Sudan University of Sciences and Technology

College of Graduate Studies

Study of the Major Flavonoids from *Balanites aegyptiaca*, *Cissus petiolata* and *Hegeina abyssinica*

دراسة الفلافونويدات الرئيسية فى أشجار الهيجليج وعرق الحجر وشاومكاده

A Thesis Submitted in Fulfillment of the Requirements for the Ph. D. Degree in Chemistry

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Dedication

Dedicated to:

- The souls of my parents
- my Sister and brothers

Acknowledgement

After thanking Almighty Allah, I would like to express my gratitude to supervisor Prof. **Mohammed Abdel Karim** for his intimate supervision, valuable advice, kind treatment and effort done during the course of this work.

Thanks are extended to Dean and staff of Chemistry Department, Faculty of Science and Technology, Omdurman Islamic University for all facilities .Thanks to the staff of Chemistry Department, Sudan University for Science and Technology for their kind help. My grateful acknowledgement also goes to Nile Valley University for funding this project.

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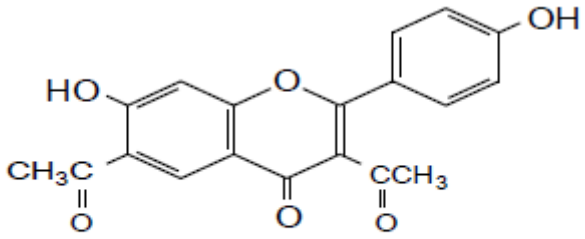
Also I would like to express my thanks to all those who made this work possible.

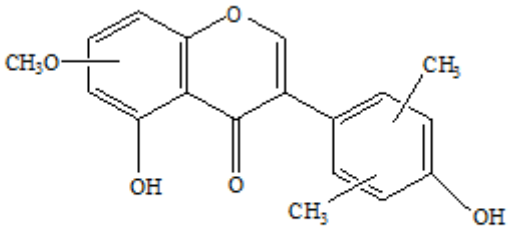
Abstract

In this study, phenolic compounds were extracted from roots of *Balanites aegyptiaca*, seeds of *Hagenia abyssinica* and roots of *Cisuss petiolata* using 95% ethanol. The crude ethanolic extracts of the studied species were subjected to paper chromatography. In this way compound I was isolated from the roots of *Balanites aegyptiaca*. The structure of this isolate was elucidated by a combination of spectral techniques (UV, IR, NMR and MS) and the following tentative structure was proposed:

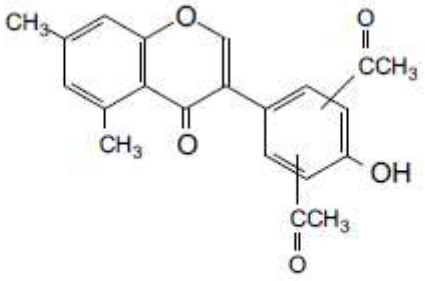


Compound I

The seeds of *Hagenia abyssinica* gave compounds II and III. The following tentative structures were proposed for these isolates:

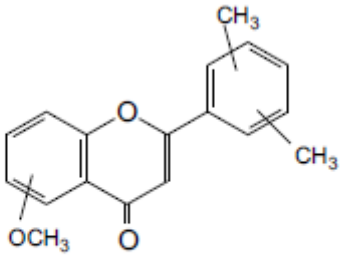


Compound II



Compound III

Compound IV was isolated from roots of *Cisuss petiolata* and The following tentative structure was suggested:

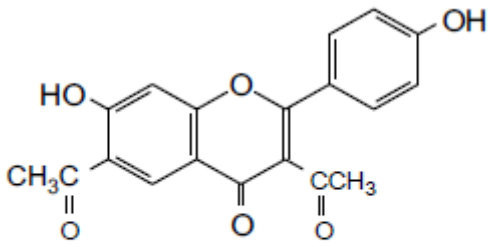


Compound IV

ملخص الدرا سه

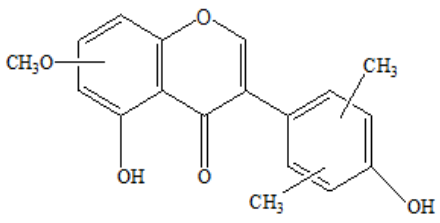
فى هذه الدراسه استخلصت المركبات الفينوليه من جذور الهيجليج، وبذور شاو مكادة وجذور عرق الحجر بواسطة 95 % إثانول .وعن طريق كروموتوغرافيا الورق تم فصل المركب I. تم تحديد التركيب باستخدام الطرق المطيافيه (طيف الاشعه فوق البنفسجيه، طيف الاشعه تحت الحمراء، الرنين النووى المغنطيسى وطيف الكتله)

واقترح التركيب المبدئ التالى:

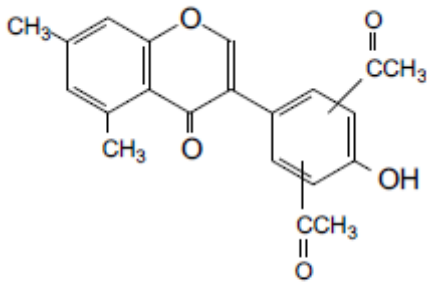


مركب I

ثم فصل المركبين II وIII من نبات شاو مكاده واقترح لهما التركيبين المبدئيين التاليين:

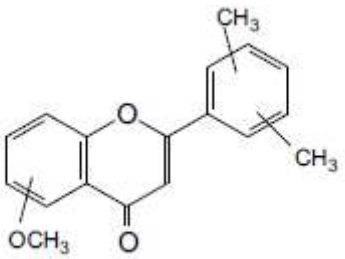


مركب II



مركب III

اما المركب IV فقد فصل من جذور نبات عرق الحجر واقترح له التركيب التالى بناء على البيانات الطيفيه:



مركب IV

Contents

Chapter One

1-Introduction

1.1- *Balanites aegyptiaca* Del.

Balanites aegyptiaca Del., also known as 'Desert date' in English, a member of the family Zygophyllaceae, is one of the most common but neglected wild plant species of the dry land areas of Africa and South Asia. This tree is native to much of Africa and parts of the Middle East. This is one of the most common trees in Sudan. It can be found in many kinds of habitat, tolerating a wide variety of soil types, from sand to heavy clay, and climatic moisture levels[1].

Balanites aegyptiaca

Balanites aegyptiaca

Balanites aegyptiaca fruits

Synonyms: *Ximenia aegyptiaca* L. (excl. *Balanites roxburghii* Planch), *Agialida senegalensis* van Tiegh., *Agialida barteri* van Tiegh., *Agialida tombuctensis* van Tiegh., *Balanites ziziphoides* Milbr. Et Schlechter, *Balanites latifolia* (van Tiegh.) Chiov.

Balanites aegyptiaca is a multibranched, spiny shrub or tree up to l0 m tall. Crown spherical, in one or several distinct masses. Trunk short and often branching from near the base. Bark dark brown to grey, deeply fissured. Branches armed with stout yellow or green thorns up to 8 cm long. Leaves with two separate leaflets; leaflets obovate, asymmetric, 2.5 to 6 cm long, bright green, leathery, with fine hairs when young. Flowers in fascicles in the leaf axils, and are fragrant, yellowish-green. Fruit is a rather long, narrow drupe, 2.5 to 7 cm long, 1.5 to 4 cm in diameter. Young fruits are green and tormentose, turning yellow and glabrous when mature. Pulp is bitter-sweet and edible. Seed is the pyrene (stone), 1.5 to 3 cm long, light brown, fibrous, and extremely hard. It makes up 50 to 60% of the fruit[1].

Flowers are small, inconspicuous, hermaphroditic, and pollinated by insects. Seeds are dispersed by ingestion by birds and animals. The tree begins to flower and fruit at 5 to 7 years of age and maximum seed production is when the trees are 15 to 25 years old.

Natural distribution is obscured by cultivation and naturalization. It is believed indigenous to all dry lands south of the Sahara, extending southward to Malawi in the Rift Valley, and to the Arabian Peninsula, introduced into cultivation in Latin America and India. It has wide ecological distribution, but is mainly found on level alluvial sites with deep sandy loam and free access to water. After the seedling stage, it is intolerant to

shade and prefers open woodland or savannah for natural regeneration. It is a lowland species, growing up to 1000 m altitude in areas with mean annual temperature of 20 to 30^{o} C and mean annual rainfall of 250 to 400 mm[1].

Extensive literature survey revealed that 'desert date' has a long history of traditional uses for wide ranges of disease. It has been experimentally proved that *B. aegyptiaca* Del possess antioxidant, antimicrobial, anticancer, diuretic, hypocholesterolemic, wound-healing, antiviral, antidiabetic, hepatoprotective, mosquito larvicidal, anti-inflammatory and analgesic, antivenin, anthelmintic, cardioprotective cum antioxidant activity, and antinociceptive properties. Bark, fruits, seeds, seed oil, and leaves of this plant are widely used in folk medicine. In recent years, emphasis of research has been on utilizing traditional medicines that have long and proven history of treating various ailments. So, further studies need to be carried out to explore *B. aegyptiaca* Del for its potential in curing and treating disease[1].

Aqueous extract of fruits showed spermicidal activity without local vaginal irritation in human being, up to 4% sperms becoming sluggish on contact with the plant extract and then immobile within 30 s; the effect was concentration-related. Protracted administration of the fruit pulp extract produced hyperglycemia-induced testicular dysfunction in dogs. Seed is used as expectorant, antibacterial, and antifungal. Fruit is used

in whooping cough, also in leucoderma and other skin diseases. Bark is used as spasmolytic[2].

The seed is used as a febrifuge[3]. Root extracts have proved 'slightly effective' against experimental malaria[4,5]. In Kenya, a root infusion is used as an emetic[1]. In asthma, about 10 gm of seed powder is taken with glass of water in the morning for 10 days[1]. Tablets are prepared from roots mixed with 'Hing' powder (*Ferula asafoetida*); by adding *Piper betle* leaf, juices are taken once with water for 9 days, soon after the menstruation to avoid unwanted pregnancy[6]. In Egyptian folk medicine, the fruits are used as an oral hypoglycemic[1] and an antidiabetic; an aqueous extract of the fruit mesocarp is used in Sudanese folk medicine in the treatment of jaundice[1]. Used in food preparations and herbal medicine, especially in Africa and some developing Countries[1]. The fresh leaf of the plant *Acalypha* is pounded with small amount of root of *B. aegyptiaca and Cissus quadrangularis,* and then soaked in water for an hour or two. It is decanted and administered intranasally and orally for nasal infections. Latex of the plant is used in epilepsy, administered through intranasal route[7]. Twigs are used as tooth brush. Fruits are used to treat dysentery and constipation. They are also used in treatment of hemorrhoid, stomach aches, jaundice, yellow fever, syphilis, and epilepsy[8]. A fruit is used to treat liver disease and as a purgative, and sucked by school children as a confectionary in some countries[1]. The seed oil is used to treat

tumors and wounds[1].Seeds are also used as anthelmintic and purgative. Ground seeds are given to camels to cure impaction and colic. The bark is used in the treatment of syphilis, round worm infections, and as a fish poison. The aqueous leaf extract and saponins isolated from its kernel cakes have antibacterial activity[9,10].

The root, bark, kernel, and fruit have been shown to be lethal to mollusks[1]. In Sudanese folk medicine, it is used to treat jaundice. Its antimalarial and molluscidal activity is well studied[11-14]. *In vitro* antiplasmodial test of the dichloromethane and methanol extract of stem bark of the plant showed antimalarial activity.

In Chad, fresh twigs are put on the fire in order to keep insects away. For intestinal worm, the fruits are dried and mashed in millet porridge and eaten[1]. In Libya and Eritrea, the leaves are used for cleaning infected wounds. In Sudan and Chad the of *B. aegyptiaca* is component of soap[1]. The use of the kernel oil for treatment of wounds has been reported from Nigeria. In Nigeria, a mixture of dried leaves powder of *B. aegyptiaca* and *Ricinus communis* is suspended in water and taken for constipation. In Somalia, the bark of root is crushed and suspended in water and taken orally for constipation[15]. Leaves of the plant contain: saponin, furanocoumarin, the flavonoids: quercetin 3-glucoside, quercetin-3-rutinoside; isorhamnetin- 3-glucoside, isorhamnetin-3-rutinoside, isorhamnetin- 3-7-diglucoside and isorhamnetin-

3-rhamnogalactoside[1].

Mesocarp of fruit contains 1.2 to 1.5% protein and 35 to 37% sugars, 15% organic acids beside other constituents like 3-rutinoside and 3-rhamnogalactoside[1], diosgenin[1]; However, kernel contains saponins present in mesocars. Balanitoside (furostanol glycoside) and 6-methyldiosgenin, balanitin-3-spirostanol glycoside have been reported from fruits (mesocarp) of *B. aegyptiaca*. Balanitin-6 and -7: Diosgenyl saponins. Some glycosides ,namely pregn-5-ene-3β,16β,20(*R*)-triol 3-*O*-(2,6-di-*O*-α-l-rhamnopyranosyl)-β-d-glucopyranoside (balagyptin), and pregn-5-ene-3β,16β,20(*R*)-triol 3-*O*-β-d-glucopyranoside, were also reported from this species[16]. The kernels contained 45.0 to 46.1% oil and protein (32.4%), oil contains mainly palmitic, stearic, oleic, and linoleic acids which were the main fatty acids[1].The oil exhibited anticancer activity against lung, liver, and brain human carcinoma cell lines. It also had antimutagenic activity against *Fasciola gigantica*-induced mutagenicity besides anthelmintic activity against hepatic worms *(Schistosoma mansoni* and *Fasciola gigantica).* Preliminary screening showed that the oil had antiviral activity against *Herpes simplex.* It also had antimicrobial activity against selected strains of Gram-positive bacteria, Gram-negative bacteria, and *Candida*[17]. Nine saponins have been reported from kernel cake of *B. aegyptiaca,* from the nine components, six saponins with molecular masses of 1196, 1064, 1210, 1224,

1078, and 1046 Da were identified, with the compound of mass 1210 Da being the main saponin (ca. 36%)[1]. The leaves and fruit kernels of *B. aegyptiaca* L. were found to contain six diosgenin glucosides including di-, tri-, and tetraglucosides.

Root It is reported to contain steroidal, saponins and the major sapogenin is yamogenin[1] beside some glycosides Balanitins 1 to 7 have also been reported from root and bark of this species[1].

Two alkaloids, namely: N-trans-feruloyltyramine and N-cis-feruloyltyramine, and three common metabolites, vanillic acid, syringic acid; and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, long-chain aliphatic compound, 10-methyl-n-heptacosane, and a sugar: diglucosyldirhamnoside, have also been reported from the stem-barks[1]. It also contains beta-sitosterol, bergapten, marmesin, and beta-sitosterol glucoside, balanitin-1,-2, and -3; balanitin-1 for example possesses a yamogenin aglycone with a branched glucose and rhamnose side-chain.

The plant acts as antioxidant against adriamycin-induced cardiotoxicity in experimental mice. Adriamycin when administered intraperitoneally, causes elevation of serum lactate dehydrogenase, creatine phosphokinase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, lipid peroxide, total nitric oxide, erythrocyte lysate superoxide dismutase (SOD), glutathione peroxidase (GPx), and plasma catalase (CAT) in mice heart tissue. Pretreatment with *B. aegyptiaca*

extract significantly (P<0.05) prevented these alterations and restored the enzyme activities to near normal levels[18]. The crude aqueous extract of root bark of *B. aegyptiaca* showed a dose-dependent inhibition of spontaneous motility (paralysis) in adult earthworms. And also possesses vermicidal activity[19]. It is reported that aqueous extract of stem bark (9 g/kg body weight) of *Albizia anthelmintica* and fruit mesocarp (9 g/kg body weight) of *B. aegyptiaca* shows significant anthelmintic activity compared with albendazole (20 mg/kg body weight) against *Fasciola gigantica* adult worm[20]. A single dose of 200 mg/kg body weight of *B. aegyptiaca* fruit mesocarp also showed activity against *Schistosoma mansoni* in infected mice when compared with praziquantel[1]. Balanitin-7 is isolated from aqueous extract of *B. aegyptiaca* seed and reported as anthelmintic agent when tested by *in vitro* means of an original anthelmintic assay, using *Caenorhabditis elegans* as a biological model[21].The methanolic extract of *B. aegyptiaca* fruits is reported to have anthelmintic action against different stages of *Trichinella spiralis* in rats compared with anthelmintic drug albendazole[22].The aqueous extract of *B. aegyptiaca* also has molluscicidal activity against *Bulinus globosus* and *Bulinus truncatus*.

The aqueous and organic leaves extracts of *B. aegyptiaca* and *Moringa oleifera* were reported to have antibacterial effect against *Salmonella typhi* isolated from blood clot culture using

the disc diffusion method. The extracts *of B. aegyptiaca* plants demonstrated the higher activity than *Moringa oleifera*. The ethanolic extracts of both plants demonstrated the highest activity whereas the aqueous extracts of both plants showed the least activity at 100 mg/ml as compared with ethanolic extracts. The activities of these plant extracts were comparable with those of antibiotics, ciprofloxacin, cotrimoxazole, and chloramphenicol, commonly used for treating typhoid fever. The antibacterial activity appears to increase when extracts of the two plants were used in combination at 100 mg/ml each. Preliminary phytochemical screening showed that plant extracts contain saponins, tannins, and phenols, and *B. aegyptiaca* possesses anthraquinones. The antibacterial activities of the extracts on *S. typhi* were reasonably stable when treated at 4, 30, 60, and 100^{o} C for 1 hour. However, it reduces significantly when the pH was altered toward alkalinity[23]. The aqueous and ethanolic extracts of leaves of six plants viz., *B. aegyptiaca* (L.) Del, *Hyptis suaveolens* Poit, *Lawsonia inermis* L., *Leucas aspera* L., *Lobelia nicotianifolia* Roth, and *Phyllanthus maderaspatana* L. were reported as antibacterial when tested individually and in combinations against five different diarrheagenic bacteria: *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella enteritidis, and Listeria monocytogenes.* Ciprofloxacin (20 ˝g) was used as antimicrobial standard. The highest antimicrobial activity was in both crude

aqueous leaf extract and crude ethanolic leaf extract of *Lobelia nicotianifolia*, when all extracts were tested individually. However, in combination, the highest activity was observed in crude ethanolic leaf extract *Lobelia nicotianifolia* + *B. aegyptiaca* against *S. aureus*[24,25].

The acetone and methanolic extracts of stem bark of plant showed an anti-venin activity against saw-scaled (*Echis carinatus*) viper venom concentration at lethal dose (0.194 mg/ml), when administered intramuscularly to Wistar albino rats. Both extracts were found to be effective at 75 and 100 mg/ml[26].

A mixture of steroidal saponins: balanitin-6 (28%) and balanitin-7 (72%), isolated from *B. aegyptiaca* kernels, demonstrated appreciable anticancer effects in human cancer cell lines *in vitro* against A549 non-small-cell lung cancer (IC50, 0.3 μM) and U373 glioblastoma (IC50, 0.5 μM) cell lines. Balantin 6/7 mixture displayed higher antiproliferative activity than etoposide and oxaliplatin,but markedly less active than taxol. This indicates that balanitin 6/7 mixture is more a cytotoxic compound than a cytostatic one. *In vitro* anticancer activities are due to partly depletion of [ATP]i, leading in turn to major disorganization of action and it does not induce an increase in intracellular reactive oxygen species. *In vivo*, balantin6/7 mixture increased the survival time of mice bearing murine L1210 leukemia grafts to the same extent reported for

vincristine[27,28].

The ethanol and petroleum ether extracts of aerial parts of *B. aegyptiaca* have been reported to have significant anti-inflammatory action on carrageenan-induced hind paw eodema in rats. The paw volume was measured plethysmometrically at 0 and 3 hours after injection and analgesic activity by using Eddy's hot plate method and tail-flick method in albino rats. The ethanol and petroleum ether extracts showed a greater anti-inflammatory and analgesic effects in comparative with the standard drugs, indomethacin and diclofenac sodium, It was also indicated that the ethanolic extract of *B. aegyptiaca* exhibited more significant activity than petroleum ether in the treatment of pain and inflammation[29].

The galls and leaf extracts of *B. aegyptiaca* showed significant xanthine oxidase, and acetylcholinesterase inhibitory activities. The total phenolics and flavonoids were measured using Folin-Ciocalteu and $AlCl_3$ reagents, respectively. Two methods, that is, FRAP (Iron (III) to Iron (II) reduction activity) and ABTS (2,2-azinobis-3-ethylbnzothiazoline-6-sulphonate) assay were used to estimate the total antioxidant capacity of the plant materials. Dichloromethane fraction of the gall and ethyl acetate fraction of the leaves were reported to have highest antioxidant activity. The antioxidant activities were correlated significantly with the total phenolic and flavonoid contents. The study also showed that *B. aegyptiaca* galls and leaves fractions exhibited a

moderate xanthine oxidase inhibitory activity compared with the acetylcholinesterase which was weakly inhibited by the tested extracts and fractions[30].

Two saponins isolated from *B. aegyptiaca* showed significant anti- inflammatory, antinociceptive activity in the carrageenin-induced eodema in the rat, and acetic acid-induced writhing in mice and antioxidant action by using *in vitro*, using a method based on the Briggs-Rauscher oscillating reaction. The saponins were intragastrically administered to animals[31].

A saponin extract and water extract from fruit kernel *of B. aegyptiaca* was investigated as a mosquito larvicide. Both extracts were tested against second and fourth instar larvae of the three mosquito species namely *Anopheles arabiensis, Culex quinquefasciatus,* and *A. aegypti,* and LC_{50} and LC_{90} values were determined. Second instar larvae were more susceptible than fourth instar larvae in all cases. The larvae of *Anopheles arabiensis* were more susceptible than *Culex quinquefasciatus* and *A. aegypti*. The saponin was more active than the water extract[32].

Various mesocarp extracts, viz. chloroform, methanol, butanol and ethyl acetate showed larvicidal activity against *A. aegypti* mosquito larvae. The highest larval mortality was found in methanolic extract. The amount of saponin is correlated with larval mortality[33].

Administration of the aqueous bark extract to biliary duct-ligated rats showed a dose-dependent significant decrease in serum bilirubin level. For three days, the animals were given different concentration of the extract intraperitoneally. The bilirubin concentration was reduced by 22.2% in the animal that received 1.2 g bark extract each day, by 31.6% in those given 2.4 g, and by 45.9% in those given 4.8 g[34,35].

The pure saponin, extracted from the fruit mesocarp, and water extracts have been reported as hypoglycemic agent when tested on albino rats in different concentrations using Daonil as a standard medication. The aqueous extract of the mesocarp of fruits was reported to have antidiabetic effect in streptozotocin-induced diabetic mice. It also inhibited *Escherichia coli*[36]

Bark aqueous extract of *B. aegyptiaca* were used in treatment of both AIDS and Leukemia. An oral administration of the aqueous extract (30% w/v given at 100 ml every 8 hours for 30 days) for the treatment of HIV patients have shown excellent results. The same was given to patients with leukemia and a good increase in platelets and a normal blood differential reading after one month was noted[37].

It seems that *B. aegyptiaca* has potent wound-healing activity, as evident from the wound contraction. The plant also possesses potent antioxidant activity by inhibiting lipid peroxidation, bleaching DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical, and protecting against oxidant injury to fibroblast cells[38].

It is reported that whole and extracted pulp of *B. aegyptiaca* fruits possess a hypocholesterolemic effect when tested on adult Albino rats[39].

The ethanol and methanolic extract of leaves of *B. aegyptiaca* dxerted diuretic effect when tested on Wistar albino rats with (150 and 300 mg/kg) oral doses. Frusemide was used as standard. Ethanol and methanol extracts showed significant ($P<0.05$) increase in the urine volume and electrolyte excretion ($P<0.001$) when compared with control[40].

1.2- *Hagenia abyssinica*

Hagenia abyssinica

Hagenia abyssinica is a slender tree up to 20 m tall, with a short trunk and thick branches; branchlets covered in silky brown hairs and ringed with leaf scars. Bark thick, brown or reddish-brown and readily peeling. No thorns or buttresses. Leaves compound, 40 cm long, in terminal tufts; leaflets pale or bright

green above, with silvery hairs below, reddish and sticky when young, 3-6 pairs plus a terminal leaflet, each about 10 cm long; margin finely toothed and fringed with long hairs; leaf stalks 12 cm long, with expanded wings formed from the stipules, densely hairy on the underside. Flowers in handsome multibranched, terminal, drooping panicles up to 60 cm long and 30 cm wide, polygamo-dioecious, female heads pinkish-red, clearly veined, bulkier than the more feathery orange-buff to whitemale heads[41]. Fruit small, dry, winged, asymmetric, single seeded, brown syncarp with a single more or less ovoid carpel and fragile pericarp.Hagenia is a monospecific genus and is most closely related to the monospecific genus Leucosidea. The specific name means 'from Ethiopia[41].

Trees have either male or female flowers. Flowering and seeding can be observed throughout the year with a break in the months with the coldest temperatures[41].

In Kenya, the species can be found on the slopes of Mt. Kenya, Mt. Elgon, the Aberdares, Cherangani Hills and the slopes of other high hills or mountains. It occurs in undifferentiated afro-montane forest (mixed Podocarpus forest, Juniperus-Podocarpus forest) and dry single dominant afro-montane forest (Juniperus forest or forest dominated by Hagenia), especially along the upper limit; often associated with Schefflera abyssinica, S. volkensii, Galiniera saxifraga, Rapanea melanophloës and with the mountain bamboo, Arundinaria alpina; at lower altitudes

often at forest margins. This is a very clear example of an afro-montaneende[41].

The plant contains a volatile oil, a bitter acrid resin, tannic acid, and a bitter principle called A Kosin and B Kosin. The principle constituent is Koso-toxin, a yellow amorphous body, possibly closely allied to filicia acid, and Rottlerin; other inactive colourless bodies are crystalline Protokosin and Kosidin[41].

H. abyssinica is a good source of firewood and charcoal. Wood is dark red, medium soft but not durable; it is used for furniture, poles, flooring, carving and cabine making[41].

The roots are cooked with meat and the soup drunk for general illness and malaria, while the dried and pounded female inflorescence is used as an anthelmintic (especially for tapeworm). Bark may be pounded, added to cold water and the liquid drunk as a remedy for diarrhoea and stomach-ache. Generally, this is a strong medicine that must not be taken in large quantities; it is sometimes taken as an abortifacient[41].

The species is employed in soil-conservation activities. Hagenia constantly sheds leaves, providing mulch and green manure. This attractive tree is suitable for planting in amenity areas[41].

1.3- *Cisuss* species

The generic name: *Cissus* is derived from a Greek word meaning "Ivy". *Cissus* is a genus of approximately 350 species of woody vines in the grape family (Vitaceae). They have cosmopolition distribution, though the majority are to be found in the tropics[42].

Cissus quadrangularis has been evaluated for potential medical uses. As source of carotenoids, triterpenoids and ascorbic acid, the extracts of the plant may have potential of medical effects including gastroprotective, activity and benefits in terms of lipid metabolism and oxidative stress. *Cissus quadrangularis* is used by the Masaya of Kenia to reieve some symptons of malaria. *Cisuss antactica*, *Cisuss alata* and *Cisuss incisa* are cultivated as garden plants[42]

Cisuss verticillata is best known for its hyperglycaemic and anti-lipemic action. In Brazilian ethnopharmacology it is called "plant insulin" and the species is used mostly against diabetes as well as abscesses, haemorrhage and epilepsy[43].

The traditional healers of South Cameron use *Cisuss petiolata* in the treatment of fibromyoma, a disease shich causes infertility[44].The antmicrobial activity of the methanolic extract of *Cisuss petiolata* was studied[45]. At a dose of 250µg/ml the extract was active against *M.tuberculosis*. Images of some Cisuss species are shown below:

Cisuss nodosa

Cisuss verticillata

Cisuss javana

1.4-The flavonoids

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. The secondary metabolites of phenolic nature including flavonoids are responsible for the variety of pharmacological activities[46,47]. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection[48]. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substituents, conjugation and degree of polymerization[49]. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions[50,51]. The chelation of metals could be crucial in the prevention of radical generation

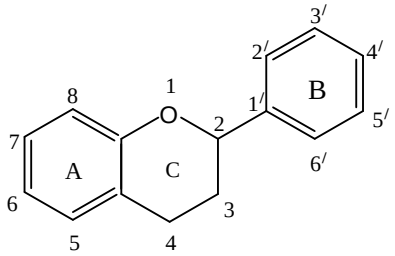
which damage target biomolecules[52,53]. As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems[54,55].

Over 8,000 varieties of flavonoids have been identified[56], which are widely distributed in the leaves, seeds, bark and flowers of plants. In the human diet, these compounds are most concentrated in fruit, vegetables, tea and coca. In plants, these compounds afford protection against ultraviolet radiation, pathogens, and herbivores[57].

Flavonoids play important biochemical and physiological roles in the various cell types or organs. Different classes of flavonoids and their conjugates have numerous functions. Due to the interaction of plant with environment, both biotic and abiotic stress conditions are expected[58].

1.4.1- Chemistry of flavonoids

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B) linked via a heterocyclic pyrane ring (C) (1).



(1)

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is an aglycone.**; a s**ix-membered ring is condensed with a benzene ring which is either an α-pyrone (flavonols and flavanones) or its dihydro derivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoids into: flavonoids (2-position) and isoflavonoids (3-position). Flavonols differ from flavanones by hydroxyl group at the 3- position and a C_2–C_3 double bond[59]. Flavonoids are often hydroxylated in positions 3, 5, 7, 2, $3'$, $4'$, and $5'$. Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose[60].

The classification of flavonoids is based initially on a study of solubility properties and colour reaction[61]. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings[62,63]. Both the oxidation state of the heterocyclic ring and the position of ring B are important in classification.

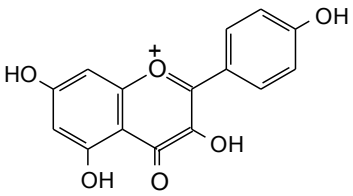
Flavonoids can be classified according to the modification of the flavonoids nucleus into nine groups: chalcones, aurons, flavanones, dihydroflavonoids, flavones, isoflavones, anthocyanins, flavonols, flavanols. Compounds belonging to the

same group differ between them by the degree and the position of hydroxylation, the presence of substituents on the nucleus and the state of their polymerization[64].

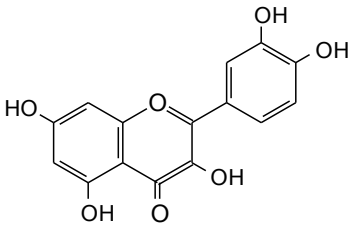
However, flavonoids can be classified according to their biosynthetic origin. Some flavonoid classes are intermediates in biosynthesis as well as end-products, which can acculmulate in plant tissues. The latter include: chalcones, flavonones, flavan-3-ols and flavan-3, 4-diols. Other classes, for instance the pro-anthocyanins, flavones and flavanonols only exist as biosynthetical end-products[65].

Anthocyanins belong to a wider class of phenolic compounds and are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts[63,66]. The most common sugar components of anthocyanins are glucose, glalactose and arabinose, which are usually conjugated to the anthocyanidin molecule via C-ring C-3 hydroxyl group[65]. The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number sugars attached to the molecule, the position of this attachment and the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule[65,67]. Some anthocyanins comprise multiple sugar moieties involving hydroxyl functionalities of the aglycone molecule other than C-3[54]. Most of anthocyanidins are partly responsible for colour variation in fruits and flower.

The most common anthocyanidins are: pelargonidin (2) cyanidin (3), and delphindin (4) and peonidin(5)[65].



(2)



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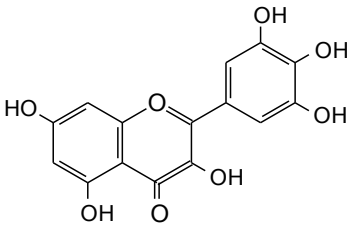

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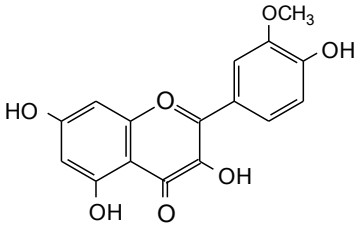
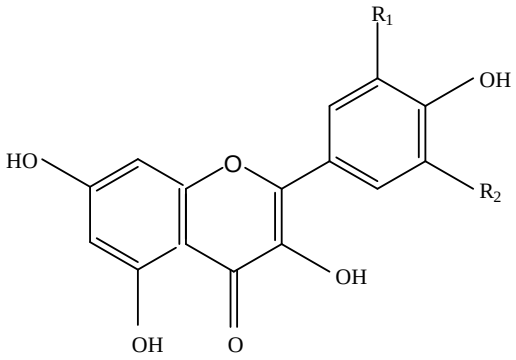


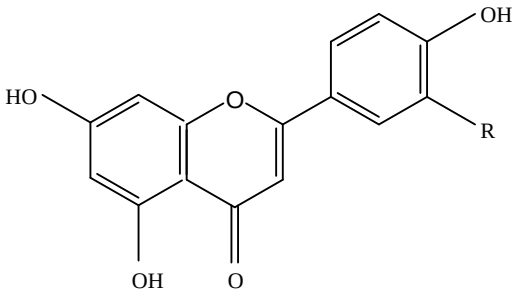
(5)

Flavonols are mainly colourless co-pigments in both cyanic an acyanic flower. They are widespread in leaves. There is a considerable number of known flavonols but only three are at all common: kaemferol (6), quercetin (7) and myricetin (8) [61].



(6), $R_1 = R_2 = H$
(7), $R_{1=}OH$, $R_2 = H$
(8), $R_1 = R_2 = OH$

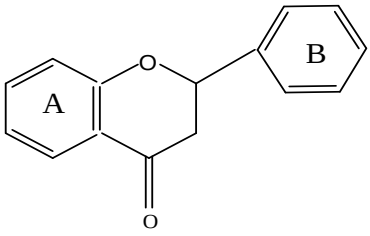
The name flavone was suggested for 2-phenylbenzopyrone. While thename flavonol has been given to 3-hydroxyflavone. There are only two common flavones, namely, apigenin (9) and luteolin (10)[61].



(9), R =H
(10), R =OH

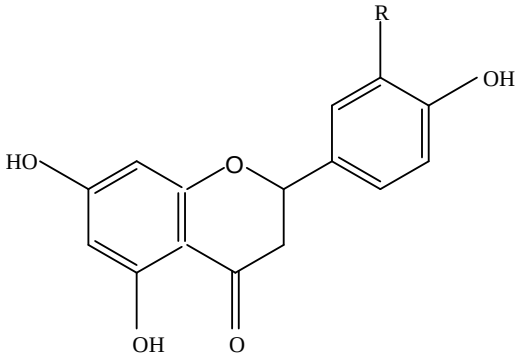
Flavone only differ from flavonols in lacking a 3-hydroxyl substitution; this affects their UV absorption, chromatographic mobitity and colour reaction and simple flavones can be distinguished from flavonols on these basis[61].

Flavanone (11) is colourless substance and hydroxylated flavanones occur either in the free form or in combination as glycosides in flowers, fruits, leaves, bark and roots and appear to be of fairly general distribution, especially in higher plant[68]. Chemically flavanones differ from flavones in being saturated between carbon 2 and 3 thus lacking the conjugation of a double bond between the carbonyl group, conjugated with the (A-ring), and the 2-phenyl group (B-ring).



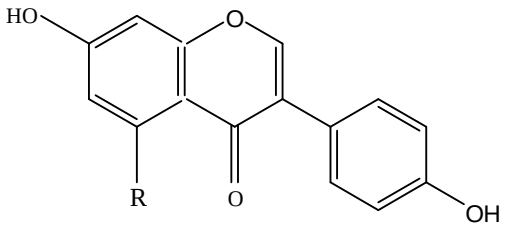
(11)

Flavanones are isomeric with chalcones and the two classes are inter-convertible *in vitro*. Chalcones are frequently found in nature together with flavanone analogues, but the converse is not always true[69]. The most common flavanones are naringenin (12), eridictyol and hespertin (13)[65].



(12), R = H
(13), R = OH

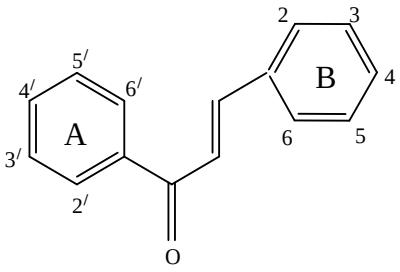
Isoflavones are isomeric with flavones but are much rarer in their occurrence[61]. In isoflavones the B-ring is substituted at C-3 instead of C-2 for the other flavonoids. Isoflavones can be divided into categories on their physiological properties. Compounds such as 7, 4′-dihydroxyisoflavones-daidzein (14) and 5, 7, 4′-trihydroxyisoflavone-genistien (15) are weak natural estrogens, present in clover[61].



(14), R = H
(15), R =OH

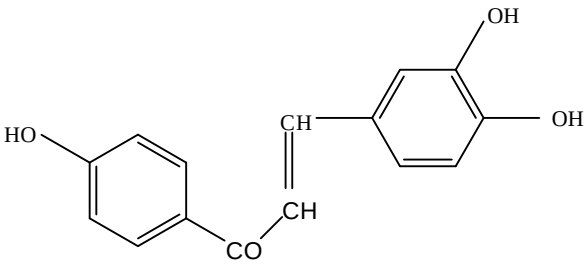
Chalcones are the open-chain flavonoids in which two aromatic rings (A and B) are linked by an aliphatic three-carbon chain which does not participate in forming a hetero ring as is usually found in other types of flavonoid compounds[68]. Naturally occurring chalcones are all hydroxylated to a greater or lesser extent. The A-ring substitution pattern is usually based upon the phloroglucinol system (2′, 4′, 6′- trihydroxybenzene). The B-ring oxiginates from a phenylpropanoid precursor and thus most commonly exhibits a 4-mono, 3, 4-di-, or 3,4, 5-trihydroxylation pattern. Structures, which vary from the common types, however, are found. It should be noted that the numbering of the position of substitution in the chalcone nucleus is reversed from

that in most other flavonoids, i.e the A-ring is numbered 2′-6′ and the B-ring 2-6 (16).



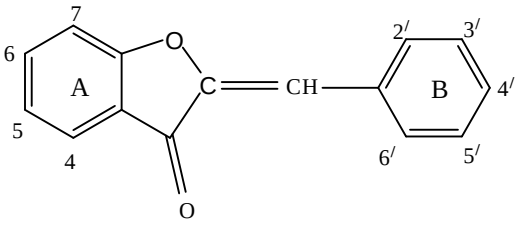
(16)

Besides their biosynthetic importance as the first isolable C_{15} precursors in flavonoid biosynthesis, the chalcones play an ecological role in nature, in relation to plant colour[69]. Atypical chalcone is butein (17) occur naturally as glycosides[61].



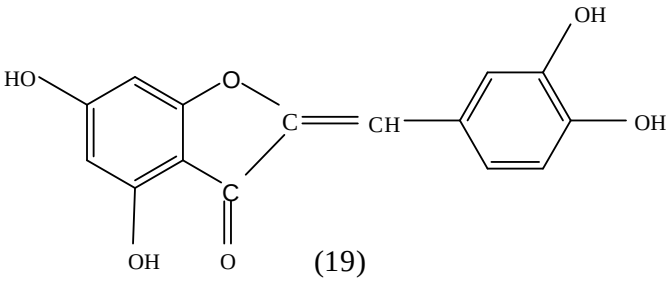
(17)

The aurones are not restricted to floral tissue but have been obtained from bark, wood and leaves as well. Chemically, aurones are based on the 2-benzylidene-coumaranone or 2-benzylidene-3(2H)-benzofuranone system (18)[70].



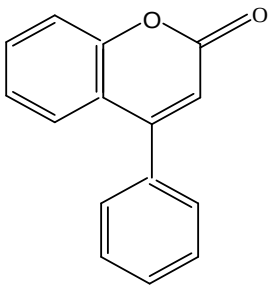
(18)

Aurones are most easily discussed by grouping them according to the number of hydroxyl groups present in the B-ring. A common aurone is aureusidin (19) occur naturally as glycosides[61].



(19)

The neoflavonoids are structurally and biogenetically closely related to the flavonoids and isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2H-1-benzopyran-2-ones) (20), 3, 4-dihydro-4-arylcoumarins (21), and neoflavenes (22)[71].



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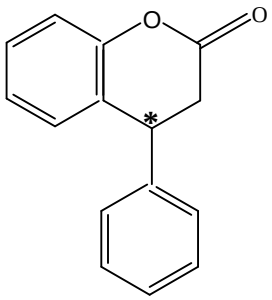
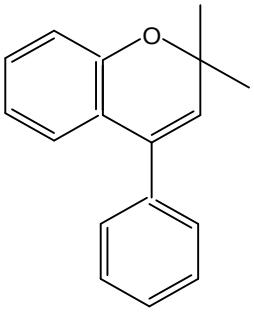
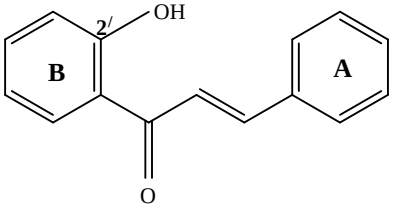


(21)



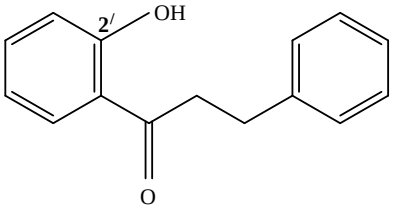
(22)

Natural products such as chalcones and aurones also contain a C_6-C_3-C_6 backbone and are considered to be minor flavonoids.



(23)

These groups of compounds include the 2/-hydroxychalcones (23), 2/-OH-dihydrochalcones (24), 2/-OH-retrochalcones (25), aurones (2-benzylidenecoumarauone) (26), and auronols (27) [71].



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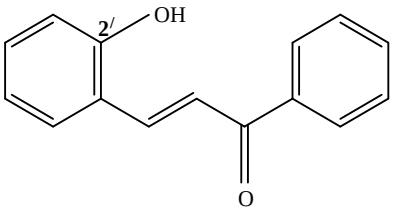

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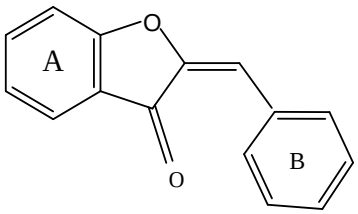

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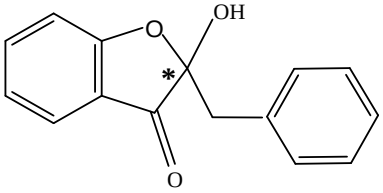
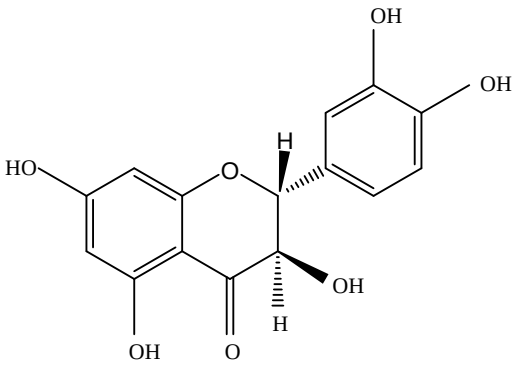


(27)

Dihydroflavonol or 3-hydroxyflavanones has wide distribution in the plant kingdom. The most common members of this group are: dihydroquercetin (28), dihydrokaempferol (29), dihydromyricetin (30)[72].

Dihydroflavonol have two asymmetric carbons at C-2 and C-3. Most dihydroflavonols are polyhydroxylated and some are partially O-methylated. Dihydroflavonols have a very wide natural occurrence. Dihydroquercetin, for example, has been found in 50 angisoperm families. The most significant biological property is their antimicrobial activity[73].



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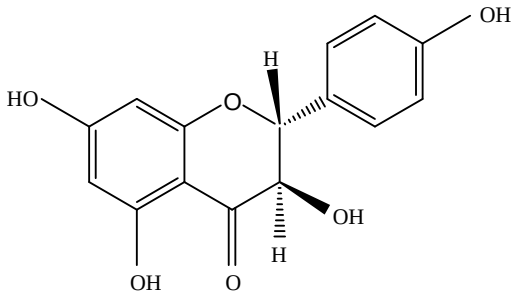

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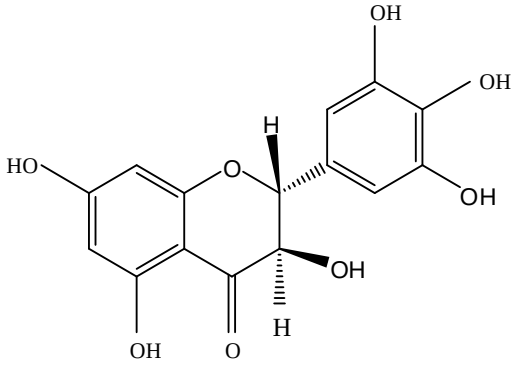


(30)

1.4.2- Optical activity of flavonoids

The flavonoids are a class of natural product that gains interest due its great variety and the number of its members. The flavonoids are often hydroxylated in positions 3, 5, 7, 3′, 4′, and 5′. Such hydroxyls are frequently methylated, acetylated, or sulphated. The actual number of flavonoids that have been found so far and for which the structure has been completely

elucidated is large, but probably does not exceed 1% of the theoretical number of possible variants. This abundance of variants is further augmented by the chirality of the subunits and their connections. Since many stereoisomers do not differ significantly in their electronic or fluorescence spectra so the optical activity of the species is often a useful antalytical parameter[74].

1.4.3- Functions of flavonoids in plants

Anthocyanin pigment present in flowers provide colour which contributes to pollination[75-77]. Flavonoids present in leaves promote physiological survival of plant by protecting it from fungal infections and UV radiations. In addition, flavonoids are involved in photosensitisation, energy transfer, respiration and photosynthesis control, morphogenesis, sex-determination, energy transfer[76].

1.4.4- Distribution of flavonoids

Flavonoids are widely distributed among the plant kingdom[76].Flavonoids are found in vegetables, fruits, nuts, seeds, stem, flowers, tea, wine etc. These are an integral part of our daily diet[54,78,79]. The dietary intake of flavonoids is estimated to be 1-2 g/day[80]. The average intake of flavonols and flavones was found to be 23 mg/day, among which, flavonol quercetin contributed 16 mg/day[75].

1.4.5- Biosynthesis of flavonoids

The flavonoid pathway is part of the larger phenylpropanoid pathway, which produces a range of other secondary metabolites, such as phenolic acids, ligins, lingnans, and stilbenes.

The key flavonoid precursors are phenylalanines, obtained via the shikimate pathway, and malonly-CoA derived from citrate produced by the TCA cycle.

Most flavonoid biosynthetic enzymes characterized to date are thought to operate in enzyme complexes located in the cytosol. Flavonoid end products are transported to various subcellular or extracellular locations, with those flavonoids involved in pigmentation generally being transported into the vacule[81].

The first flavonoids, the chalcones, are formed from HCA-CoA esters, usually 4-coumaroyl-CoA, in three sequential reactions involving the "extender" molecule malonyl-CoA. In a few species, caffeoyl-CoA and feruloyl-CoA may also be used as substrates for chalcone formation[81].

4-Coumaroyl-CoA is produced from the amino acid phenylalanine by what has been termed the general phenylpropanoid pathway, through three enzymatic conversions catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate: CoA ligase (4CL)[81].

Malonyl-CoA is formed from acetyl-CoA by acetyl-CoA carboxylase (ACC). Acetyl-CoA may be produced in

mitochondria, plastids, peroxisomes, and the cytosol by a variety of routes. It is the cytosolic acetyl-CoA that is used for flavonoid biosynthesis, and it is produced by the multiple subunit enzyme ATP- citratelyase that converts citrate, ATP, and Co-A to acetyl-CoA, oxaloacetate, ADP, and inorganic phosphate[82].

Many other compounds are involved in flavonoid biosynthesis in some species, for example, as donors for methylation or aromatic or aliphatic acylation. For intact plants, these are generally accepted to be available in the cell for the reaction to proceed if the appropriate modification activity is present[81].

The most important enzyme-catalzed reaction leading to the production of chalcones and 6′-deoxchalcones(34) is gives in scheme I. Central to this scheme is the biosynthesis of 2′,4′,6′,4-tetrahydroxychalcone, which is also known by the trivial names of chalconaringenin or naringenin chalcone (35).

This compound is formed by sequential condensation of three molecules of malonyl co enzyme A (malonyl-CoA) (31) with one of P-coumarolyl –CoA (32), a reaction catalyzed by chalcone synthase[83]. The final step is believed to be the cyclizaton of tetraketide precursor (33) (scheme I).

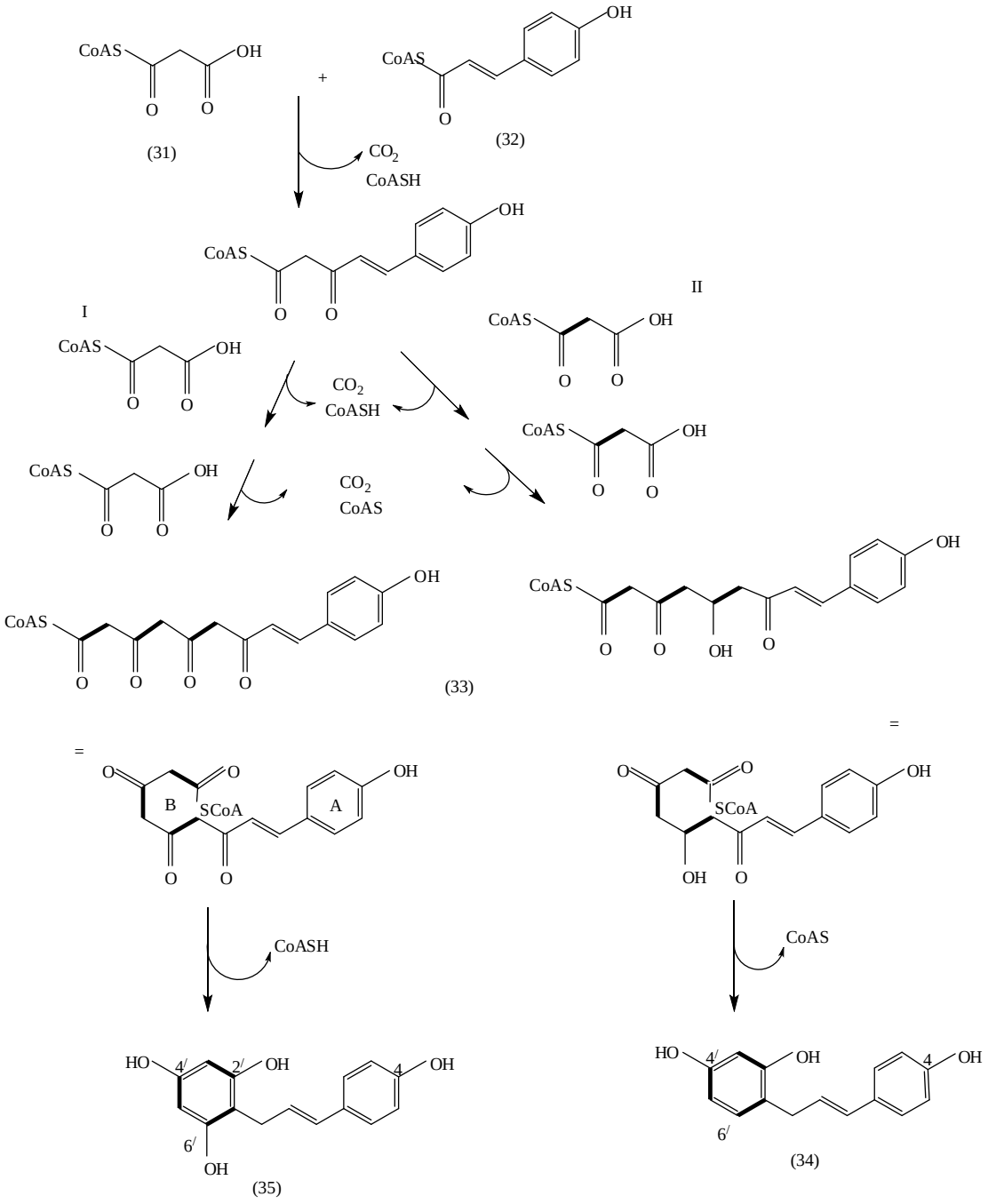
The production of chlacones as natural product represents the convergence of two biosynthetic pathways, the acetate (leading to the A-ring) and the shikimate (leading to the B-ring) respectively.

An enzyme involved in aurones biosynthes has been identified for the firt time[84]. The aglycones of aurone glycosides such as the 6-O-glucoside of aureusidin (4,6,3′,4′-tertrahydroxyaurone) (38) and bracteatin (4,6,3′,4′,5′-pentahydroxyaurones) (39) are now known to be formed through the action of aureusidin synthase on the chalcone precursors, chalconaringenin (2′,4′,6′,4-tetrahydroxychalcone) (36) and 2′,4′,6′,3,4-pentahyroxychalcone(37) (scheme II)[85-87].

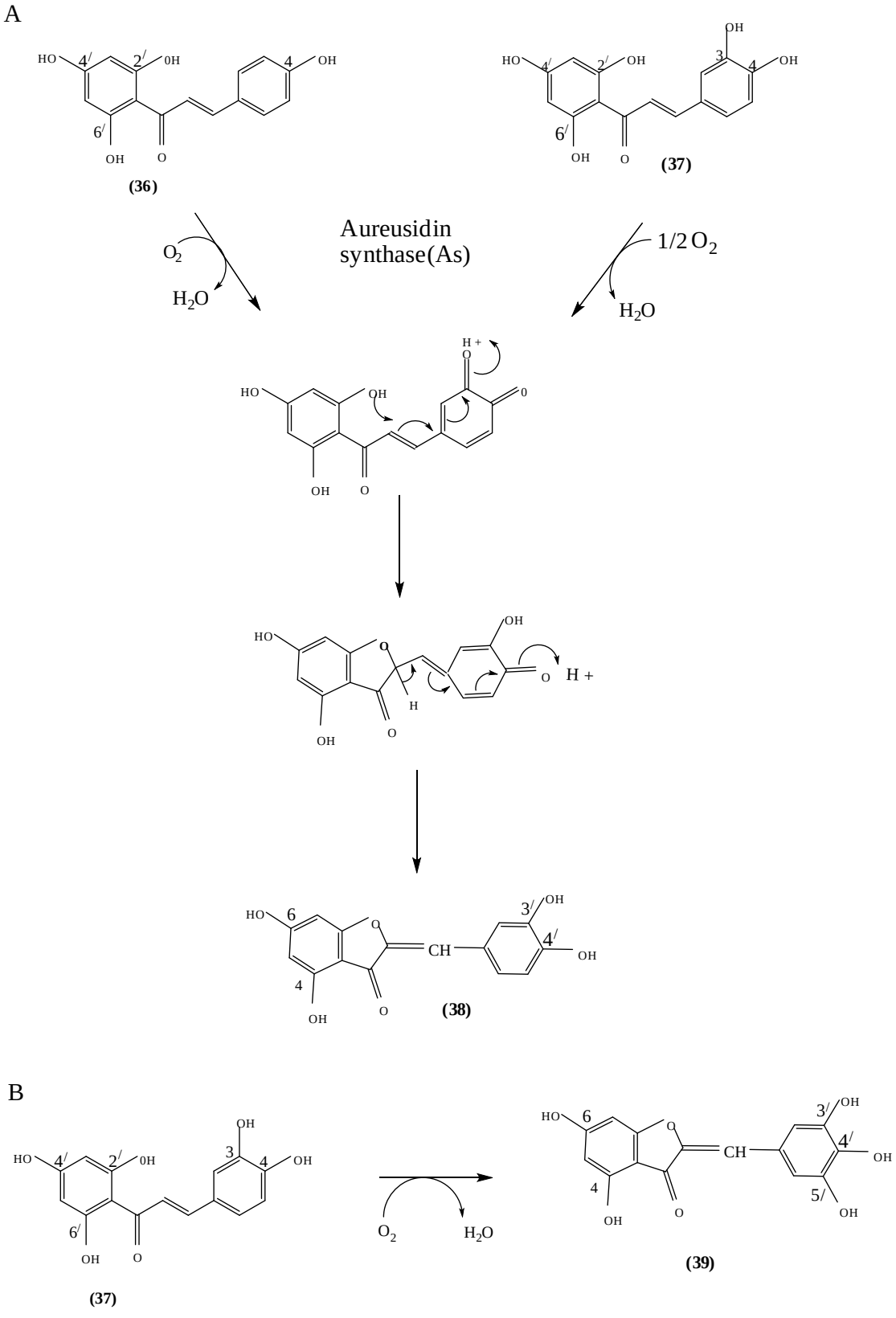
Two important features of the mechanism involving aureusidin synthase (scheme II) are the ortho-hydroxylation of the chalcone 4-pentahydroxy chalcone, presumably by the initial transformation of the o-dihydroxy B-ring of the latter to an o-diquinone. The biosynthetic origin of aurones with only one or no hydroxyl groups in the B-ring is unknown at present. Flavanone are formed from chalcones (40) by isomerization. There is evidence for the *in vitro* and *in vivo* existence of equilibrium between flavanones and the corresponding chalcones[88].

The inter conversion between chlcones and flavanone is catalyzed *in vivo* by enzyme as chalcone isomerase. The important feature of this enzymatic reaction is the stereo specificity, apparent in (S) chirality of C-2 in flavanone derivative. Therefore, it is not accidental that all the flavanones found in nature have the (S) configuration at C-2 and are levorotatory. With chalcones having at least two free hydroxyl

groups at C-2 and C-6, the equilibrium in aqueous solution is completely and rapidly shifted to the flavanone (41) (scheme III).



Scheme I: Biosynthesis of chalcones

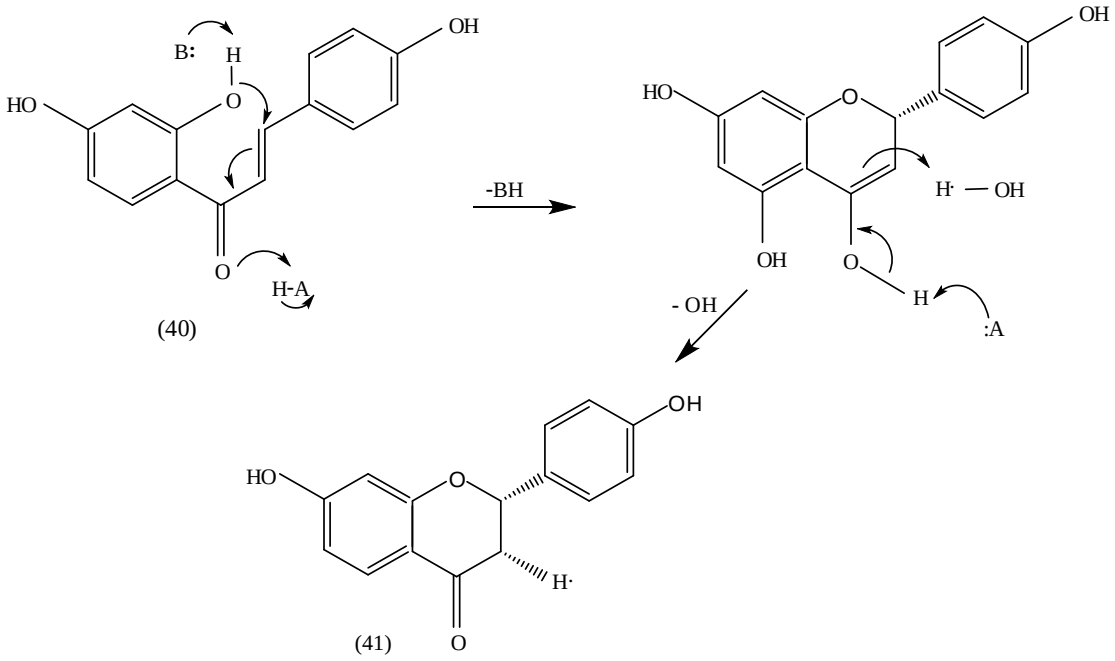


Scheme II: Biosynthesis of aurones

The stabilization energy of the strong hydrogen bond between carbonyl group and the ortho-phenolic hydroxyl group greatly influence the position of equilibrium and the inter conversion rate. When only one hydroxyl is available, either for the cyclization or for hydrogen bonding, the system ends to remain in the open form (chalcone form).

The key step in isoflavone formation is the 2, 3-migration of the aryl side chain of a flavonane - chalcone intermediate. An enzyme activity catalyzing this transformation was found in microsomal preparation from elicitor-challenged soybean cell suspension cultures. It transforms (2S)-narigenin (flavanone) (41) into genisten[89] (43) (scheme IV).

It was found that two enzymatic steps are involved in this transformation. The first step comprises oxidation and rearrangement of naringenin to 2-hydroxy-2,3-dihydrogenistein (42). This step is strictly dependant on NADPH and molecular oxygen. The second enzyme which catalyses the elimination of water from the 2-hydroxyisoflavanone is in the soluble fraction and not yet been characterized[90]. For the following reasons, (2S)-flavanones and not the chalcones are very probably the actual substrates:

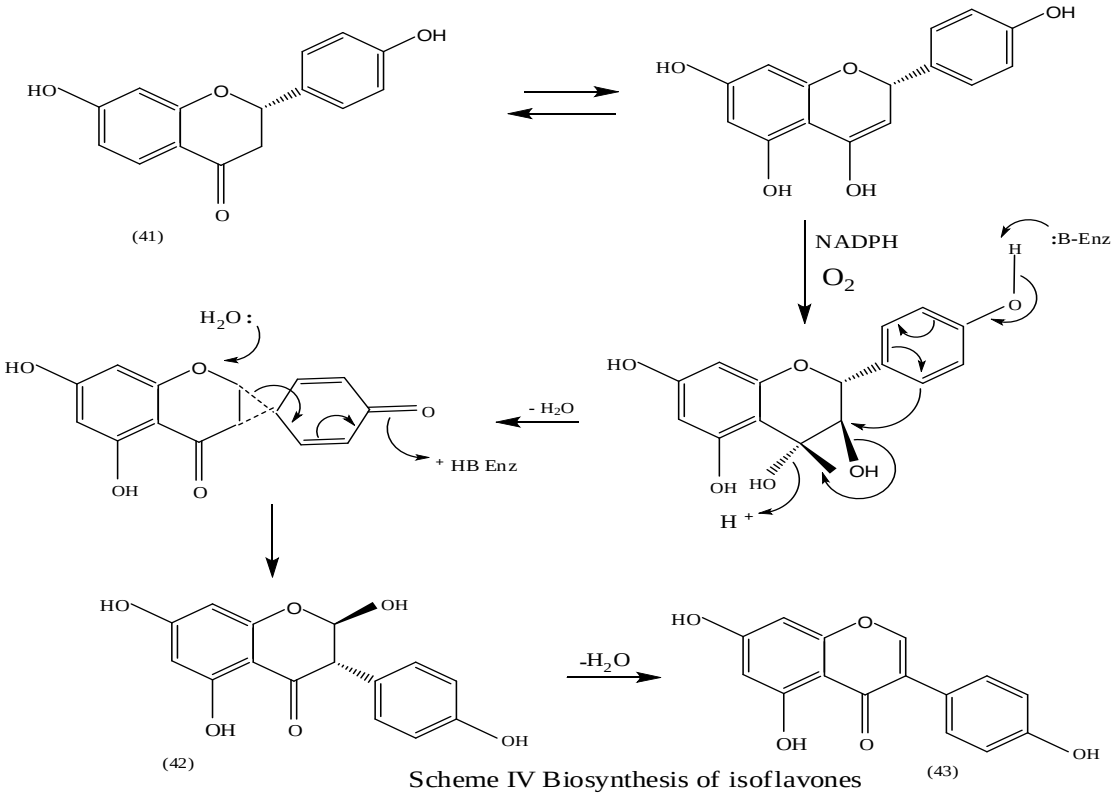


Scheme III Biosynthesis of flavanones

- A stereospecific incorporation of (2S)-naringenin into biochanin A (5,7-dihydroxy-4′-methoxyisoflavone)[91].
- Only the (2S) but not the (2R) enantiomer acts as asubstrate *in vitro*[91].
- The equilibrium of 4,2′,4′,6′-tetrahydroxychalcones is at least 1000:1 in favour of the flavanones[92].

The hypothetical path way from (2S)-flavanone to isoflavone (scheme IV) is consistent with the participation of NADPH and molecular oxygen. The sequence of events may be initiated by an epoxidation step, protonation and subsequent cleavage of the epoxide would render appositive charge to the B-ring. Keto-enol tautomerism, as indicated by proton exchange at C-3 in flavanones[93] which allows homoallyic interaction between C-3 and C-1′, and rearrangement of the structure then take place.

Addition of hydroxyl in to C-2 leads to the 2-hydroxy isoflavanone intermediate, which is transformed to the isoflavone by elimination of water molecule.

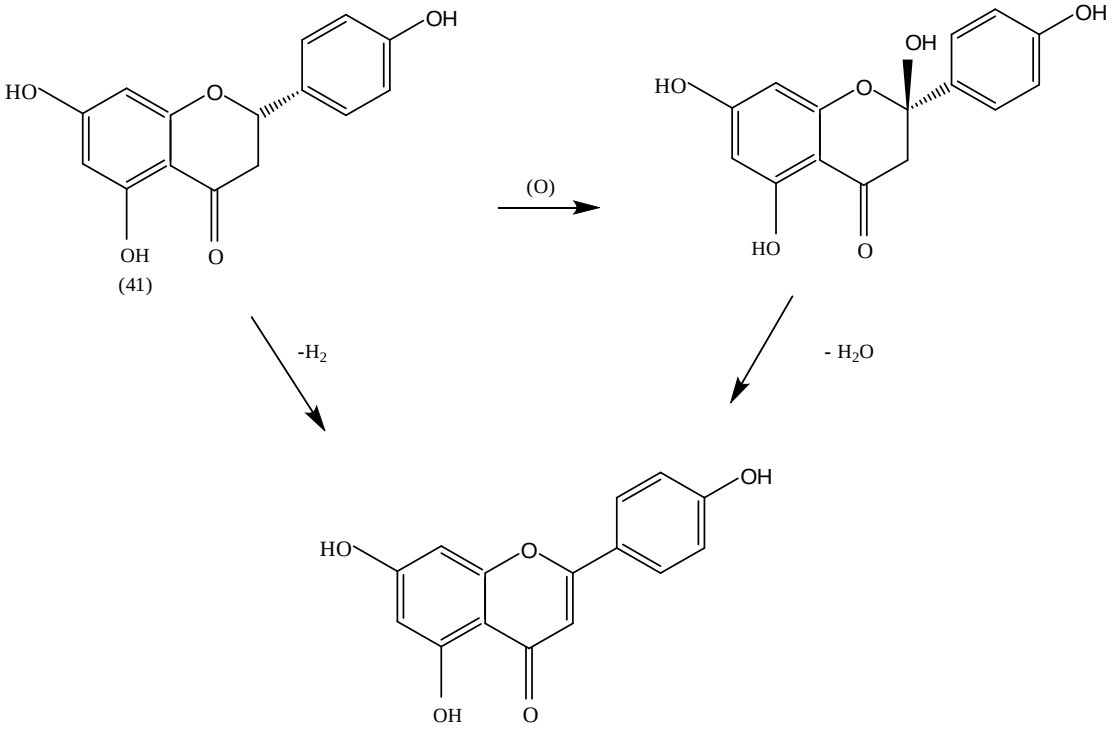


Scheme IV Biosynthesis of isoflavones

The *in vitro* conversion of flavanones to flavones was first observed in parsley plants[94]. The reaction has been studied in more detail in parsley cell suspension cultures[95] and in antirrhinum flower[96]. The parsley enzyme requires 2-oxoglutarate, Fe^{+2} and possibly ascorbate as Co-factors. This Co-factor requirement would classify it as belonging to the 2-oxoglutarate dependant dioxygenases[97]. Ascorbate stimulates this and other 2-oxoglutarates-dependant dioxygenases involved in the flavonoid pathway and, in addition, exhibits a remarkable stabilizing effect on the enzyme activity[98]. In antirrhinum[96] and

in other plants including Verbena[99], Dahlia, Streptocarpus and Zinnia[100], oxidation of flavanone to flavone is catalyzed by a microsomal enzyme requiring NADPH as Co-factor.

Both the parsly and the flower enzyme catalyzed the reaction from (2S)-naringen (flavanone) to apigenin (flavone) (scheme V). The mechanism of double bond formation is still unclear. It has been suggested that 2-hydroxyflavanone is formed in the first step, and water is then eliminated via a dehydrates[95,96]. However, no such 2-hydroxy intermediated has yet been observed even with a nearly homogenous enzyme protein[98]. On the other hand, 2-hydroxyflavones certainly exist as plant metabolites and indeed are the substrates in C-glycosyl flavones formation[101].

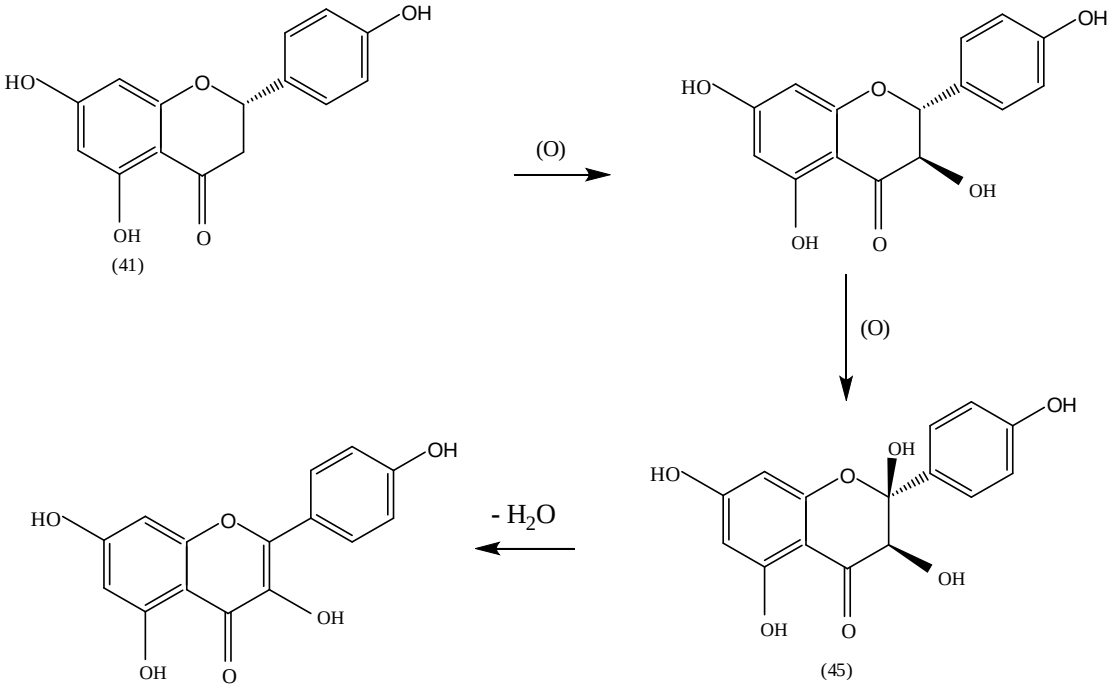


Scheme V Biosynthesis of flavones

Enzymatic conversion of dihydroflavonols to flavonols was first observed with enzyme preparations from parsley cell suspension cultrures[95]. Synthesis of flavonols was found to be catalyzed by a soluble 2-oxoglutarate-dependant oxygenase.

Flavonol synthesis, most probably, proceeds via a 2-hydrroxy intermediate such as hydroxyl-dihydrokaempferol (45) with subsequent dehydration, giving rise to the respective flavonols (46)[102] (scheme VI).

Flavonol synthase has also been demonstrated in flower extracts from Mattiola[103] and Petunia[104]. As in parsley, flavonol formation in these flowers is catalyzed by a soluble 2-oxoglutarate-dependant dioxygenase.



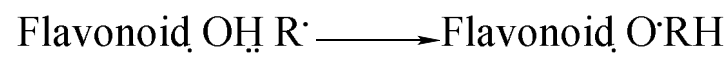
Scheme VI Biosynthesis of flavonols

1.4.6- Biological activities of flavonoids

Flavonoids have been reported to exert wide range of biological activities. These includes: anti-inflammatory, antibacterial, antiviral, antiallergic[54,58,105] , cytotoxic antitumour, treatment of neurodegenerative diseases, vasodilatory action[105-108]. In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation[54,108,109]. These are also reported to inhibit variety of enzymes like hydrolases, hyalouronidase, alkaline phosphatise, arylsulphatase, CAMP phosphodiesterase, lipase, α-glucosidase, kinase[110].

Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers[111]. This activity is attributed to their hydrogen-donating ability. Indeed, the phenolic groups of flavonoids serve as a source of a readily available ‘‘H” atoms such that the subsequent radicals produced can be delocalized over the flavonoid structure[65].

Free radical scavenging capacity is primarily attributed to high reactivities of hydroxyl substituents that participate in the reaction[65] as shown :



Flavonoids inhibit lipid peroxidation *in vitro* at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to a peroxy radical, thus, forming flavonoids radical, which, further reacts with free radicals thus terminating propagating chain[54112]. Naturally, the organism has developed a defence against toxic substances such as peroxynitrite and nitrous acid. An important mechanism is catalyzed by the enzyme superoxide dismutase (SOD), which converts two superoxide anions to H_2O_2 and O_2[74].

Flavonoids are known to be synthesized by plants in response to microbial infection; thus it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid rich plant extracts from different species have been reported to possess antibacterial activity[113-116] Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity[117].

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to

inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes[118.119].

Catechins, the most reduced form of the C-3 unit in flavonoid compounds, have been extensively researched due to their antimicrobial activity. These compounds are reported for their *in vitro* antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria[120-121]. The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyltransferases in *S. mutans*, probably due to complexing activities[120,122]. Robinetin, myricetin, and (−)-epigallocatechin are known to inhibit DNA synthesis in *Proteus vulgaris.* The B ring of the flavonoids may interchelate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria[123]. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3′,4′-pentahydroxy-flavone against *Escherichia coli* DNA gyrase[124].

Naringenin and sophoraflavanone G have intensive antibacterial activity against methicilline resistant *Staphylococcus aureus* (MRSA) and *Streptococci*. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner layers of membranes[125]. The correlation between antibacterial activity and membrane

interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells. The 5,7-dihydroxylation of the A ring and 2′,4′-or 2′,6′- dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity[126]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C-8 and C-10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class[127].

Osawa et al.[128] have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2′ and 4′ positions inhibited the growth of *S. mutans* and *Streptococcus sobrinus*[123]. Haraguchi and colleagues[126] studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus.* They observed that licochalcone A inhibited incorporation of radioactive precursors into macromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome *c* in the bacterial respiratory electron transport chain[126]. There are many examples that lend support to the

process of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents[129-131].

Flavonoids have been ascribed a wide range of beneficial properties related to human health, including cancer[132-137] ; cardiovascular diseases[138] ; including coronary heart disease[133,135,139] and atherosderosis[139] ; inflammation[135,141] ; and other diseases in which an increase in oxidative stress has been implicated[142-145].Their ability to act as classical electron-(hydrogen-) donating antioxidants *in vitro* has been intensively reported[146-148] and used to explain their protective effects against oxidative stress. Structurally important features that define this antioxidant activity are the hydroxylation pattern, in particular a 3′, 4′-dihydroxycatechol structure in the B-ring and the presence of 2,3 unsaturation in conjugation with a 4-oxo-function in the C-ring[148].

The antioxidant efficacy of flavonoids has been described for protection against oxidative damage to avariety of cellular biomolecules. For example, flavonoids inhibit the oxidation of low-density lipoprotein[149-153].

And deoxyribon nucleic acid (DNA)[154-157] *in vitro*. In addition, flavonoids are effective scavengers of reactive nitrogen species in the form of peroxynitrite[158-161] and limit dopamine oxidation mediated by peroxynitrite in structure-dependent way involving oxidation or nitration of the flavonoid ring system[162]. Furthermore, their antioxidant properties have also been

attributed to their abilities to chelate transition metal ions[163-166] and their potential to quench singlet oxygen[167,168].

A number of studies have investigated the ability of flavonoids to protect against neuronal death induced by oxidative stress[169-173]. For example, flavonoid such as epicatechin and quercetin have been shown to reduce the neurotoxicity induced by oxidized low-density lipoprotein[173-176].

There has been an equivalent amount of interest in the ability of flavonoids to protect against oxidative injury, particularly that induced by hydrogen peroxide, in a variety of cell systems. For example, the flavanol epicatechin has been show to evoke strong protection against cell damage, caspase-3activation, and binding of annexin v-cy3.18 (an early marker of opoptosis) induced by hydrogen peroxide in dermal fibroblasts[177,178] and primary cortical neurous[178]. Ouercetin and another flavonol, kaempferol, as well as catechin and flavone taxifolin, have been observed to suppressthe cytotoxicity of O_2 and H_2O_2 to chinese hamster V79 cells, as assessed by the ability of the flavonoids to prevent the decrease in the number of cell coloies induced by the oxidants[179].

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells,

release of ROS, RNS, and proinflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders[180].

The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals. Certain members of flavonoids significantly affect the function of the immune system and inflammatory cells[181]. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein kinases[182,183]. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules significantly affect the function of the immune system and

inflammatory cells[181]. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein kinases[182,183]. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules[184]. Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of the anti-inflammatory effect of flavonoid is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Certain flavonoids are potent inhibitors of the production of prostaglandins, a group of powerful proinflammatory signaling molecules[185].

Reversal of the carrageenan induced inflammatory changes has been observed with silymarin treatment. It has been found that quercetin inhibit mitogen stimulated immunoglobulin secretion

of IgG, IgM, and IgA isotypes *in vitro*[186]. Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10mM concentration[187]The effect of flavonoid on platelets has been related to the inhibition of arachidonic acidmetabolism by carbon monoxide[188].

Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

The liver is subject to acute and potentially lethal injury by several substances includingphalloidin (the toxic constituent of the mushroom, *Amanita phalloides*), CCl4, galactosamine, ethanol, and other compounds. Flavonoids have also been found to possess hepatoprotective activity. In a study carried out to investigate the flavonoid derivatives silymarin, apigenin, quercetin, and naringenin, as putative therapeutic agents against microcrystin LR-induced hepatotoxicity, silymarin was found to be the most effective one[189].The flavonoid, rutin and venoruton, showed regenerative and hepatoprotective effects in experimental cirrhosis[190].

Ulcer is a commonly occurring disease in developed countries and its occurrence is emerging with increase in modernisation of living standards. The stress hormones like epinephrine, norepinephrine, and ACTH, causes a vasoconstriction in the integument and the periphery, whereas, they dilate the vessels of muscles, heart, and brain. But, if the duration of this state gets

prolonged, then blood supply to major organs, e.g., stomach, intestine, liver, kidneys, and skin get reduced and thus failing to satisfy demand for oxygen, antibodies and other agents that are required to maintain a healthy condition. As a result, the stress hormones increases the glandular secretion which denatures proteins in plasma membranes and catalyses the hydrolysis of polysaccharide moieties of proteoglycans in the protective mucous coat covering the luminal surface of the stomach and the upper intestine to a perilous extent during prolonged stress. When the walls of the blood vessels supplying oxygen, nutrients, and protective substances to this area gets sufficiently weakened, then slight mechanical insults easily cause ruptures, resulting in leakage of blood into the tissue. Such events start inflammation and repair processes in which eicosanoids, e.g., PGs, participate[74]. Flavonoids are favourable, effective, and usually innocuous substitutes for the classical therapeutic agents. It has also been reported that flavonoids protect against gastric cancer. Similar to aspirin, acylated flavonoids may transfer their acyl group to the side chain hydroxyl group of serine in the active site of COX[74]. Flavonoids glycosides of *Ocimum basilicum* decreased ulcer index and thus inhibit gastric acids in aspirin-induced ulcers. Quercetin, Kaempferol, rutin when administered intraperitoneally (25-100 mg/kg) inhibited dose-dependent gastric damage produced by ethanol in rats[191].

Deregulated proliferation appears to be a hallmark of increased susceptibility to neoplasia. Cancer prevention generally is associated with inhibition, reversion, or delay of cellular hyperproliferation. Most flavonoids have been demonstrated to inhibit proliferation in many types of cultured human cancer cell lines, whereas they have little or no toxicity to normal human cells. For example, Kandaswami and coworkers reported antiproliferative effects of four citrus flavonoids (quercetin, taxifolin, nobiletin, and tangeretin, at 2-8 μg/ml for 3–7 days) on squamous cell carcinoma HTB43 [191]. Kuo showed antiproliferative potency of five flavonoids and two isoflavonoids (0–100 μM) on colon carcinoma HY29 and Caco-2 cell lines, with the induction of apoptosis[192].

1.5- Aim of this study

This study was aimed to:

- Screening the roots of *Balanites aegyptiaca*, *Psidium guajava* and seed of *Hegeina* ***abyssinica*** for different phytochemicals.
- Extraction of flavonoids from plants species.
- Isolation of flavonoids via different chromatographic tools.
- Elucidation of structure via sensitive analytical tools.
- Evaluation of antimicrobial activity of different extracts.

Chapter Two

2-Materials and Methods

2.1- Materials

2.1.1- Plant material

The roots of *Balanites aegyptiaca* were collected from Singa, Gadaref State in October 2013. The roots of *Cisuss petiolata* were collected from Kordofan- west Sudan- during November 2013.The seeds of *Hagenia abyssinica* were collected from Niala- west Sudan .The plants were kindly authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum.

2.1.2- Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV- Visible Spectrophotometer. NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer. Mass spectra were run on a Joel Mass Spectrometer (JMS- AX500).

2.2- Methods

2.2.1- Preparation of reagents for phytochemical screening.

2.2.1.1-Flavonoid test reagents

- Aluminium chloride solution

(1 g) of aluminum chloride was dissolved in (100 ml) methanol.

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in (100 ml) distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in (100 ml) methanol.

2.2.1.2- Alkaloid test reagents

Maeyer reagent

- **Mercuric chloride solution**: (1.36 g) in (60 ml) distilled water.

- **Potassium iodide solution**: (5 g) in (10 ml) distilled water

The two solutions were combined and then diluted with distilled water up to (100 ml).

Wagner reagent

(1.27 g) iodine and (2 g) of potassium iodide in (100 ml) distilled water.

2.2.2- Preparation of plant extract for phytochemical screening

(100 g) Of powdered air- dried plant material were extracted with 80% aqueous methanol (soxhelt) until exhaustion.This prepared extract (PE) was used for phytochemical screening.

2.2.3- Phytochemical screening

The prepared extract of each plant was screened for major secondary constituents.

2.2.3.1- Test for unsaturated sterols and for triterpenes

(10 ml) of the (PE) of each plant were separately evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with (10 ml) chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphite. (5 ml) portion of the solution was mixed with (0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

2.2.3.2- Test for flavonoids

(20 ml) of the (PE) of each plant were separately evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in (30 ml) of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To (3 ml) of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.

- To (3 ml) of the filtrate few drops of aluminium chloride solution were added.
- To (3 ml) of the filtrate few drops of potassium hydroxide solution were added.

2.2.3.3- Test for alkaloids

(10 ml) of the (PE) of each plant were separately evaporated to dryness on water bath and (5 ml) of 2 N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Maeyer reagent were added to the other portion few drops of Wagner reagent were added.

2.2.3.4- Test for tannins

(10 ml) of (PE) of each plant were separately evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v) of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled, filtrated and the volume adjusted to (10 ml) with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

2.2.3.5- Test for Saponins

(1 g) of dried powdered plant material was placed in a clean test tube. (10 ml) of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand.

2.2.4- Extraction of flavonoids

(1 kg) of powdered shade-dried plant material was macerated with 95% ethanol (5 L) for 48hr. at room temperature with occasional shaking and then filtered off . The extraction process was repeated two more times with the same solvent. Combined filtrates were concentrated under reduced pressure using rotatory evaporator at 40 $^{\circ}$C until all ethanol was removed yielding a crude product.

2.2.5-Isolation of flavonoids

The ethanolic extract of *Balanites aegyptiaca* was dissolved in (100 ml) water and partitioned successively with chloroform, ethyl acetate and n-butanol.

Preparative paper chromatography was carried out for ethyl acetate fraction using BAW (Butanol: Acetic acid: Water 4:1:5 upper layer) as solvent system. The developed chromatograms were dried, observed in UV light (λ= 366, 254 nm) and then determined with smooth lines using a pencil.

The equivalent bands from each paper was then cut out into small strips and slurred with methanol. After several hours of contact with occasional shaking, the liquid was filtrated and evaporated to dryness. In this way compounds I (R_f 0.87) was isolated.

Using the same procedure, the n-butanol fraction of *Hagenia abyssinica* gave compound II (R_f 0.66). The ethyl acetate fraction of this species yielded compound III (R_f 0.72).In the same way compound IV (R_f 0.68) was isolated from the ethanolic extract of *Cissus peiolata.*

2.2.6- The UV spectrum of the isolated flavonoids in presence of UV shift reagents

2.2.6.1- The UV spectrum of the isolated flavonoids in presence of NaOMe

Three drops of NaOMe were added to a solution of the flavonoid in methanol (2 ml) and the UV spectrum was immediately recorded.

2.2.6.2- The UV spectrum of the isolated flavonoids presence of aluminium chloride

Six drops of the stock solution of aluminium chloride were added to a solution the flavonoid in methanol (2 ml) and UV spectrum was immediately recorded.

2.2.6.3- The UV spectrum of the isolated flavonoids in presence of $AlCl_3$/HCl

Three drops of the stock solution of hydrochloric acid were added to the solution in (2.2.6.2) and the spectrum was immediately recorded.

2.2.6.4- The UV spectrum of the isolated flavonoids in presence of NaOAc

Exess coarsely powdered anhydrous NaOAc was added with shaking to a cuvette containing (2-3 ml) of the solution of flavonoids in methanol and the UV spectrum was recorded after 2 minutes.

2.2.6.5- The UV spectrum of the isolated flavonoids in presence of NaOAc/H_3BO_3

Sufficient powdered anhydrous H_3BO_3 was added with shaking to a cuvette containing the solution (2.2.6.4) to give a saturated solution. The UV spectrum was recorded after 2 minutes.

Chapter Three

3-Results and Discussion

3.1 Phytochemical screening

The species *Balanites aegyptiaca*, *Cissus petiolata* and *Hegeina abyssinica* were subjected to phytochemical screening and the results are depicted in table (1).

Table (1): phytochemical screening

Phytochemical species	flavonoids	alkaloids	saponins	steriods	tannis
Balanites aegyptiaca	+ve	-ve	+ve	-ve	+ve
Cissus petiolata	+ve	+ve	+ve	-ve	-ve
Hegeina abyssinica	+ve	+ve	+ve	-ve	+ve

3-1Characterization of compound I

Compound I was isolated as yellow powder from the roots of *Balanites aegyptiaca*. The IR spectrum of compound I (Fig.1) showed: v(KBr) 540, 918 (C-H bending), 1047 (C-O) 1417 (C=C, Ar.), 1622 (C=O) 2925 (C-H, aliphatic), 3386 cm^{-1} (OH).

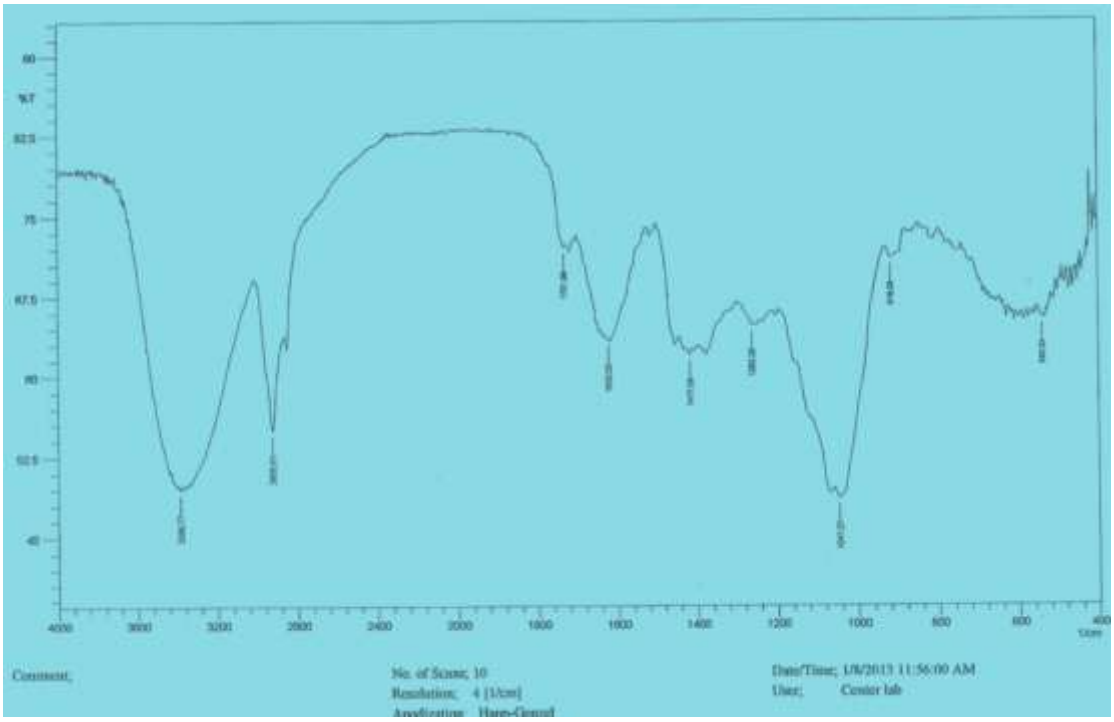
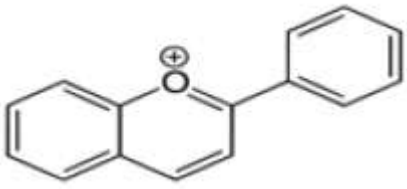
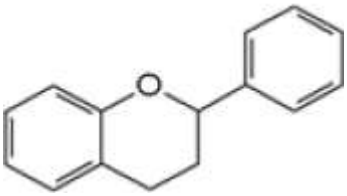


Fig.1: IR spectrum of compound I

Since a (C=O) stretching was detected in the IR, then compound I is evidently neither a catechin nor an anthocyanin.

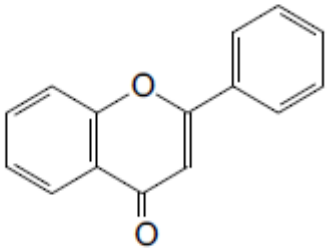


Anthocyanin

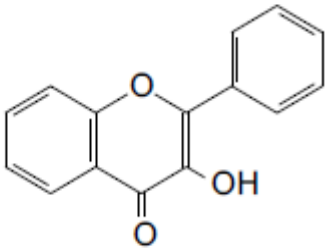


Catechin

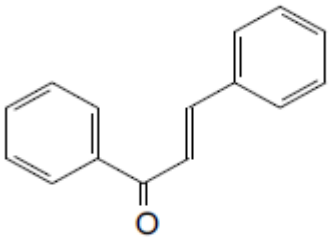
Compound I is thus one of the following classes:



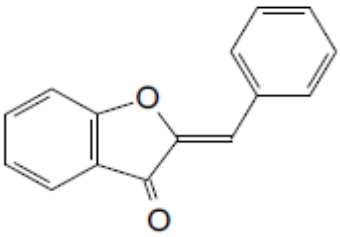
Flavone



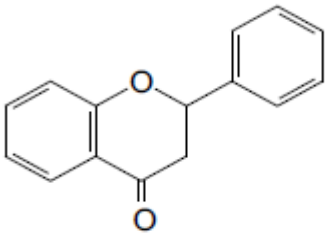
Flavonol



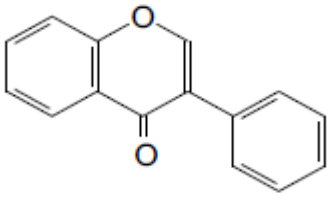
Chalcone



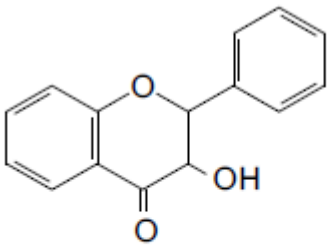
Aurone



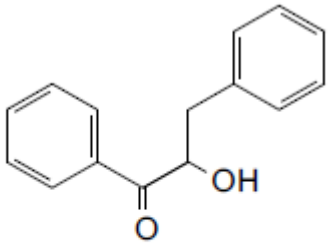
Flavanone



Isoflavone



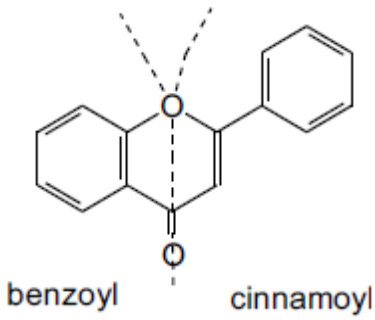
Dihydroflavonol



Dihydrochalcone

UV is a spectral technique that can differentiate between those flavonoids with unsaturation at carbon 2 (flavones, flavonols, chalcones and aurones) and other classes which are characterized by loss of conjugation between rings A and B (flavanones, isoflavones, dihydorchalcones and dihydroflavonols).

Flavonoids with conjugation between A and B rings show two absorption bands in the UV spectrum: band I (due to cinnamoyl chromophore) and band II (due to benzoyl chromophore). Band I occur in the range: 300- 400 nm, while band II occur in the range: 220 – 290 nm.



Flavonoids like flavanones, isoflavones, dihydrochalcones and dihydroflavonols lacking conjugation between rings A and B afford only band II.

The UV spectrum of compound I gave λmax (MeOH) 275, 335nm (Fig. 2) a pattern which is characteristic of flavones[193].

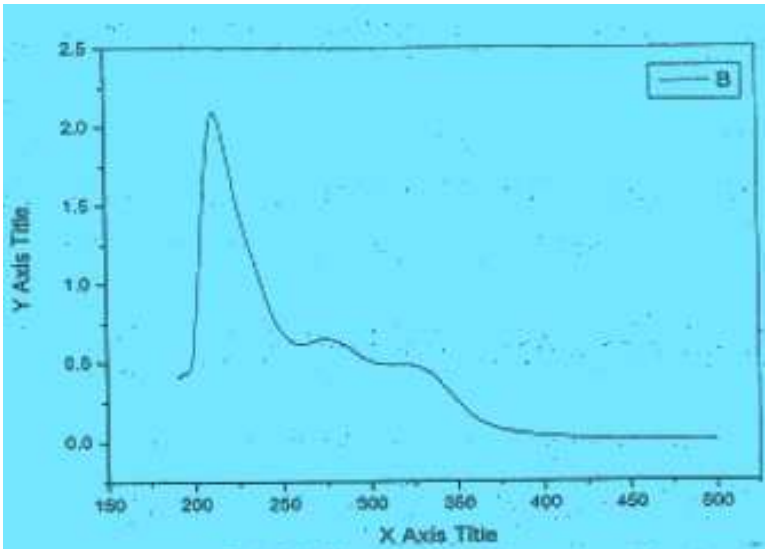


Fig.2: UV spectrum of compound I

The UV shift reagents (sodium methoxide, sodium acetate, aluminium chloride and boric acid/sodium acetate) can provide characteristic bathochromic shifts diagnostic of specific hydroxylation pattern. Sodium methoxide is strong base. It is diagnostic of 3-OH and 4` -OH groups. In both cases it induces a bathochromic shift, but with decrease intensity in case of 3-OH function. When the shift is accompanied by an increase in intensity, both hydroxyl groups are present.

Addition of sodium methoxide to a methanolic solution of compound I, induced a 38nm bathochromic shift without decrease in intensity indicating a 4`-OH function (Fig.3).

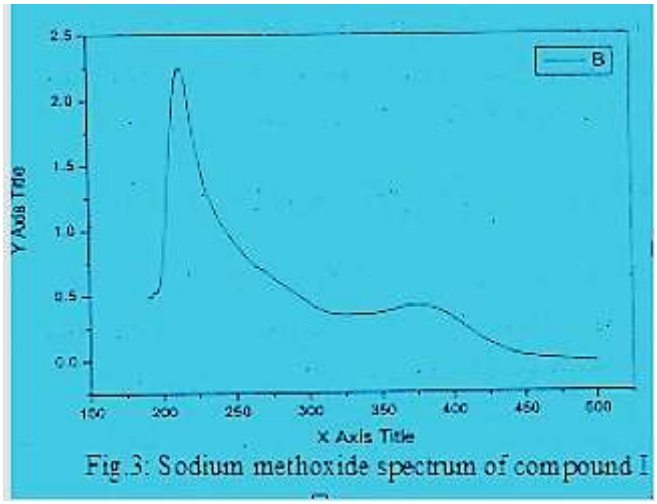


Fig.3: Sodium methoxide spectrum of compound I

The sodium acetate spectrum showed a 5nm bathochromic shift diagnostic of a 7-OH function (Fig.4). No detectable bathochromic shifts were observed in the aluminium chloride (Fig.5) and boric acid (Fig.6) spectra. Such data clearly indicate absence of 5- and 3-hydroxyl functions as well as catechol systems.

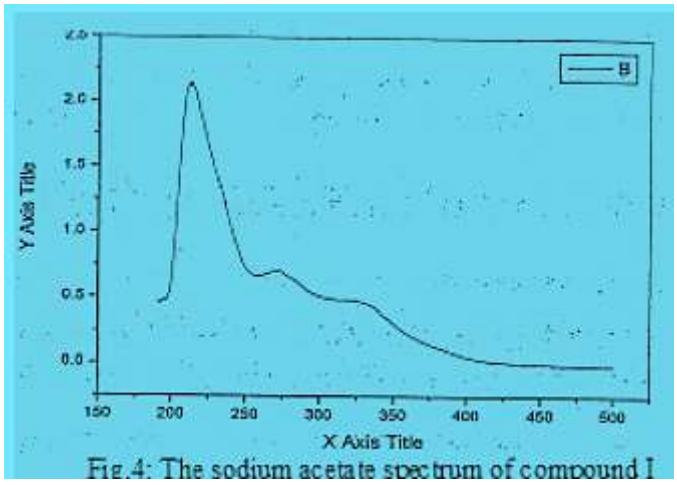
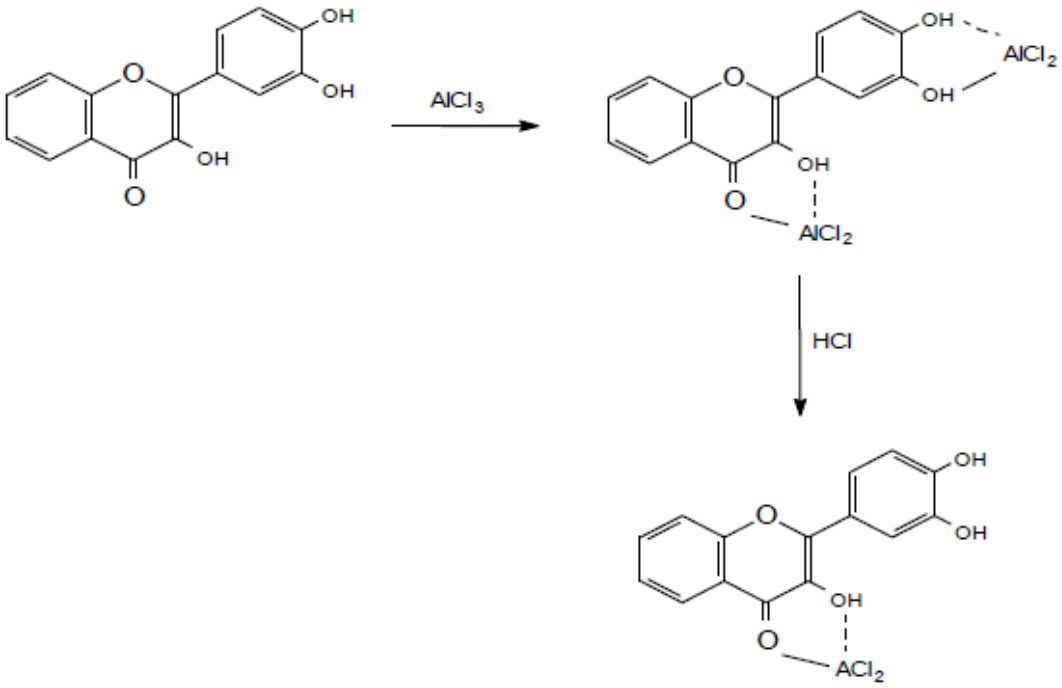


Fig.4: The sodium acetate spectrum of compound I

Aluminum chloride is a useful diagnostic tool for 3-OH, 5-OH groups and catechol moieties where it gives bathochromic shifts, when added to a methanolic solution of the flavonoid. Catechols afford acid – labile complexes with aluminium chloride, while the 3-OH (or 5-OH) form acid – stable complexes with participation of the 4-keto function[194,195]. Such complexes acid – stable are shown below:



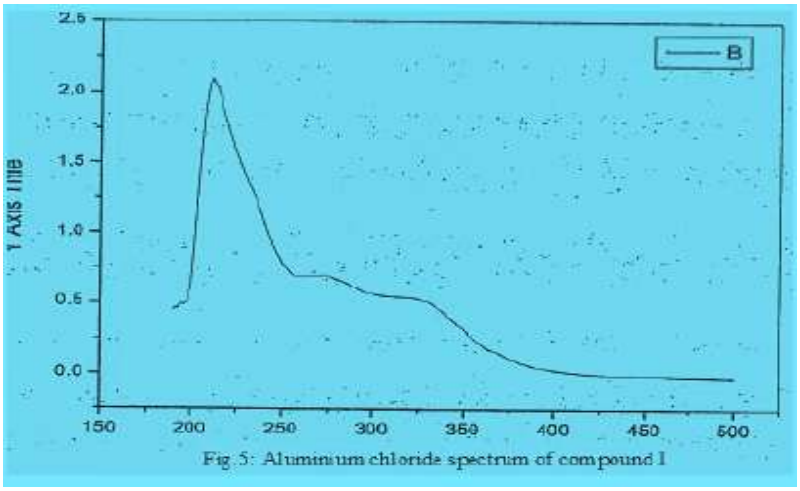


Fig 5: Aluminium chloride spectrum of compound I

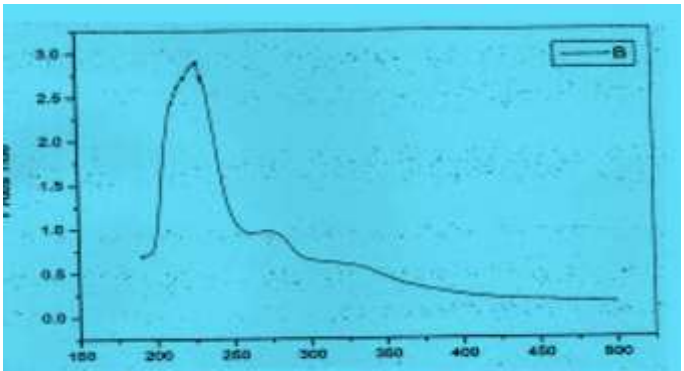


Fig.6: Boric acid spectrum of compound I

Sodium acetate, as a shift reagent, only ionizes the more acidic 7-OH group and as such it is diagnostic of 7-OH function. On the other hand, boric acid is diagnostic of catechol systems. It induces bathochromic shifts, where it chelate with ortho – dihydroxy systems at all locations on flavonoid nucleus, except at $C_{5,6}$[193].

The ^1H NMR spectrum (Fig.7) showed δ2.40 (6H) assigned for two aromatic acetyl groups. The NMR did not reveal any signals for C_6-H and C_3-vinylic proton, hence the two acetyl functions were assigned for these positions. The resonances at δ7.60 (^1H) and 8.01 (^1H) ppm account for C_8 and C_5 protons respectively. The C_5 proton usually resonates at lower field near 8 ppm relative to the other A ring protons due to the deshielding influence of the 4-keto function. The mass spectrum (Fig.8) gave m/z 340 for M^+ + 2H. Fragments >m/z340 corresponds to the glycoside whose sugar was not identified in this study.

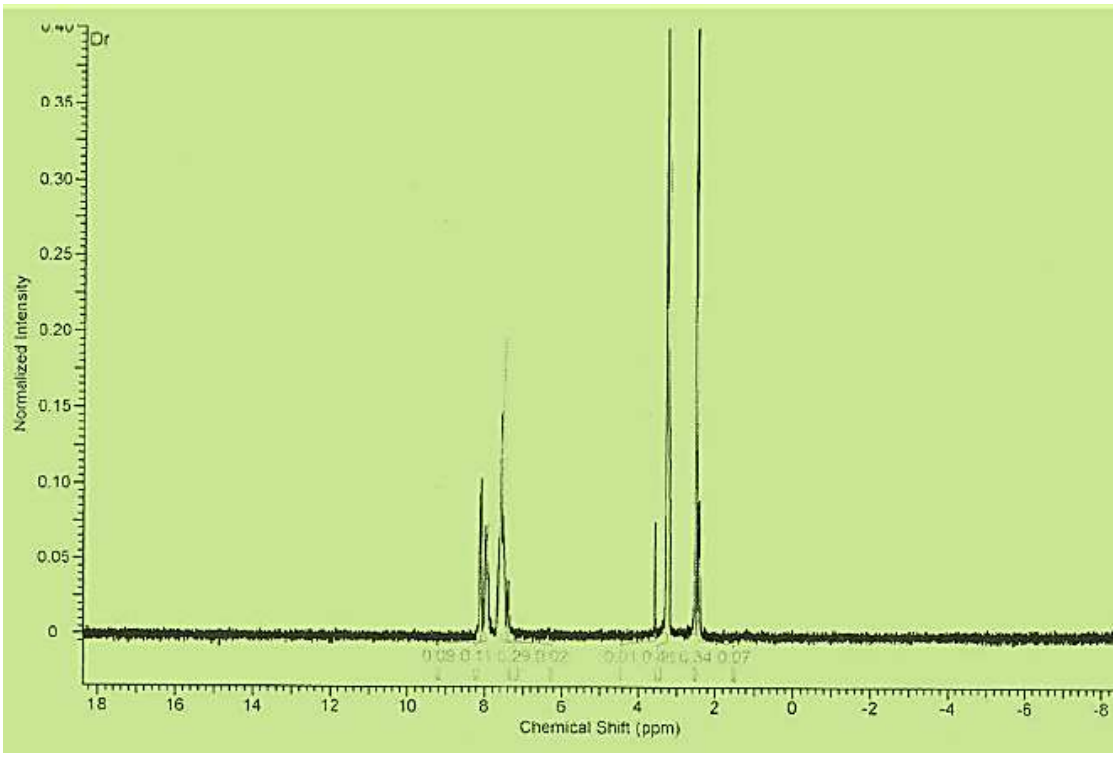


Fig. 7: ^{1}HNMR spectrum of compound I

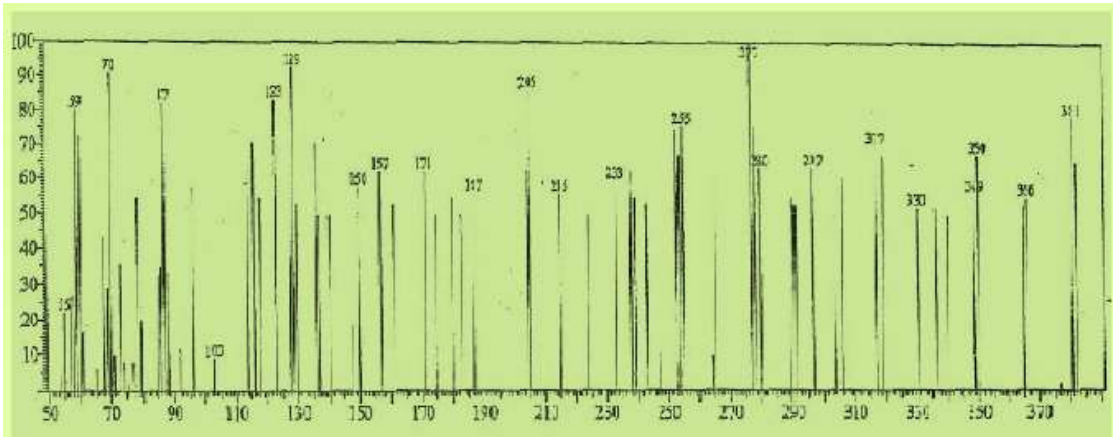
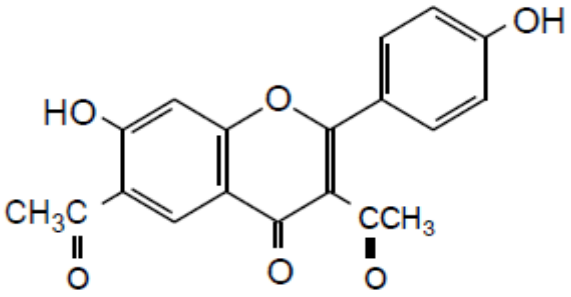
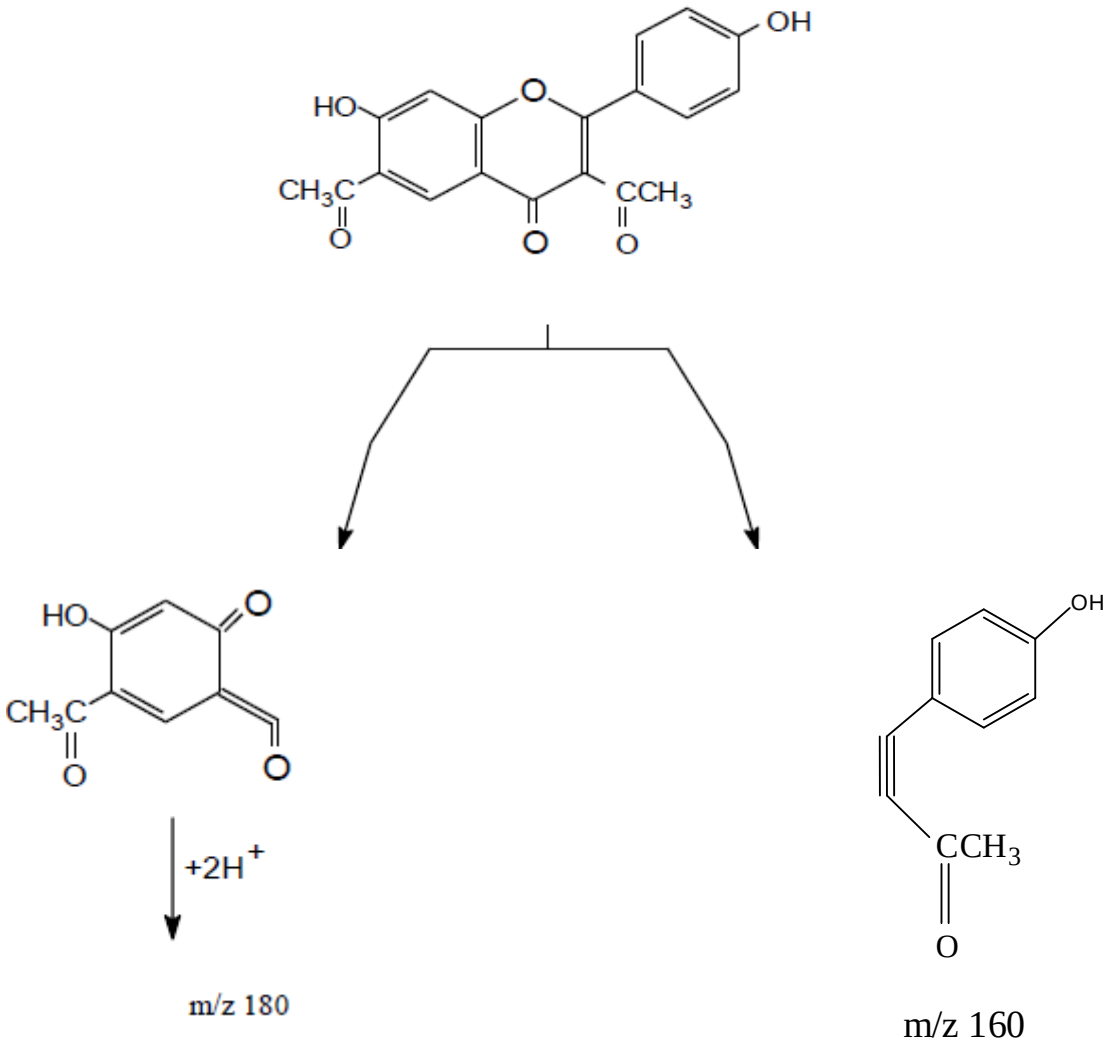


Fig.8: Mass spectrum of compound I

On the basis of the above argument, the following structure was proposed for compound I:



Some important fragments revealed by the retro Diels-Alder fission (scheme VII), and resulting from intact A and B rings, lend evidence for the proposed substitution pattern of these rings.



Scheme VII: Retro Diels- Alder fission of compound I

3.2- Characterization of compound II

Compound II was isolated from the seeds of *Hagenia abyssinica* by paper chromatography.

The IR spectrum of compound II (Fig.9) showed: v(KBr) 543, 609, 943 (C- H, Ar. bending), 1027(C-O), 1380,1411(C=C, Ar.), 1737(C=O) 2923 (C-H, Aliph.) and 3406cm^{-1} (OH) .

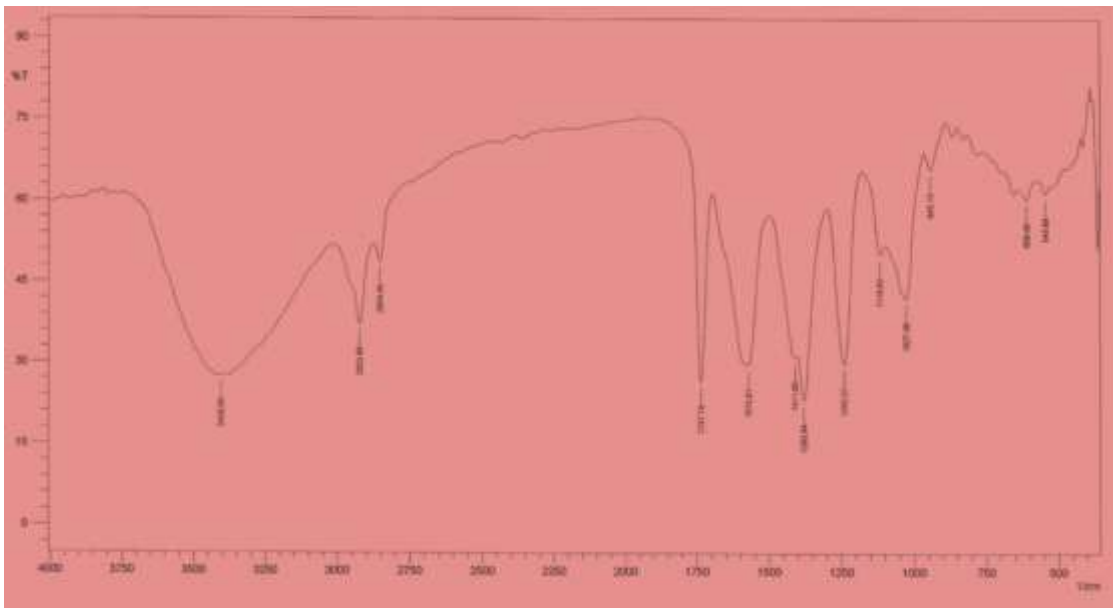


Fig.9: IR spectrum of compound II

The UV spectrum (Fig.10) gave λmax 256, 293, 320 (sh.) nm which is consistentant with the absorption of isoflavones. These compounds consistently show a shoulder in the long wave side of the spectrum in the range 300-340 nm.

The sodium methoxide spectrum (Fig.11) revealed a 64 nm bathochromic shift in band I without decrease in intensity indicating a 4`-OH function.

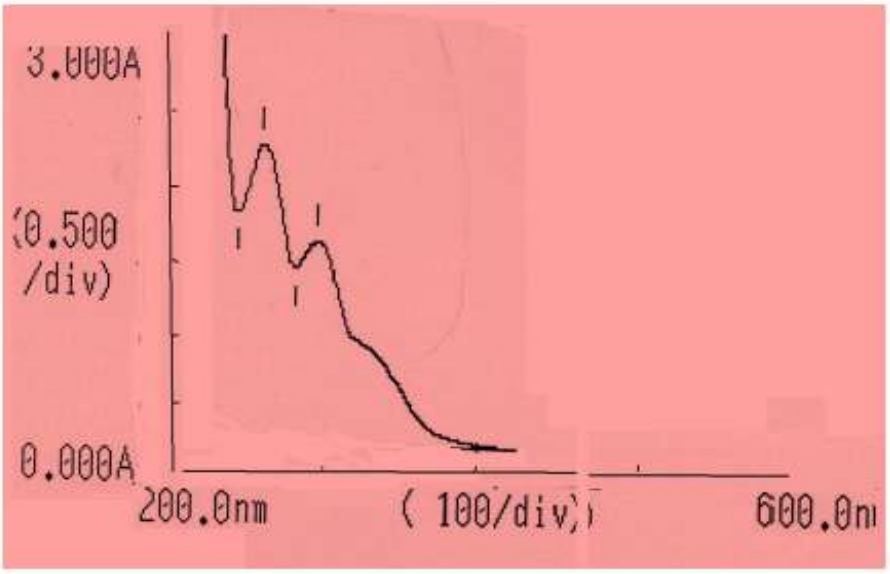


Fig.10: UV spectrum of compound II

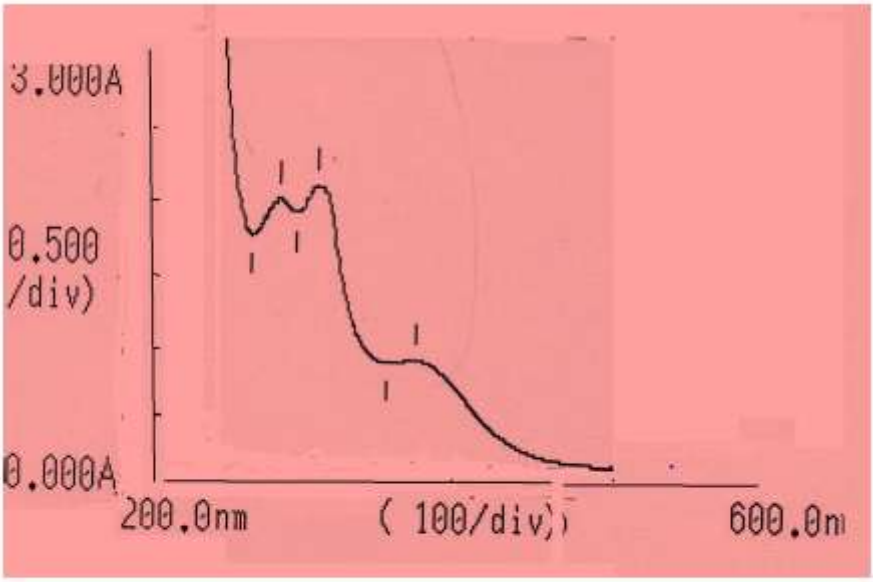


Fig.11: Sodium methoxide spectrum of compound II

No bathochromic shift was detected in the sodium acetate spectrum (Fig.12) indicating absence of a 7-OH function.

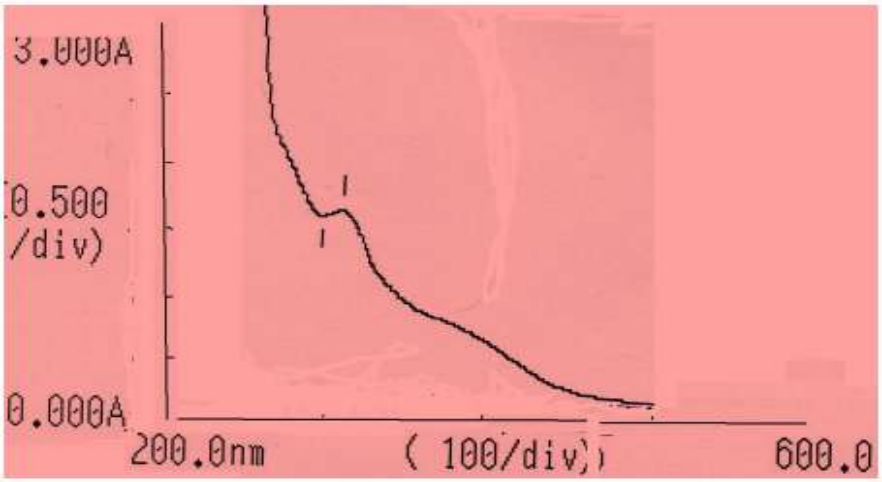


Fig.12: Sodium acetate spectrum of compound II

The aluminium chloride spectrum (Fig.13) gave a bathochromic shift indicative a 5-OH function (the spectrum was stable in HCl) Fig.14. No bathochromic shift was detected in the boric acid spectrum (Fig.15). This suggests absence of catechol systems.

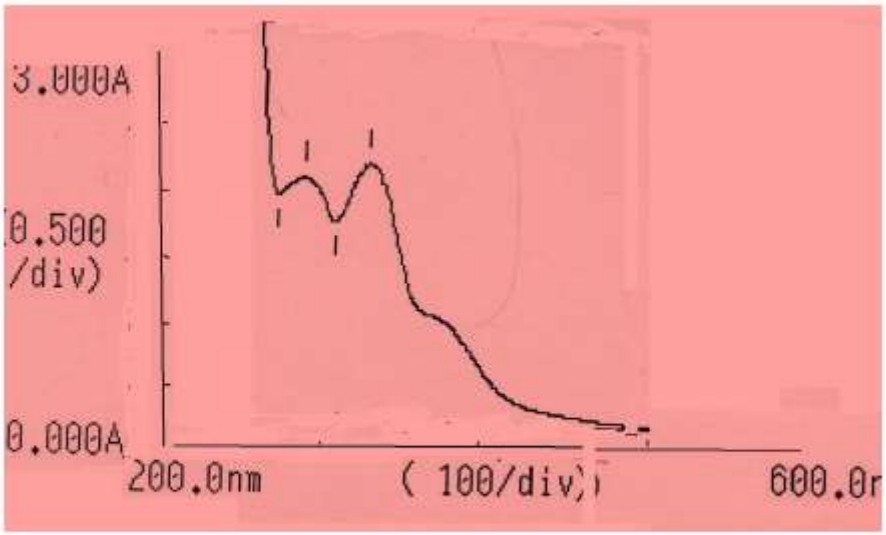


Fig.13: Aluminium chloride spectrum of compound II

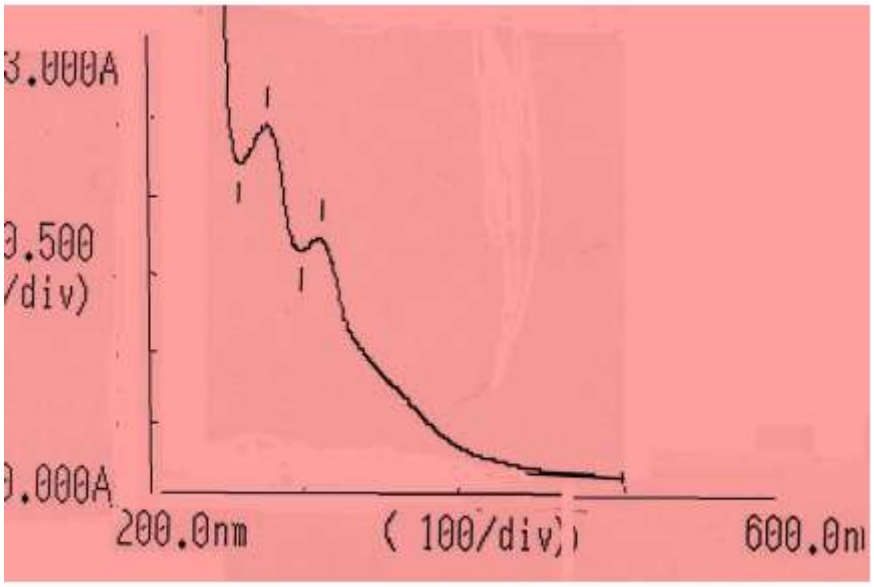


Fig14: Aluminium chloride / HCl spectrum

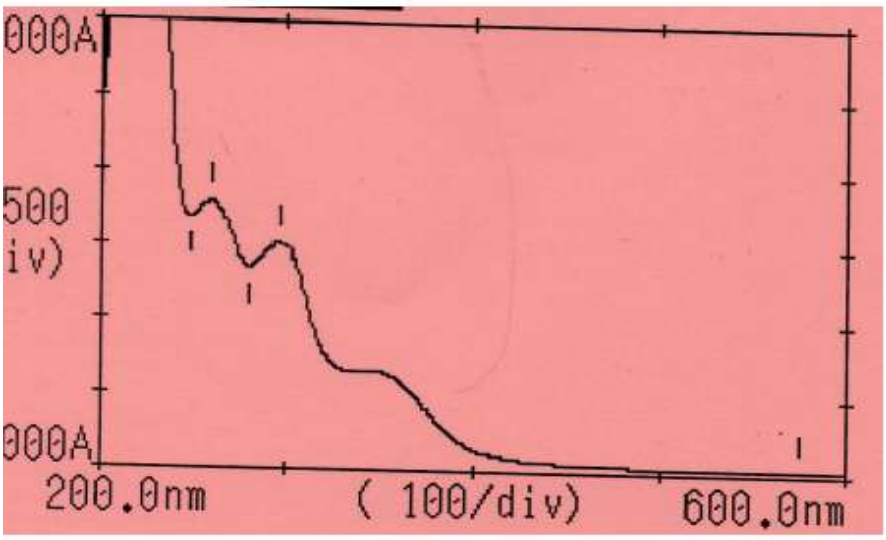
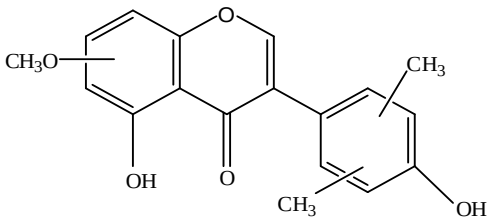


Fig. 15 : Boric acid spectrum of compound II

The ^{1}H NMR spectrum (Fig.16) showed δ1.23 (6H) due to two methyl groups. The signal at δ3.75(s, 3H) was assigned for a methoxyl function, while the doublet at δ6.75(1H) ppm is consistent with the signals of C_6- proton. The multiple at 7.26 - 7.33 ppm account for B ring protons. The mass spectrum (Fig.17) gave m/z313 for M^{+} + H (aglycone). Fragments >

m/z313 corresponds to the glycoside whose sugar was not identified in this study. Thus the following tentative structure was proposed for aglycone of compound (II):



Compound II

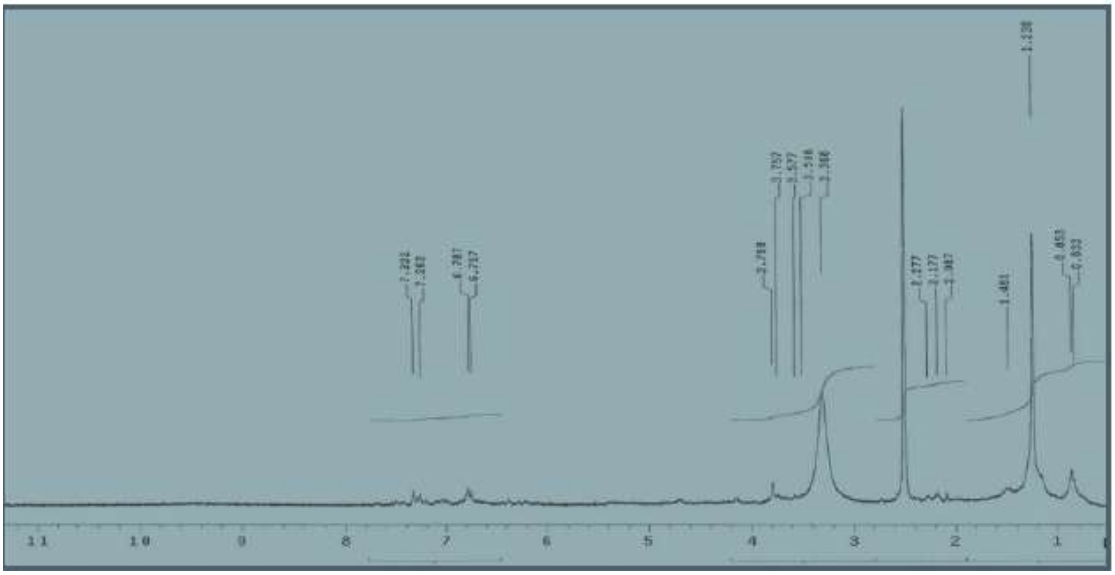


Fig.16: ^{1}H NMR spectrum of compound II

The fact that the B ring is substituted by two methyl functions is supported by the retro Diels-Alder fission (scheme VIII). This scheme also lends evidence for the proposed substitution pattern of ring A. The ions m/z146 and m/z166 corresponding to intact B and A rings respectively were recorded in the electron beam.

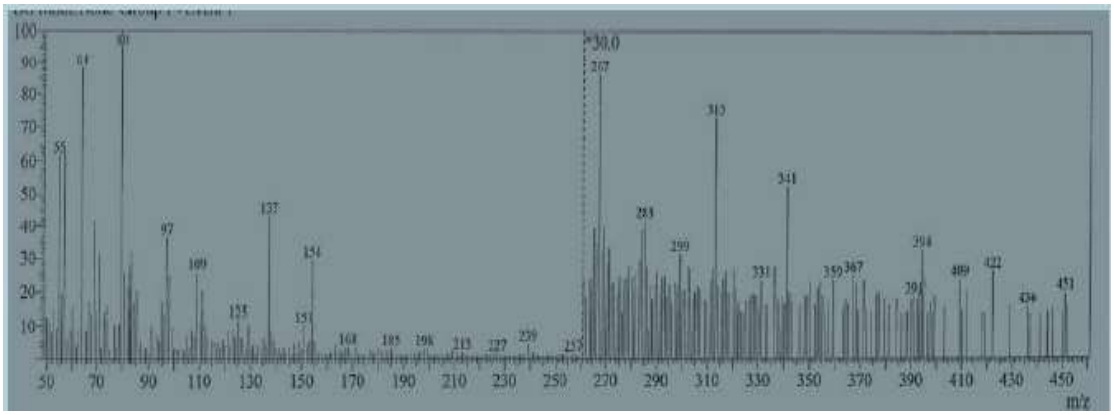
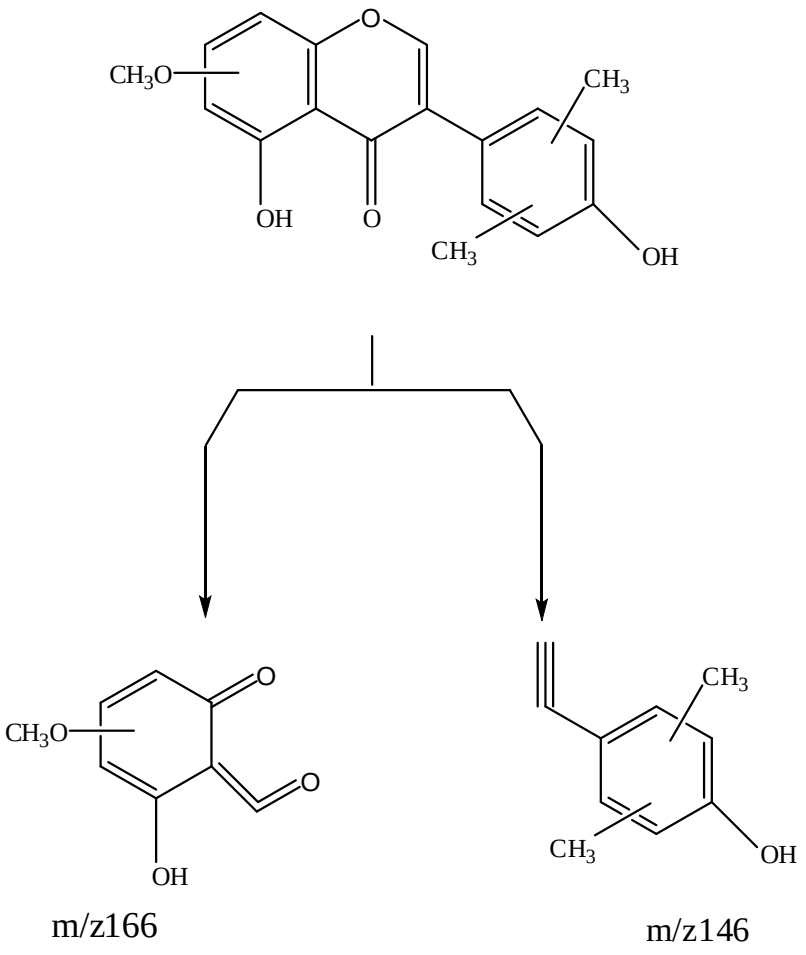


Fig.17: Mass spectrum of compound II



Scheme VIII: Retro Diels-Alder fission of compound II

3.3- Characterization of compound III

Compound III was isolated from *Hagenia abyssinica*- as yellow powder - by paper chromatography. The IR spectrum (Fig. 18) showed: ν(KBr) 673, 825 (C-H, Ar., bending), 1452,1514(C=C, Ar.),1724 (C=O), 2923(C-H, aliph.) and 3259cm^{-1} (OH).

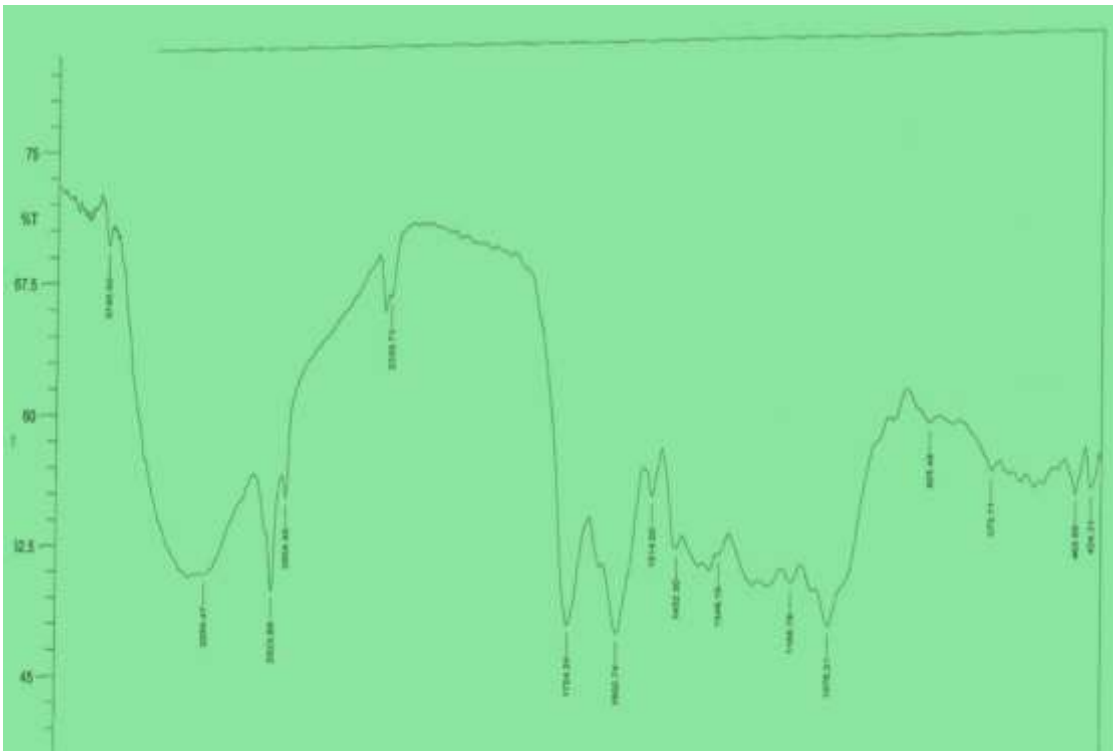


Fig.18: IR spectrum of compound III

In the UV compound III absorbs (Fig.19) at λmax 288, 339(sh.). The appearance of such shoulder is a characteristic feature of isoflavones.

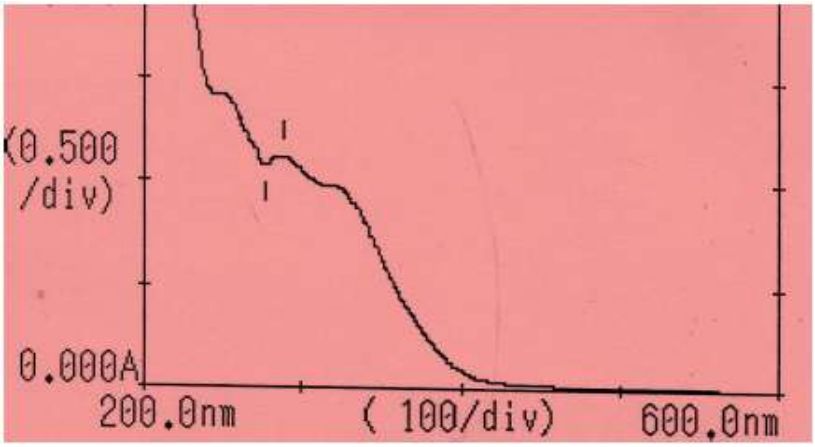


Fig.19 : UV spectrum of compound III

The sodium methoxide spectrum (Fig. 20) gave a bathochromic shift without decrease in intensity indicating a 4`-OH function.

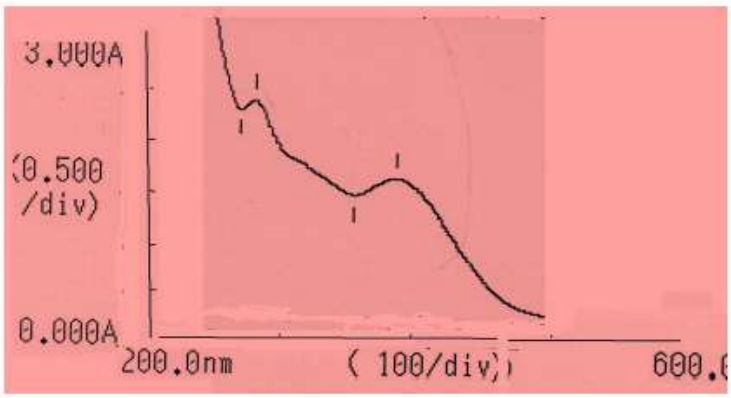


Fig.20: Sodium methoxide spectrum of compound III

The aluminium chloride spectrum did not reveal (Fig. 21) any bathochromic shift indicating absence of a 5-OH function. Also the sodium acetate spectrum (Fig. 22) did not show any bathochromic shift diagnostic of a 7-OH function. Also the boric acid spectrum (Fig. 23) did not reveal any bathochromic shift diagnostic of catechol systems.

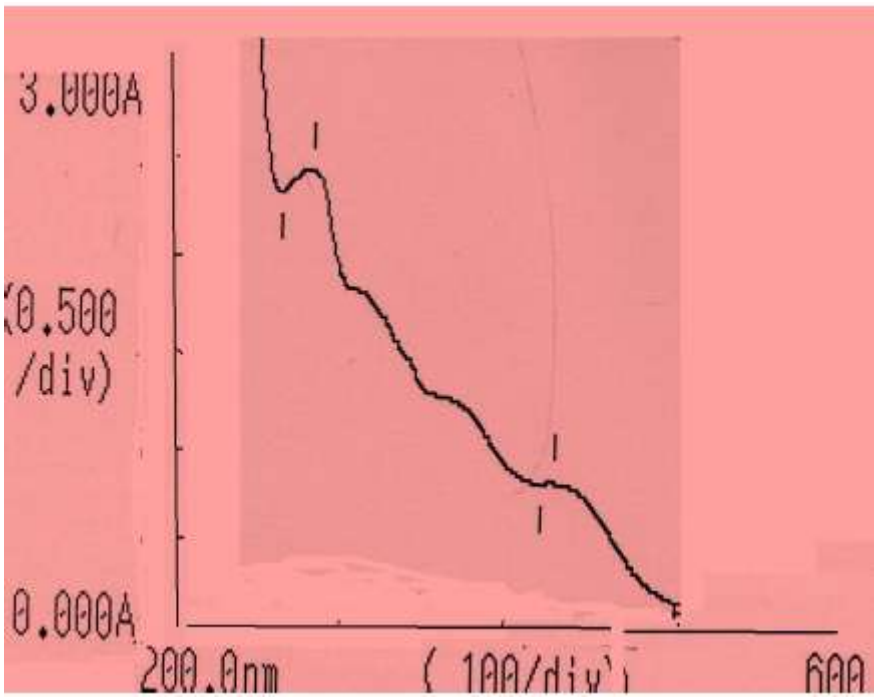


Fig.21 : Aluminium chloride spectrum of compound III

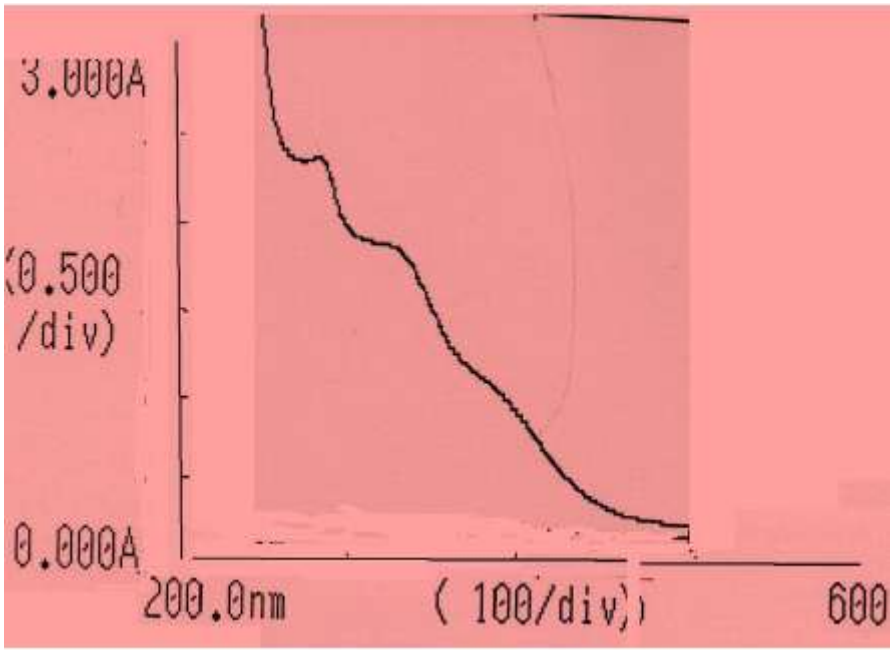


Fig.22 : Sodium acetate spectrum of compound III

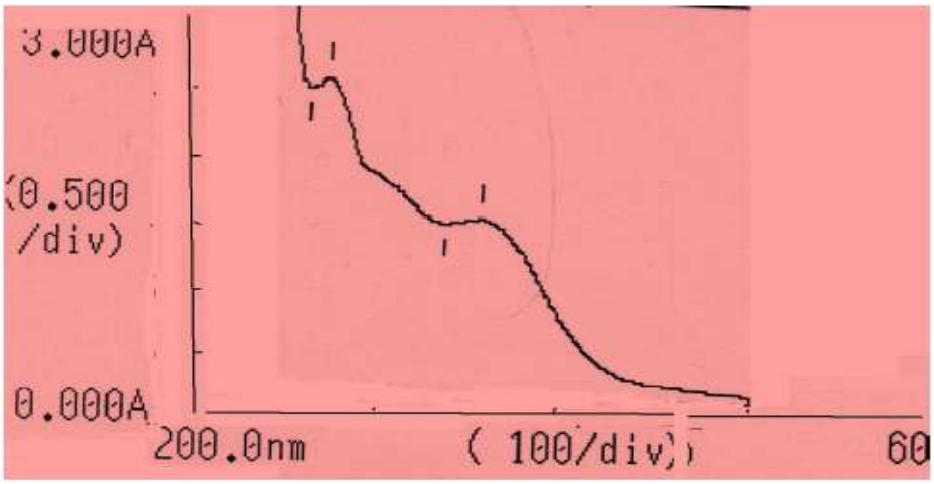
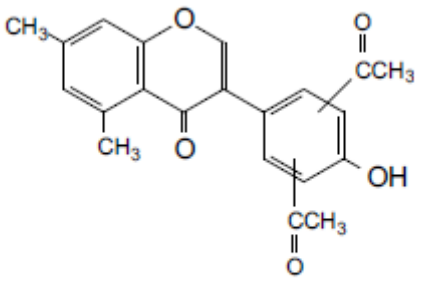


Fig. 23 : Boric acid spectrum of compound III

The ^{1}H NMR spectrum (Fig24) showed: δ1.08 (6H) assigned for two methyl groups. On the basis of the retro Diels –Alder fission (scheme III) these methyl groups were assigned for the A ring. Since the NMR spectrum showed only signals for C_6 and C_8 protons, they were cited at the 5 and 7 positions. The signals at δ2.13 (6H) account for two acetyl groups which were assigned for the B ring on the basis of the retro Diels-Alder cleavage (scheme III). The resonances at δ6.20 (1H), 6.83(1H) are due to C_6- and C_8- protons respectively. The C_8 proton resonates at lower field relative to C_6 –H due to the deshielding influence of the neighboring oxygen at C_1.The signals at δ7.01 and δ7.45 accounts for the B ring protons. The high resolution mass spectrum (Fig.25) gave m/z350 for M^{+}+ 1(aglycone). Fragments >m/z350 corresponds to the glycoside whose sugar was not identified in this study. On the basis of the above cumulative data the following tentative structure was assigned

for the aglycone of this isoflavone. The retro Diels-Alder fission (scheme IX) cited evidence for the proposed substitution pattern where the ions m/z149 and m/z 201corresponding to intact A and B rings respectively, were detected in the electron beam.



Compound III

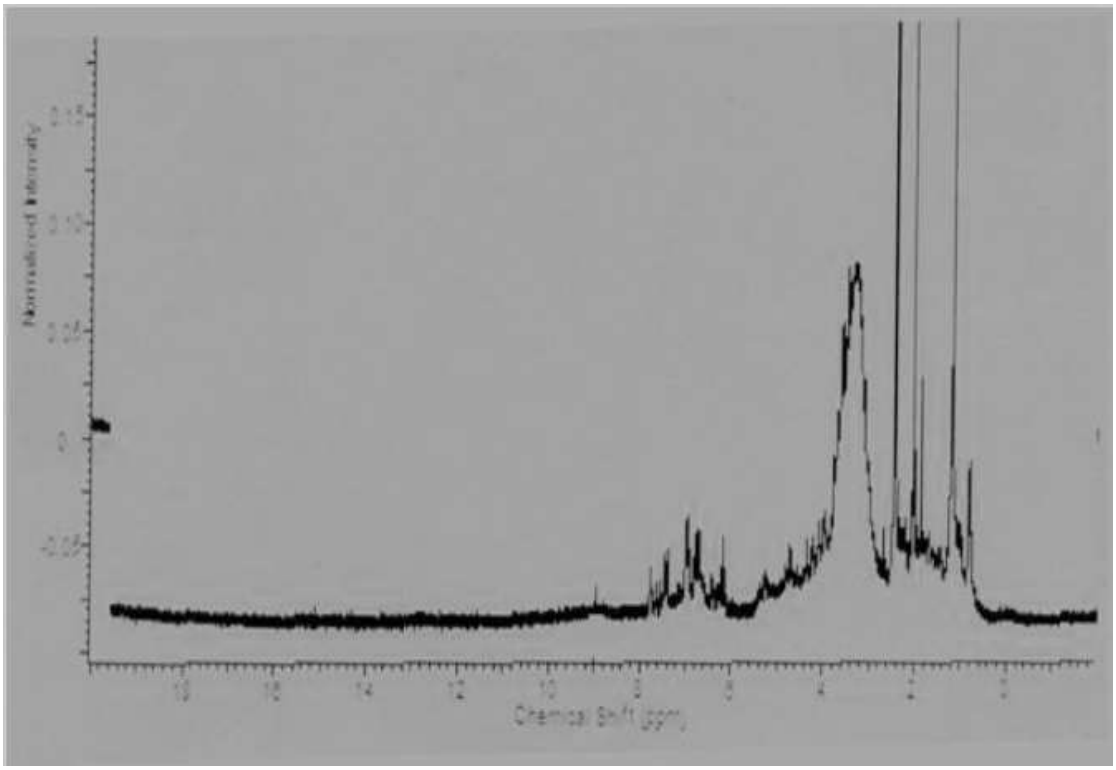


Fig. 24 : ^{1}HNMR spectrum of compound III

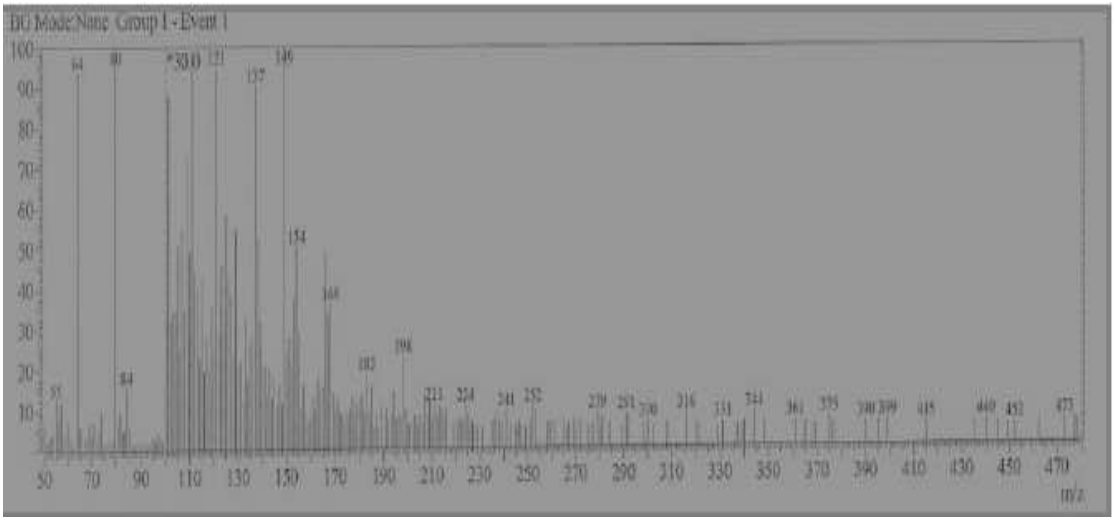
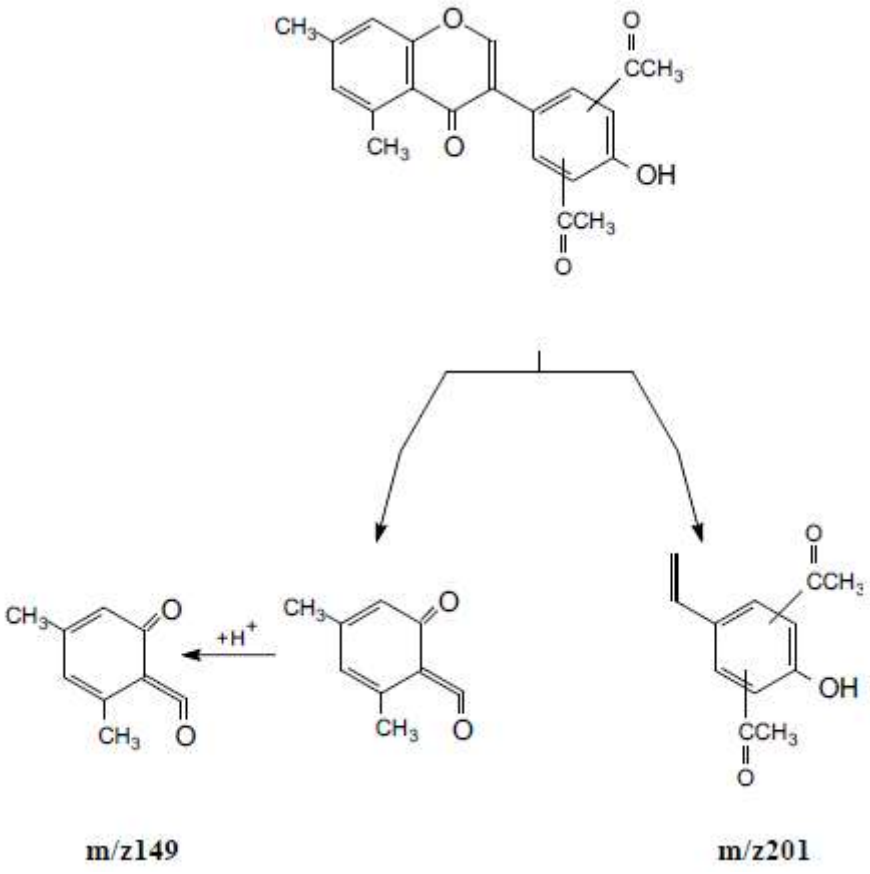


Fig. 25 : Mass spectrum of compound III



Scheme IX: Retro Diels- Alder fission of compound III

3.4- Characterization of compound IV

Compound IV was isolated from the roots of *Cisuss petiolata*. The IR spectrum (Fig.26) showed: ν(KBr) 698, 854(C-H, Ar.,bending),1037 (C-O), 1483, 1515 (C=C, Ar.), 1741(C=O), 2923(C-H, Aliph.) and 3325cm^{-1} (OH).

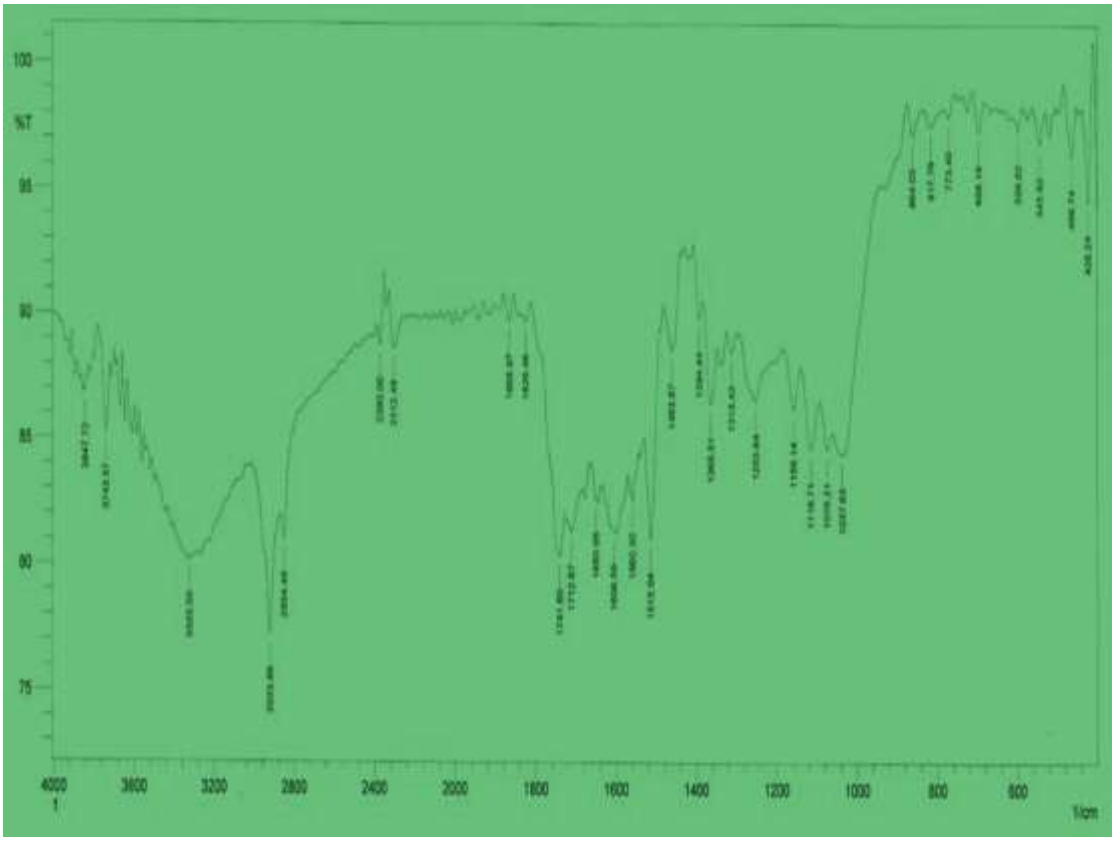


Fig. 26: IR spectrum of compound IV

In the UV compound IV absorbs (Fig. 27) at λmax 285, 337 nm. This spectrum is characteristic of flavones.

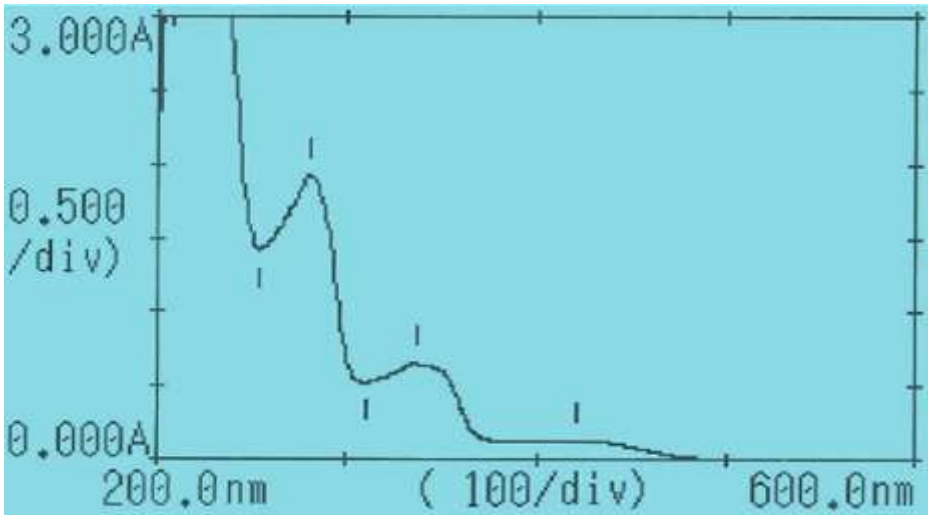


Fig.27 : UV spectrum of compound IV

The sodium methoxide spectrum (Fig.28) gave no a bathochromic shift characteristic of 3- and 4`-OH function.

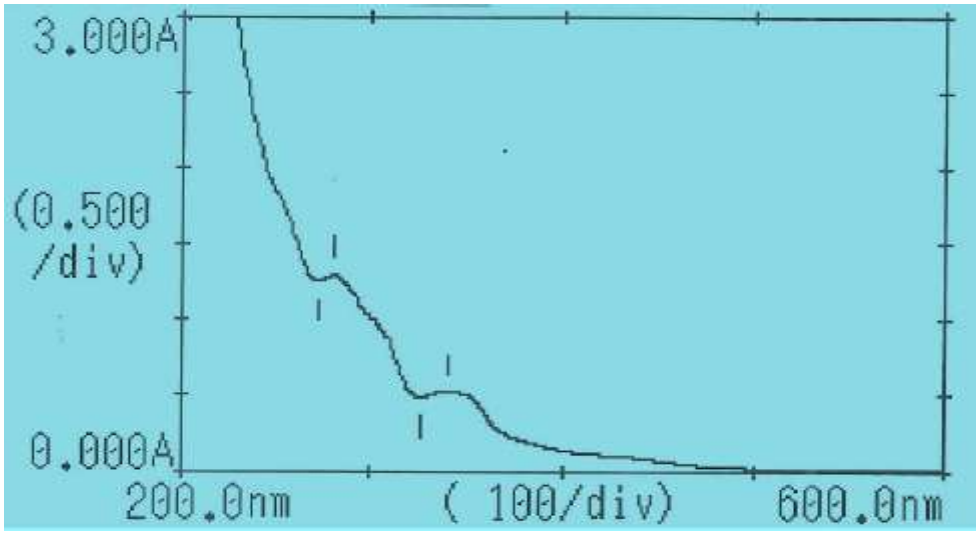


Fig.28 : Sodium methoxide spectrum of compound IV

When a methanolic solution of compound IV was treated with aluminium chloride, the spectrum did not reveal (Fig.29) any bathochromic shift diagnostic of 3-,5-OH and catechol systems.

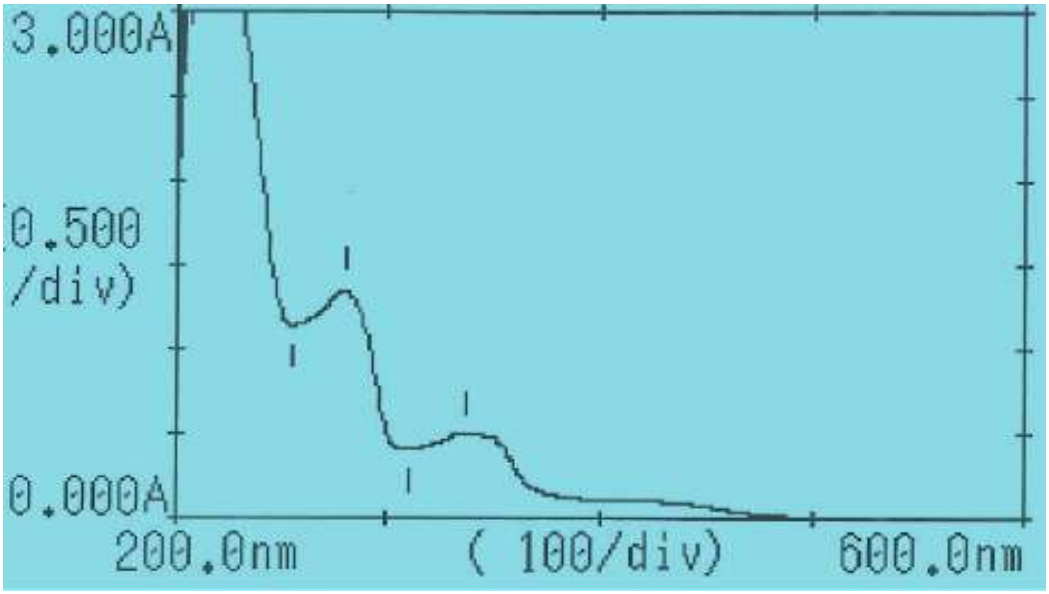


Fig.29 : Aluminium chloride spectrum of compound IV

Also the sodium acetate spectrum (Fig.30) did not show any bathochromic shift diagnostic of a 7-OH function.

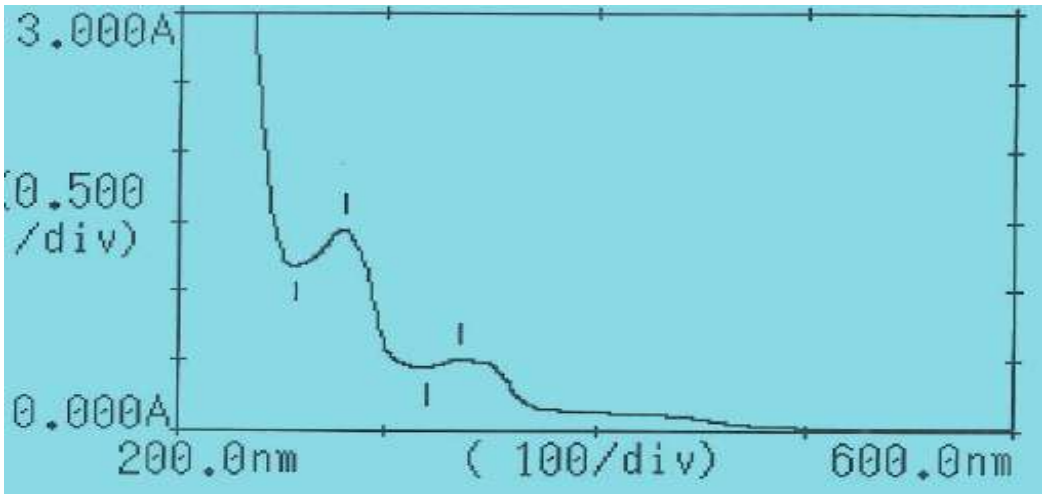


Fig.30 : Sodium acetate spectrum of compound IV

The 1H NMR spectrum (Fig.31) showed δ1.05 (6H) assigned for two methyl groups. The signal at δ3.65 (3H) account for a methoxyl function. The resonances at δ6.50 (1H), 6.91(1H) are due to C_6- and C_8- protons respectively.

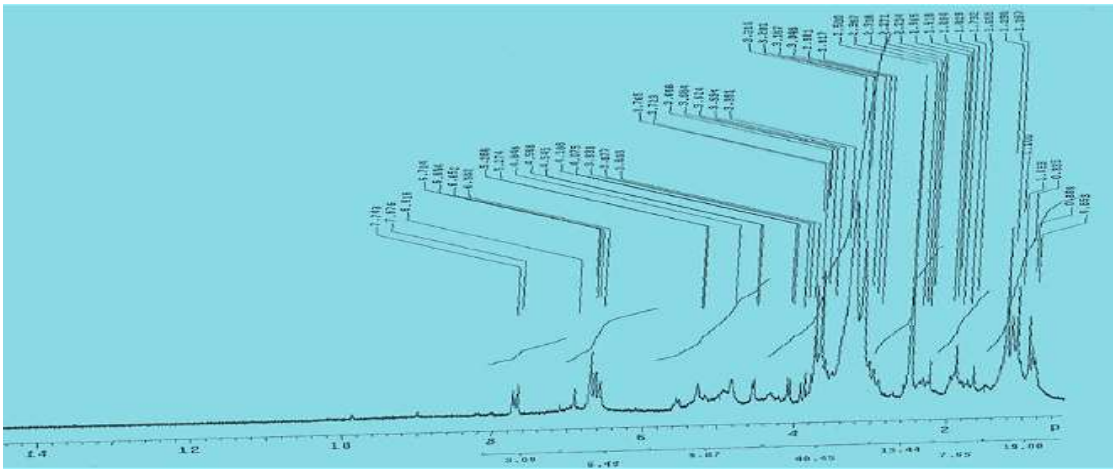


Fig.31 : ^{1}HNMR spectrum of compound IV

The signals at δ7.67 accounts for the B ring protons. The high resolution mass spectrum (Fig.32) gave m/z280 for M^{+} + H (aglycone). Fragments > m/z280 corresponds to the glycoside whose sugar was not identified in this study. Other important fragments; m/z150, m/z129 resulting from the retro Diels-Alder fission (scheme X) site evidence for the proposed substitution pattern of rings A and B.

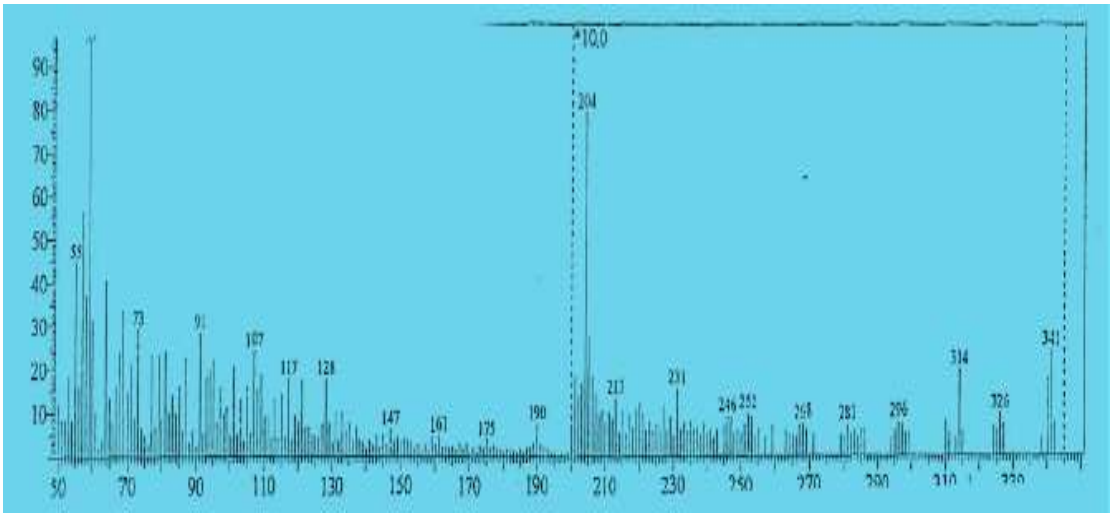
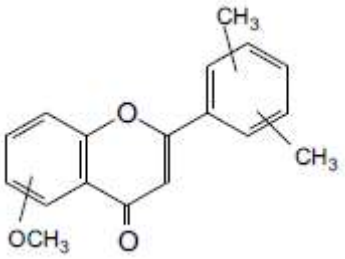
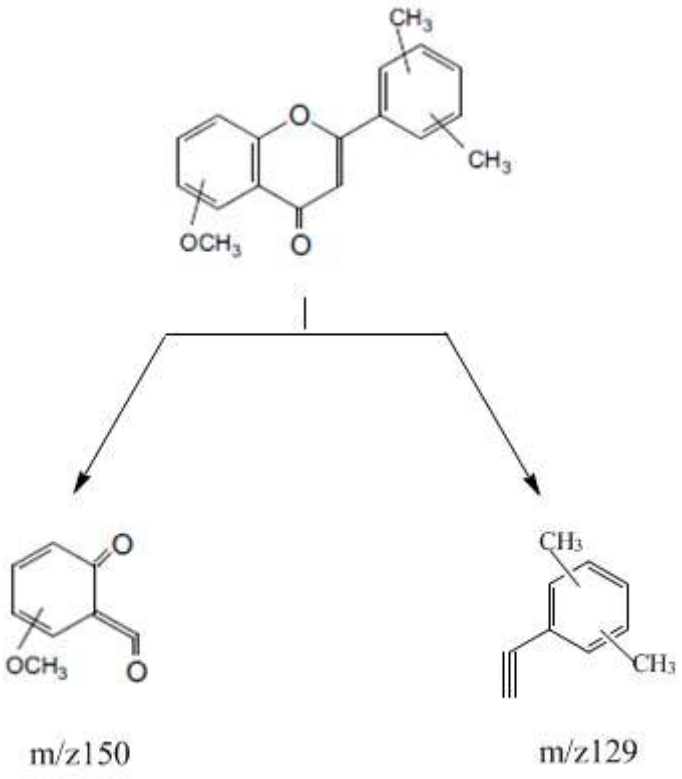


Fig.32 : Mass spectrum of compound IV

On the basis of the above cumulative data the following tentative structure was suggested for the aglycone of this flavone:



Compound IV



Scheme X: Retro Diels-Alder fission of compound IV

Recommendation

- The structures of the isolated phytochemicals may futher be confirmed by a future ^{13}C NMR and two dimension NMR (^{1}H - ^{1}H Cosy NMR, HMBC and HSQC).

- The crude plant extractives and the isolated flavonoids may be screened for their anti-inflammatory, antimalaril, anti-bacterial, antifugal, anticacer antioxidant activity.

- Other phytochemicals (Steroids, alkaloids, saponins and tannins) of the studied may be investigated.

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