MS-ESI: t_R = 16.73 min, m/z calcd 563.26, m/z found 564.18 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 564.2597, found 564.2658.

Compound 11f: Di-tert-butyl 5-hydroxy-6-oxo-8-phenyl-2-(2,4,4-trimethylpentan-2-ylamino)-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 90: 10. It was obtained as a yellowish powder. Yield = 65 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f 0.71, melting point: 82 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.68 (s, 1H), 8.77-8.61 (br s, 1H), 8.09-8.05 (dd, 2H, *J*=1.64, 8.06 Hz), 7.55-7.48 (m, 3H), 6.75 (s, 1H), 6.53 (s, 1H), 4.98 (s, 1H), 1.85-1.73 (q, 2H, *J*=15.12, 16.75 Hz), 1.52 (s, 9H), 1.50 (s, 3H), 1.47 (s, 3H), 1.27 (s, 9H), 0.97 (s, 9H) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.84 (s), 171.99 (s), 168.67 (s), 164.06 (s), 161.14 (s), 160.47 (s), 154.74 (s), 153.53 (s), 132.15 (d), 130.94 (s), 129.08 (d, 2C), 126.57 (d, 2C), 108.02 (s), 105.75 (d), 101.87 (s), 99.60 (d), 80.81 (s), 79.28 (s), 73.53 (s), 56.20 (s), 53.94 (t), 37.49 (d), 31.76 (s), 31.68 (q), 31.49 (q, 3C), 31.23 (q), 28.49 (q, 3C), 27.96 (q, 3C) ppm.

LC-MS-ESI: t_R = 19.68 min, m/z calcd 619.31, m/z found 620.73 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 620.3223, found 620.3194.

Compound 12f: Dimethyl 5-hydroxy-6-oxo-2-(pentylamino)-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 90: 10. It was obtained as a yellowish powder. Yield = 25 %.

TLC (petrol ether: ethyl acetate = 7:3), R_f = 0.63, melting point: 167 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.78 (s, 1H), 8.77-8.5 (br s, 1H), 8.11-8.98 (m, 2H), 7.59-7.53 (m, 3H), 6.74 (s, 1H), 6.52 (s, 1H), 5.08 (s, 1H), 3.75 (s, 3H), 3.59 (s, 3H), 3.49-3.26 (m, 2H), 1.66-1.54 (m, 2H), 1.45-1.33 (m, 4H), 0.93-0.87 (t, 3H *J*= 7.19 Hz) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.66 (s), 172.95 (s), 169.38 (s), 164.63 (s), 161.13 (s), 160.64 (s), 154.61 (s), 153.78 (s), 132.16 (d), 130.94 (s), 129.24 (d, 2C), 126.65 (d, 2C), 108.22 (s), 106.10 (d), 100.39 (s), 99.88 (d), 71.08 (s), 52.41 (q), 51.10 (q), 41.05 (t), 35.37 (d), 29.95 (t), 28.93 (t), 22.29 (t), 13.92 (q) ppm.

LC-MS-ESI: t_R = 15.83 min, m/z calcd 493.17, m/z found 494.53 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 494.1814, found 494.1788.

Compound 13f: Diethyl 5-hydroxy-6-oxo-2-(pentylamino)-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 85: 15. It was obtained as a yellowish powder. Yield = 33 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.61, melting point: 138 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.74 (s, 1H), 8.77-8.45 (br s, 1H), 8.10-8.05 (dd, 2H, *J*=1.69, 7.95 Hz), 7.58-7.50 (m, 3H), 6.76 (s, 1H), 6.52 (s, 1H), 5.08 (s, 1H), 4.28-4.20 (m, 1H), 4.18-4.12 (m, 1H), 4.10-3.98 (m, 2H), 3.48-3.37 (m, 2H), 1.66-1.58 (m, 2H), 1.40-1.33 (m, 4H), 1.32-1.28 (t, 3H, *J*= 7.44 Hz), 1.12-1.07 (t, 3H, *J*= 7.14 Hz), 0.92-0.87 (t, 3H, *J*= 7.14 Hz) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.69 (s), 172.91 (s), 169.01 (s), 164.38 (s), 161.03 (s), 160.59 (s), 154.71 (s), 153.73 (s), 132.18 (d), 130.91 (s), 129.16 (d, 2C), 126.67 (d, 2C), 108.18(s), 105.98 (d), 100.57 (s), 99.87 (d), 71.29 (s), 61.10 (t), 59.49 (t), 41.04 (t), 36.07 (d), 29.98 (t), 28.94 (t), 22.30 (t), 14.56 (q), 14.10 (q), 13.93 (q) ppm.

LC-MS-ESI: t_R = 19.59 min, m/z calcd 521.2, m/z found 522.63 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 522.2127, found 522.2098.

Compound 14f: Dimethyl 2-(butylamino)-5-hydroxy-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 90: 10. It was obtained as a yellowish powder. Yield = 55 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.65, melting point: 77 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.78 (s, 1H), 8.78-8.57 (br s, 1H), 8.11-8.03 (m, 2H), 7.61-7.53 (m, 3H), 6.75 (s, 1H), 6.53 (s, 1H), 5.08 (s, 1H), 3.75 (s, 3H), 3.60 (s, 3H), 3.51-3.36 (m, 2H), 1.63-1.58 (m, 2H), 1.45-1.38(m, 2H), 0.98-0.91 (t, 3H *J*= 7.34) ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.66 (s), 172.96 (s), 169.39 (s), 164.63 (s), 161.14 (s), 160.66 (s), 154.61 (s), 153.79 (s), 132.16 (d), 130.94 (s), 129.25 (d, 2C), 126.65 (d, 2C), 108.23 (s), 106.11 (d),100.40 (s), 99.88 (d), 71.09 (s), 52.41 (q), 51.10 (q), 40.74 (t), 35.96 (d), 32.32 (t), 29.67 (t), 13.71 (q) ppm.

LC-MS-ESI, t_R = 15.22 min, m/z calcd 479.16, m/z found 480.57 $[M+H]^+$

HRMS: $[M+H]^+$, calcd 480.1658, found 480.1653.

Compound 15f: Diethyl 2-(butylamino)-5-hydroxy-6-oxo-8-phenyl-4,6-dihydropyrano[3,2-g]chromene-3,4-dicarboxylate

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 85: 15. It was obtained as a yellowish powder. Yield = 33 %.

TLC (petrol ether: ethyl acetate = 7: 3), R_f = 0.68, melting point: 147 °C.

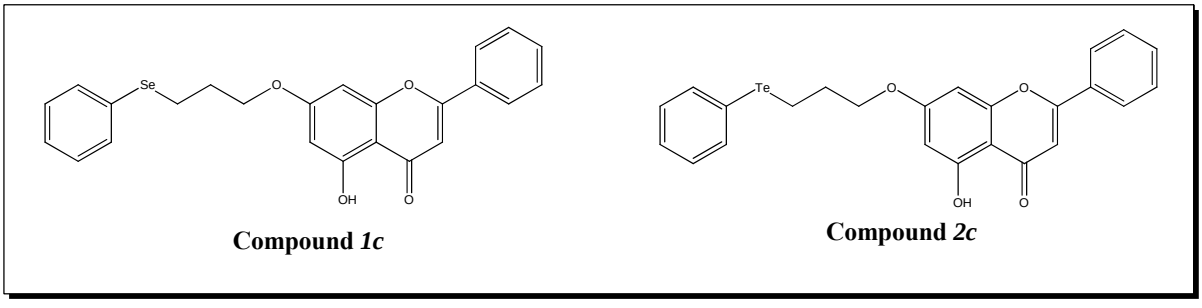
^{1}H NMR ($CDCl_3$, 500 MHz): 12.78 (s, 1H), 8.76-8.50 (br s, 1H), 8.09-8.05 (dd, 2H, *J*=1.99, 7.51 Hz), 7.58-7.53 (m, 3H), 6.76 (s, 1H), 6.53 (s, 1H), 5.08 (s, 1H), 4.27-4.21 (m, 1H), 4.19-4.13 (m, 1H), 4.09-3.98 (m, 2H), 3.49-3.37 (m, 2H), 1.64-1.57 (m, 2H), 1.47-1.39 (m, 2H), 1.32-1.28 (t, 3H, *J*= 7.07 Hz), 1.12-1.08 (t, 3H, *J*= 7.29 Hz), 0.96-0.92 (t, 3H, *J*= 7.29 Hz) ppm.

^{13}C NMR ($CDCl_3$, 125.79Hz): 182.95 (s), 173.17 (s), 169.27 (s), 164.64 (s), 161.29 (s), 160.87 (s), 154.97 (s), 153.98 (s), 132.43 (d), 131.17 (s), 129.42 (d, 2C), 126.93 (d, 2C), 108.44(s), 106.24 (d), 100.83 (s), 100.13 (d), 71.55 (s), 61.37 (t), 59.75 (t), 40.97 (t), 36.32 (d), 32.62 (t), 20.22 (t), 14.81 (q), 14.35 (q), 13.97 (q) ppm.

LC-MS-ESI, t_R = 17.78 min, m/z calcd 507.19, m/z found 508.63 $[M+H]^+$.

HRMS: $[M+H]^+$, calcd 508.1971, found 508.1953.

3.4. Synthesis of 5-hydroxy-2-phenyl-7-(3-(phenylselanyl)propoxy)-4H-chromen-4-one and 5-hydroxy-2-phenyl-7-(3-(phenyltellanyl)propoxy)-4H-chromen-4-one



Compound *1c* **Compound *2c***

Fig 29: *Chemical structure of compounds* ***1c*** *and* ***2c****.*

Compound 1c: 5-Hydroxy-2-phenyl-7-(3-(phenylselanyl)propoxy)-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 9: 1. It was obtained as a yellow powder. Yield = 57 %.

TLC (petrol ether: ethyl acetate = 8: 2): R_f = 0.45, melting point: 71 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 13.71 (s, 1H), 7.90-7.81 (dd, 2H, *J*= 1.26, 8.42 Hz), 7.56-7.49 (m, 5H), 7.29-7.19 (m, 3H), 6.64 (s, 1H), 6.45-6.42 (d, 1H, *J*= 2.20 Hz), 6.33-9.29 (d, 1H, *J*= 2.01 Hz), 4.14-4.08 (t, 2H, *J*= 5.80 Hz), 3.10-3.05 (t, 2H, *J*= 6.95 Hz), 2.23-2.15 (m, 2H), ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.49 (s), 164.77 (s), 163.88 (s), 162.05 (s), 157.66 (s), 132.68 (d), 131.74 (d), 131.27 (s), 129.08 (d, 2C), 129.01 (d, 2C), 126.97 (d, 2C), 126.19 (s), 105.80 (d), 105.67 (s), 98.60 (d), 92.99 (d), 67.41 (t), 29.42 (t), 23.89 (t) ppm.

HRMS: $[M]^+$, calcd 452.0526, found 452.0528.

Compound 2c: 5-Hydroxy-2-phenyl-7-(3-(phenyltellanyl)propoxy)-4H-chromen-4-one

The compound was purified by column chromatography on silica gel with petrol ether: ethyl acetate = 9: 1. It was obtained as a yellow powder. Yield = 68 %.

TLC (petrol ether: ethyl acetate = 8: 2): R_f = 0.45, melting point: 112 °C.

^{1}H NMR ($CDCl_3$, 500 MHz): 12.67 (s, 1H), 7.90-7.81 (dd, 2H, *J*= 1.55, 7.49 Hz), 7.75-7.79 (dd, 2H, *J*= 1.83, 1.57 Hz), 7.56-7.47 (m, 3H), 7.26-7.13 (m, 3H), 6.64 (s, 1H), 6.43-6.41 (d, 1H, *J*= 2.05 Hz), 6.31-9.28 (d, 1H, *J*= 2.25 Hz), 4.09-4.03 (t, 2H, *J*= 6.09 Hz), 3.06-3.00 (t, 2H, *J*= 7.05 Hz), 2.32-2.23 (m, 2H), ppm.

^{13}C NMR ($CDCl_3$, 125 Hz): 182.38 (s), 164.77 (s), 163.64 (s), 162.10 (s), 157.76 (s), 138.45 (d, 2C), 131.82 (d), 131.35 (s), 129.23 (d, 2C), 129.08 (d, 2C), 127.79 (d), 126.28 (d, 2C), 111.04 (s), 106.00 (d), 98.90 (s), 93.00 (d), 69.40 (t), 30.91 (t), 4.01 (t) ppm.

HRMS: $[M\text{-}C_6H_5Te]^+$, calcd 295.0970, found 295.0929.

3.5. Cell culture

The HL-60 cell line as a model to measure the antioxidant/cytotoxic activities of compounds in cell culture

Human promyelocytic leukemia HL-60 cells were used in this study as a model to investigate the antioxidant and cytotoxic activities of natural and synthetic flavonoids in cell culture. The compounds were tested at six different concentrations, *i.e.* at 10, 40, 80, 120, 160 and 200 μM. The cytotoxic activity was estimated by incubation of the HL-60 cells (at a concentration of 9 x 10^5 cells per mL), with the various compounds – all at different concentrations for 24 hours. The antioxidant power of compounds was evaluated by measuring the capacity of compounds to protect HL-60 cells from oxidative stress induced by exogenous hydrogen peroxide (H_2O_2). The cell suspensions (9 x 10^5 cells/1 mL) were treated with the various compounds (at different concentrations) in the presence of 5 mM of H_2O_2 (final concentration). After 24 hrs of incubation, the viability of the cells was measured by the MTT assay.

The MTT cell viability assay was first described by Mosmann *et al.* [181]. The MTT assay is a colourimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product (***Fig 30***). The cells are then treated with an organic solvent (DMSO) and the released, solubilised formazan reagent is quantified photometrically at 550 nm. Since the reduction of MTT can only occur in metabolically active cells, the extend of MTT reduction is a measure of the viability of the cells [182].

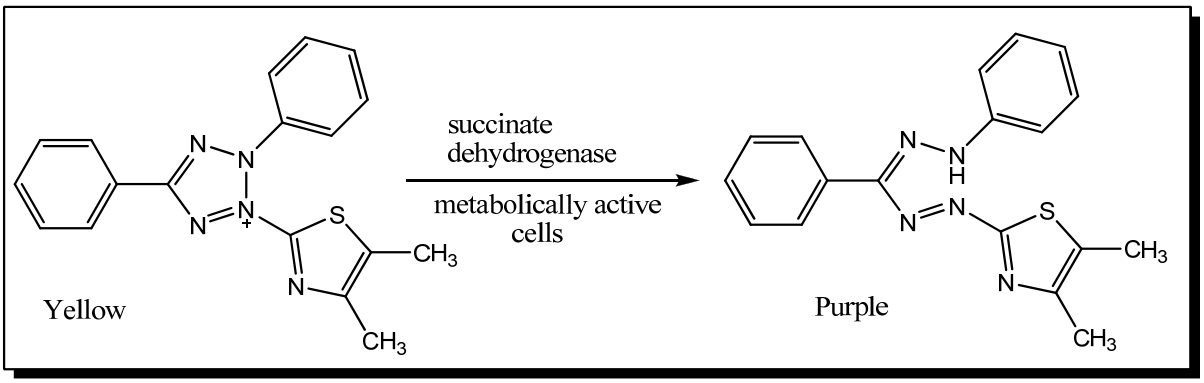


Fig 30: *The basic chemistry associated with the MTT assay: reduction of a yellow tetrazolium salt to a blue formazan by succinate dehydrogenase in metabolically active cells.*

Results displayed in ***Table 17*** and ***18*** summarize the cytotoxic and antioxidant activities of the natural and synthetic compounds when used at 200 μM concentration. The DPPH scavenging activity and aromatase inhibition activity are also listed.

Quercetin and chrysin, which are the chemical precursors of the synthetic flavonoids were used as controls for cytotoxic and antioxidant activity as well as aromatase inhibitor activity. Furthermore, taxifolin, epicatechin, α-tocopherol (vitamin E), and trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as controls in the cytotoxic and antioxidant assay.

Table 17: *A summary of biological activities associated with the isolated natural products. Please note that the viability in the two cell culture assays is set at 100 % for either the untreated cell control or the oxidatively stressed untreated cell control. The experimental details and discussion of these results are provided in the text.*

Compound	Viability[1] of cells after treatment with 200 μM of compounds	Viability[2] of cells after treatment with 200 μM of compounds + 5 mM of H_2O_2	DPPH scavenging activity IC_{50} in μM	Aromatase inhibition activity IC_{50} in μM
1d	140.93 ± 7.56	240.56 ± 7.56	41.61 ± 2.27	*m*
2d	127.14 ± 1.65	255.98 ± 2.82	51.66 ± 3.35	194.21 ± 9.55
3d	85.57 ± 6.75	122.60 ± 8.65	173.21 ± 3.79	*m*
4d	103.50 ± 2.59	175.22 ± 6.43	88.72 ± 5.53	*m*
5d	78.45 ± 2.28	210.74 ± 8.48	102.62 ± 2.85	20.90 ± 7.32
1r	70.95 ± 4.99	137.31 ± 5.01	109.17 ± 8.16	19.99 ± 5.42
2r	32.20 ± 0.31	63.43 ± 2.96	*m*	*m*
3r	27.93 ± 0.72	91.73 ± 3.19	*m*	35.24 ± 2.50
4r	25.53 ± 0.30	77.15 ± 5.26	*m*	21.52 ± 1.77
5r	26.63 ± 4.95	97.23 ± 7.29	*m*	*m*
quercetin	153.48 ± 1.15	267.03 ± 17.22	35.00 ± 1.71	105.25 ± 4.38
chrysin	25.15 ± 1.30	79.47 ± 5.42	*m*	11.50 ± 0.05
taxifolin	80.19 ± 3.04	116.92 ± 3.86	86.07 ± 0.98	*m*
epicatechin	114.21 ± 6.03	171.66 ± 6.82	91.15 ± 7.90	*m*
vit E	133.25 ± 8.75	212.08 ± 4.54	85.36 ± 0.88	n.d.
trolox®	111.44 ± 2.28	185.52 ± 1.77	135.12 ± 3.41	n.d.
aminoglutethemid	n.d.	n.d.	n.d.	36.47 ± 0.26

[1] viability of untreated cells is set as 100 %.
[2] viability of untreated cells (in the presence of 5 mM H_2O_2) is set as 100 %.
m = IC_{50} larger than 300 μM
n.d. = not determined
The data is presented as a mean of at least two independent measurements ± standard deviation.

Table 18: *A summary of biological activities associated with the synthetic compounds. Please note that the all viability in the two cell culture assays is set at 100 % for either the untreated cell control or the oxidatively stressed untreated cell control. The experimental details and discussion of these results are provided in the text.*

Compound	Viability[1] of cells after treatment with 200 μM of compounds	Viability[2] of cells after treatment with 200 μM of compounds + 5 mM of H_2O_2	DPPH scavenging activity IC_{50} in μM	Aromatase inhibition activity IC_{50} in μM
1m	65.19 ± 7.51	85.64 ± 7.21	*m*	87.53 ± 7.48
2m	39.42 ± 1.09	87.21 ± 5.76	*m*	*m*
3m	63.12 ± 0.19	101.45 ± 0.42	*m*	5.90 ± 1.86
4m	87.81 ± 5.05	123.25 ± 6.19	*m*	*m*
5m	25.84 ± 1.06	90.44 ± 7.77	*m*	69.69 ± 9.85
6m	86.20 ± 5.12	111.57 ± 6.01	*m*	178.37 ± 9.55
7m	32.84 ± 2.62	97.30 ± 6.55	*m*	*m*
10m	57.06 ± 2.21	195.40 ± 8.72	49.42 ± 2.88	261.35 ± 3.25
11m	54.15 ± 2.87	200.63 ± 7.51	110.01 ± 3.71	11.24 ± 1.34
12m	35.52 ± 1.30	96.50 ± 7.34	n.d.	*m*
13m	22.18 ± 6.41	99.82 ± 3.27	n.d.	28.25 ± 1.99
14m	31.99 ± 0.10	197.91 ± 4.10	58.56 ± 6.65	*m*
15m	33,90±1,51	104.42 ± 5.65	*m*	204.86 ± 3.57
16m	26.92 ± 1.27	112.88 ± 6.64	*m*	15.63 ± 3.92
17m	88.68 ± 1.10	98.92 ± 2.70	*m*	*m*
18m	82.22 ± 3.16	108.04 ± 0.05	*m*	*m*
19m	82.00 ± 7.15	101.11 ± 4.22	*m*	*m*
20m	86.75 ± 1.95	109.69 ± 7.60	*m*	*m*
21m	88.49 ± 3.48	103.73 ± 7.38	*m*	193.22 ± 11.16
1f	93.90 ± 8.88	80.30 ± 5.18	*m*	*m*
2f	28.70 ± 1.99	79.83 ± 3.24	*m*	*m*
3f	69.80 ± 4.98	n.d.	n.d.	*m*

Compound	Viability[1] of cells after treatment with 200 μM of compounds	Viability[2] of cells after treatment with 200 μM of compounds + 5 mM of H_2O_2	DPPH scavenging activity IC_{50} in μM	Aromatase inhibition activity IC_{50} in μM
4f	60.19 ± 3.32	n.d.	n.d.	*m*
5f	85.07 ± 1.00	n.d.	n.d.	*m*
6f	48.31 ± 1.68	n.d.	n.d.	166.61 ± 11.33
7f	11.88 ± 1.09	49.00 ± 2.33	*m*	187.44 ± 4.48
8f	72.56 ± 1.76	85.83 ± 4.67	*m*	79.41 ± 4.47
9f	n.d	n.d.	n.d.	*m*
10f	64.38 ± 4.74	95.62 ± 5.04	*m*	*m*
11f	26.15 ± 2.26	n.d.	n.d.	*m*
12f	20.69 ± 1.48	103.58 ± 7.42	*m*	*m*
13f	34.50 ± 1.64	88.62 ± 3.57	*m*	*m*
14f	22.46 ± 2.60	n.d.	*m*	26.35 ± 1.01
15f	n.d	n.d.	*m*	36.49 ± 2.44
1c	95.71 ± 1.23	101.61 ± 4.88	*m*	*m*
2c	88.51 ± 1.99	95.62 ± 8.92	*m*	*m*

[1] viability of untreated cells is set as 100 %.
[2] viability of untreated cells (in the presence of 5 mM H_2O_2) is set as 100 %.
m = IC_{50} larger than 300 µM
n.d. = not determined
The data is presented as a mean of at least two independent measurements ± standard deviation.

3.6.Nematode assay

Several compounds were selected (these compounds were chosen based on a distinct cytotoxicity profile in the cell culture assays) to investigate their nematocidal activities. The nematode assay was devised in order to measure the toxicity of the compounds on the intact complete organisms. *Steinernema feltiae* was used as a model for a living organism. This nematode belongs to the beneficial nematodes that seek out and kill the immature stages of harmful soil-dwelling insects before they become adults.

Three natural products with highly cytotoxic activity against HL-60 cell line ***3r***, ***4r*** and ***5r*** were selected for this test, together with six synthetic flavonoids namely ***2f***, ***7f***, ***11f***, ***12f***, ***13f*** and ***14f***.

All compounds were tested at four different concentrations, *i.e.* at 50, 100, 200, 400 μM, with readings taken after 24 and 48 hrs of exposure. The results in ***Table 19*** indicate that virtually none of these compounds were toxic against *Steinernema feltiae* at the concentration used.

Table 19: *Nematode viability after 24 and 48 hours of exposure to 200 and 400µM of tested compounds.*

Compound	24 hrs of exposure (untreated control = 100%)		48 hrs exposure (untreated control = 100%)	
	200 µM	400µM	200 µM	400µM
3r	99.10 ± 3.2	98.34 ± 3.3	98.17 ± 4.4	98.19 ± 3.1
4r	96.09 ± 3.0	96.64 ± 1.6	94.37 ± 4.8	95.9 ± 0.8
5r	95.99 ± 2.1	94.74 ± 2.5	87.15 ± 3.7	82.89 ± 4.2
2f	99.17 ± 1.4	94.59 ± 1.3	99.58 ± 0.7	98.00 ± 1.1
7f	100.00 ± 0.0	84.62 ± 3.1	93.22 ± 4.5	85.70 ± 3.8
11f	94.08 ± 2.1	95.90 ± 1.6	86.72 ± 2.2	91.37 ± 3.3
12f	99.65 ± 0.6	99.31 ± 1.2	98.62 ± 1.2	98.20 ± 3.0
13f	98.00 ± 6.2	98.04 ± 1.4	97.81 ± 1.2	96.19 ± 3.1
14f	94.32 ± 3.2	92.46 ± 1.7	90.56 ± 1.5	87.07 ± 2.5

The data is presented as a mean of at least two independent measurement ± standard deviation.

3.7. Aromatase assay

One of the main approaches in controlling postmenopausal hormone-dependent breast cancer involves the reduction of plasma and tissue levels of estrogen via the inhibition of aromatase (a key enzyme of their biosynthetic process) [183]. Aromatase is a multi enzymatic complex formed by cytochrome P450 XIX (CYP19) and NADPH-cytochrome P450 reductase. It catalyzes the conversion of androgens to estrogens through the aromatization of the **A**-ring of androgen substrates (see Introduction) (***Fig 12***). The enzyme has been considered as a particularly attractive target for the treatment of hormone-dependent breast cancer [183].

Aminoglutethimide is an anti-steroid drug marketed worldwide under the tradename Cytadren® by Novartis. It blocks the production of steroids derived from cholesterol and is clinically used in the treatment of Cushing's syndrome and metastatic breast cancer. It is also a drug abused by body builders. This compound was used as benchmark and control.

Natural and synthetic flavonoids showed a wide range of inhibition against the aromatase enzyme, and therefore may help us understand the structural activity features necessary to produce effective aromatase inhibitors. ***Tables 17*** and ***18*** summarize the results of the aromatase assay.

3.8. 2,2-dipheny-1-picryl hydrazyl (DPPH) radical scavenging activity

Scavenging of the stable 2,2-dipheny-1-picryl hydrazyl (DPPH) radical by suspected antioxidants is a widely used method to estimate and compare antioxidant activities in a relatively short period of time compared to other more extensive methods. The ability of antioxidants to scavenge DPPH radical is thought to be related to their hydrogen donating abilities [184].

DPPH can generate stable free radicals in ethanolic solution. It has a dark purple colour in methanol absorbing light at 517 nm. When a suspected antioxidant is added to this DPPH solution, the purple colour may decolourize. Since decrease in absorbance at 517 nm is directly proportional to the antioxidant potency of the compounds, absorbance can be measured by following the colour of the sample at this wave length [185].

The scavenging ability of flavonoid compounds was measured at five different concentrations, *i.e.* at 10, 50, 100, 200, 400 μM. Based on the concentration of the compounds needed to reduce the absorbance at 530 nm, an IC_{50} value was estimated and summarised in ***Table 17*** and ***18*** for the natural isolated and synthetic compounds.

3.9. Electrochemical results

Cyclic voltametry (CV) and differancial pulse polarography (DPP) of chromene-flavone compounds and reference compounds were investigated on the glassy carbon working electrode. These compounds exhibited various oxidation and reduction potentials which are summarized in ***Table 20***.

Table 20: *Oxidation and reduction potentials of chromene-flavones were measured by CV and DPP on a glassy carbon electrode. Please note that only clear, visible and reproducible peak signals are listed and not all compounds exhibited all types of Epa_1, Epa_2, Epa and Epc. Although CV produces oxidation and reduction signals, the distance ΔE implies that these do not form a reversible or even quasi-reversible redox couple, therefore the redox potential $E_{1/2}$ cannot be calculated. The experimental details and the discussion of the results are provided in the text.*

Compound	Oxididation potential [mV] measured by DPP		Oxidation potential [mV] measured by CV	
	***E*pa$_1$**	***E*pa$_2$**	***E*pa**	***E*pc**
phenol	+40	+520	+340 +800	-320
catechol	+250	-	+300	-45
resorcinol	+20	+600	+740	-260
epicatechin	+120	-	+240	-45
taxifolin	+135	-	+350	-35
chrysin	+130	+800	+200 +1050	-
quercetin	+160	+990	+150 +970	-
cyanidin	-27	+200	+25 +290	-525 +170
1f	-	-	-	-
2f	-	-	-	-
3f	-160	+830	+245 +1010	-
4f	-140	+850	-	-
5f	-120	+880	-	-
6f	-	-	-	-
7f	-	-	-	-
8f	-220	+877	-	-
9f	-130	+810	+900	-
10f	-120	+790	-	-
11f	-140	+1230	-	-
12f	-130	+920	+930	-
13f	-160	+1320	-	-
14f	-210	+840	-	-
15f	-160	+850	-	-

These potentials were measured either by DPP (left column) or Cyclic Voltammetry (right column).

*E*pa$_1$: first (*i.e.* lower) oxidation potential.

*E*pa$_2$: second (*i.e.* higher) oxidation potential.

*E*pa: oxidation potential.

*E*pc: reduction potential.

Chapter IV: Discussion

4.1.Structure elucidation of new natural products

Two new natural compounds, *i.e.* ***4r*** and ***5r*,** were isolated from the roots of *Robinia pseudoacacia* (Black locust). Structure elucidation of the new as well as known compounds was confirmed by complete NMR analysis (1D and 2D) and mass spectroscopy, and also through comparison with published spectra of related compounds.

4.1.1. Compound *4r*

Compound ***4r*** was isolated from the ethyl acetate extract of *Robinia pseudoacacia* roots and was purified by column chromatography using dichloromethane: methanol (95: 5). It was obtained as a yellow amorphous powder with a melting point of 102 °C.

The ^{1}H NMR spectrum of compounds ***2r***, ***3r*** and ***4r*** revealed an AB-system (J_{AB} around 15.50 Hz) centred at δ_H 8.04-8.07 (H-α) and δ_H 7.84-7.98 (H-β) (***Fig 31***) indicating the (*E*)-geometry of a *trans* double bond and suggesting the chalcone nature of the compounds [173, 186]. The ^{1}H and ^{13}C NMR spectra of ***4r*** in addition to DEPT spectra (***Fig 31*** and ***32***), which had close similarities to that of compound ***2r,*** indicated the chalcone pattern of compound ***4r*** with a hydroxyl group at position 2′ of the **B**-ring (***Table 12*** and ***13***). The hydrogen bonded phenolic hydroxyl group appeared as a singlet at δ_H 12.39 ppm in ^{1}H NMR. It is strongly chelated by a vicinal carbonyl group, indicative of an OH group at C-2′.

There were three aromatic proton signals for the **A**-ring appearing as a multiblet at δ_H 7.00 ppm and integrated for two protons (H-4 and H-6), and at δ_H 7.12 ppm, integrated for one proton (H-5) with a doublet of a doublet and a coupling constant *J*=2.68, 6.65 Hz (***Table 12*** and ***Fig 31***). Signals for two methoxy groups appeared at δ_H 3.84 and δ_H 3.81, integrated for six protons were also observed in the ^{1}H NMR of compound ***4r***. Their presence was also confirmed by ^{13}C NMR signals at δ_C 61.00 (C-2) and δ_C 56.00 (C-3) (***Table 12*** and ***13*** and ***Fig 31*** and ***32***). Placement of the methoxy groups at positions C-2 and C-3 of compound ***4r*** is suggested by the C-2 and C-3 resonances. There are significant downfield shifts δ_C + 25.27 for C-3 and upfield shifts δ_C -6.43 for C-1 and δ_C -15.57 for C-4 compared to compound ***2r***.

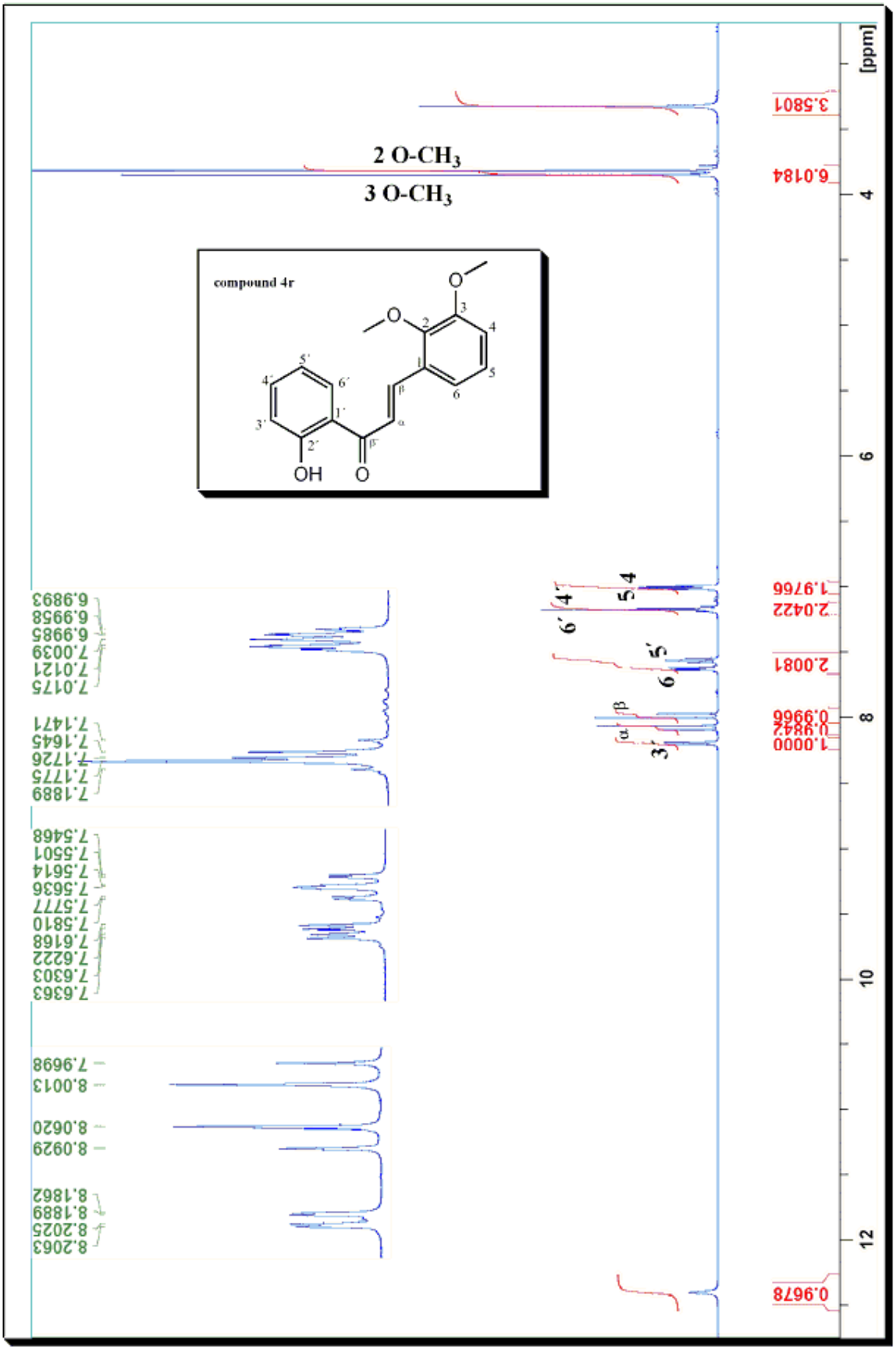


Fig 31: *^{1}H NMR spectrum of compound* ***4r****, with partial spectrum enlargement to enable the visualization of peak multiplicity.*

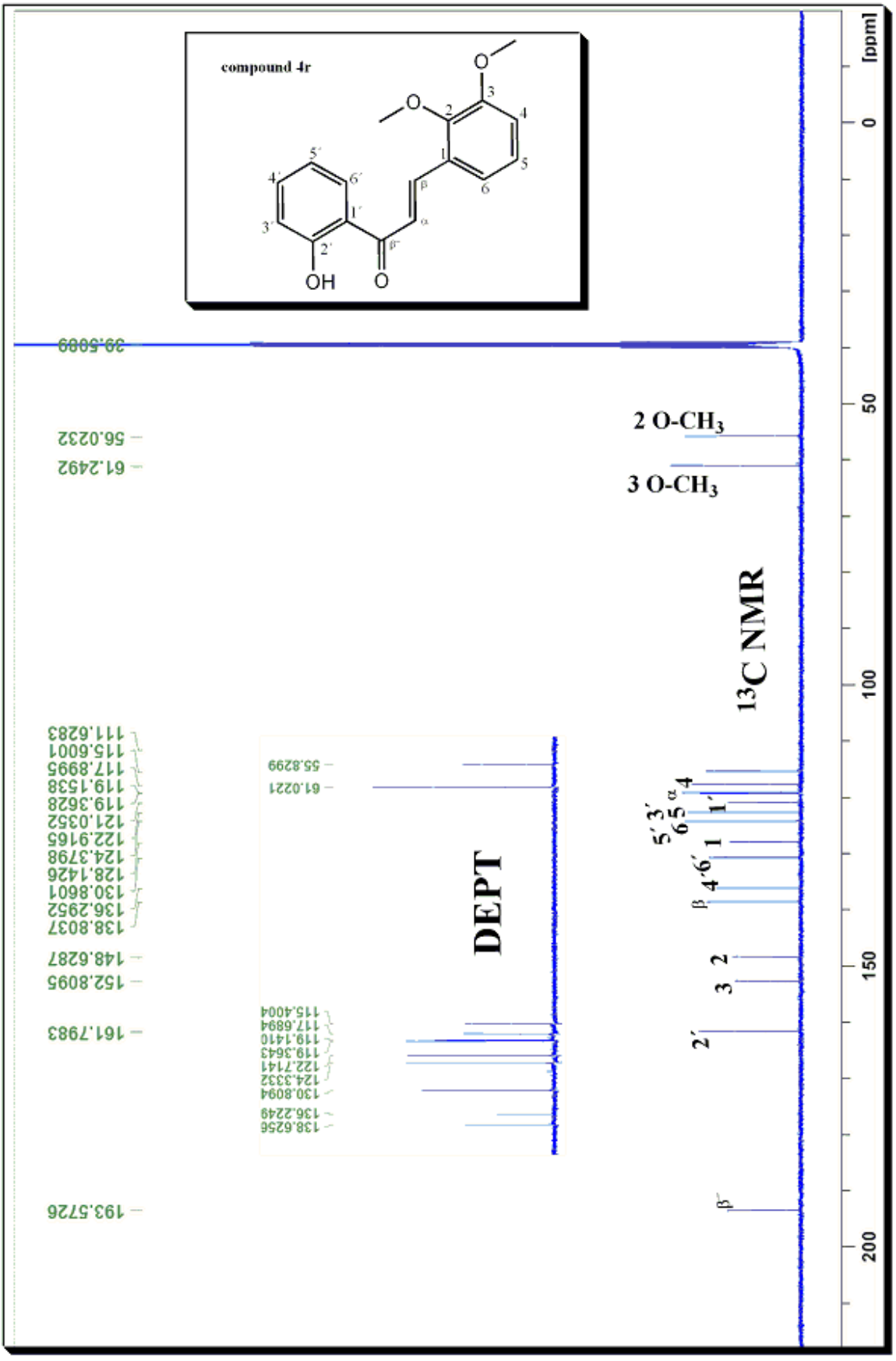


Fig 32: *^{13}C and DEPT NMR spectra of compound* ***4r****.*

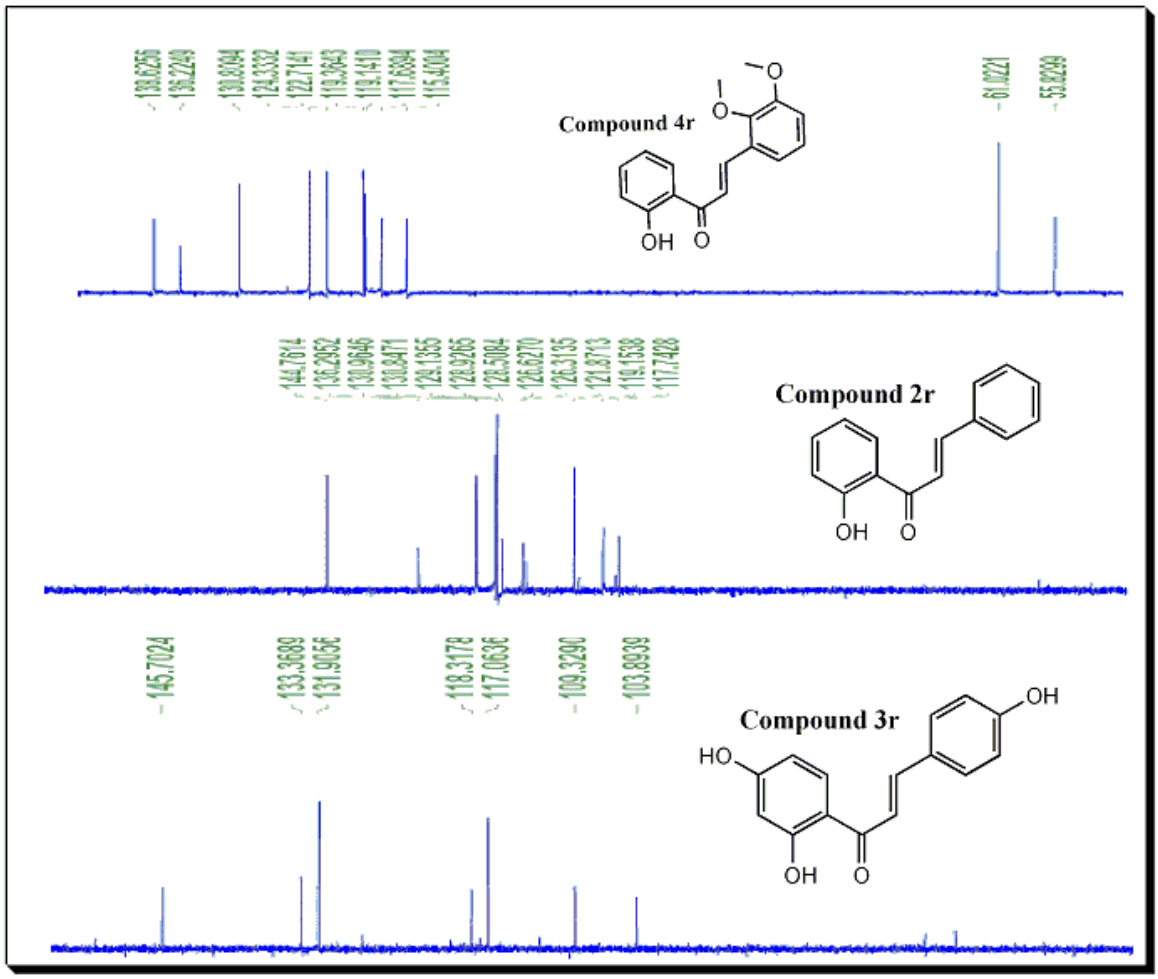


Fig 33: *DEPT spectra of compounds* ***2r****,* ***3r*** *and* ***4r****.*

A combined analysis of ^{13}C and DEPT NMR spectra indicates the presence of two primary carbons (δ_C 55.82 and δ_C 61.02 ppm), nine tertiary and six quaternary carbon atoms in compound ***4r***. There are nine tertiary and six quaternary carbon atoms in ***3r***, compared to eleven tertiary and four quaternary carbon atoms in ***2r*** (***Fig 33***). This provides evidence that compound ***4r*** is a chalcone with three substituents, two of them methoxy groups.

Two dimensional NMR in conjunction with HMBC showed a strong coupling correlation between the methoxy proton at δ_H 3.84 ppm and δ_C 153.77 ppm (C-3), and at δ_H 3.81 ppm with δ_C 148.51ppm (C-2) (***Fig 34***).

Through interpretation of this spectral data and with additional comparison to data previously published [173, 174-176], the structure of compound ***4r*** was determined to be 2´-hydroxy-2,3-dimethoxy chalcone, and to our knowledge is a previously unknown natural product.

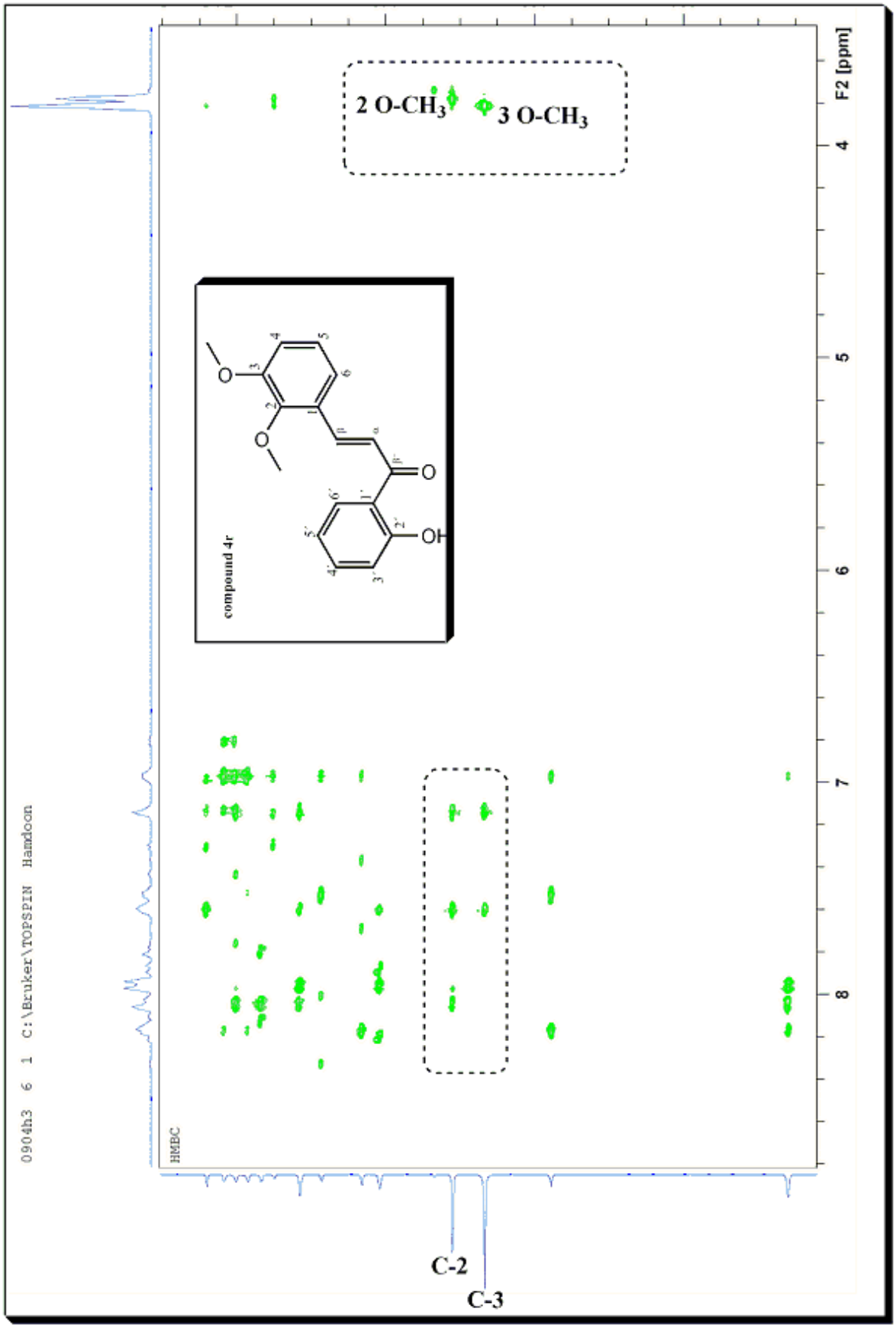


Fig 34: *2-D HMBC-NMR spectrum of compound* ***4r****.*

4.1.2. Compound *5r*

Compound ***5r*** was isolated from the ethyl acetate extract of *Robinia pseudoacacia* roots. It was purified by column chromatography using petroleum benzene: ethyl acetate (80: 20). It gave an orange precipitate with Dragendorff's reagent (potassium bismuth iodide, special precipitating reagent for alkaloids) within 24 hours. Compound ***5r*** was obtained as a white amorphous powder with a melting point of 174°C.

Compound ***5r*** showed a molecular ion peak at 269.1$[M]^+$ compatible with the molecular formula of $C_{16}H_{15}NO_3$. The ^1H NMR spectra of compound ***5r*** (***Fig 35***) measured at 500 MHz exhibited the characteristic pattern of a medicarpin schaffold [177, 178]. The AB-X system of the two benzene rings was clearly visible due to the presence of one proton doublet at δ_H 7.36 ppm, with a J = 8.50 Hz (H-5), one proton doublet of a doublet at δ_H 6.43 ppm,with a J = 2.50, 8.50 Hz (H-6), one proton doublet at δ_H 6.38 ppm, with a J = 2.50 Hz (H-8), one proton doublet at δ_H 7.11 ppm, with a J = 8.45 Hz (H-2´), one proton doublet of a doublet at δ_H 6.53 ppm, with a J = 2.20, 8.45 Hz (H-3´), one proton doublet at δ_H 6.43 ppm, with a J = 2.20 Hz (H-5´), which indicates the presence of a hydroxyl group at position C-7 and a methoxy group at the C-4´ position.

The only difference between the ***5r*** and medicarpin is the presence of one broad singlet proton at δ_H 4.99 ppm. In the two dimensional H-H COSY and H-C HMQC NMR spectra (***Fig 38***) there is no correlation between this proton and other protons or carbons within the compound. The chemical shift of this proton at δ_H 4.99 ppm and publication comparison suggest that this proton is attached to a nitrogen group [179, 180]. The NH proton (**δ_H 4.99**) correlation is clearly visible in the H-C HMBC NMR spectra (***Fig 37***) with C-5´, C-6´ and C-10. This spectrum provides evidence that the NH group is attached to C-6´.

The ^{13}C NMR spectra of compound ***5r*** in comparison with DEPT spectra (***Fig 36***) confirm the presence of one primary, one secondary, eight tertiary and six quaternary carbon atoms, and reveal a distinct pattern similarity to medicarpin except for small shifts for carbons C-2, C-3 and C-4.

The spectral data of NMR, together with spectral calculation, publication comparison, and reaction of ***5r*** with Dragendorff's reagent, assigen the ***5r*** chemical structure shown in ***Fig 35*** and identified compound ***5r*** as a new natural product isolated for the first time from *Robinia pseudoacacia*.

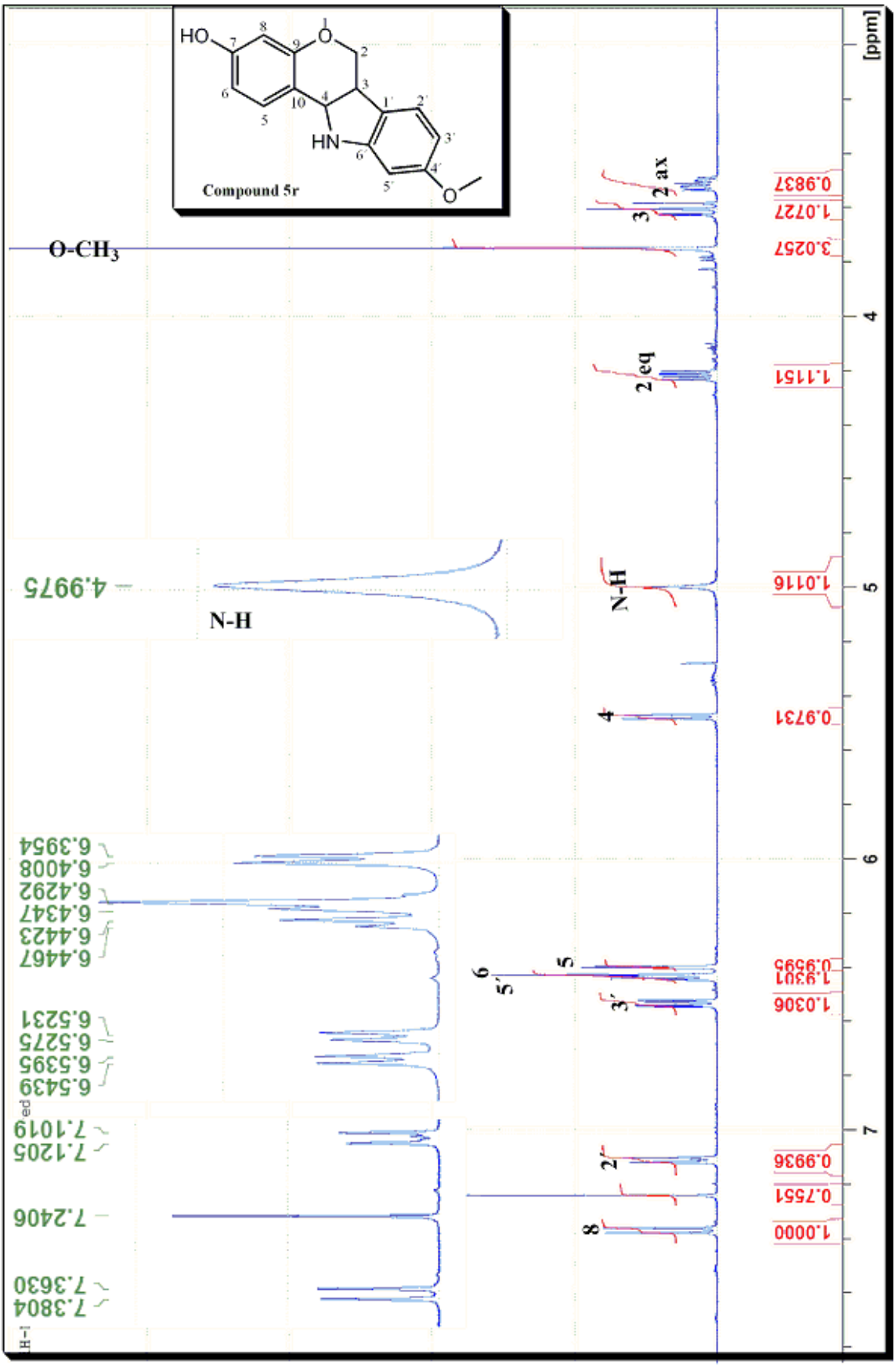


Fig 35: *^{1}H NMR spectrum of compound* ***5r*** *with partial spectrum enlargement to enable the visualization of peak multiplicity.*

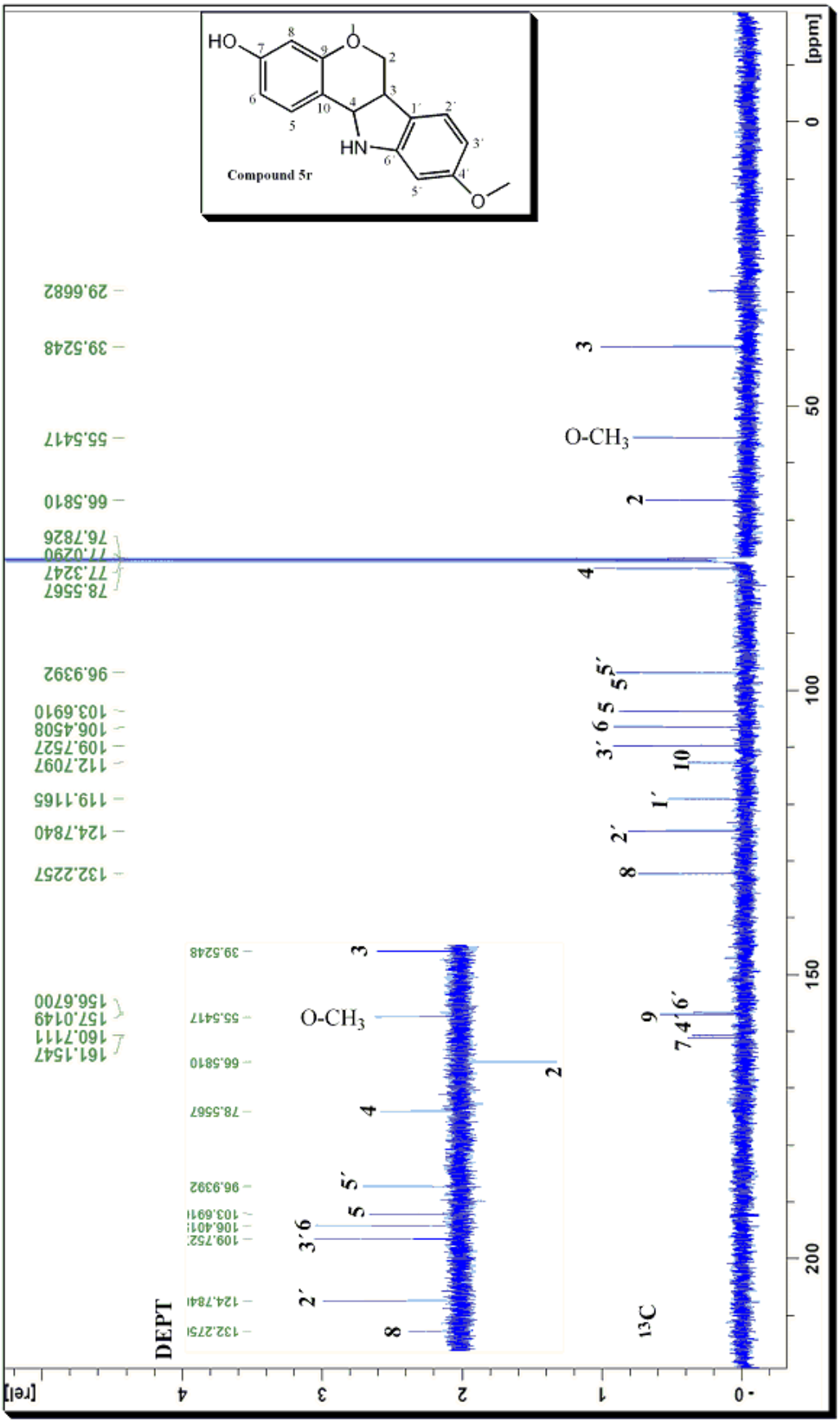


Fig 36: *^{13}C NMR and DEPT NMR spectra of compound* ***5r****.*

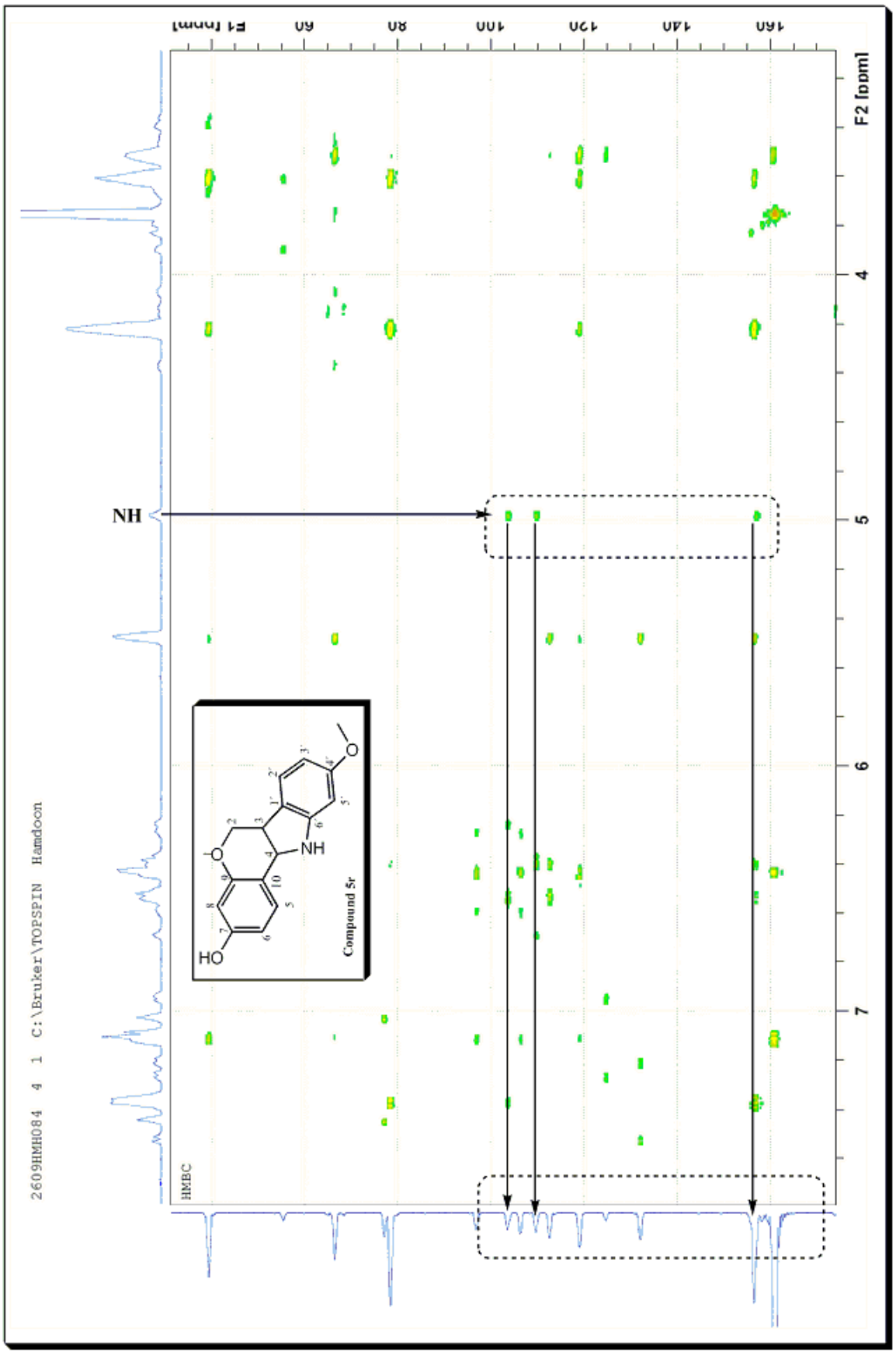


Fig 37: *2-D HMBC-NMR spectrum of compound* ***5r****.*

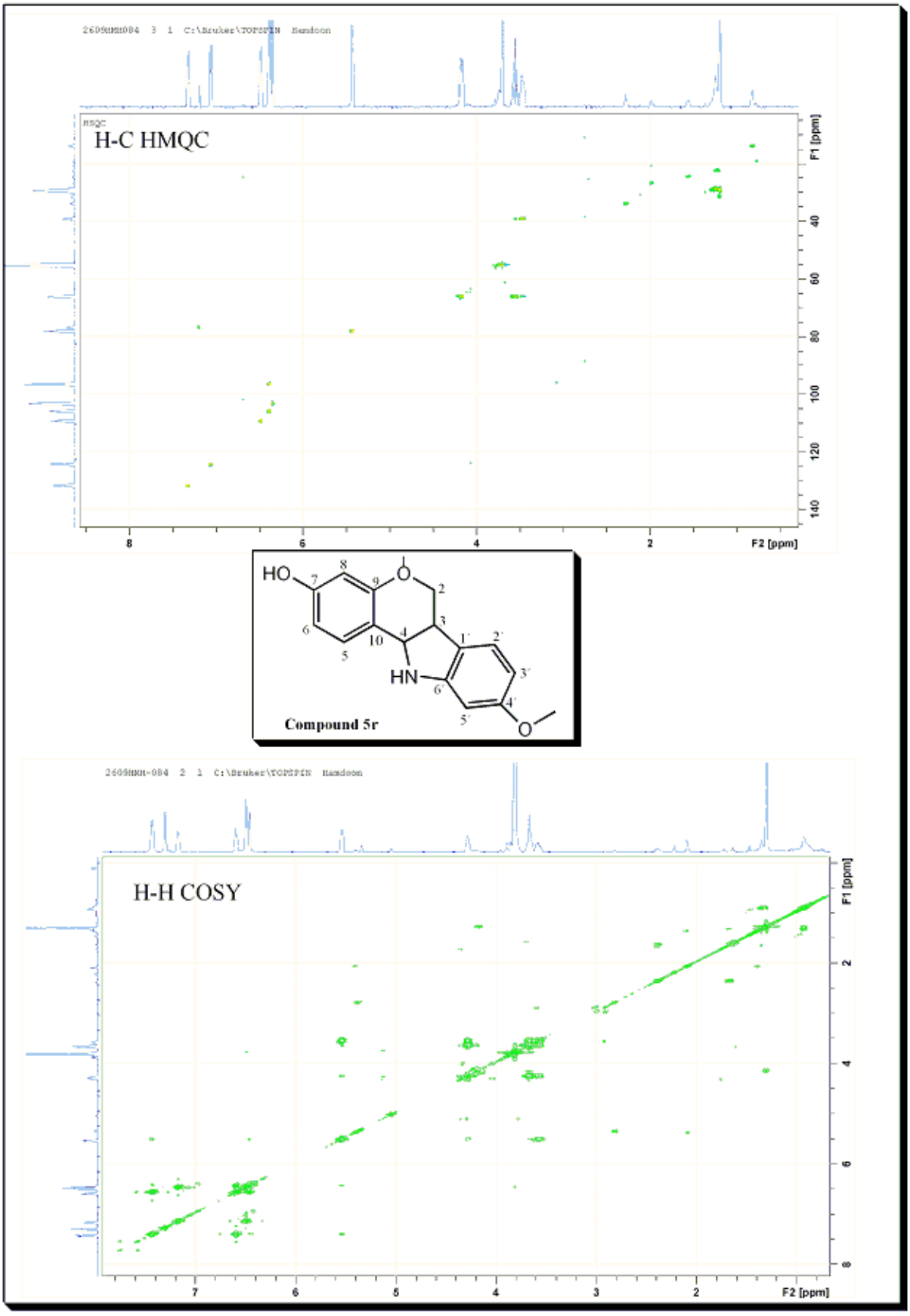


Fig 38: *2-D COSY & HMQC-NMR spectra of compound* ***5r****.*

4.2.Mannich reaction

Because the coupling of three components (chrysin or quercetin, aldehyde, and amine) can be achieved in a one-pot reaction, a Mannich-type aminoalkylation has been an attractive method for introducing a side chain to various phenols [187].

Chrysin and quercetin are two phenolic compounds (***Fig 5***) and representative flavones present in many plants and fruits. They are associated with important roles in the plants and also exhibit highly interesting biological activities in animals and humans. The benefits they provide such as a distinct antioxidant activity have been discussed in thousands of publications and in many books.

Although there are structural similarities between chrysin and quercetin, there are also significant differences in biological activities. Three additional hydroxyl groups in quercetin make it a strong antioxidant and provide nutritive properties even towards cancerous cells (the viability of the HL-60 leukaemia cells was 153% after treatment with 200 μM of quercetin (***Table 17***)). Chrysin, on the other hand has been reported as a powerful aromatase inhibitor [65], and also has notable anticancer activity (HL-60 cells viability was just 25% (***Table 17***)). It is therefore possible that the addition of an amino alkyl moiety via a Mannich type reaction may enhance or decrease one of the previously mentioned biological activities.

Structural analysis indicates that the **A**-ring of chrysin and quercetin, especially at positions C-6 and C-8, may be more reactive towards electrophiles than the **C**-ring (***Fig 39***). Therefore, a Mannich reaction operating under relatively mild conditions may provide a potential site-selective derivation procedure to achieve chrysin and quercetin aminoalkylation.

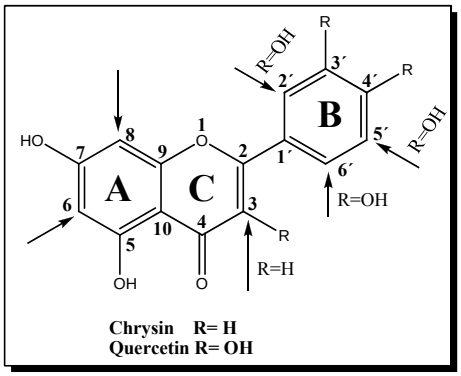


Fig 39: *Possible sites for modification via a Mannich type reaction.*

As part of such a Mannich reaction, carbons C-3 of chrysin and C-2′, 5′ and 6′ in quercetin could also potentially react (***Fig 39***). Previous studies on electrophilic reactions of chrysin, quercetin and other similar flavonoids (such as naringenin), under a variety of conditions, indicate that mixtures often result through substitution at different positions [188, 189]. In order to acquire single pure Mannich adducts, one frequently adopted approach is to protect one or more phenolic hydroxyl groups prior to the Mannich reaction. Such an indirect approach, however, usually requires extensive and time consuming protection and de-protection steps. Consequently, it would be advantageous to find a regioselective Mannich reaction, which would allow the addition of extended functionalized alkyl groups without the need for protecting groups.

There is a distinguished chemical and electronic difference between the various hydroxyl groups of chrysin and quercetin. In essence, position C-6 in flavonoid substrates is the most reactive site for electrophillic reactions [190].

Regioselectivity of the Mannich reaction has been reported in the case of naringenin (a dihydroflavone), with the temperature and time of reaction controlling the substitution pattern [190]. There are many other factors we found that also affect the selectivity of the reaction and product yield; among these factors, the most important are the reaction solvent, and the type of flavonoid substrate.

The influence of the solvent was apparent in the preparation of compounds ***6m*** and ***7m*** from chrysin, ethylglyoxalate and morpholin. When using ethanol as a solvent, product ***6m*** (substitution at position C-6) was obtained with 80% yield and ***7m*** (disubstitution at positions C-6 and C-8) with 12% yield (***Table 15***). In contrast, the yield of ***6m*** was 45%, and ***7m*** was 39%, when DMF was used as a solvent (***Fig 40***).

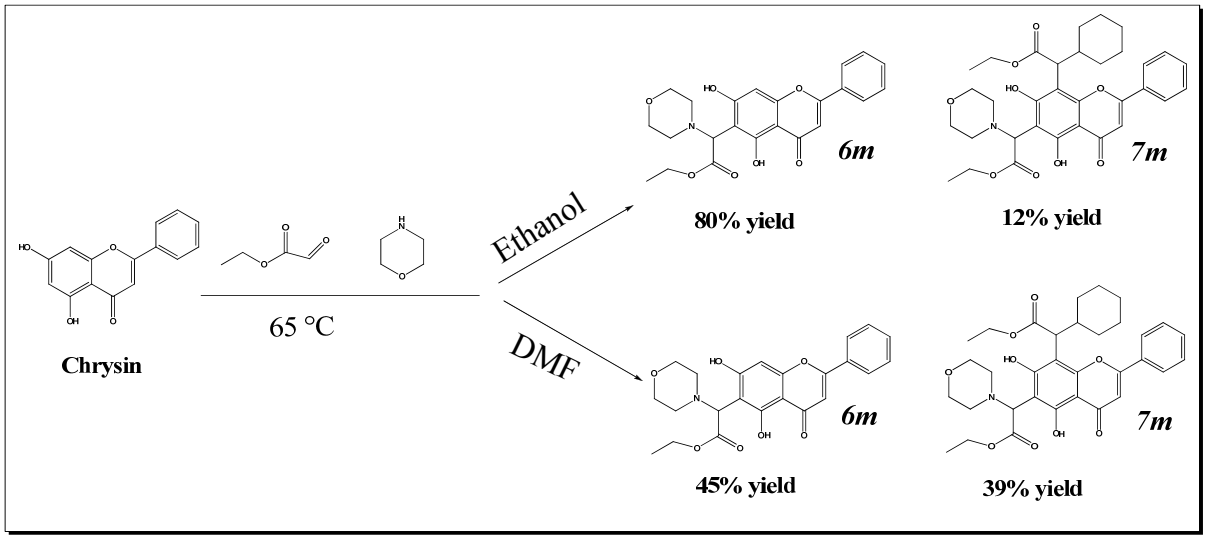


Fig 40: *Solvent effect on the Mannich reaction of chrysin with ethylglyoxalate and morpholin.*

Table 15 points towards a large difference in Mannich reaction conditions between chrysin and quercetin substrates. Reactions involving chrysin normally need a temperature ranging from 55 °C to 65 °C, and 8 to 24 hours reaction time. It was found that in the case of quercetin, the reaction conditions were less demanding (temperature ranging from room temperature to 40 °C, with 4 to 8 hours reaction time). It was also noted that the quercetin reaction produced a mixture of many products, thus increasing difficulties with the isolation and purification procedures. For this reason only three quercetin products, *i.e.* ***10m***, ***11m***, and ***14m,*** in yields of 72, 36 and 39 % respectively, were synthesised. The results obtained with quercetin may be due to the presence of a hydroxyl group at position **3** (in ring **C**) and the catechol structure of ring **B**, which may increase the possibility of different substitution patterns. These hydroxyl groups may also enhance the effect of the carbonyl group in position C-4 and the reaction proceeds under milder condition.

The results summarized in ***Table 15*** for compounds ***15m, 16m, 17m, 18m, 19m, 20m, 21m*** indicate that the use of benzaldehyde or its derivatives in the reaction leads to morpholin decomposition and primary amine formation. There is no clear reason for this behavior in the literature of Mannich reactions. One possibility is that sterical hindrance may play a role, as the incorporation of three bulky groups is not straight forward (***Fig 41***). The structures of these compounds were confirmed by ^{1}H, ^{13}C NMR spectra in addition to LC-MS and HR-MS. At first, it was thought that the reaction would be an Aldol condensation [191] and would not involve the amine at all. HR-MS spectra of compounds ***15m*** and ***16m*** for $C_{22}H_{17}NO_5$ $[M-CH_3+H]^+$ (calcd = 375.1106, found = 375.1076), however, suggest the presence of an amine.

A proton signal ranging from δ_H 5.04- δ_H 4.49 ppm indicate the attachment of a singlet proton to a methylene bridge between two phenyl groups (***Fig 41***), and proximally to an amino group. In contrast, proximity of the proton to a hydroxyl group instead of an amino group would result in a downshift of this proton to approximately δ_H 5.80 ppm [192,193].

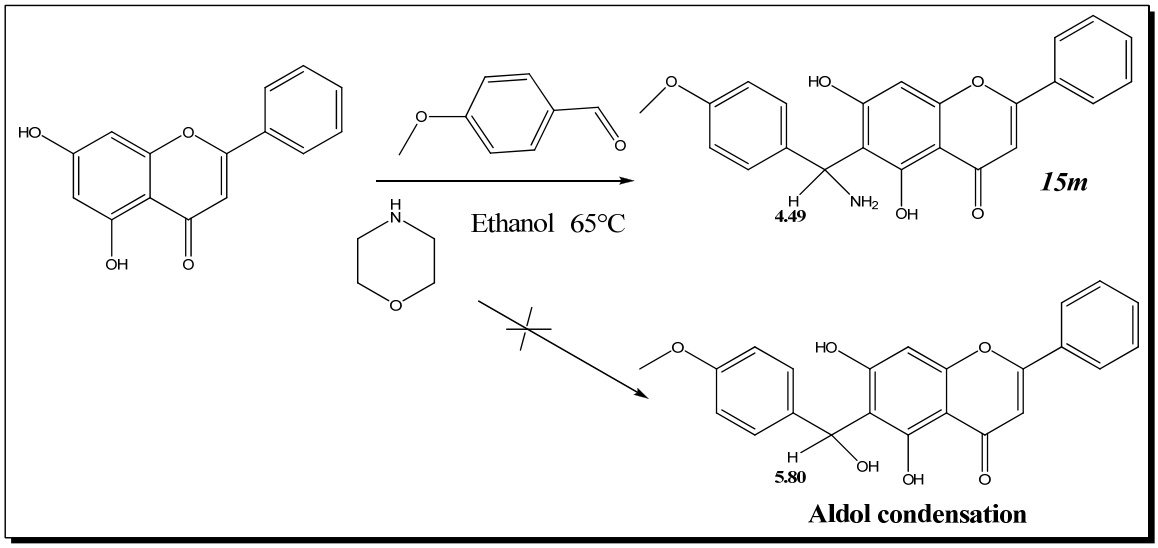


Fig 41: *Suggested structure of the products obtained from the reaction of chrysin with p-methoxy benzaldehyde and morpholin. The chemical shifts of the proton attached to the methylene carbon are shown.*

Aldol condensation only took place during the preparation of compound ***12m***. The secondary amine used in this reaction (phthalimide (amide)) did not participate in the product formation. Condensation may take place as a part of a competing reaction [191] and only formaldehyde was incorporated, which led to the presence of a hydroxy methyl group attached to chrysin (***Fig 42***).

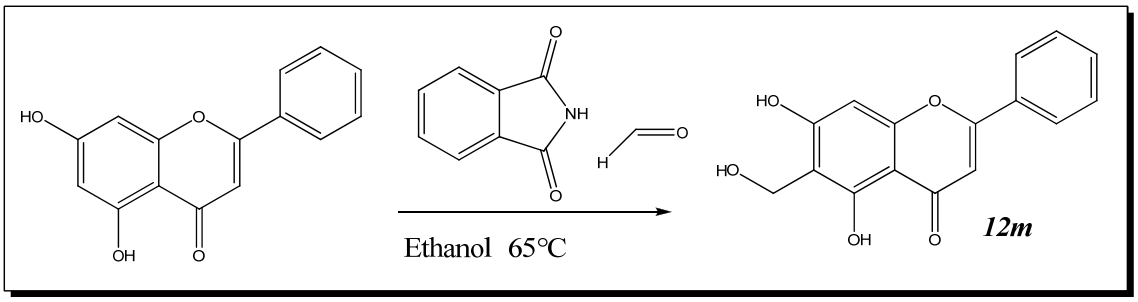


Fig 42: *Reaction of chrysin with paraformaldehyde in the presence of phthalimide. Please note that phthalimide did not participate in this reaction.*

Overall, 21 compounds were obtained via the Mannich reaction pathway, with yields varying from 10 % (*e.g.* ***12m***), to 86 % (***4m***). The wide range of diverse structures that were

obtained via the Mannich reaction may provide a better understanding of the flavonoid reaction behaviours in such a Mannich reaction. They may also allow us to study the biological behaviour of flavonoids in more detail.

4.3. Chromene-flavone reaction

The three-component reaction between chrysin, isonitrile and acetylene dicarboxylate has been employed successfully to synthesize 15 different tricyclic chromene-flavone compounds ***1f-15f*** (***Fig. 27, Table 16***). Different derivatives of isonitrile and acetylene dicarboxylate were used in the reaction and therefore products differ in the amine side chain and dicarboxylate ester moieties. Product yields were high in general and ranged from 25% (for ***12f***) to 85% (for ***9f***). The chemical structures of all compounds were confirmed by ^{1}H and ^{13}C NMR in addition to LC-MS and HR-MS spectroscopy. Two dimensional NMR was used to identify which hydroxyl group of chrysin was involved in the reaction, and also to determine the site of ring closure (ring **D**).

Diagnostic protons were present in all compounds. One proton singlet was found at δ_H 12.50-12.75 ppm, one proton broad singlet at δ_H 8.96-8.25 ppm, two proton doublets of a doublet at δ_H 8.08-7.98 ppm, three proton multiplets at δ_H 7.59-7.50 ppm, one singlet proton at δ_H 6.75-6.73 ppm, one singlet proton at δ_H 6.54-6.53 ppm, and one singlet proton at δ_H 5.08-4.98 ppm (***Fig 44***).

When considering the structure of chrysin, there are, in theory, three different structural possibilities for the new chromene ring (ring **D**) (***Fig 43***).

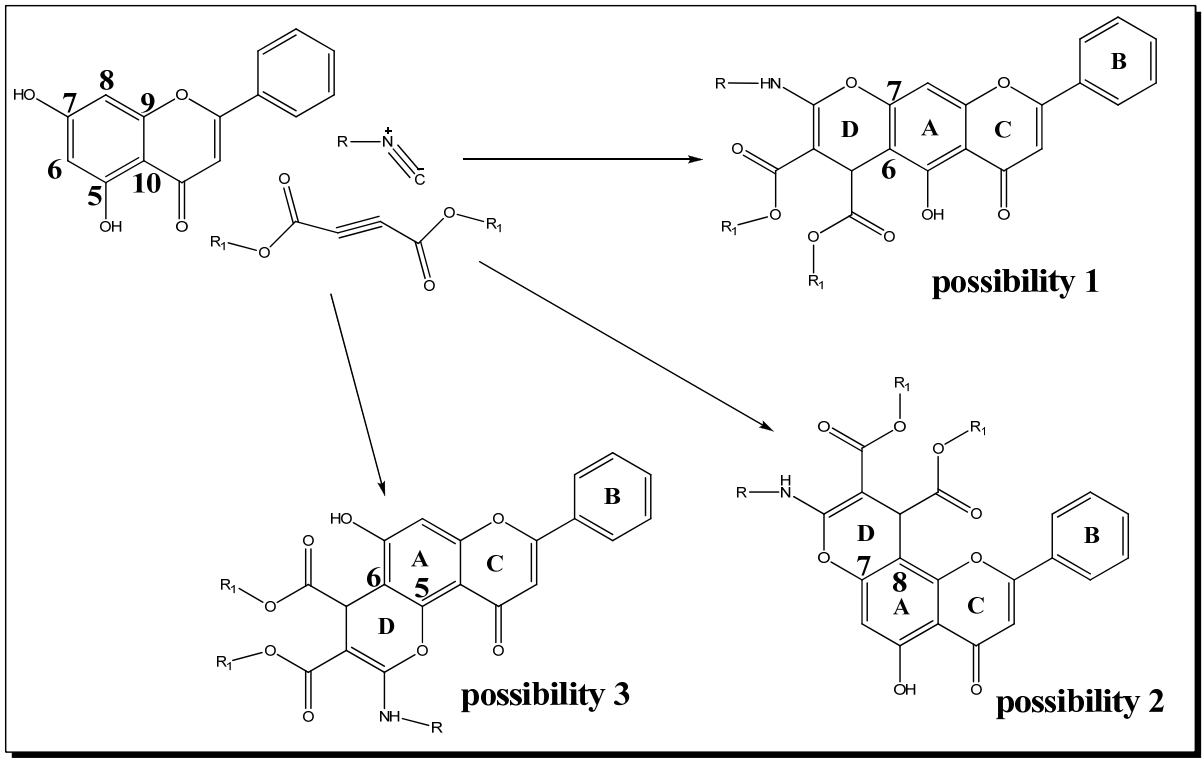


Fig 43: *Possible structures of the chromene-flavones, based on theoretical considerations.*

^{1}H NMR spectra of all chromene-flavone compounds revealed a singlet proton at approximately δ_H 12.70 ppm that can be unambiguously assigned to the chelate proton 5-OH, with another singlet at δ_H 6.50 ppm that is better fitted to H-8 than H-6 (***Fig 44***). 2D, H-C HMBC NMR spectra of compounds ***5f*** and ***10f*** show a strong correlation between the proton at δ_H 12.73 ppm (5-OH) and carbon numbers C-5 at δ_C 164 ppm, C-10 at δ_C 100 ppm and C-6 at δ_C 108 ppm, and another correlation between the proton at δ_H 6.50 (H-8) with C-6 at δ_C 108 ppm, C-7 at δ_C 169 ppm, C-9 at δ_C 161 ppm and C-10 at δ_C 100 ppm (***Fig 45***), thus providing evidence for 6,7-annulation of the chromene ring.

More evidence comes from the ***5f*** structure prediction using NMR prediction software (ACDLabs 7.0). Predicted values (***Fig 46***, ***47*** and ***48***) were found to be at δ_H 11.7 ± 3.5 ppm for 5-OH and δ_H 6.82 ± 0.37 ppm for H-8, thereby supporting the structure of possibility 1 (***Fig 43***). Prediction values for the structure in possibility 2 yield a singlet proton at δ_H 6.71 ± 0.04 ppm (values for proton H-6) which is incompatible with actual spectra of compound ***5f***. Prediction of the structure in possibility 3 yields a singlet proton at δ_H 7.34 ± 3.15 ppm for 7-OH and δ_H 6.65 ± 0.12 ppm for H-8, which is also incompatible with the actual spectra of compound ***5f***.

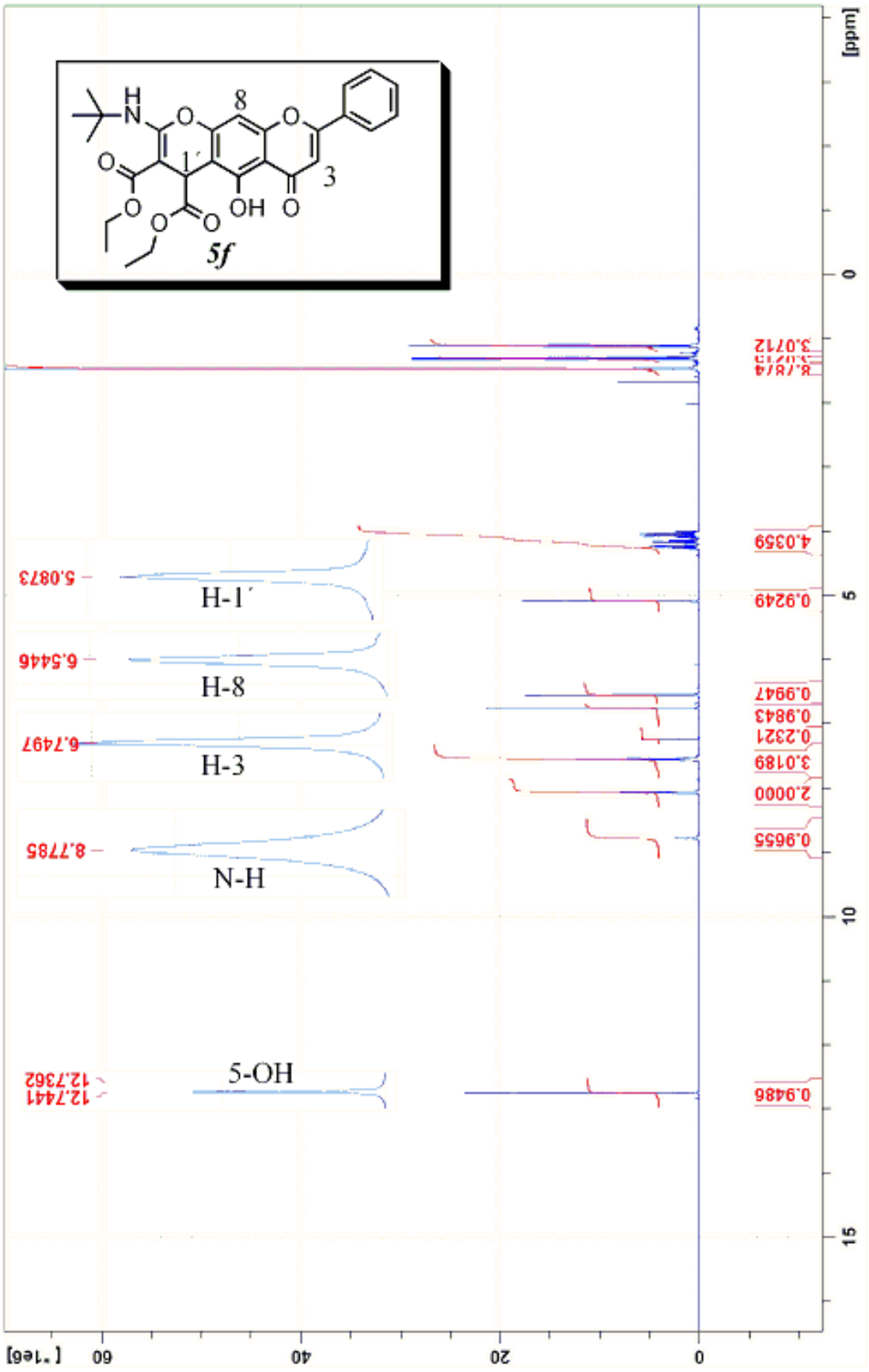


Fig 44: *^{1}H NMR spectrum of compound* ***5f****, with partial enlargement*

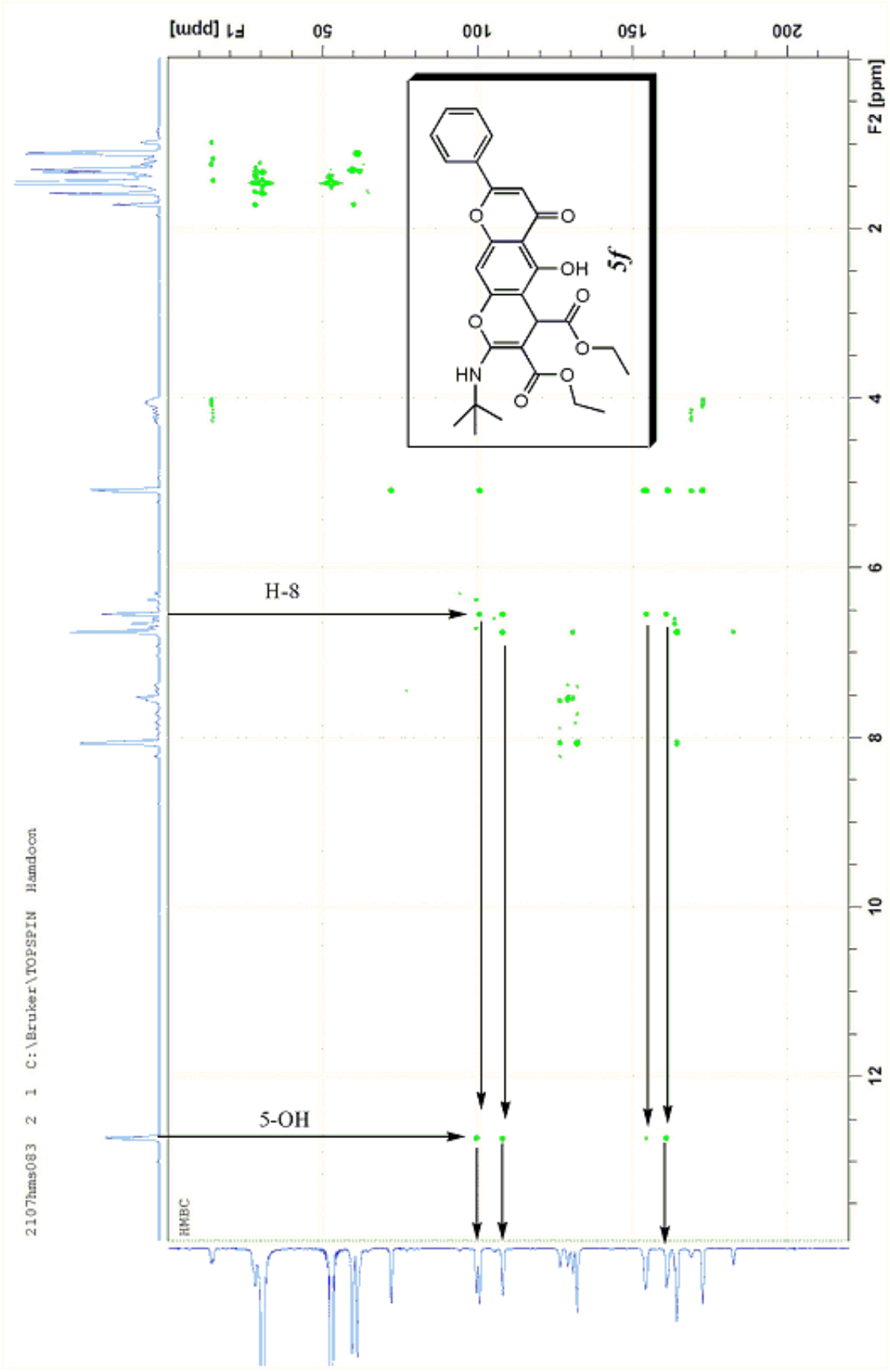
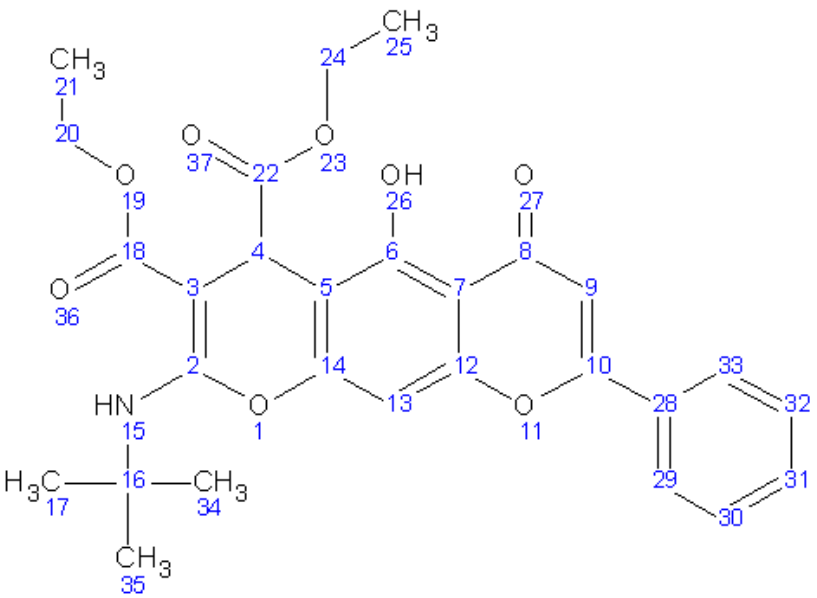


Fig 45: *HMBC spectrum of compound* ***5f****.*



Group	nH	Shift	Error
4	1	5.887	0.78
9	1	6.7	0.03
13	1	6.823	0.37
15,26	2	11.718	3.46
17,34,35	9	1.182	0.03
20	2	4.088	0.12
21	3	1.168	0.15
24	2	4.233	0.02
25	3	1.248	0.04
29	1	7.9	0.04
30	1	7.53	0.02
31	1	7.54	0.05
32	1	7.53	0.02
33	1	7.9	0.04

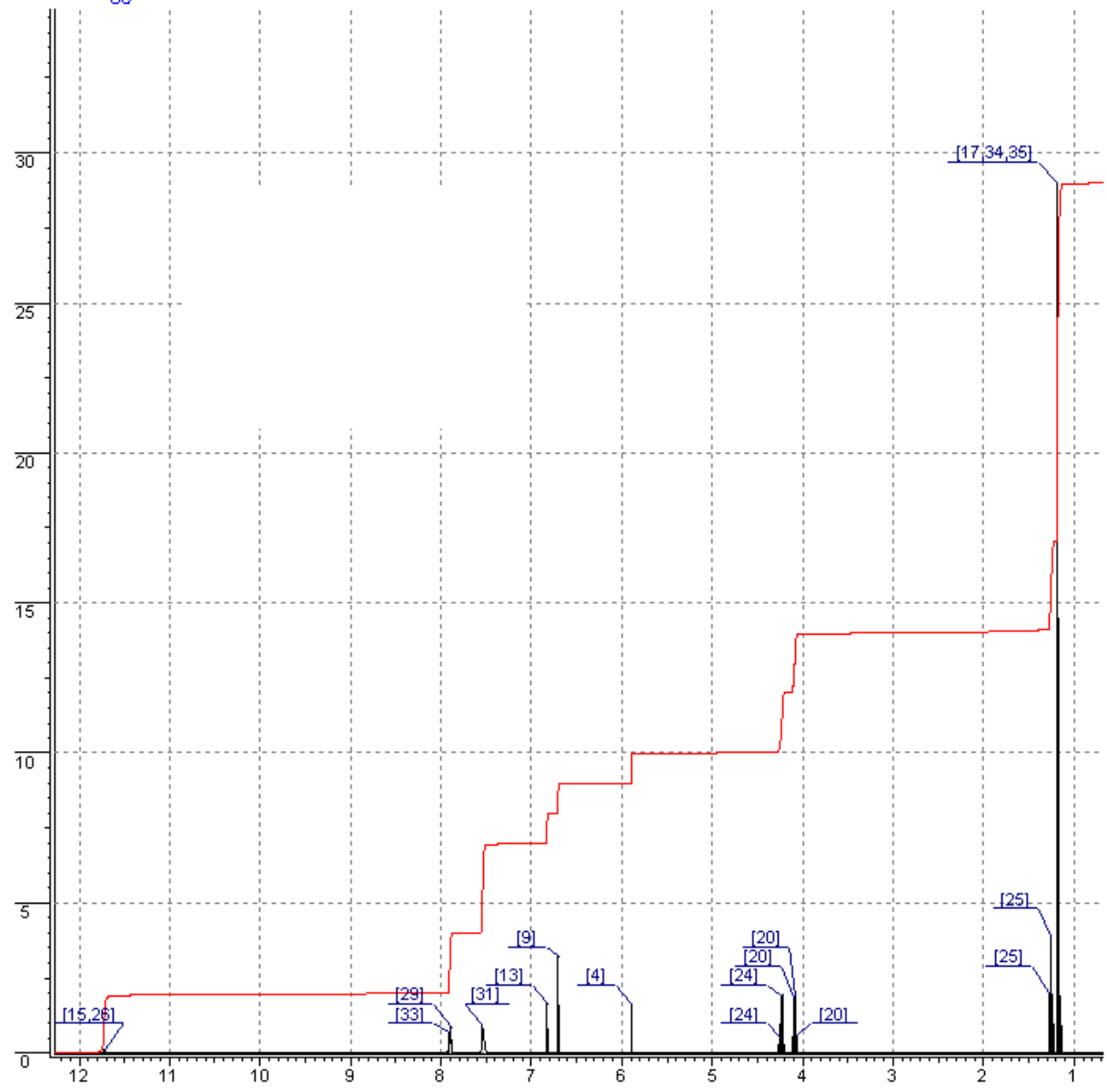
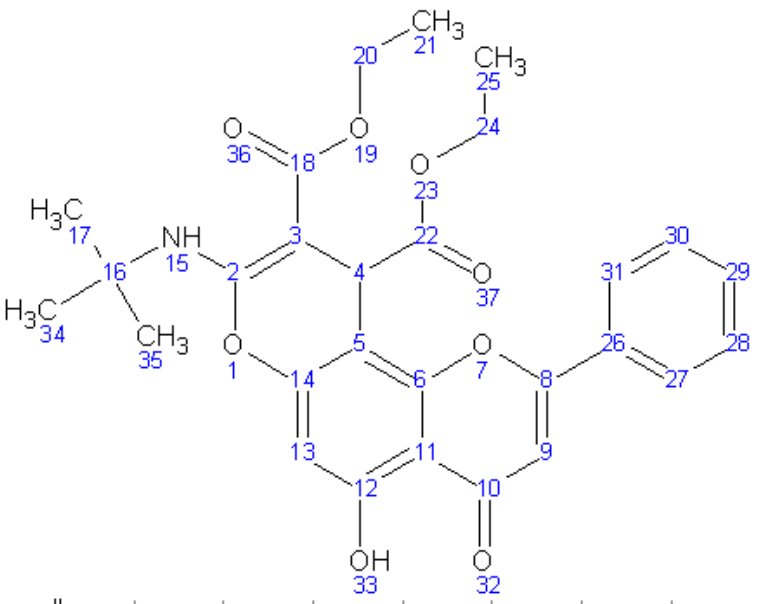


Fig 46: *^{1}H-NMR data predicted for the structure resulting from possibility 1.*



Group	nH	Shift	Error
4	1	5.764	0.78
9	1	6.624	0.08
13	1	6.712	0.04
15,33	2	10.747	3.15
17,34,35	9	1.182	0.03
20	2	4.088	0.12
21	3	1.168	0.15
24	2	4.233	0.02
25	3	1.248	0.04
27	1	7.652	0.12
28	1	7.36	0.07
29	1	7.425	0.03
30	1	7.36	0.07
31	1	7.652	0.12

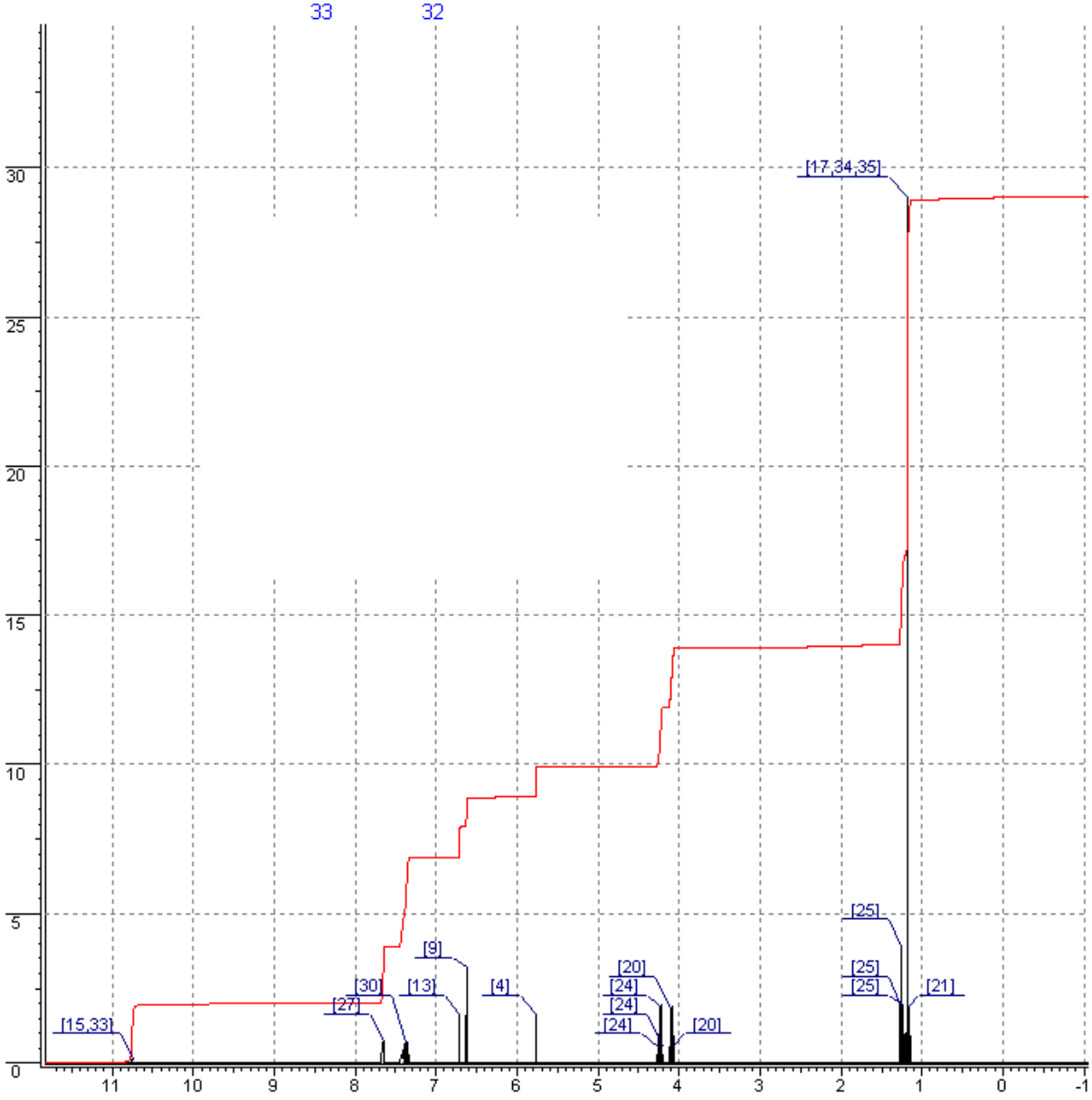
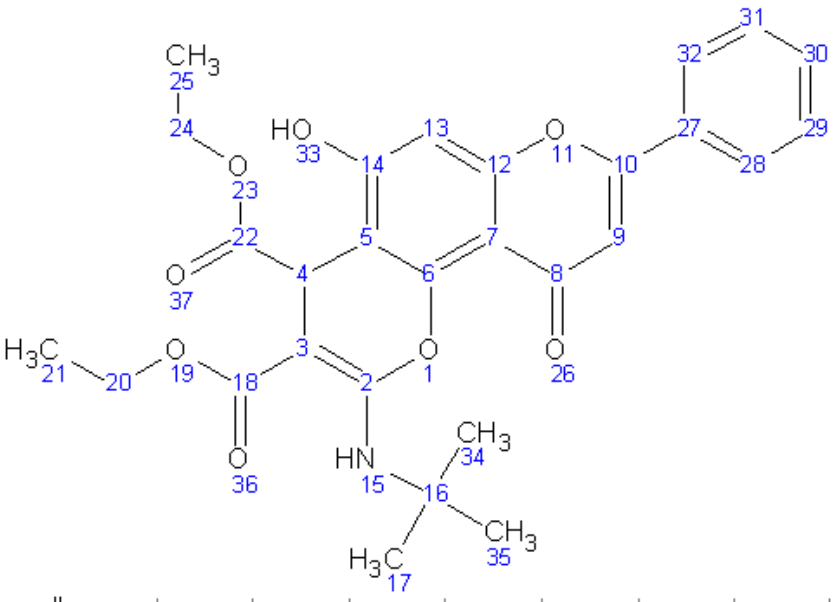


Fig 47: *^{1}H-NMR data predicted for the structure resulting from possibility 2.*



Group	nH	Shift	Error
4	1	5.887	0.78
9	1	6.589	0.08
13	1	6.657	0.12
15,33	2	7.342	3.15
17,34,35	9	1.182	0.03
20	2	4.088	0.12
21	3	1.168	0.15
24	2	4.233	0.02
25	3	1.248	0.04
28	1	7.889	0.03
29	1	7.52	0.02
30	1	7.545	0.05
31	1	7.52	0.02
32	1	7.889	0.03

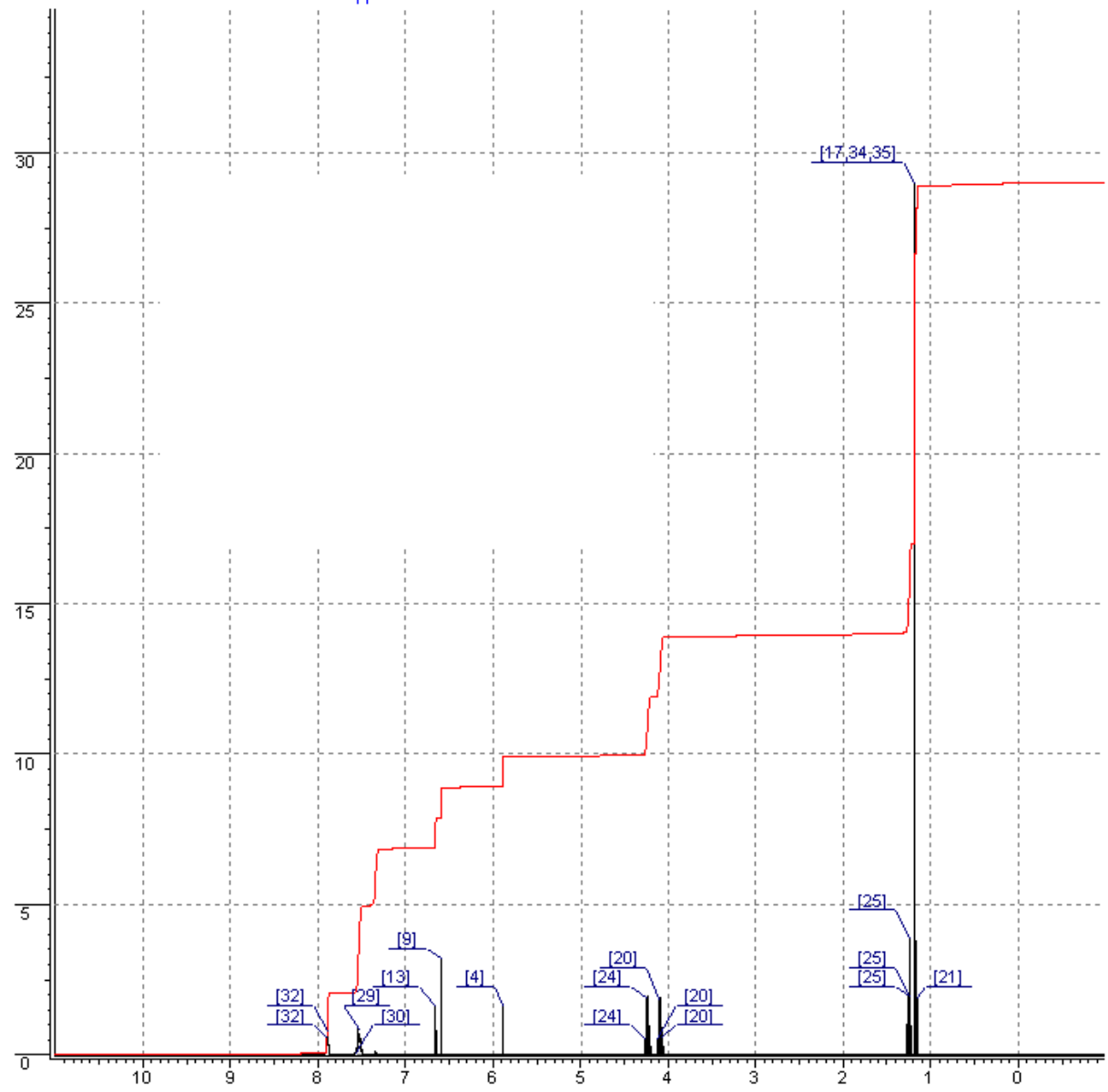


Fig 48: *^{1}H-NMR data predicted for the structure resulting from possibility 3.*

4.4. Cell culture

The cancer HL-60 cell line as a model to measure the antioxidant/ cytotoxic activities of compounds

Cytotoxicity and antioxidant activities of isolated natural and synthetic flavonoid derivatives were measured using cultured human promyelocytic leukemia HL-60 cells. Compounds were tested at six different concentrations ranging from 10 μM to 200 μM. A concentration of 200 μM was found to be most suitable to differentiate between activities of compounds. Viability of the cells was measured in the MTT assay. The purple colour of formazan was determined spectrophotometrically at 550 nm (reference at 690 nm) (***Fig 30***). Chrysin, quercetin, taxifolin, epicatechin, vitamin E and trolox® were used as controls and for comparison. The cytotoxicity of compounds was calculated as the percentage of cell viability readjusted according to the negative control (viability of cells in the negative control was set as 100 %). The antioxidant activity of compounds was calculated as the percentage of cell survival in the presence of hydrogen peroxide (the oxidativily stressed cell control was set as 100 %).

Among the natural products used, compounds ***2r***, ***3r***, ***4r*** (natural chalcone products), ***5r*** and chrysin exhibited the strongest cytotoxic activities against the HL-60 cells with a survival of 32, 28, 25, 27 and 25 %, respectively, as shown in ***Table 17***. The same compounds showed no antioxidant activity against H_2O_2 induced oxidative damage on HL-60 cells. Compounds ***2r***, ***3r***, ***4r*** and chrysin even increased the damage caused by hydrogen peroxide by 36, 8, 23 and 20 %, respectively (***Fig 49***).

In contrast, compounds ***3d***, ***5d***, ***1r*** and taxifolin showed a weak cytotoxicity (survival 85, 78, 71 and 80 % respectively) combined with a relatively weak antioxidant activity (the survival of the oxidatively stressed cells was 122 % for ***3d***, 137 % for ***1r*** and 117 % for taxifolin (please note that the oxidatively stressed control was set as 100 %)). Compound ***5d*** showed a good antioxidant activity with a protection compared with vitamin E and trolox® (survival of the cells was 211, 212, 185 % respectively) (***Fig 49***).

All other natural compounds showed no cytotoxic effect. Some of them, however, exhibited a strong antioxidant activity. In the presence of 5 mM of H_2O_2, the damaging effect of this oxidant was reduced by 240, 256, 175, 267 and 172 %, when 200 μM of compounds ***1d***, ***2d***, ***4d***, quercetin and epicatechin were used respectively (***Fig 49***).

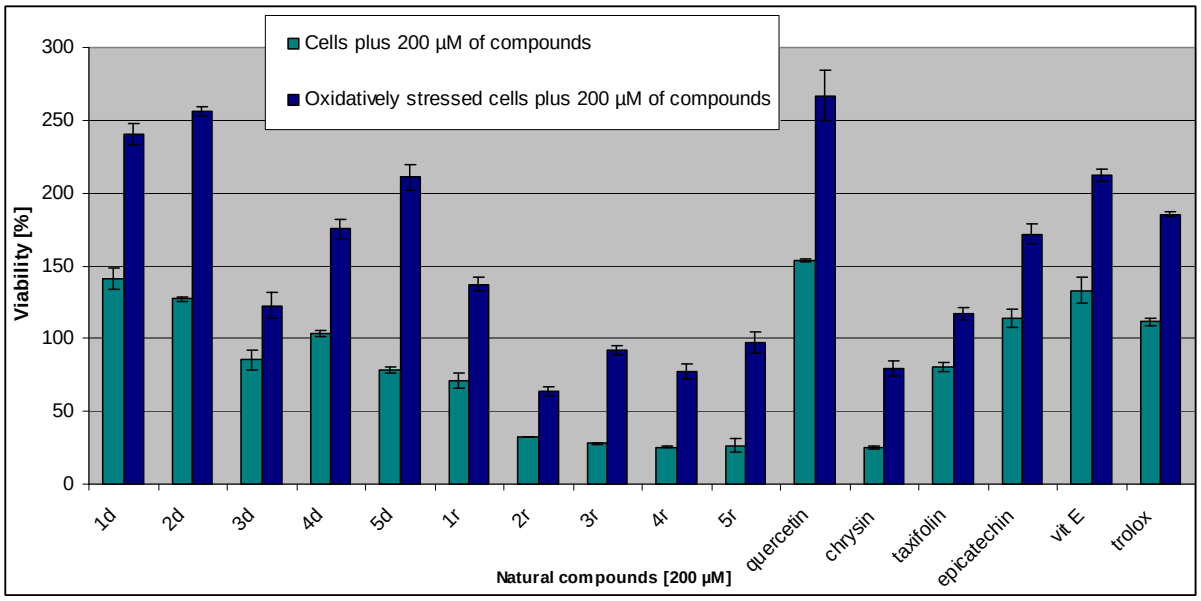


Fig 49: *Cytotoxic versus antioxidant activity of natural products as well as controls at 200 µM. Please note that the viability in the two cell culture assays is set at 100 % for either the untreated cell control or the oxidatively stressed untreated cell control. The data is presented as a mean value ± standard deviation.*

Table 17 also reflects a strong proliferative effect associated with compound ***1d***, ***2d***, ***4d***, quercetin, epicatichen, vitamin E and trolox®, with a cell survival of 141, 127, 103, 153, 114, 133, and 111 %, respectively (***Fig 49***). This proliferative effect towards HL-60 cells may be related to a strong antioxidant activity, which reduced the level of oxidative stress and therefore increases the survival of the cancer cell. Alternatively, these compounds may also serve as a nutrient to the cells.

Comparison of compounds ***10m***, ***11m*** and ***14m,*** which are synthetic products derived from quercetin, reveal a clear difference in the cytotoxic and antioxidant activities. Viability of the cells was 57, 54 and 32 % for ***10m***, ***11m*** and ***14m*** respectively, but 153 % for quercetin. In contrast, antioxidant activity of these compounds was also found to be reduced considerably when compared to their parent compound (195, 200 and 198 % for ***10m***, ***11m*** and ***14m***, respectively, 267 % for quercetin) (***Fig 50***).

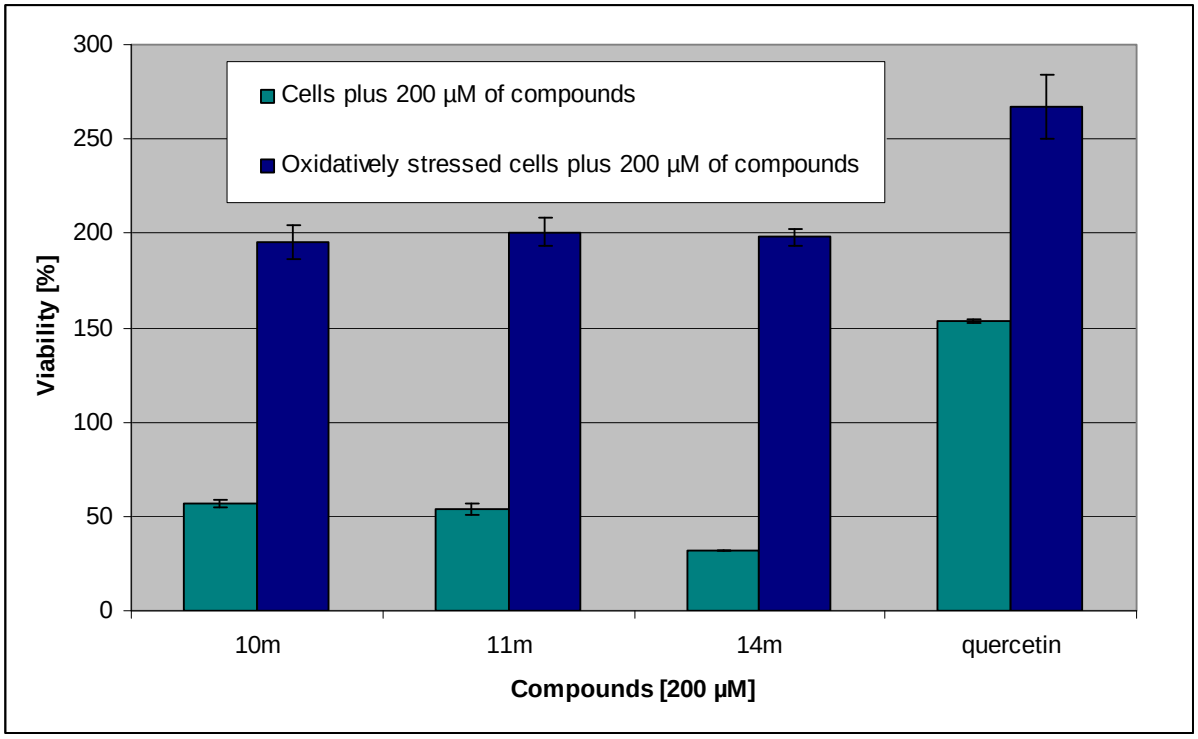


Fig 50: *Cytotoxic versus antioxidant activity of quercetin as well as* ***10m****,* ***11m*** *and* ***14m*** *(synthetic products derived from quercetin) at 200 µM. Please note that the viability in the two cell culture assays is set at 100 % for either the untreated cell control or the oxidatively stressed untreated cell control. The data is presented as a mean value ± standard deviation.*

Synthetic compounds derived from chrysin exhibited a wide range of cytotoxic activities (***Table 18***): survival of HL-60 was 94 % for ***1f*** and just 12 % for ***7f***. In contrast, and as expected, all of these compounds showed no antioxidant activity. Some of them even enhanced the damaging effects of H_2O_2 on the HL-60 cells, with cell survival of 85, 80, 80, 49 and 86 % for ***1m***, ***1f***, ***2f***, ***7f*** and ***8f***, respectively. This effect may be due to an increase in the oxidative stress within the cancer cells triggered by these chrysin derivatives.

The antioxidant activity observed for some of the compounds may help better understand the role of antioxidant flavonoid compounds in protecting human cells against oxidative stress. The results also indicate that there is an inverse relationship between cytotoxic activity against HL-60 cells on the one hand, and an increased antioxidant activity on the other.

The results presented in ***Table 18*** further indicate that addition of alkylamine moieties to quercetin by a Mannich type reaction causes a significant increase of cytotoxic activity, with a decrease of antioxidant activity. Chromene-flavones show large differences in cytotoxicity that

may be due to the presence of an alkyl chain on the nitrogen and the different dicarboxylate moieties.

4.5.Nematode assay

Steinernema feltiae is a beneficial nematode that seeks out and kills the immature stages of harmful soil-dwelling insects before they become adults. It has been used as an intact animal model that may provide detailed insight into the toxicity of the selected compounds.

Steinernema feltiae nematodes were incubated with selected compounds and numbers of living nematodes were counted under a microscope at 0, 24 and 48 hours. The results presented in ***Table 19*** indicate that most compounds exhibited no significant toxicity against *Steinernema feltiae*, when used at two different concentrations of 200 μM and 400 μM. After 24 hours of incubation, only compounds ***5r*** (survival was 96 % at 200 μM and 95 % at 400 µM), and ***14f*** (survival was 94 % for 200 µM and 92 % for 400 µM) showed a weak toxicity.

Despite this, it can still be envisaged that the majority of these compounds have little or no effect on the nematodes, and therefore may not be toxic to higher animals and humans; further research would be required for confirmation of this in more detail.

4.6.Aromatase assay

Estrogens are involved in numerous physiological processes including the development and maintenance of the female sexual organs, the reproductive cycle and various neuroendocrine functions. These hormones also have crucial roles in certain disease states, particularly in mammary and endometrial carcinomas. Cancer is the leading cause of death among women between the ages of 30 and 54, with breast and uterine cancers comprising 28 % and 10 % of cancer related deaths [111], respectively.

Aromatase is a cytochrome P450 enzyme (CYP19), a unique member of a super-family of microsomal enzymes that catalyzes the aromatization of androgens (testosterone and androstenedione) to estrogens (estradiol and estrone, respectively) through the aromatization of the **A**-ring of androgen substrates. Not surprisingly, aromatase has been considered a particularly attractive target for the treatment of hormone-dependent breast cancer [183].

Aminoglutethimide has been used in the studies as a comparative control. This compound is an anti-steroid drug marketed around the world under the trade name Cytadren® by Novartis. It blocks the production of steroids derived from cholesterol and is clinically used in the treatment of Cushing's syndrome and metastatic breast cancer. It is also a drug abused by body builders.

The mechanism of aromatase inhibition by flavonoids is based on the fact that rings **A** and **C** of flavonoids mimic the aromatase substrate rings **C** and **D** of a genuine aromatase substrate (see ***Fig 51***) [194]. The addition of another ring to the flavonoids (ring **D**) may further enhance the mimetic properties of these flavonoids, therefore increasing their aromatase inhibiting effect (***Fig 51***).

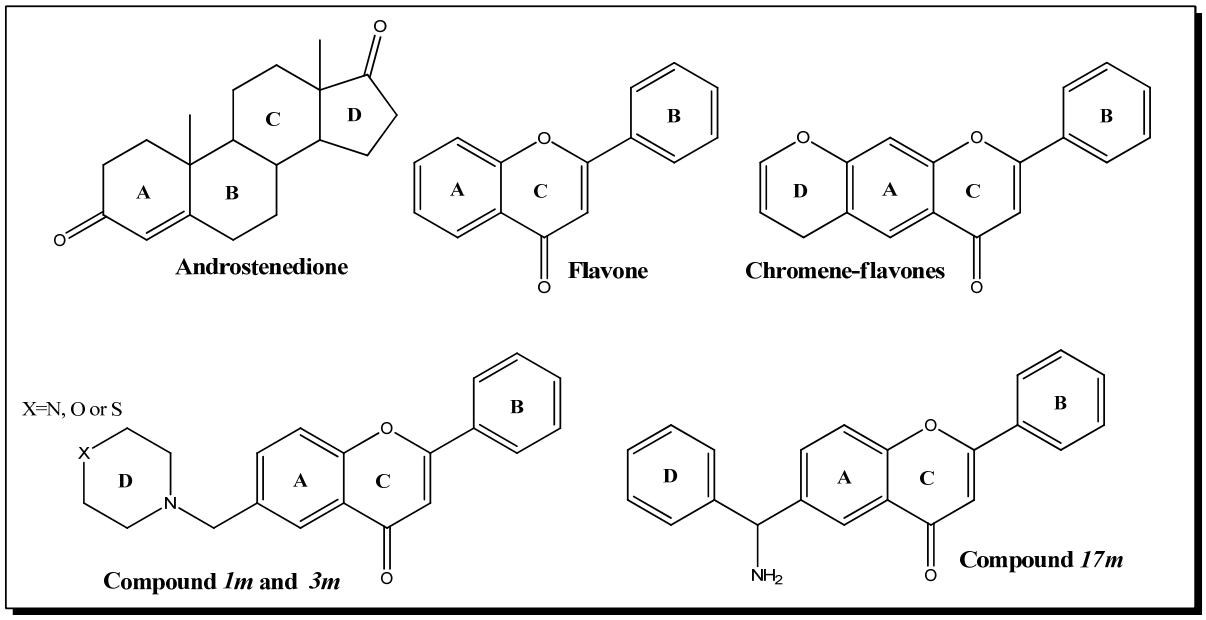


Fig 51: *Structural similarities between the aromatase substrates on one hand and flavonoids on the other. These similarities may explain the ability of flavonoids to inhibit aromatase in a competitive manner.*

Chrysin has been is reported to be a strong aromatase inhibitor which served as bench mark [65]. It was used in the experiments together with quercetin, taxifolin and epicatechin as controls.

Among the natural products employed, chrysin was found to be the strongest inhibitor of aromatase. Most of the isolated natural products also exhibited a significant inhibiting activity; compounds ***5d***, ***1r***, ***3r*** and ***4r*** (***Table 17***), with an IC_{50} equal to 20.9 ± 7.32, 19.9 ± 5.42, 35.2 ± 2.50 and 21.5 ± 1.77 μM, respectively, showed even stronger effects than aminoglutethimide and quercetin (IC_{50} = 36.4 ± 0.26 and 105.2 ± 4.38 μM respectively) (see

Fig 52). In contrast, compound ***2d*** was a relatively weak inhibitor with an IC_{50} of 194.2 ± 9.55 μM. IC_{50} values of the other natural products, including taxifolin and epicatechin, were larger than 300 μM.

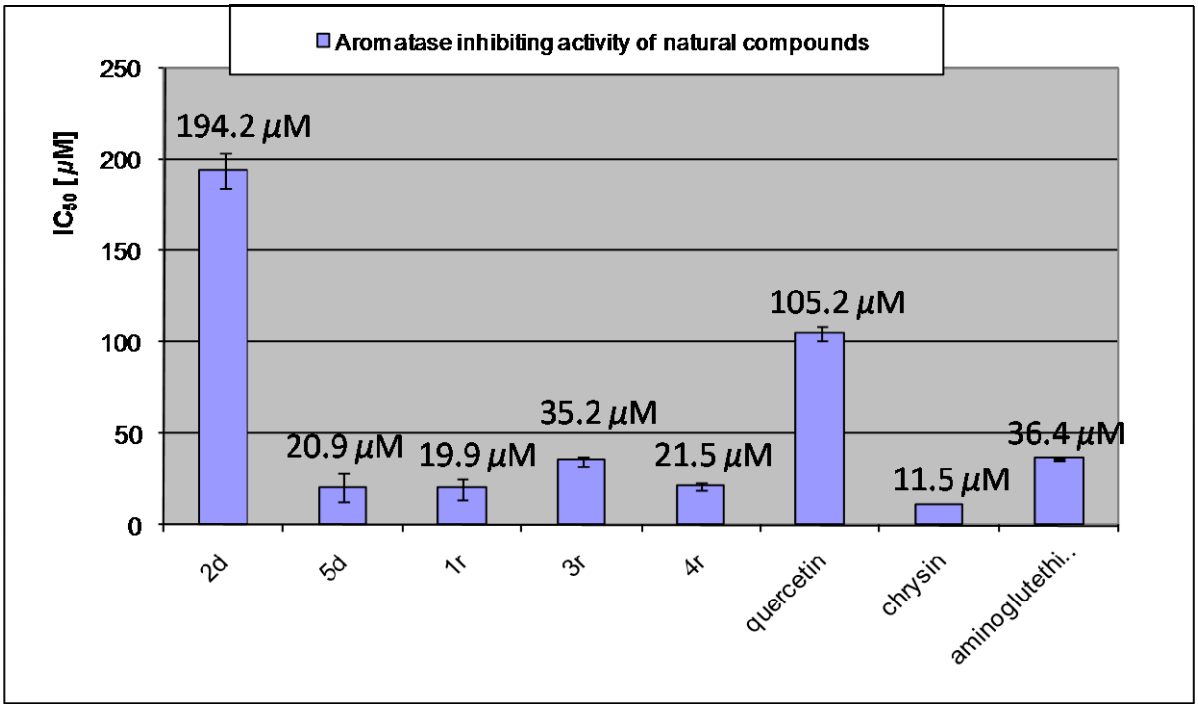


Fig 52: *Aromatase inhibiting activity of natural products, presented as IC_{50}. The data presented as a mean value ± standard deviation.*

Some of the chrysin-based synthetic flavonoid derivatives proved to be stronger aromatase inhibitors compared to chrysin itself. Compounds ***3m*** and ***11m*** for instance, showed IC_{50} values of 5.9 ± 1.86 and 11.2 ± 1.34 μM respectively. Many synthetic flavonoids showed activities better than aminoglutethimide, such as compound ***13m*** (IC_{50} = 28.2 ± 1.99 μM), ***16m*** (IC_{50} = 15.6 ± 3.92 μM), ***14f*** (IC_{50} = 26.3 ± 1.01 μM) and ***15f*** (IC_{50} = 36.4 ± 2.44 μM). Some compounds only showed moderate activities, such as ***1m***, ***5m*** and ***8f*** with IC_{50} values of 87.5 ± 7.48, 69.6 ± 9.85 and 79.4 ± 4.47 μM, respectively. Weak activities were observed for some compounds such as ***6m*** and ***6f,*** with IC_{50} values equal to 178.3 ± 9.55 and 166.6 ± 11.35 μM, respectively.

The results discussed previously and displayed in ***Tables 17*** and ***18*** indicate that 2,3-dihydroflavones (***5d*** and ***1d***), chalcones (***3r*** and ***4r***), in addition to thiomorpholin drivatized chrysin (***3m***, ***11m***) and some chromene-flavones (***14f***) exhibit good inhibitor activity against aromatase compared to benchmark compounds. These flavone-derivatives may have a potential use in breast cancer therapy.

4.7.DPPH radical scavenging activity

The scavenging ability of compounds was examined by using the stable 2,2-dipheny-1-picryl hydrazyl (DPPH) free radical as a model. With antioxidant compounds present in the solution of DPPH, the purple colour of DPPH decolourizes and this decline is measured at 530 nm. The degree of decolourisation is directly proportional to the antioxidant potency of the compound tested.

The DPPH assay was performed with all compounds that had previously shown activity in the antioxidant assay based on the HL-60 cell line. A few chromene-flavones were also tested. Our aim was to investigate the antioxidant activities of the compounds *in vitro* by a direct chemical reaction and to compare these results with the ones obtained in cell culture.

The purple DPPH free radical reacts with flavonoids. It attracts an electron or hydrogen atom ($H^{\cdot}$) and converts to a stable, colourless compound, while the flavonoid is converted to a flavonoid radical (***Fig 53***).

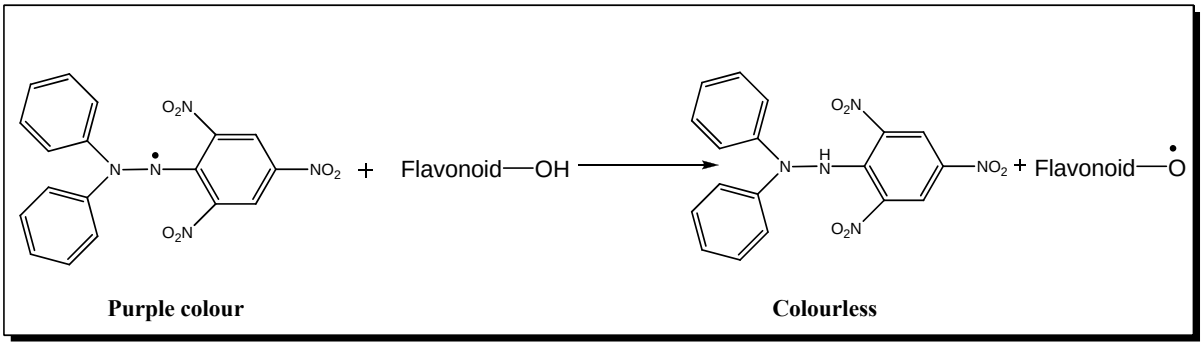


Fig 53: *Reaction of the DPPH free radical with flavonoids.*

Known antioxidant compounds, such as quercetin, taxifolin, epicatechin, vitamin E and trolox® were also used in this assay. Out of all these compounds, quercetin exhibited the highest scavenging activity with an IC_{50} equal to 35.0 ± 1.71 μM, which is in good agreement with HL-60 cell protection from H_2O_2 (267 %) observed for this apparently antioxidant compound. Compounds ***1d***, ***2d***, ***4d***, ***5d*** and ***1r*** are natural products with IC_{50} values in the DPPH assay equal to 41.6 ± 2.27, 51.6 ± 3.35, 88.7 ± 5.53, 102.6 ± 2.85 and 109.1 ± 8.16 μM, respectively, which is also compatible with the results obtained in HL-60 cell culture,

where these compounds act as protectants against H_2O_2 (240, 256, 175, 210 and 137 %, respectively) (***Table 17***).

Among all new synthetic compounds, only compounds derived from quercetin showed scavenging activity against the DPPH free radical, and even then, the activity was weaker than that of quercetin itself (***Table 18***) Although these results were compatible with the results obtained in cell culture, they do not point towards any particulary promising antioxidant activity of these compounds.

4.8. Antioxidant structure activity relationship

Through analysis of the results obtained in cell culture (protection of the HL-60 cells against damage caused by H_2O_2 effect) and in the DPPH assay, it is possible to deduce the relationship between the structure of the compounds and their antioxidant potential. Quercetin, for instance, has an almost ideal structure to develop antioxidant activity (***Table 17*** and ***18***).

In addition to the presence of five free hydroxyl groups, the **B**-ring of quercetin has a catechol type 3´,4´-dihydroxy benzene structure, as well as the 3-hydroxyl and 4-keto groups present in the **C**-ring, and the double bond between C2 and C3. Together, these features may explain the antioxidant strength of quercetin (***Fig 54***).

Taxifolin has the same number and distribution of free hydroxyl groups as quercetin, but differs with regard to the saturation of the **C**-ring (absence of a double bond between C2 and C3). Yet this compound showed only one third of the antioxidant activity of quercetin in the DPPH assay. Epicatechin also has the same number of hydroxyl groups, yet it exhibited weak antioxidant activity in the DPPH assay, probably due to the absence of the 4-keto group (***Fig 54***).

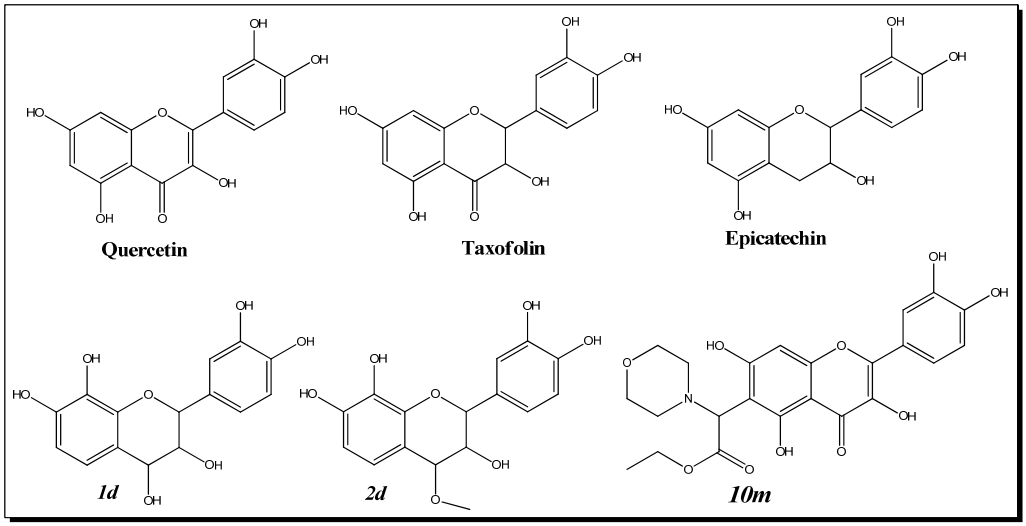


Fig 54: *Structures of compounds with antioxidant activity.*

Compounds ***1d*** and ***2d*** also lack the double bond between carbons 2 and 3, yet, at the same time, these compounds showed a higher DPPH scavenging activity (IC_{50} = 41 and 51 μM, respectively) as well as better HL-60 cell protection (240 and 256 %, respectively) when compared to epicatechin and taxifolin. The **A**-rings of ***1d*** and ***2d*** possess *ortho* dihydroxy groups (catechol-type) which may assist in the electron or hydrogen ($H^{\cdot}$) donating properties of these compounds, and increase the stability of the resulting flavonoid radical (***Fig 54***).

Derivatisation of quercetin with amino alkyl derivatives is found to reduce the antioxidant and scavenging activity. The addition of hydrocarbon chains, which do not bear antioxidant groups, yet that are lacking in more conjugation and free hydroxyl groups, may be the cause of the weak activity associated with quercetin derivatives.

In summary, the number and positioning of free hydroxyl groups is a very important factor which exerts a significant effect on the antioxidant activity of individual flavonoids. The presence of a double bond between the C-2 and C-3 together with the keto group at position 4, also appear to be a significant factor of good antioxidant activity. These factors enable flavonoids to form stable free radicals.

4.9.Electrochemical properties

Differential pulse polarography (DPP) was employed to study the redox behaviour of the compounds and to compare them to other flavonoids of different oxidation states. Overall,

all chromene-flavones studied exhibit a similar redox behaviour, which is dominated by a major oxidation current of around +800 to +900 mV *vs.* SSE, with ***11f*** and ***13f*** at higher potentials (+1230 and +1320 mV, respectively). The underlying oxidation process is irreversible, as confirmed by the absence of a reduction current in Cyclic Voltammetry (CV). The electrochemical behavior of the chromene-flavones is therefore similar to that of the parent flavone chrysin, whose DPP also shows an oxidation signal at around +800 mV. Both chromene-flavones and chrysin also exibit small oxidation currents at around -150mV and +100 mV, respectively. The major oxidation currents observed for ***1f-15f*** and chrysin may be assigned to the phenolic ring system **A**. Although the **A**-ring in chrysin is formally a resorcinol and not just a phenol (in contrast to ***1f-15f***), the *meta*-substitution in the resorcinol ring implies that its redox behaviour is more similar to the one of a phenol (oxidation to a phenyl radical at +520 mV) than of a hydroquinone (oxidation to *ortho*- or *para*-quinone). Such hydroquinone redox chemistry is present in other flavonoids, however, which contain a catechol moiety (**B**-ring) and whose redox behaviour differs from the one of chromene-flavones. In general, these flavonoids exhibit a (mostly quasi-reversible) redox signal between +100 and +200 mV, which is due to catechol redox chemistry and endows these compounds with particular antioxidant properties. Epicatechin shows a major signal in DPP at +120 mV, taxifolin at +135 mV, the anthocyanidin cyanidine at +200 mV and quercetin at +160 mV, which turns these natural products into good electron donors and antioxidants. The catechol substituent and the redox chemistry associated with it (signals at around +150 to +250 mV) are notably absent in the chromene-flavones and in chrysin, and these compounds are quite cytotoxic.

Overall the electrochemical studies therefore confirm and to some extent also rationalise the observations made in cell culture and in the *in vitro* assay. In particular, it is possible to explain some of the apperent “contradictions” between cytotoxicity (of chrysin derivatives) and antioxidant activity (of catechol-bearing derivatives).

Chapter V: Conclusion and Outlook

Two new natural products in addition to eight well known ones have been isolated from the roots of two *Leguminosae* plants (*Delonix regia*, growing in Egypt, and *Robinia pseudoacacia*, growing in Germany). The isolation and purification procedures included extraction and successive partitions of the water soluble part with ethyl acetate and *n*-butanol, followed by silica gel and Sephadex column chromatography.

Twenty-one chrysin and quercetin derivatives were synthesized via Mannich type reactions. The reaction conditions were monitored tightly to obtain the desired regioselective products at an acceptable yield and to avoid complications with the isolation and purification. Type of the flavonoid, reaction solvent, the bulk of the reactant and the reaction time and temperature were the most important factors affecting the yield and selectivity of these reactions. Our results indicate that the highly hydroxylic subistituted flavone (quercetin) is more reactive in the Mannich reaction. This creates problems with the synthesis of a regioselective product in relatively good yield, and additional problems with the isolation and purification of products are encountered.

The addition of a further ring to chrysin (ring **D**) has been successfully achieved by the reaction of chrysin with an isonitrile and acetylene dicarboxylate. Fifteen new chromene-flavone compounds have been produced by this method with a good yield ranging from 25% for ***12f*** to 85% for ***9f***. The chromene-flavone structures were confirmed by 1D and 2D NMR data and mass spectroscopy (LC-MS and HR-MS), and compared to the prediction calculation with the computational structure prediction program ACDLabs 7.0.

A large number of flavonoid compounds (10 natural products and 38 synthetic products in addition to chrysin, quercetin, epicatechin and taxifolin) were tested for antioxidant and anticancer potency using *in vitro* and *in vivo* assays. The diversity of these compounds was useful for the investigation of the structure antioxidant activity relationships of these compounds. In addition to comparison of the cytotoxic activity and aromatase inhibition, the results of the nematode assays also provided information about the selectivity and toxicity of the compounds towards cells and intact organisms.

The natural product quercetin, in addition to compounds ***1d*** and ***2d,*** (***Fig 55***) exhibited the highest antioxidant potency *in vivo* (protection of the HL-60 cells against H_2O_2 induced

oxidative damage) with 267, 240 and 256 % protection respectively (please note that the oxidatively stressed cell control is set as 100 %). The results obtained from the *in vitro* DPPH assay also pointed towards high antioxidant potency for the aforementioned compounds (with IC_{50} values of 35.0, 41.6 and 51.6 μM respectively). The results obtained for ***1d*** and ***2d*** revealed that ***2d*** (with a methoxy group at position 4 of the 2,3-dihydroxyflavan nucleus), has more protecting power in the cell culture assay compared to ***1d***. At the same time, ***1d***, with one more free hydroxyl group (***Fig 55***), showed more activity in the *in vitro* DPPH assay (see ***Table 17***).

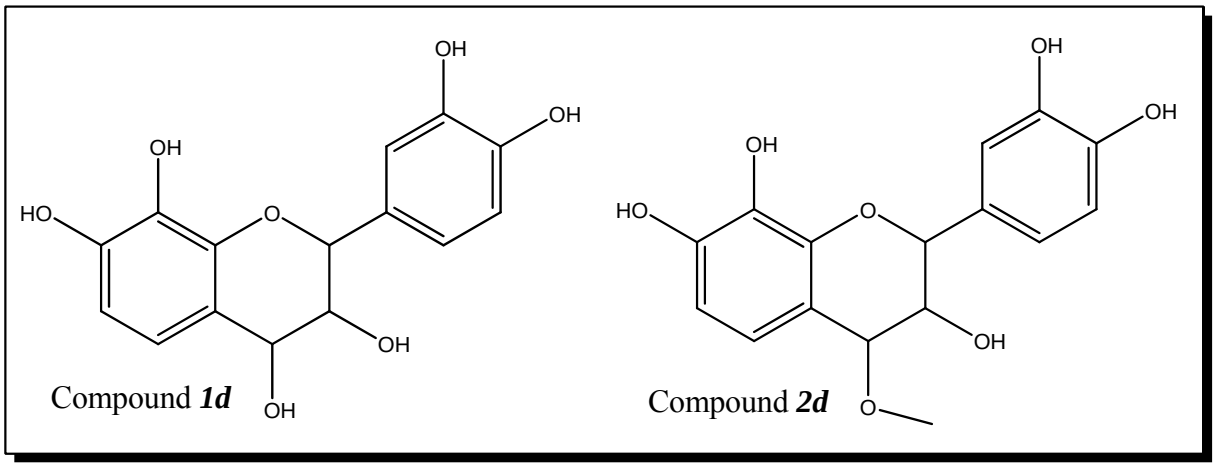


Fig 55: *Structure of compounds* ***1d*** *and* ***2d****, which appear to act as promising antioxidants.*

Compounds ***10m*** and ***14m*** (***Fig 56***), which are quercetin derivatives, showed strong scavenging of the DPPH radical with IC_{50} values of 49.4 and 58.6 μM, respectively. Nonetheless, these compounds exhibited relatively weak protective effects against H_2O_2 induced oxidative damage in cell culture (195 and 198 % protection, respectively).

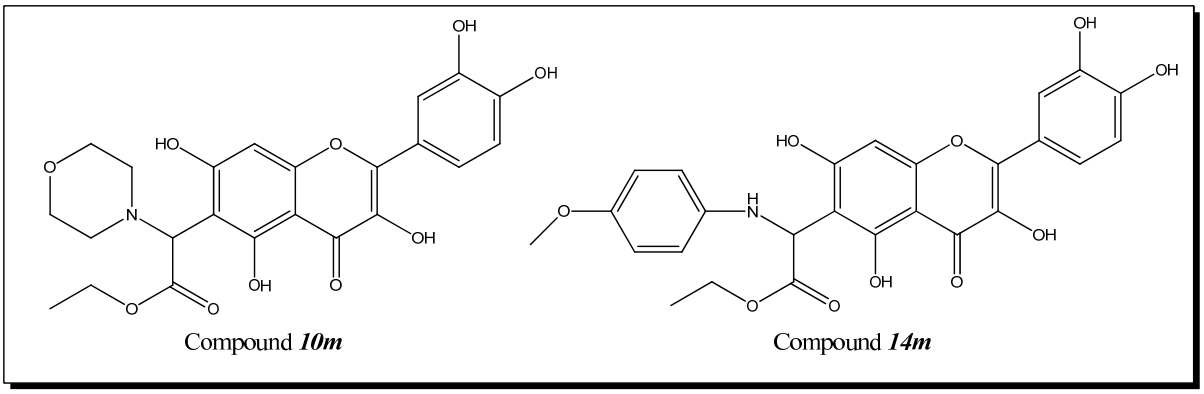


Fig 56: *Structure of compounds* ***10m*** *and* ***14m****, which is quercetin derivative.*

It is possible to derive a relationship between the antioxidant and the cytotoxic activity of the compounds: In essence, powerful antioxidant compounds appear to have little or no cytotoxic effects. Compound ***4d*** and epicatechin showed moderate antioxidant activities in addition to cell protective effects. At the same time, these two compounds showed no cytotoxic activity in the HL-60 cell assay. Other examples for this "antioxidant" relation are quercetin, ***1d*** and ***2d***, which all showed a significant increase in the proliferation of the cells compared to the controls.

In contrast, ***7f***, a chromene-flavone was found to have the strongest cytotoxicity with a mortality rate of 88 % in the HL-60 cells. Compounds ***3r***, ***4r*** and ***5r***, in addition to chrysin, ***5m***, ***13m***, ***16m***, ***2f***, ***11f***, ***12f*** and ***14f***, showed a strong cytotoxic activity, while most of the other synthetic compounds showed only a moderate cytotoxicity. Results of the nematode assay for some of these compounds showed a very weak nematocidal activity. Together, these cytotoxicity (and antioxidant) results may be useful in building a concept regarding the selectivity of the compounds towards the cancer cells.

Some of the natural products showed considerable inhibiting potency towards the aromatase enzyme. Compounds ***5d***, ***1r***, ***3r*** and ***4r*** seem to be even more effective than aminoglutethimide. A point we must bear in mind is that compounds ***5d*** and ***1r*** were only weakly cytotoxic, which again may provide an entry point for selectivity, in this case for the treatment of breast cancer.

Some of the synthetic compounds were also effective aromatase inhibitors, especially ***3m***, ***11m***, ***13m***, ***16m***, ***14f*** and ***15f***, which exhibited the highest activity among all compounds (some of them were even more active than chrysin). Compound ***11m,*** a derivative from quercetin, showed higher activity against aromatase than quercetin itself. Although the

quercetin core-structure does not appear to inhibit aromatase particularly well, ***11m*** indicates that more effective aromatase inhibition may still be built on the quercetin core.

In the future, it will be important to develop a method to remove the side chain acetyl groups from the chromene-flavone compounds, and in doing so, perhaps enhance the bioavailability and solubility of these compounds. Various methods were implemented in an attempt to remove this ester link, but without noticeable results. Removal of parts of the nitrogen side chain may also be beneficial (***Fig 57***).

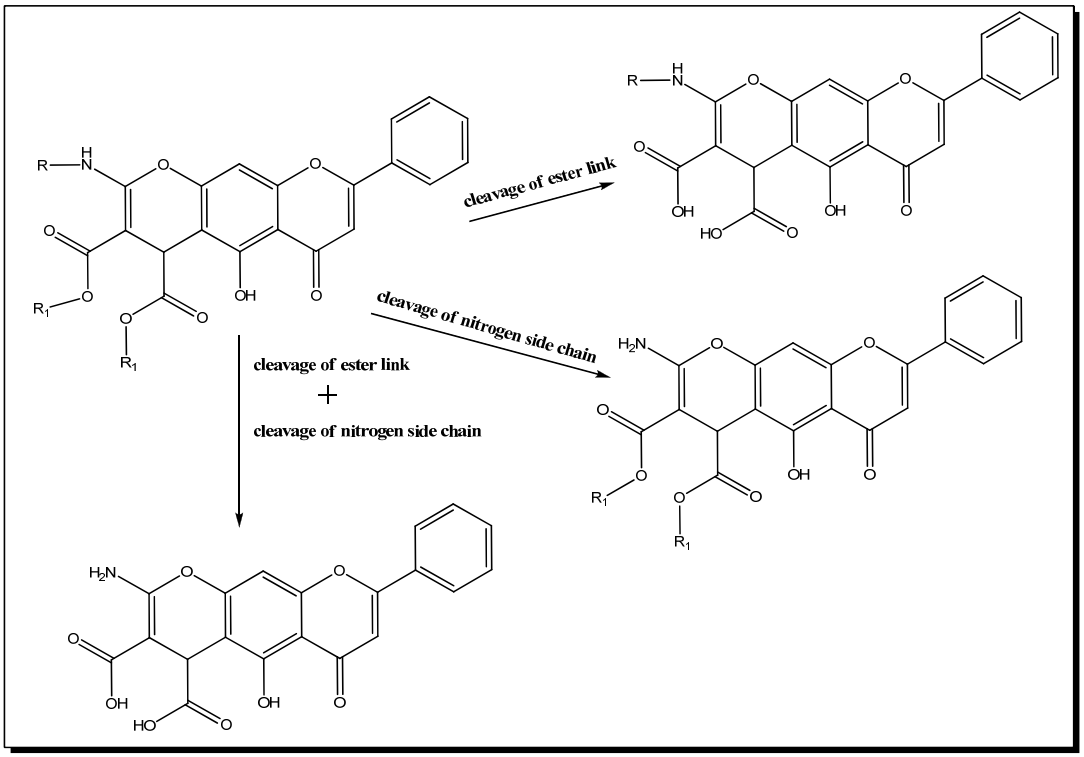


Fig 57: *Suggested removal of some side chain groups from the chromene-flavones in order to increase activity and improve the pharmacokinetic profile of the compounds.*

Removal of this nitrogen side chain from the chromene-flavones will provide a new type of structurally-related substances which could prove to be useful as enzyme inhibitors and/or anti-cancer agents. This is a possible direction to be taken in the future, which would combine advanced synthetic chemistry with more extensive and in-depth biological testing.

References

1. G. Di Carlo, N. Mascolo, A.A. Izzo, F. Capasso, Flavonoids: Old and new aspects of a class of natural therapeutic drugs, *Life Sci*., **1999**, 65, 337–353.
2. T. Swain, J.B. Harborne, T.J. Mabry, H. Mabry (Eds.), The Flavonoids, Chapman and Hall, London, UK, **1975**, p. 1096.
3. C. Remésy, C. Manach, C. Demigne, O. Texier, F. Regerat, in: J. Vercauteren, C. Chèze, J. Triaud (Eds.), Polyphenol 96, 18th International Conference on Polyphenols, INRA Éditions, (France), **1996**, p. 251.
4. M.G.L. Hertog, E.J.M. Feskens, D. Kromhout, P.C.H. Hollman, M.B. Katan, Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study, *The Lancet*, **1993**, 342, 1007-1011.
5. S. Renaud, M. de Lorgeril, Wine, alcohol, platelets, and the French paradox for coronary heart disease, *The Lancet*, **1992**, 339, 1523-1525.
6. B. Simini, Serge Renaud: from *French paradox* to Cretan miracle, *The Lancet*, **2000**, 355, 48.
7. R. Brouillard, A. Cheminat, Flavonoids and plant color. *Prog. Clin. Biol. Res.*, **1988**, 280, 93–106.
8. J. Kuhnau, The flavanoids. A class of semi-essential food components: their role in human nutrition, *World Rev. Nutr. Diet.*, **1976**, 24, 117-191.
9. H. De Groot, U. Raven, Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundam. Clin. Pharma. Col.*, **1998**, 12, 249-255.
10. J.B. Harborne, The flavonoids advanced in research since 1986, Chapman &Hall, London, New York, Tokyo, **1994**, 499-514.
11. J. Bruneton, Pharmacognosy, phytochemistry of medicinal plant, 2nd edition, TEC & DOC., London, Paris, NewYork, **1999**, 316-319.
12. J.F. Hammerstone, S.A. Lazarus, H.H. Schmitz, Procyanidin content and variation in some commonly consumed foods, *J. Nutr.*, **2000**, 130, 2086S–2092S.
13. C. Santos-Buelga, A. Scalbert, Proanthocyanidins and tannin-like compounds in human nutrition, occurrence, dietary intake and effects on nutrition and health, *J. Food Sci. Agr.*, **2000**, 80, 1094–1117.
14. D.A. Balantine, S.A. Wiseman, L.C.M. Bouwens, The chemistry of tea flavonoids, *Crit. Rev. Food Sci. Nutr.*, **1997**, 37, 693–704.

15. N.C. Cook, S. Samman, Flavonoids: Chemistry, metabolism, cardioprotective effects, and dietary sources, *J. Nutr. Biochem.*, **1996**, 7(2), 66–76.
16. H.J. Leese, G. Semenza, On the identity between the small intestinal enzymes phlorizin hydrolase and glycosylceramidase, *J. Biol. Chem.*, **1973**, 248(23), 8170–8173.
17. A.J. Day, F.J. Canada, J.C. Diaz, P.A. Kroon, R. McLauchlan, C.B. Faulds, M.R. Morgan, G. Williamson, Dietary flavonoid and isoflavone glycosides are hydrolyzed by lactase site of lactase phlorizin hydrolase, *FEBS Lett*, **2000**, 468, 166–170.
18. L.B. Daniels, P.J. Coyle, Y.B. Chiao, R.H. Glew, Purification and characterization of a cytosolic broad specificity beta-glucosidase from human liver, *J. Biol. Chem.*, **1981**, 256(24), 13004–13013.
19. V. Gopalan, A. Pastuszyn, W.R. Galey, R.H. Glew, Exolytic hydrolysis of toxic plant glucosides by guinea pig liver cytosolic beta-glucosidase, *J. Biol. Chem.*, **1992**, 267(20), 14027–14032.
20. J.P.E. Spencer, G. Chowrimootoo, R. Choudhury, E.S. Debnam, S.K. Srai, C. Rice-Evans, The small intestine can both absorb and glucuronidate luminal flavonoids, *FEBS Lett*, **1999**, 458, 224–230.
21. G. Cao, R.L. Prior, Anthocyanins are detected in human plasma after oral administration of an elderberry extract, *Clin. Chem.*, **1999**, 45, 574–576.
22. I. Erlund, G. Alfthan, J. Maenpaa, A. Aro, Tea and coronary heart disease: the flavonoid quercetin is more bioavailable from rutin in women than in men, *Arch. Intern. Med.*, **2001**, 161(15), 1919–1920.
23. J.M. Gee, M.S. Dupont, M.J. Rhodes, I.T. Johnson, Quercetin glucosides interact with the intestinal glucose transport pathway, *Free Radic. Biol. Med.*, **1998**, 25(1), 19–25.
24. K.R. Markham, L.J. Poter, flavonoids in green algae (*Chlorophyta*), *phytochemistry*, **1969**, 8(9), 1777-1781.
25. R. Marchelli, L.C. Vining, the biosynthetic origin of chloroflavonin and flavonoid antibiotic from *Aspergillus candidus*, *Can. J. Biochem.*, **1973**, 51 (12), 1624-1629.
26. R. Sanduja, G.E. Martin, A.J. Weinheimer, M. Alam, M.B. Hossain, D. van der Helm, secondary metabolite of coelenterate *Echinophora labellosa*, *J. heterocycl. Chem*. **1984**, 21, 845-848.
27. J.B. Harborne (Ed), Phytochemical dictionary of Liguminosae, volume 1, "plant and their constituents", Chapman and Hall, London, **1994**.

28. T.E. Wills, "text book of pharmacognosy" CBS publisher & distributor, India, 5th ed. **1985**, 198-199.
29. G.E. Trease, W.C. Evans, "Pharmacognosy", Bailliere Tindal, London, 11th ed., **1978**, 103-104.
30. S. Al-Bahry, A.E. Elshafie, M. Deadman, A. Al Sa'di, A. Al Raeesi, Y. Al Maqbali, First report of *Ganoderma colossum* on *Ficus altissima* and *Delonix regia* in Oman, *Plant Pathology*, **2005**, 54(2), 245-245.
31. E.F. Gilman, D.G. Watson, *Delonix regia* –US Forest Service Fact Sheet, TS-228, **1993**, 1-3.
32. F. Adje, Y.F. Lozano, E. Meudec, P. Lozano, A. Adima, G.A. Nzi, E.M. Gaydou, Anthocyanin Characterization of Pilot Plant Water Extracts of *Delonix regia* Flowers, *Molecules*, **2008**, 13(6), 1238-1245.
33. N.A.M. Saleh, M.S. Ishak, Anthocyanins of some leguminosae flowers and their effect on colour variation. *Phytochemistry,* **1976**, 15, 835-836.
34. A.H.S. Abou Zeid, Phytochemical and biological investigation of *Delonix regia* Raf. Leaves, *Bulletin of the Faculty of Pharmacy (Cairo University)*, **2002**, 40(3), 175-187.
35. P.P. Joy, J.S. Mathew, B.P. Skaria, Medicinal plants. *Trop. Horticult*., **2001**, 2, 449-632.
36. M.V. Jyothi, S.N. Mandayan, N.C. Kotamballi, N. Bhagyalakshmi, Antioxidative efficacies of floral petal extracts of *Delonix regia* Raffin, *Int. J. Biomed. Pharmaceut. Sci.*, **2007**, 1, 73-82.
37. R.H. Sammour, A.R. El-Shanshoury, Antimicrobial activity of legume seed proteins, *Bot. Bull. Acad. Sin*., **1992**, 33, 185-190.
38. F. Aqil, I. Ahmad, Broad-spectrum antibacterial and antifungal properties of certain traditionally used Indian medicinal plants, *World J. Microbiol. Biotechnol.*, **2003**, 19 (6), 653-657.
39. L.H. Bailey, Most Commonly Grown in the Continental United States of Canada, *Manual of Cultivated Plants*, **1991**, 9581, 559–560.
40. T.B. Myron, J.T. Hugh, Root Development as a factor in the Success or Failure of Windbreak Trees in the Southern High Plains, *Journal of Forestry*, **1938**, 36, 790–803.
41. A. Rabijns, C. Verboven, P. Rougé, A. Barre, E.J. Van Damme, W.J. Peumans, C.J. DeRanter, Structure of A legume Lectin From the Bark of *Robinia pseudoacacia* and its Complex with N. acetyl Galactesamine, *Proteins*, **2001**, 44(4), 470–478.

42. J. Wantyghem, N. Platzer, M. Giner, C. Derappe, Y. Goussault, Structure analysis of the carbohydrate chain of glycopeptides isolated from *Robinia pseudoacacia* Seed Lectins, *Carbohydrate research*, **1992**, 236, 181-193.
43. C. Baoliang, K. Junei, N. Toshihiro, Triterpene Glycosides from the Bark of *Robinia pseudoacacia* L. II, *Chem. Pharm. Bull*., **1993**, 41(3), 553–556.
44. A.L. Smith, C.L. Camphell, M.P. Diwakar, J.W. Hanover, R.O. Miller, Extracts from black locust as wood preservatives: A comparison of the methanol extract with pentachlorphenol and chromated copper arsenate, *Holzforschung*, **1989**b, 43, 421–423.
45. A.L. Smith, C.L. Camphell, D.B. Walker, J.W. Hanover, Extracts from black locust as wood preservatives: extraction of decay resistance from black locust heartwood, *Holzforschung*, **1989**a, 43, 293–296.
46. Trees, Structure and Function, **1996**, 11 (5), 316–321.
47. J.C. Huntley, *Robinia pseudoacacia* L. black locust. In: Burns, Russell M., Honkala, Barbara H., technical coordinators. Silvics of North America. Volume 2. Hardwoods. Agric. Handb. 654. Washington, DC: U.S. Department of Agriculture, Forest Service, **1990**, 755-761.
48. Trees, Structure and Function, **1994**, 8 (4), 165–171.
49. M.D. Tindale, D.G. Raux, Phytochemical survey of the Australian species of Acacia, *Phytochemistry*, **1969**, 8, 1713-1727.
50. J.B. Harborne, Comparative Biochemistry of the Flavonoids, Academic Press, London, **1967**.
51. T. Iwashina, J. Kitajima, Chalcone and flavonol glycosides from *Asarum canadense* (*Aristolochiaceae*), *Phytochemistry*, **2000**, 55(8), 971–974.
52. S.E. Drewes, D.G. Roux, Condensed tannins. 15. Interrelationships of flavonoid components in wattle-bark extract, *Biochem. J.*, **1963**, 87, 167-172.
53. D.G. Roux, E. Paulus, Condensed tannins. 13. Interrelationships of flavonoid components from the heartwood of *Robinia pseudoacacia*, *Biochem. J.*, **1962**, 82, 324-330.
54. K. Freudenberg, L. Hartmann, Inhaltsstoffe der *Robinia pseudacacia*, *Justus Liebigs Annalen der Chemie,* **1954***,* 587 (3), 207-212.
55. H. Pachéco, Recherches sur la biochimie comparée des flavanonols dans les végétaux supérieurs, *Bull. Soc. chim. biol.*, **1957**, 39, 971-987.
56. L.J. Putman, P.E. Laks, M.S. Pruner, Chemical constituents of black locust bark and their biocidal activity. *Holzforschung*, **1989**, 43, 219–224

57. L.V. Alegrio, R. Braz-Filho, O.R. Gottlieb, Diarylheptanoids and isoflavonoids from *Centrolobium* species, *Phytochemistry*, **1989**, 28, 2359-2362.
58. F. Tian, J.L. McLaughlin, Bioactive Flavonoids from the Black Locust Tree, *Robinia Pseudoacacia*, *Pharmacutical-Biology*, **2000**, 38(3), 229–234
59. K. Yongsoo, H.O. Geon-seung, M. Kim-Cheng, Y. Kwon, S.H. Geon, C.M. Kim, Isoflovonoids from Root Cortex of *Robinia pseudoacacia*, *Nat. Prod. Sci.*, **1989**, 6(3), 139–141.
60. J.W. McClure, Physiology of flavonoids in plants, in Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships (V. Cody, E. Middleton, J.B. Harborne eds), Alan R. Liss, Inc., New York, **1986**, 77–85.
61. D.A. Smith, S.W. Banks, Formation and biological properties of isoflavonoid phytoalexins, in Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships (V. Cody, E. Middleton, J.B. Harborne eds), Alan R. Liss, Inc., New York, **1986**, 113–124.
62. P. Knekt, R. Jarvinen, A. Reunanen, J. Maatela, Flavonoid intake and coronary mortality in Finland: a cohort study, *Br. Med. J.*, **1996**, 312(7029), 478–481.
63. L.P. Balant, M. Wermeille, L.A. Griffith, Metabolism and pharmacokinetics of hydroxyethylated rutosides in animals and man, *Q. Rev. Drug Metab. Drug Interact*., 1984, 5(1), 1-24.
64. P. Ferenci, B. Dragosics, H. Dittrich, H. Frank, L. Benda, H. Lochs, S. Meryn, W. Base, B. Schneider, Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver, *J. Hepatol.*, **1989**, 9(1), 105–113.
65. R. Monteiro, I. Azevedo, C. Calhau, Modulation of Aromatase Activity by Diet Polyphenolic Compounds, J. Agric. Food Chem. 2006, 54(10), 3535–3540.
66. G. Cao, E. Sofic, R.L. Prior, Antioxidant and prooxidant behavior of flavonoids, *Free Radical Biology & Medicine*, **1997**, 22(5), 749–760.
67. W. Bors, W. Heller, C. Michel, M. Saran, Flavonoids as Antioxidants, Determination of radical Scavenging efficiencies, *Methods Enzymol*, **1990**, 186, 343–355.
68. O.F. Cristián, E. de Lamirande, C. Gagnon, Positive role of reactive oxygen species in mammalin sperm capacitation: Triggering and modulation of phosphorylation events, *Free Radical Biology & Medicine,* **2006**, 41, 528–540.
69. A. Catherine, R. Evans, N.J. Miller, G. Paganga, Structure-Antioxidant Activity Relationships of Flavonoids and Phenolic Acids, *Free Radic. Biol. Med.*, **1996**, 20, 933–956.

70. S. Jovanovic, I. Jankovic, L. Josunovic, Electron-Transfer, Reactions of Alkylperoxy Radicals, *J. Am. Chem. Soc.*, **1992**, 114, 9018–9022.
71. P. Wardman, Reduction Potentials of One-Electron Couples Involving Free Radicals in Aqueous Solution, *J. Phys. Chem. Ref. Data. Ser.*, **1989**, 18, 1637–1755.
72. Y. Hanasaki, S. Ogawa, S. Fukui, The Correlation Between Active Oxygens Scavenging and Antioxidative Effect of Flavonoids, *Free Radic. Biol. Med*., **1994**, 16, 845–850.
73. Y. Tsajimoto, H. Hashizume, M. Yamazaki, Superoxide Radical Scavenging Activity of Phenolic Compounds, *Int. J. Biochem.*, **1993**, 25, 491–494.
74. S. Jovanovic, S. Steenken, Y. Hara, M. Simic, Reduction potentials of flavonoid and model phenoxyl radicals. Which ring in flavonoids is responsible for antioxidant activity?, *J. Chem. Soc. Perkin.*, **1996**, 2**,** 2497-2504.
75. W. Heimann, F. Reiff, Beziehung zwischen chemischer konstitution und antioxygener wirkung bei flavonolen, *Fette Seifen Anstr-Mittel*, **1953**, 55. 451–458.
76. M. Lurdes, M.T. Fernandez, M. Santos, R. Rocha, M.H. Florencio, K.R. Jennings, Interactions of Flavonoids with Iron and Copper Ions: A Mechanism for their Antioxidant Activity, *Free Radical Research*, **2002**, 36(11), 1199–1208.
77. P. Dobsak, J. Siegelova, J.E. Wolf, L. Rochette, J.C. Eicher, J. Vasku, Prevention of apoptosis by deferoxamine during 4 h of cold cardioplegia and reperfusion: in vitro study of isolated working rat heart model, *Pathophysiology,* **2002**, 9, 27–32.
78. G. Giuseppe, J.O. Peter, Toxicity and cancer protective properties of flavonoids, *Free Radical Biology & Medicine*, **2004**, 37(3), 287–303.
79. J.A. Manthey, N. Guthrie, K. Grhmann, Biological properties of Citrus flavonoids pertaining to cancer and inflammation, *Curr. Med. Chem*., **2001**, 8, 135–153.
80. L. Bravo, Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance, *Nutr. ReV*., **1998**, 56, 317–333.
81. C. Kandaswami, E. Perkins, D.S. Soloniuk, G. Drzewiecki, E. Middleton, Antiproliferative effects of Citrus flavonoids on a human squamous cell carcinoma in Vitro, *Cancer Lett.*, **1991**, 56, 147–152.
82. M. Piantelli, A. Rinelli, E. Macri, N. Maggiano, L.M. Larocca, A. Scerrati, R. Roselli, M. Iacoangeli,. G. Scambia, A. Capella, F.O. Ranelletti. Type II estrogen binding sites and antiproliferative activity of quercetin in human meningiomas, *Cancer,* **1993**, 63, 193–198.

83. S.M. Kuo, Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells, *Cancer Lett.*, **1996**, 110, 41–48.
84. S. Kuntz, U. Wenzel, H. Daniel, Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines, *Eur. J. Nutr*., **1999**, 38, 133–142.
85. G. Agullo, L. Gamet-Payrastre, Y. Fernandez, N. Anciaux, C. Demigne, C. Remesy, Comparative effects of flavonoids on the growth, viability and metabolism of a colonia adenocarcinoma cell line (HT29 cells), *Cancer Lett.*, **1996**, 105, 61–70.
86. J.A. Manthey, N. Guthrie, Antiproliferative activities of Citrus flavonoids against six human cancer cell lines, *J. Agric. Food Chem.,* **2002**, 50, 5837–5843.
87. G. Agullo, L.G. Payrastre, S. Manenti, C. Viala, C. Remesy, H. Chap, B. Payrastre, Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition, *Biochem. Pharmacol.*, **1997**, 53, 1649–1657.
88. H.C. Huang, H.R. Wang, L.M. Hsieh, Antiproliferative effect of baicalein, a flavonoid from Chinese herb, on vascular smooth muscle cell, *Eur. J. Pharmacol.*, **1994**, 251, 91–93.
89. F. Casagrande, J. Darbon, Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: Regulation of cyclin-dependet kinases CDK2 and CDK1, *Biochem. Pharmacol.*, **2001**, 61, 1205–1215.
90. J.C. Le Bail, F. Varnat, J.C. Nicolas, G. Habrioux, Estrogenic and antiproliferative activities on MCF-7 human breast cancer cells by flavonoids, *Cancer Lett.*, **1998**, 130, 209–216.
91. D.M. Brownson, N.G. Azios, B.K. Fuqua, S.F. Dharmawardhane, T.J. Mabry, Flavonoids effects relevant to cancer, *J. Nutr.*, **2002**, 132, 3482S–3489S.
92. H. Liu, D.C. Radisky, C.M. Nelson, H. Zhang, J.E. Fata, R.A. Roth, M. Bissell, Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2, *Proc. Natl. Acad. Sci. U.S.A*, **2006**, 103, 4134–4139.
93. S. Caltagirone, C. Rossi, A. Poggi, F.O. Ranelletti, P.G. Natali, M. Brunetti, F.B. Aiello, M. Piantelli, Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential, *J. Cancer*, **2000**, 87, 595–600.
94. R.T. Greenlee, T. Murray, S. Bolden, P.A. Wingo, Cancer statistics, *CA Cancer J. Clin.*, **2000**, 50, 7-33.

95. M. Stockler, N.R.C. Wilcken, D. Ghersi, Systematic reviews of chemotherapy and endocrine therapy in metastatic breast cancer, *Cancer Treat. Rev.*, **2000**, 26, 151-68.
96. S.E. Bulun, E.R. Simpson, Regulation of aromatase expression in human tissues, *Breast Cancer Res.Treat.,* **1994**, 30, 19-29.
97. S. Sebastian, S.E. Bulun, A Highly Complex Organization of the Regulatory Region of the Human CYP19 (Aromatase) Gene Revealed by the Human Genome Project, *J. Clin. Endocrinol. Metab.*, **2001**, 86, 4600-4602.
98. E.R. Simpson,T. Price, J. Aitken, M. Mahendroo, G. Means, M. Kilgore, The aromatase enzyme: from cloning to cancer, *Princess Takamatsu Symp.,* **1990**, 21, 75-87.
99. G.D. Means, M.S. Mahendroo, C.J. Corbin, J.M. Mathis, F.E. Powell, C.R. Mendelson, E.R. Simpson, Structural analysis of the gene encoding human aromatase cytochrome P-450, the enzyme responsible for estrogen biosynthesis, *J. Biol. Chem.*, **1989**, 264, 19385–19391.
100. K. Toda, M. Terashima, T. Kawamoto, H. Sumimoto, Y. Yokoyama, I. Kuribayashi, Y.Mitsuuchi, T. Maeda, Y. Yamamoto, Y. Sagara, *Eur. J. Biochem.,* **1990**, 193, 559-565.
101. N. Harada, K. Yamada, K. Saito, N. Kibe, S. Dohmae, Y. Takagi, Structural characterization of the human estrogen synthetase (aromatase) gene, *Biochem. Biophys. Res. Commun.*, **1990**, 166, 365-372.
102. G.D. Means, M.W. Kilgore, M.S. Mahendroo, C.R. Mendelson, E.R. Simpson, Tissue specific promoters regulate aromatase cytochrome P450 gene expression in human ovary and fetal tissues, *Mol. Endocrinol*., **1991**, 5, 2005-2013.
103. M.S. Mahendroo, G.D. Means, C.R. Mendelson, E.R. Simpson, Tissue-specific expression of human P450arom: the promoter responsible for expression in adipose tissue is different from that utilized in placenta, *J. Biol. Chem.,* **1991**, 266, 11276-11281.
104. T. Sun, Y. Zhao, D.J. Mangelsdorf, E.R. Simpson, Characterization of a Region Upstream of Exon I.1 of the Human CYP19 (Aromatase) Gene That Mediates Regulation by Retinoids in Human Choriocarcinoma Cells, *Endocrinology*, **1998**, 139, 1684-1691.
105. Y. Zhao, C.R. Mendelson, E.R. Simpson, Characterization of the sequences of the human CYP19 (aromatase) gene that mediate regulation by glucocorticoids in adipose stromal cells and fetal hepatocytes, *Mol. Endocrinol.*, **1995**, 9, 340-349.

106. Y. Zhao, J.E. Nichols, S.E. Bulun, C.R. Mendelson, E.R. Simpson, Aromatase P450 Gene Expression in Human Adipose Tissue, *J. Biol. Chem*., **1995**, 270, 16449-16457.
107. Y. Zhao, J.E. Nichols, R. Valdez, C.R. Mendelson, E.R. Simpson, Tumor necrosis factor-alpha stimulates aromatase gene expression in human adipose stromal cells through use of an activating protein-1 binding site upstream of promoter 1.4, *Mol.Endocrinol.*, **1996**, 10, 1350-1357.
108. E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, M.M. Hinshelwood, G.S. Lorence, B. Amarneh, Y. Ito, C.R. Fisher, M.D. Michael, Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr. Rev.*, **1994**, 15, 342–355.
109. E.R. Simpson, M.S. Mahendroo, G.D. Means, M.W. Kilgore, C.J. Corbin, C.R. Mendelson, Tissue-specific promoters regulate Aromatase cytochrome P450 expression, *J. Steroid Biochem. Mol. Biol.*, **1993**, 44, 321–330.
110. J.T. Kellis, L.E. Vickery, Purification and characterization of human placental aromatase cytochrome P-450, *J. Biol. Chem*., **1987**, 262, 4413–4420.
111. W.R. Brueggemeier, J.C. Hackett, E.S. Diaz-Cruz, Aromatase Inhibitors in the Treatment of Breast Cancer, *Endocrine Reviews*, **2005**, 26(3), 331–345.
112. H.A. Harvey, A. Lipton, R.J. Santen, Aromatase: new perspectives for breast cancer, *Cancer Res.*, **1982**, 42, 3261s–3467s.
113. L. Banting, H.J. Smith, M. James, G. Jones, W. Nazareth, P.J. Nicholls, M.J. Hewlins, M.G. Rowlands, Structure-activity relationships for non-steroidal inhibitors of aromatase, *J. Enzym Inhib.*, **1988**, 2, 215–229.
114. R.W. Brueggemeier, Biochemical and molecular aspects of aromatase, *J. Enzym Inhib.*, **1990**, 4, 101–111.
115. R.W. Brueggemeier, Aromatase inhibitors—mechanisms of steroidal inhibitors, *Breast Cancer Res. Treat.*, **1994**, 30, 31–42.
116. P.A. Cole, C.H. Robinson, Mechanism and inhibition of cytochrome P-450 aromatase, *J. Med. Chem.*, **1990**, 33, 2933–2942.
117. A.M. Brodie, V.C. Njar, Aromatase inhibitors and breast cancer, *Semin. Oncol.*, **1996**, 23, 10-20.
118. A. Brodie, Q. Lu, B. Long, Aromatase and its inhibitors, *J. Steroid Biochem. Mol. Biol.*, **1999**, 69, 205–210.
119. R.J. Santen, H.A. Harvey, Use of aromatase inhibitors in breast carcinoma, *Endocr. Relat Cancer*, **1999**, 6, 75–92.

120. G. Cocconi, First generation aromatase inhibitors—aminoglutethimide and testololactone, *Breast Cancer Res. Treat.*, **1994**, 30, 57–80.
121. K. Hoffken, Experience with aromatase inhibitors in the treatment of advanced breast cancer, *Cancer Treat. Rev.*, **1993**, 19 (Suppl B), 37–44.
122. H. Adlercreutz, Phytoestrogens: epidemiology and a possible role in cancer protection, *Environ Health Perspect*, **1995**, 103, 103–112.
123. J.T. Kellis, L.E. Vickery, Inhibition of human estrogen synthetase (aromatase) by flavones, *Science*, **1984**, 225, 1032–1034.
124. A.R. Ibrahim, Y.J. Abul-Hajj, Aromatase inhibition by flavonoids, *J. Steroid. Biochem. Mol. Biol.*, **1990**, 37, 257–260.
125. Y.C. Kao, C. Zhou, M. Sherman, C.A. Laughton, S. Chen, Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: a site-directed mutagenesis study, *Environ Health Perspect*, **1998**, 106, 85–92.
126. C. Pouget, C. Fagnere, J.P. Basly, G. Habrioux, A.J. Chulia, Design, synthesis and evaluation of 4-imidazolylflavans as new leads for aromatase inhibition, *Bioorg. Med. Chem. Lett.*, **2002**, 12, 2859–2861.
127. M. Recanatini, A. Bisi, A. Cavalli, F. Belluti, S. Gobbi, A. Rampa, P. Valenti, M. Palzer, A. Palusczak, R.W. Hartmann, A new class of nonsteroidal aromatase inhibitors: design and synthesis of chromone and xanthone derivatives and inhibition of the P450 enzymes aromatase and 17-α-hydroxylase/C17,20-lyase, *J. Med. Chem.*, **2001**, 44, 672–680.
128. R.W. Brueggemeier, J.A. Richards, S. Joomprabutra, A.S. Bhat, J.L. Whetstone, Molecular pharmacology of aromatase and its regulation by endogenous and exogenous agents, *J. Steroid Biochem. Mol. Biol.*, **2001**, 79, 75–84.
129. D.J. Manglesdorf, C. Thummel, M. Beato, P. Herrlich, G. Shutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Charmbon, R.M. Evans, The nuclear receptor superfamily: the second decade, *Cell*, **1995**, 83, 835-839.
130. J.M. Beekman, G.F. Allan, S.Y. Tsai, M.J.O. Tsai, B.W. Malley, Transcriptional activation by the estrogen receptor requires a conformational change in the ligand binding domain, *Mol. Endocrinol.*, **1993**, 267, 19513-19520.
131. D. Barton, C. Loprinzi, D. Wahner-Roedler, Hot flashes: aetiology and management, *Drugs Aging*, **2001**, 18(8), 597-606.

132. S.C. Manolagas, S. Kousteni, R.L. Jilka, Sex Steroids and Bone, *Recent Prog. Horm. Res.*, **2002**, 57, 385-409.
133. M.J. Stampfer, G.A. Colditz, W.C. Willett, J.E. Manson, B. Rosner, F.E. Speizer, C.H. Hennekens, Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the nurses' health study, *N. Engl. J. Med*., **1991**, 325, 756.
134. D.A. Shewmon, J.L. Stock, C.J. Rosen, K.M. Heiniluoma, M.M. Hogue, A. Morrison, E.M. Doyle, T. Ukena, V. Weale, S. Baker, Tamoxifen and estrogen lower circulating lipoprotein(a) concentrations in healthy postmenopausal women, *Arterioscler. Thromb.,* **1994**, 14, 1586-1593.
135. M.A. Limouzin-Lamothe, N. Mairon, C.R. Joyce, M. Le Gal, Quality of life after the menopause: influence of hormonal replacement therapy, *Am. J. Obstet. Gynecol*., **1994**, 170, 618-624.
136. I.H. Russo and J. Russo, Role of hormones in mammary cancer initiation and progression, *J. Mamm. Gland Biol. Neoplasia*, **1998**, 3, 49–61.
137. P. Cos, T. De Bruyne, S. Apers, D. Vanden Berghe, L. Pieters, A.J. Vlietinck, Phytoestrogens: recent developments, *Planta Med.*, **2003**, 69, 589–599.
138. S.B. Katzenellenbogen, J.A. Katzenellenbogen, Estrogen receptor transcription and transactivation: estrogen receptor-alpha and estrogen receptor beta–regulation by selective estrogen receptor modulators and importance in breast cancer, *Breast Cancer Res.*, **2000**, 2, 335–344.
139. E. Minami, M. Taki, S. Takaishi, stereochemistry of *cis* and *trans*-hinokiresional and their estrogen like activity, *Chem. Pharm. Bull.*, **2000**, 48(3), 389-392.
140. J.C. Le Bail, Y. Champavier, A.J. Chulia, G. Habrioux, Effects of phytoestrogens on aromatase, 3beta and 17beta-hydroxysteroid dehydrogenase activities and human breast cancer cells, *Life Sci.*, **2000**, 66(14), 1281–1291.
141. Y.J. Moon, X. Wang, M.E. Morris, Dietary flavonoids: effects on xenobiotic and carcinogen metabolism, *Toxicol. In Vitro*, **2006**, 20(2), 187–210.
142. C.R. Sirtori, A. Arnoldi, S.K. Johnson, Phytoestrogens: end of a tale?, *Ann. Med.*, **2005**, 37(6), 423–438.
143. J. Allan, R. Robinson, Accessible derivative of chromonol, *J. Chem. Soc*., **1924**, 2192–2194.
144. H.S.Mahal, K.Venkataraman, *Curr. Sc.*, **1933**, 4, 214—216.
145. T.S. Wheeler, "Organic Syntheses," Collective Vol. IV, 2nd ed. By N. Rabojohn, John Wiley & Sons, New York, **1967**, p. 478—481.

146. M. Iinuma, K. Iwashima, S. Matsuura, Synthetic Studies on Flavone Derivatives. XIV. Synthesis of 2',4',5'-Trioxygenated Flavones, *Chem. Pharm. Bull.*, **1984**, 32(12), 4935—4941.
147. A. Hercouet, M. LeCorre, A simple synthesis of chromene, *Synthesis*, **1982**, 597—598.
148. T.S. Kukhareva, V.A. Karsnova, M.P. Koroteev, Electrophilic subistitution in dihydroquercetin system. Aminomethylation, *Russian journal of organic chemistry*, **2004**, 40(8), 1190-1193.
149. T.G. van Aardt, P.S. van Heerden, D. Ferreira, The First Direct Synthesis of Pterocarpans via Aldol Condensation of Phenylacetates with Benzaldehydes, *Tetrahedron Letters*, **1998**, 39, 3881-3884.
150. A.R. Ibrahim, Y.J. Abul-Hajj, Microbiological transformation of chromone, chromanone, and ring A hydroxyflavones, *Journal of Natural Products*, **1990**a, 53, 1471–1478.
151. A.R. Ibrahim, Y.J. Abul-Hajj, Microbiological transformation of flavone and isoflavone, *Xenobiotica*, **1990**b, 20, 363–373.
152. A.R. Ibrahim, A.M. Gatal, J.S. Mossa, F.S. El-Feraly, Glucose- conjugation of the flavones of Psiadia arabica by Cunninghamella elegans, *Phytochemistry*, **1997**, 46, 1193–1195.
153. I.O. Anyanwutaku, E. Zirbes, J.P.N. Rosazza, Isoflavonoids from streptomycetes: origins of genistein, 8-chlorogenistein, and 6,8-dichlorogenistein, *Journal of Natural Products*, **1992**, 55, 1498–1504.
154. P. Janeiro, O. Corduneanu, A. Maria, O. Brett, Chrysin and taxifolin electrochemical mechanisms, *Electroanalysis*, **2005**, 17(12), 1059-1064.
155. S.V. Jovanovic, S. Steenken, M.G. Simic, Y. Hara, in Flavonoids in Health and Disease (Eds: C. A. Rice-Evans, L. Packer), Marcel Dekker, New York **1998**, p 137.
156. A.S. Pannala, T.S. Chan, P.J.O. Brien, C.A. Rice-Evans, Flavonoid B-ring chemistry and antioxidant activity: Fast reaction kinetics, *Biochem. Biophys. Res. Comm*., **2001**, 282, 1161-1168.
157. H. Hotta, S. Nagano, M. Ueda, Y. Tsujino, J. Koyama, T. Osakai, Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation, *Biochim. Biophys. Acta*, **2002**, 1572(1), 123-132.

158. H. Hotta, H. Sakamoto, S. Nagano, T. Osakai, Y. Tsujino, Unusually large numbers of electrons for the oxidation of polyphenolic antioxidants, *Biochim. Biophys. Acta*, **2001**, 1526(2), 159-67.
159. P. Zuman, J.J.M. Holthuis, *Recueil des Travaux Chimiques des Pays-Bas*, **1988**, 107, 403.
160. A. Maria, O. Brett, M.E. Ghica, Electrochemical Oxidation of Quercetin, *Electroanalysis*, **2003**, 15, 1745-1750
161. G. Konig, H. Rimpler, Iridoid glucosides in Avicennia marina, *Phytochemistry*, **1985**, 24, 1245–1248.
162. C. Mannich, W. Krosche, Ueber ein Kondensationsprodukt aus Formaldehyd, Ammoniak und Antipyrin, *Archiv der Pharmazie*, **1912**, 250, 647–667.
163. P.M. Dewick, Medicinal Natural Products "A Biosynthetic Approach", John Wiley & Sons, Ltd, **2002**, 18-19.
164. E.R. Alexander, E.J. Underhill, Studies on the Mechanism of the Mannich Reaction. I. Ethylmalonic Acid, A Methynyl Compound, *J. Am. Chem. Soc.*, **1949**, 71(12), 4014–4019.
165. A. Ouchi, S. Liu, Z. Li, S.A. Kumar, T. Suzuki, T. Hyugano, H. Kitahara, Factors Controlling Photochemical Cleavage of the Energetically Unfavorable Ph−Se Bond of Alkyl Phenyl Selenides, *J. Org. Chem.*, **2007**, 72(23), 8700-8706.
166. E.A. Thompson, P.K. Siiteri, Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during Aromatization of Androstenedione, *J. Biol. Chem.*, **1974**, 249, 5364-5372.
167. J. W. Clark-Lewis, I. Dainis, Flavan derivatives. XI. Teracacidin, melacacidin, and 7,8,4'-trihydroxyflavonol from Acacia sparsiflora, and extractives from Acacia orites**,** *Australian Journal of Chemistry*, **1964**, 17 (10), 1170-1173.
168. W. Jyh-Horng, T. Yu-Tang, C. Shih-Chang, W. Sheng-Yang, K. Yueh-Hsiung, S. Lie-Fen, C. Shang-Tzen, Effect of Phytocompounds from the Heartwood of *Acacia confusa* on Inflammatory Mediator Production, *J. Agric. Food Chem.,* **2008**, 56, 1567–1573.
169. S.K. Sadhu, P.M. Phattanawasin, S.K. Choudhuri, T.O.M. Ishibashi, A new lignan from Aphanamixis polystachya, *J. Nat. Med.*, **2006**, 60, 258–260.
170. B. Nay, V. Arnaudinaud, J. Peyrat, A. Nuhrich, G. Deffieux, J. Merillon, J. Vercauteren, Total Synthesis of Isotopically Labelled Flavonoids, ^{13}C-Labelled (±)-Catechin From Potassium [^{13}C]Cyanide, *Eur. J. Org. Chem*., **2000**, 1279-1283.

171. M.J. Jung, S.S. Kang, H.A. Jung, G.J. Kim, J.S. Choi, Isolation of Flavonoids and a Cerebroside from the Stem Bark of *Albizzia julibrissin*, *Arch. Pharm. Res.*, **2004**, 27(6), 593-599.
172. M.J.C. Falcao, Y.B.M. Pouliquem, M.A.S. Lima, N.V. Gramosa, L.V. Costa-Lotufo, G.C.G. Militao, C. Pessoa, M.O. De Moraes, E.R. Silveira, Cytotoxic flavonoids from Platymiscium floribundum**,** *J. Nat. Prod.*, **2005**, 68 (3), 423-426.
173. N. De Tommasi, S. Piacente, C. Pizza, Flavonol and Chalcone Ester Glycosides from Bidens, *J. Nat. Prod*., **1998**, 61, 973-977.
174. Y.H. Kim, J. Kim, H. Park, H.P. Kim, Anti-inflammatory Activity of the Synthetic Chalcone Derivatives: Inhibition of Inducible Nitric Oxide Synthase-Catalyzed Nitric Oxide Production from Lipopolysaccharide-Treated RAW 264.7 Cells, *Biol. Pharm. Bull.*, **2007**, 30(8), 1450—1455.
175. C. Franco, B. Adriana, M. Fedele, S. Daniela, C. Paola, G. Arianna, B. Olivia, T. Paola, C. Roberto, T.L. Francesco, A. Stefano, O. Francesco, L. Thierry, T.L.A. Francesco, Synthesis, Biological Evaluation and 3D-QSAR of 1,3,5-Trisubstituted-4,5- Dihydro-(1H)-Pyrazole Derivatives as Potent and Highly Selective Monoamine Oxidase A Inhibitors., *Current Medicinal Chemistry*, **2006**, 13, 1411-1428.
176. M. Sanchez-Gonzalez, J.P.N. Rosazza, Microbial Transformations of Chalcones: Hydroxylation, O-Demethylation, and Cyclization to Flavanones, *J. Nat. Prod*., **2004**, 67, 553-558.
177. A. Major, M. Nogradi, B. Vermes, M. Kajtar-Peredy, Synthese der naturlichen Tsoflav-3-ene Haginin A, B und D., *Liebigs Ann. Chem.*, **1988**, 555–558.
178. Y. Goda, F. Kiuchi, M. Shibuya, Inhibitor of prostagland biosynthesis from *Dalbergia odorifera*, *Chem. Pharm. Bull.*, **1992**, 40(9), 2452-2457.
179. J.S. Yadav, B.V. Subba Reddy, G. Kondaji, Intermolecular imino-Diels-Alder reaction in [bmim] BF4 ionic medium: Green protocol for the synthesis of tetrahydrochromanoquinolines, *J. of molecular catalysis A: Chemical*, **2006**, 258, 361-366.
180. G. Sabitha, E.V. Reddy, J.S. Yadav, Bismuth(III) chloride-Catalyze intramolecular hetro Diels-Alder reaction: Application to the synthesis of tetrahydrochromano[4,3-b]quinoline derivative, *Synthesis*, **2001**, 10, 1979-1984.
181. T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods*, **1983**, 65, 55-63.

182. L.M. Green, J.L. Reade, C.F. Ware, Rapid Colormetric Assay for Cell Viability: Application to the Quantitation of Cytotoxic and Growth Inhibitory Lymphokines, *J. Immunol. Methods*, **1984**, 70(2), 257-268.

183. S. Gobbi, A. Cavalli, A. Rampa, F. Belluti, L. Piazzi, A. Paluszcak, R.W. Hartmann, M. Recanatini, A. Bisi, Lead Optimization Providing a Series of Flavone Derivatives as Potent Nonsteroidal Inhibitors of the Cytochrome P450 Aromatase Enzyme, *J. Med. Chem.,* **2006**, 49, 4777-4780.

184. J. Baumann, G. Wurn, F.V. Bruchlausen. Prostaglandin synthetase inhibiting O^{-}_{2} radical scavenging properties of some flavonoids and related phenolic compounds. Deutsche Pharmakologische Gesellschaft Abstracts of the 20th spring meeting, Naunyn-Schmiedebergs Abstract No: R27 cited in *Arch. Pharmacologia*, **1979**, 307, R1–R77.

185. M. Hosny, A.J. Holly, K.U. Amanda, J.N.P. Rosozza, Oxidation, reduction and methylation of carnosic acid by Nocardia, *J. Nat. Prod.*, **2002**, 65, 1266–1269.

186. T. Iwashina, J. Kitajima, Chalcone and flavonol glycosides from *Asarum canadense* (*Aristolochiaceae*), *Phytochemistry*, **2000**, 55, 971–974.

187. Y. Omura, Y. Taruno, Y. Irisa, M. Morimoto, H. Saimoto, Y. Shigemasa, Regioselective Mannich reaction of phenolic compounds and its application to the synthesis of new chitosan derivatives, *Tetrahedron Letters*, **2001**, 42, 7273–7275.

188. X. Zheng, W. Meng, Y. Xu, J. Cao, F. Qing, Synthesis and anticancer effect of chrysin derivatives, *Bioorganic & Medicinal Chemistry Letters*, **2003**, 13(5), 881-884.

189. P. Yaipakdee, L.W. Robertson, Enzymatic halogenation of flavanones and flavones, *Phytochemistry*, **2001**, 57, 341-347.

190. L. Chen, T.S. Hu, J. Zhu, H. Wu, Z.J. Yao, Application of a Regioselective Mannich Reaction on Naringenin and its Use in Fluorescent Labeling, *SYNLETT*, **2006**, 8, 1225–1229.

191. M.M. Garazd, Y.L. Garazd, A.S. Ogorodniiehuk, V.V. Shilin, A.V. Turov, V.P. Khilya, Mannich reaction in the series of 7-hydroxy-3-phenoxychromones and their derivatives, *Chemistry of Natural Compounds*, **1998**, 34,(4), 442-447.

192. M. Fontes, X. Verdaguer, L. Sola, M.A. Perica, A. Riera, 2-Piperidino-1,1,2-triphenylethanol: A Highly Effective Catalyst for the Enantioselective Arylation of Aldehydes, *J. Org. Chem.*, **2004**, 69, 2532-2543.

193. K. Suzuki, K. Kondo, T. Aoyama, Asymmetric Synthesis of Diarylmethanols by Rh(I)-Catalyzed Arylation of Aromatic Aldehydes with Arylboronic Acid, *SYNTHESIS*, **2006**, 8, 1360–1364.
194. R. Monteiro, I. Azevedo, C. Calhau, Modulation of Aromatase Activity by Diet Polyphenolic Compounds, *J. Agric. Food Chem*., **2006**, 54, 3535-3540

Curriculum Vitae

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Objectives:

It is my ambition to investigate the chemical and biological properties of natural products, with particular focus on compounds extracted from plants.

The importance of Flavonoids and phenolic natural products in plant defense is widely acknowledged. There is also significant potential for the beneficial application of these natural products in agriculture and medicine. Research into the structural modification of flavonoids could provide knowledge that could be implemented in the development of treatments against modern diseases such as AIDS, Hepatitis and certain types of Influenza.

Synthetic chemistry is often necessary for the reproduction of naturally-occurring compounds in cases where there are difficulties in isolation, extraction and purification, or if the natural product is very rare. Through synthetic chemistry it is also possible to enhance or modify these natural compounds, thus producing novel drugs for further investigation.

Isolation of natural products, biological assays and synthesis of active compounds or modification to improve their biological activity will be the main focus of my work in the future.

Education:

August 1998: Bachelor of the pharmaceutical sciences, Faculty of pharmacy, Al-Azhar University, Cairo, Egypt.

July 2004: Master degree “Antioxidant activities of some plants belong to family leguminosea”, Faculty of pharmacy, Al-Azhar University, Cairo, Egypt.

Work Experience:

1) Five years as a demonstrator in the Department of Pharmacognosy, Faculty of pharmacy, Al-Azhar University, from 2000 to 2004.

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3) Ph D student in the Department of Bioorganic Chemistry, Faculty of Pharmacy, Saarland University, Germany, from 2007 up to now. During which I have developed my practical knowledge of synthetic chemistry and biological assays (for example, Aromatase assays and

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