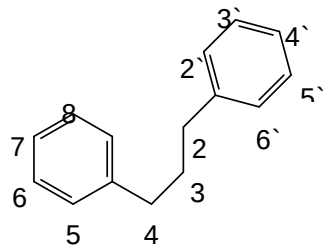
1- Introduction

1.1. General approach

Flavonoids comprise a large group of plant secondary metabolites characterized by a diphenylpropane structure (C6-C3-C6). They are widely distributed throughout the plant kingdom and are commonly found in fruits, vegetables and certain beverages. Numerous preclinical and some clinical studies suggest that flavonoids have potential for the prevention and treatment of several diseases. Some epidemiological studies support a protective role of diets rich in foods with flavonoids and a reduced risk of developing cancer and cardiovascular diseases[1-6.] Preclinical *in vitro* and *in vivo* investigations have shown plausible mechanisms by which flavonoids may confer cancer and cardiovascular protection[7]. In addition to their preventive potential, certain flavonoids may be useful in the treatment of several diseases. Some evidence supporting the therapeutic potential of flavonoids comes from the study of plants used in traditional medicine to treat a wide range of diseases, which has shown that flavonoids are common bioactive constituents of these plants [7-10].

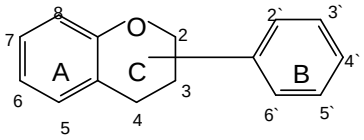
1.2- Properties and classification of flavonoids

Chemically the flavonoids are polyphenolic compounds possessing 15 carbon atoms, two benzene rings joined by a linear three – carbon chain (1).



(1)

The skeleton above can be represented as a C_6-C_3-C_6 system.The chemical structure of flavonoids is based on a C-15 skeleton with a chromare ring bearing a second aromatic B ring in position 2, 3 or 4 (2)[11].



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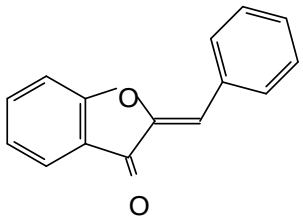
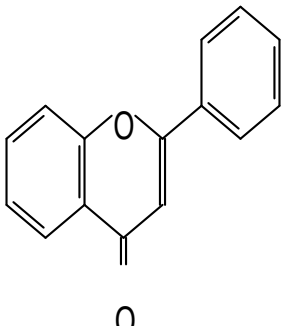
In a few cases, the six- member heterocyclic ring C occurs in an isomeric open form or replaced by a five – memberd ring (3).



The oxygen bridge involving the central carbon atom (C2) of The 3 C-chain occurs in a rather limited of cases, where the resulting heterocyclic is of the furan type.

Various subgroups of flavonoids are classified according to the substitution pattern of ring C. Both the oxidation state of the heterocyclic ring and the substitution pattern are important in the classification[12].

The flavonoids are all structurally derived from the parent substance flavone (4), and all share a number of properties in common[13].



(4)

Flavonoids are mainly water – soluble compounds. They can be extracted with 70% ethanol and remains in the aqueous layer, following partition of this extract with petroleum ether. Flavonoids dissolve in alkalis, giving yellow solutions with addition of acid, the solution become colorless. Flavonoids are phenolic and hence they change color when treated with base or with ammonia, thus they are easily detected on chromatograms or in solution. Table (1) shows the color properties of flavonoids in visible and ultraviolet light[14].

Flavonoids are generally present in plants bound to sugar as glycosides and any one flavonoid aglycone may occur in a single plant in several glycosidic combinations. For this reason, when analyzing flavonoids, is better to hydrolyse plant extracts before considering the complexity of glycosides that may be present in the original extract **[14]**.

Flavonoids are present in all vascular plants, but some classes are more widely distributed than others; while flavones and flavonols are found in only a few plant families**[14]**.

Table1: The color properties of flavonoids in visible and ultraviolet light[14]

Visible color	Color UV light		Indication
	Alone	With ammonia	
Orange Red Mature	Dull orange. Red of mauve. Flouriest yellow cerise or pink.	Blue Blue	Anthocyanidin 3-glycosides Mostanthocyanidin. 3,5-Di glycosides.
Bright Yellow	Dark brown or black. Bright yellow or yellow green	Dark brown or black. Dark red or Bright orange. Bright orange or red.	6-Hydroxylatedflavonols and flavons; Some chalcone glycoside. Most chalconse Auronnes.

Very pale yellow	Dark brown	Bright yellow or yellow brown. Vivid yellow green. Dark brown.	Most flavonols glycosides. Most flavone glycoside. Biflavonoyls and un usually substituted flavones
None	Dark mauve. Faint blue. Dark mauve.	Faint brown Intense Blue Pale yellow or Yellow green.	Most isoflavnes and flavonodis. 5-desoxyflavononnes. Flavenones and flavonol 7-glycosides

Flavonoids are present in plants as mixtures and it is very rare to find only a single flavonoid component in a plant tissue .In addition, there are often mixtures of different flavonoid classes. The colored anthocyanins in flower petals have flavones as important co-pigments, such flavones are essential for the full expression of anthocyanin color in floral tissues.

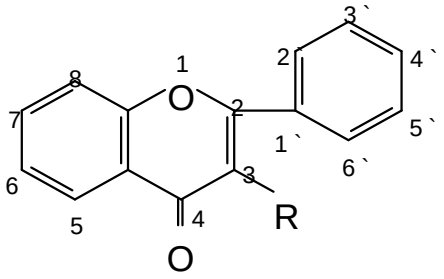
Classification of flavonoid type in plant tissue in based initially on a study of solubility properties and color reactions. This is followed by one – dimensional chromatographic examination of hydrolyzed plant extract and two dimensional chromatography of direct alcoholic extract. Finally, the flavonoids can be separated by chromatographic procedures and the individual components

identified by chromatographic and spectral comparison with known marker[15].

1.3- The Flavonols

Flavonols (5,R= OH) are simply flavones (5, R = H) in which the 3- position is substituted by a hydroxyl. Flavones are widely distributed in the plant kingdom, usually in the form of glycosides[16].

Flavonols are practically ubiquitous in woody angiosperms and appear less frequently in angiosperms. There are no records of flavonoids occurring in bacteria and algae. And chloeflavonin (6) is so far the only fully characterized flavonol isolated from strain of *Aspergillums candidus*[17].



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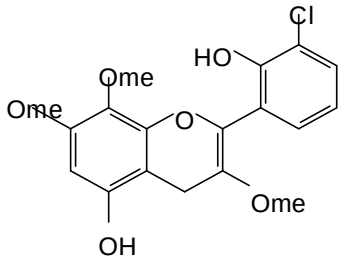
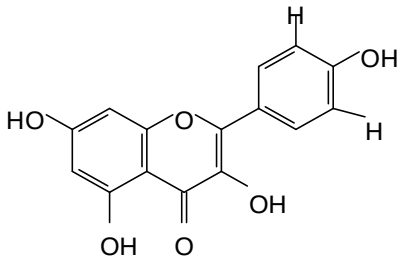


(6)

The hydroxylated pattern of chloeflavonin (6), although not a common one, incorporates features usually associated with flavonols from higher plants. Kaempferol (7), querctin(8) and less frequently, myrecetin(9) occur singly or jointly in a vast proportion of analyzed species [18]. Altogether with the comparatively rare galangin (10) they form the fundamental structural types from which all other naturally occurring flavonols may be derived, this derivation implies a modification of the oxygenation pattern and /or in etherification.



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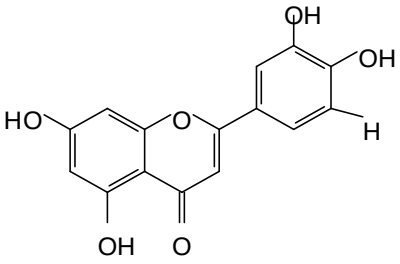

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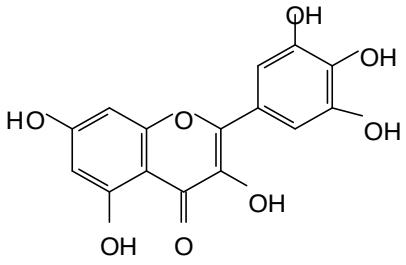

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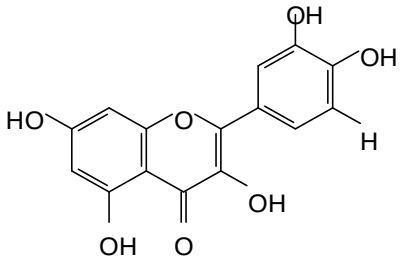
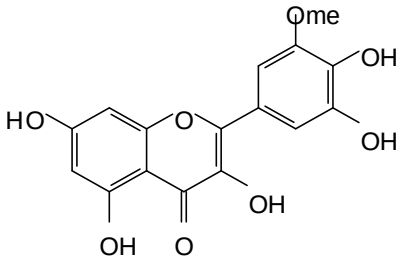


(10)

Modification of the oxygenation pattern of galangin, kaempferol, quercetin and myricetin leads to derivatives of relatively more restricted occurrence and, consequently, of enhanced value as chemosystematic markers[19] .

Isorhamnetin (11) (quercetin 3- methyle ether) is also of widespread occurrence and together with kacmpferol, quercetin and myricicetin, exists in the plant cell in glycosidic form, as part of the hydrophilic flavonol fraction. With increasing O-methylation, flavonols become lipophilic and are less prone to glycolysation. The relative amounts of flavonol glucoside to aglycone present depends additionally on the stage of growth and the plant organ analyzed, and is even subject to variation according to the analytical method used [20-22].



(11)

The number of different ethers derived from a particular hydroxylated flavonol is not proportional to the number of

positions available for etherfications but is roughly, related to its frequency of distribution in nature. To quote only some examples all possible mono methyl ether of kaempferol (four) and of quercetin (five) have already been isolated. By contrast, few mono methyl esters of 6- hydroxyl kaempferol (2 out of 5) , 6- hydroxyl quercetin (2 out of 6), 8- hydroxykaempferol (2 out of 5), 8- hydroxykaempferol (2 out of 6) were isolated.

1.4- The Flavones

The flavones, which are also known as the anthoxanthins, are often yellow pigments, which occur, in the higher plant and in young tissues, where they occur in the cell sap[23]. Flavones occur naturally in the free state or as glycosides (the aglycone is the anthoxanthidin and the sugar is glycose or rhamnose) or associated with tannins.

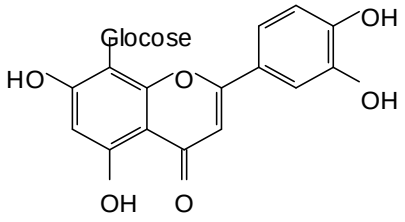
Chemically, the flavons are very closely related to the anthocyanins, the flavones are hydroxylated derivatives of flavone (2-phenyl-4- chromone) (4), which may be partially alkylated[16].

In almost all cases position 5 and 7 are hydroxylated and frequently one or more of position 3, 4 and 5.They are abundant in the Polygonaceac, Rutacae, Legumunosae, Umelliferae, and Compositae[23].

Pure flavones, which is colourless, occurs on the surface of some species of pirmula. Many flavones are phenolic or methoxy

derivatives and form sap- soluble glycosides. The intensity of their yellow colour increases with the number of hydroxyl groups and with increase of pH [23].

Flavones occurring as glycosides is less than in the case of flavonols. They also occur remarkably with sugar bond by a carbon – carbon bond. A series of such glycosyl flavones have been described[19], one example being orienthin (12). The carbon --carbon bond is very resistant to acid hydrolysis, so that it is relatively easy to distinguish these C–glucosides from O- glycosides, which are more readily hydrolysed[24].

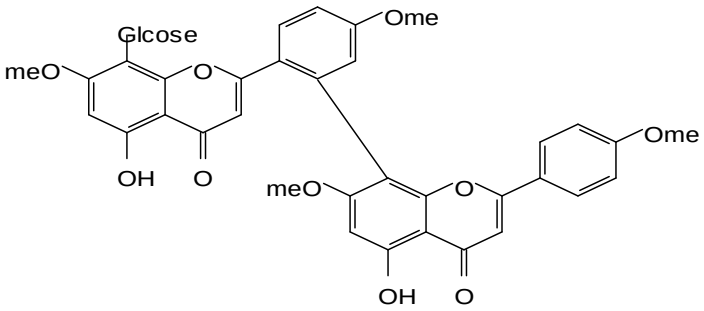


(12)

1.5-Biflavonyl

One other structural variant in the flavone series must be mentioned, the biflavonyl, These dimeric compounds are formed by carbon – carbon or carbon – oxygen coupling between flavones (usually apigenin units. Most also carry O – methyl substituent's, a typical example being kayoflavone (13). Biflavonyl occur almost

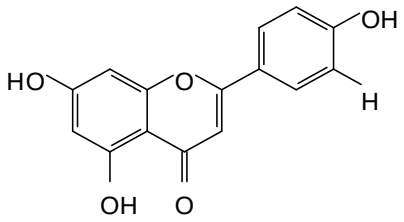
exclusively in the gymnosperms, but they have been found occasionally in angiosperms and method for their identification are of general importance[23].



(13)

1.6- Hydroxy flavones

Apigenin (14) and luteolin (15), free and as glycosides, are most widely occurring flavones. The A-ring of great majority of flavones is derived from phloroglucinol (16) and the B – ring is oxygenated in the 4 or 3, 4 or 3, 4, 5- position as expected from their established acetate – shikimate biosynthetic origin[24].



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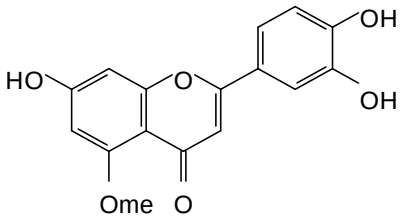

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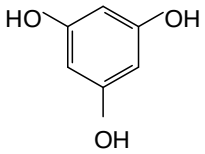
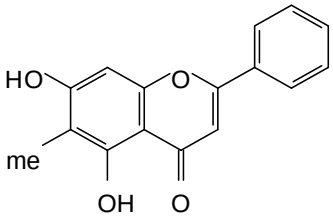


(16)

1.7- C- Methyl flavones

Only four C- methyl flavones have been found as natural products: strobochrysin (6- methyl chrysin) (17), 5- hydroxyl – 7, 4- dimethoxy – 6- methyl flavone (18), and the 6, 8- dimethyl derivative (cucalyptin) (19), Also 4, 5- dihydroxy -7- methoxy - 6,8- dimethyl- flavone (sideroxilin) (20) was reported[25].



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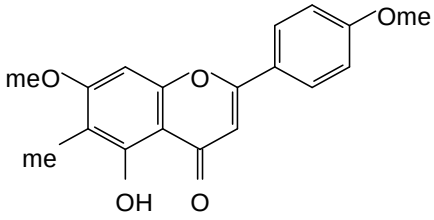

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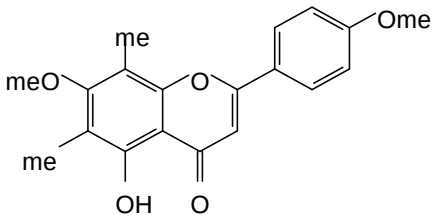

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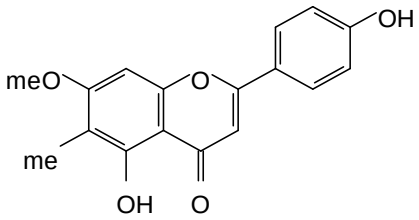
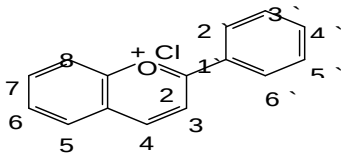


(20)

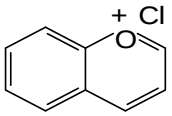
1.8- Anthocanyanins

Anthocyanins are the most important, and widespread group of colouring matters in plants. These intensely coloured water-soluble pigments are responsible for nearly all the pink, scarlet, red, mauve, violet, and blue colours in petals, leaves and fruits of higher plants[23]. The various shades of colour exhibited by all flowers are due to a very small number of different compounds. Furthermore, these differe compounds were shown to contain the same carbon skeleton and different only in the nature of the substituted groups. The anthocyanins pigments are amphoteric and their acid salts are usually red, their metallic salts are usually blue in neutral solution[6.]. In addition to anthocyanins, the colour of flowers depends on the presence of a co-pigment such as flavones, flavonols, etc, and to metal chelation, particularly with iron and aluminium. The colour (due to chelation) of the anthocyanins is affected to a large extent only when the molecule contains two hydroxyl groups in the ortho – postion[16].The anthocyanins are glycosides of anthocyanidins and are based on the flavylium salt structure (flavylium cation) (21)[19].



(21)

The fundamental nucleus in anthocyanidins is benzopyrylium chloride (22), but the parent compound is 2- phenyl benzopyrylium chloride or flavylium chloride (23)[16].



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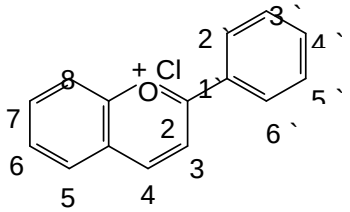
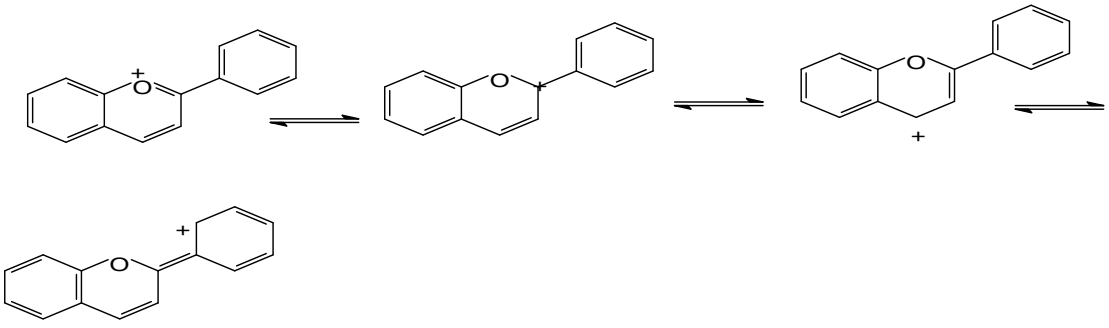
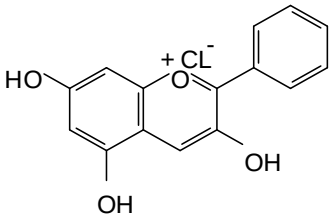


(23)

The flavlium cation can be represented as a number of resonating structures e. g:



For convenience, flavylium salts will be represented as oxonium salts. Most of the anthocyanidins are derivatives of 3, 5, 7- tri hydroxyl flavylium chioride (24). Thus the hydroxylation patterns in the natural anthocyanidins fall into the three basic groups of pelaryonidin (25), cyandin (26) and delphinidin (27)[19].



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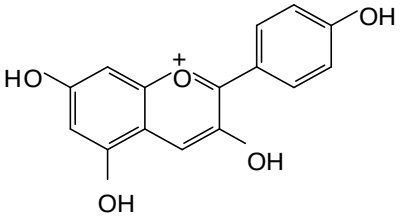

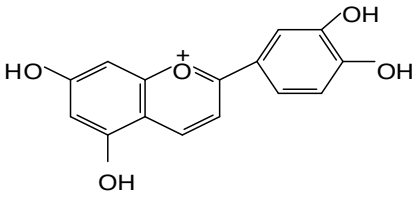

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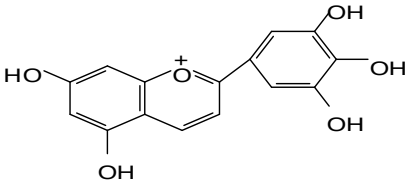

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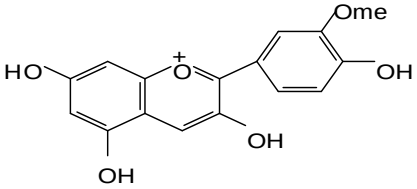
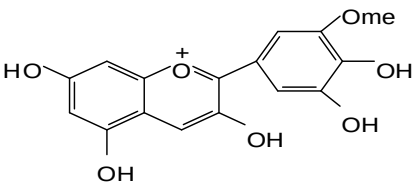
Three anthocyanidin methyl ethers are also quite common: peonidin (28) derived from cyanidine, petunidin (29) and malvidin (30)[13].





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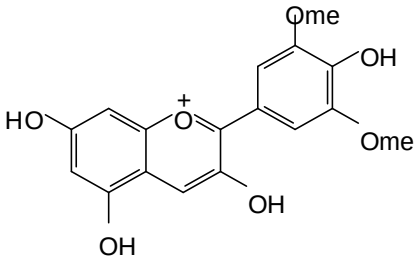

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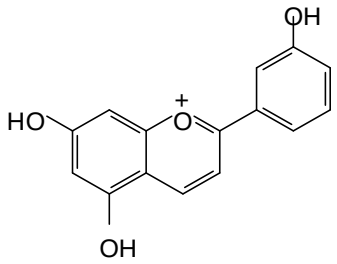
A far less common type is the 3- deoxyanthocyanidine group (the 3- hydroxyl group is absent), e. g. luteolindin (3- deoxycanidin) (31) [26].



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1.9-Metabolism of Flavonoids in Humans

The absorption of the dietary flavonoids liberated from the food by chewing will depend on its physicochemical properties such as molecular size, configuration, lipophilicity, solubility, and pKa. The flavonoid can be absorbed from the small intestine or has to go to the colon before absorption. It may depend upon structure of flavonoid, that is, whether it is glycoside or aglycone. Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as *β*-glycosides. Aglycans can be easily absorbed by the small intestine,

while flavonoid glycosides have to be converted into aglycone form[27]. The hydrophilic flavonoid glucosides such as quercetin are transported across the small intestine by the intestinal Na+-dependent glucose co transporter (SGLT1) [27.] An alternative mechanism suggests that flavonoid glucosides are hydrolyzed by lactase phloridzin hydrolase (LPH), a β-glucosidase on the outside of the brush border membrane of the small intestine. Subsequently, the liberated aglycone can be absorbed across the small intestine [28]. The substrate specificity of this LPH enzyme varies significantly in a broad range of glycosides (glucosides, galactosides, arabinosides, xylosides, and rhamnosides) of flavonoids [29]. The glycosides which are not substrates for these enzymes are transported towards the colon where bacteria have ability to hydrolyze flavonoid glycosides, but simultaneously they will also degrade the liberated flavonoid aglycones [30]. Since absorption capacity of the colon is far less than that of the small intestine, only trivial absorption of these glycosides is to be expected. After absorption, the flavonoids are conjugated in the liver by glucuronidation, sulfation, or methylation or metabolized to smaller phenolic compounds[31]. Due to these conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins[32]. Depending on the food source bioavailability of certain flavonoids differs markedly; for example, the absorption of quercetin from onions is fourfold greater than that from apple or tea [33]. The flavonoids secreted with bile in intestine and those that cannot be

absorbed from the small intestine are degraded in the colon by intestinal micro flora which also breakdown the flavonoid ring structure. Oligomeric flavonoids may be hydrolyzed to monomers and dimers under influence of acidic conditions in the stomach. Larger molecules reach the colon where they are degraded by bacteria. The sugar moiety of flavonoid glycosides is an important determinant of their bioavailability. Dimerization has been shown to reduce bioavailability. Among all the subclasses of flavonoids, isoflavones exhibit the highest bioavailability [34]. After ingestion of green tea, flavonoid content is absorbed rapidly as shown by their elevated levels in plasma and urine. They enter the systemic circulation soon after ingestion and cause a significant increase in plasma antioxidant status [35].

1.10- Biological Activities of Flavonoids

1.10.1- Antioxidant Activity

Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants. The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability [14,36]. The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and

giving rise to a relatively stable flavonoids radical[37]. Mechanisms of antioxidant action can include suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation, scavenging ROS, and upregulation or protection of antioxidant defenses [38,39]. Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth [40]. Lipid peroxidation is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanisms . Free metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13–1.0V) such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation [39,41]. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at specific positions of different rings of flavonoid structures[42]. Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation *in*

vitro[43]. Because of oxidation on the B ring of flavonoids having catechol group a fairly stable orthosemiquinone radical is formed which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals exhibit weak scavenging potential[44]. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features[45]. The flavonoid heterocycle contributes to antioxidant activity by permitting conjugation between the aromatic rings and the presence of a free 3-OH. Removal of a 3- OH annuls coplanarity and conjugation which compromises scavenging ability[46]. It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a 3`,4`-catechol, elucidating the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature. Generally O-methylation of hydroxyl groups of flavonoids decreases their radical scavenging capacity[45]. Occurrence, position, structure, and total number of sugar moieties in flavonoid (flavonoids glycosides) play an important role in antioxidant activity. Aglycones are more potent

antioxidants than their corresponding glycosides. There are reports that the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased[47]. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety. In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7-position[48]. Increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion. Tetramers exhibit greater activity against peroxynitrite and superoxide mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers[49].

1.10.2-Hepatoprotective Activity

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities[50]. Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. glutamate-cysteine ligase catalytic subunit (Gclc) expression, glutathione, and ROS levels are reported to be decreased in liver of diabetic mice. Anthocyanins have drawn increasing attention because of their preventive effect against various diseases[51].

1.10.3- Antibacterial Activity

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 [39,41,52,53]. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity[54]. 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[55,56]. Catechins, the most reduced form of the C3 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[57,58]. The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyl transferases in *S. mutans*, probably due to complexing activities[57,59]. Robinetin, myricetin, and (−)-

epigallocatechin are known to inhibit DNA synthesis in *Proteus vulgaris*, suggested that 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[60]. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3ˈ,4ˈ- pentahydroxyflavone against *Escherichia coli* DNA gyrase[61]. 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[62]. 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 2ˈ,4ˈ-or 2ˈ,6ˈ-dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity[63]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class[64]. Osawa et al. have shown that 5-hydroxyflavanones and 5- hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2ˈ and 4ˈpositions inhibited the

growth of *S. mutans* and *Streptococcus sobrinus*[65]. Haraguchi and colleagues[63]. 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[63]. There are many examples that lend support to the prowess of phyto constituents derived from edible and medicinal plants as potent antibacterial agents[66-68]

1.10.4- Anti-Inflammatory Activity

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[69]. 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[70]. 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[71,72]. 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[73]. 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[74]. 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*[75]. Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10mM concentration[76]. The effect of flavonoid on platelets has been related to the inhibition of arachidonic acid metabolism by carbon monoxide[77]. Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

1.10.5-Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemopreventive agents[78]. Consumption of onions and/or apples, two major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach and breast. The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods[79]. Several mechanisms have been proposed for the effect of flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities[80]. Major molecular mechanisms of action of flavonoids are given as follows:

(1) down regulation of mutant p53 protein,

(2) cell cycle arrest,

(3) tyrosine kinase inhibition,

(4) inhibition of heat shock proteins,

(5) estrogen receptor binding capacity,

(6) inhibition of expression of Ras proteins.

Mutations of p53 are among the most common genetic abnormalities in human cancers. The inhibition of expression of p53 may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines[81]. Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the transduction of growth factor signals to the nucleus. Their expression is thought to be involved in carcinogenicity via an ability to override normal regulatory growth control. Drugs inhibiting tyrosine kinase activity are thought to be possible antitumor agents without the cytotoxic side effects seen with conventional chemotherapy. Quercetin was the first tyrosine kinase inhibiting compound tested in a human phase I trial[82]. Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses. Flavonoids are known to inhibit production of heat shock proteins in several malignant cell lines, including breast

cancer, leukemia, and colon cancer[81]. Recently it has been shown that the flavanol epigallocatechin- 3-gallate inhibited fatty acid syntheses (FAS) activity and lipogenesis in prostate cancer cells, an effect that is strongly associated with growth arrest and cell death[83]. In contrast to most normal tissues expression of FAS is markedly increased in various human cancers. Up regulation of FAS occurs early in tumor development and is further enhanced in more advanced tumors[84]. Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included P-388 leukemia cells, gastric cancer cells (HGC-27, NUGC-2,NKN-7, andMKN-28), colon cancer cells (COLON320DM) human breast cancer cells, human squamous and gliosarcoma cells, and ovarian cancer cells[81]. Markaverich[85] proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent[86]. Barnes[87] has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models. In a study to determine effects of isoflavones genistein, daidzein, and biochanin A on mammary carcinogenesis, genistein was found to suppress the development of chemically induced mammary cancer without reproductive or endocrinological toxicities. Neonatal

administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats[88]. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats[89]. The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll[90]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity[91]. A carbonyl function at C-4 of the flavone nucleus was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects[92]. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10- epoxide-2 on mouse skin[93]. Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk [94]. It is well known that due to oxidative stress cancer initiation may take place and thus potent antioxidants show potential to combat progression of carcinogenesis. Potential of antioxidant as an anticancer agent depends on its competence as an oxygen radical inactivator and inhibitor[95]. Therefore diets rich in radical scavengers would diminish the cancer-promoting action of some radicals[96].

1.11- Isolation of flavonoids

The separation and purification of plant constituents is mainly carried out using one or other, or combination of the following chromatographic techniques:

paper chromatography (PC); thin- layer chromatography (TLC), and high performance liquid chromatography (HPLC).The choice of the technique depends largely on the solubility properties and volatility of the compound to be separated. All the above techniques can be used both in micro and macro- scale[97].

1.11.1- Paper chromatography (PC)

The technique of (PC) is common in the field of flavonoid analysis and separation [98,99]. (PC) is suitable for the separation of complex mixtures of all types of flavonoids and their glycosides. It is convenient for isolation of both small and relatively large amounts, and is associated with the low cost of necessary equipment and materials [100].One of the main advantages of PC is the great convenience of carrying out separation simply on sheets of filter paper, which serve both as the medium for the separation and as the support.Another advantage is the considerable re-produiblity of R_f values on paper, so that such measurements are valuable parameters for use in describing new plant compounds. Indeed, for substances such as the anthocyanins, which do not have other

clearly defined physical properties the R_f is the most important mean of describing and distinguishing the different pigments [101].

Most flavonoids appear as coloured spot on paper chromatogram. when viewed in UV-light and when fumed with ammonia they often produce significant changes in these colures. Compounds appear as coloured or UV- fluorescent spots, after reaction with a chromogenic reagent which is used either as spray or as a dip [97].

1.11.2- Thin layer chromatography (TLC)

TLC is technique which has developed rapidly. However, it is also complementary to PC in that it provides new media for separation of flavonoids on small scale and permits the use of a wider variety of detecting Reagents [102].The special advantages of TLC compared to PC include: speed, versatility and sensitivity. The greater speed of TLC is due to the more compact nature of adsorbent when working with labile compounds. Finally, the sensitivity of TLC is such that separation on less than milligram amount of material can be achieved. Detection of compounds on TLC plates is normally carried out by spraying procedure. One advantage over PC is that glass plates may be sprayed with conc(H2SO4) which is a useful detection reagent for steroids and lipids[97].TLC is an ideal technique for the screening of drugs because of its low cost, easy

maintenance and selectivity of detection reagents. TLC on silica gel is very favorable for the analysis of flavonoid [103,104].

On the other hand, the separation may be achieved by high performance- thin layer chromatograph (HP- TLC) which represents a combination of TLC and HP. This technique has a large applicability in the field of plant material analysis and stability tests of extracts and finished products.

The separation of polyphenols (natural anti- oxidant) present in plant extracts may be performed by (HP- TLC) which is a competitive analytical method. It has many advantages, such as lower cost short analysis time, the possibility of mutable detection, and specific derivatization of the some components.[105]Two dimensional TLC conditions were also chosen and applied in the separation of investigated plant extracts[106].

1.11.3- Gas chromatography

Two types of gas- chromatography are encountered .Gas-solid chromatography (GSC) and gas- liquid chromatography (GLC). GLC finds widespread use in all fields of science, where its name is usually shortend to gas chromatography (GC)[107]. GC is no more complicated than other chromatographic procedures. The main variables are the nature of stationary phase of column and the temperature of operation, this are varied according to the polarity

and volatility of the compounds being separated. Most frequently, GC is automatically linked to mass spectroscopy (MS) and the combined GC-MS apparatus has emerged as one the most important techniques for phytochemical analysis.

1.11.4-High performance liquid chromatography (HPLC)

HPLC is analogous to GLC in its sensitivity and ability to provide both quantitative and qualitative data in a single operation. HPLC is mainly used for those classes of compounds which are non-volatile, e.g. Higher terpenoids, phenolics of all types, alkaloids lipids and sugars [97].Various techniques have been used for the determination of natural anti- oxidants including HPLC [108,109].

HPLC is gaining increasing importance for the analysis of plant extracts. The qualitative analysis which produces a “fingerprint” chromatogram obtained under standard conditions can be very useful for quality control of phytochemicals. Although TLC is a powerful and simple technique used for this purpose. There are situations in which it can produce doubtful results. HPLC can also be a useful tool in chemosystematics helping for example, to characterize species on the basis of their secondary metabolite contents [110].

HPLC has been used in a number of occasions for the analysis of flavonoids in plants. In one study [111], HPLC was used to

distinguish species based on the quantitative variation of flavonoids among them, in other study[112], It was used for the quantitative analysis of flavonoid aglycones.

The literature is replicate with chromatographic systems which are capable of measuring flavonoid content across one, two and three of the five common sub- classes of flavonoids found in foods. However many foods and mixed diets, in particular, contain members of all five sub- classes of flavonoids [113]. Advantages claimed for HPLC analysis include :

(i) Short analysis time, (ii) high resolution, (iii) no derivatization is required, (iv) no risk of thermal decomposition and (v) easy quantification[97].

Where twenty years ago the principal chromatographic methods for analysis of flavonoids were TLC and PC, in last decade HPLC has taken over almost completely separation of widely differing compounds [114,115].

Natural products are often obtained by some conventional solvents extraction and separation techniques, such as using organic solvents to extract and column chromatography, including silica gel and (HPLC), to isolate. However, some organic solvents are unfriendly to environment and the conventional separation methods are usually tedious, time consuming and needing multiple

steps and worse still the sample are adsorbed on the stationary phase irreversibly.

1.12- Spectroscopic methods

In identifying a plant constituent, once it has been isolated and purified, it is necessary first to determine the class of compound and then to find out which particular substance exist in that class. It should travel as a single spot in several TLC and / or PC systems. The class of compound is usually clear from its response to colour test, its solubility and R_f properties. Complete identification depends on measuring other properties and then comparing these data with those in the literature. These properties include melting point, boiling point, R_f and optical rotation (for optically active compounds). However, equally informative data on plant substances are its spectral characteristics. These include ultra violet (UV), infrared (IR), nuclear magnetic resonance (NMR), and mass spectroscopy (MS).

A known plant compound can usually be identified on the above basis. If a new compound is present, all the above data should be sufficient to characterize it [116].

1.12.1. Mass Spectrometry

Mass Spectrometry (MS) has proved to be one of the most effective techniques in biomedical research, in special when complex matrices of

biological samples must be analyzed. The main advantages of MS are its high sensitivity, which allows analysis of compounds present in the micro gram scale, and high specificity, as it is able to separate molecules of the same molecular weight but different atomic composition, and sometimes even to differentiate stereoisomeric compounds. It is easy to couple separation techniques such as liquid and gas chromatography which is an excellent advantage. Sample preparation may also be critical, but that varies from sample to sample; Never the less , some general guidelines have been reviewed[117].

Gas Chromatography (GC) is one of the key techniques for the separation of organics and when coupled to MS, it is the most common techniques of structural identification. However, flavonoids are largely nonvolatile, and need to be derivatized also they are usually thermally unstable. Both these characteristics have led to the establishment of Liquid Chromatography, in particular High Performance (HPLC), as the fundamental separation technique for flavonoids. Consequently, LC-MS coupling is routinely used for the overall structure elucidation of flavonoids[118,119].

Various ionization techniques are available, and each has their own specificities which make them more or less useful depending on the molecules under study and on the aim of such study. The ones most applied to flavonoid research are hereafter presented.

Fast-Atom Bombardment (FAB) and Liquid Secondary Ion Mass Spectrometry (LSIMS) are ionization techniques used in Secondary Mass Ion Spectrometry (SIMS) in which secondary ions emitted by sample irradiation with a beam of energetic (primary) ions are analyzed. Typically, these techniques are able to produce ions from polar compounds with molecular weights up to 10 kDa, but they require the analyte to be dissolved in a matrix which may lead to the formation of more complex spectra. Also being soft techniques, ion abundance is low, and it has been used essentially to identify flavonoid glycosides and for molecular weight determination.[120]

Electrospray Ionization (ESI) is a technique in which ions are generated by solvent evaporation under a high voltage potential, and can be applied directly, by infusion of the sample with a flow-controlled syringe, or coupled to separation techniques such as LC or capillary electrophoresis. In both cases, a steady liquid stream enters the system, allowing multiple analyses to be performed over a relatively large period of time. ESI interfaces are mostly coupled to quadrupole mass spectrometers. Both are simple and robust equipment's able to produce either positive or negative ions, and their main limitation is the relatively limited *m/z* range, usually below 2 kDa. In Atmospheric Pressure Chemical Ionization (APCI) sources ionization occurs via a corona discharge on a heated solvent spray, which produces solvent-derived primary ions that will, in turn, ionize the solute.[121] Both ESI and APCI

use atmospheric pressure and high collision frequency, and thus generate large amounts of ions as they involve solvent evaporation, the decomposition of the analytes is reduced, and full scans show limited fragmentation. The main disadvantage of both these techniques is that some HPLC solvents interfere with the ionization process, and thus chromatographic separations need to be specifically designed.[117]

Matrix-Assisted Laser Desorption/Ionization (MALDI) is a soft ionization technique in which the analytes are co-crystallized with a matrix; this mixture is deposited on a plate upon which a laser beam is aimed. The laser discharge ultimately leads to analyte ionization and projection from the matrix and onto the analyzer. Typical matrices are derivatives of 4-hydroxycinnamic acid, and also 2,5-dihydroxybenzoic acid (2,5DHB). [121]

Due to the structural similarity between these matrices and the flavonoids, only recently has MALDI been applied to flavonoid structural elucidation, using a FT-ICR spectrometer, and a significant, although still informative, number of flavonoid-matrix clusters are observable[122].

Mass analysis is the second step in a mass spectrum experiment. Following ion generation, mass analyzers measure the mass-to-charge ratio, *m/z*, of the ions, by using a combination of electromagnetic fields. There are many types of mass analyzers, as there are of ion sources and detectors. The most common are of the ion type and of the quadrupole

type, which analyze *m/z* ratios by the resonance frequency and by the trajectory stability, respectively, and time-of-flight (TOF) analyzers, which measure ion velocity (or flight time). More recent resonance frequency analyzers, namely Fourier Transform (FT) ion cyclotron resonance (FT-ICR) and FT orbitraps, are now starting to be applied to flavonoids[123]. A recent work has compared the performance of different mass analyzers coupled to the same ion source and it was concluded that fragmentation patterns are transferable among different mass analyzers, only the relative abundances are changed; although applied to cyano dyes[123], the conclusions also apply to other classes of compounds, such as flavonoids, where different mass analyzers lead to similar fragmentation patterns.

Tandem mass spectrometry, usually abbreviated MS/MS, or MSn for *nth* order fragmentation is any method that involves at least two stages of mass analysis, in conjunction with a fragmentation process. Most commonly, a mass analyzer is used to isolate a precursor ion, which is then fragmented to yield product ions (and, eventually, neutral fragments) that will be detected in the second mass analysis – a typical MS2 experiment. This can, at least conceptually, be expanded with further successive modification and detection steps, giving rise to MS3,… ,MSn. However, as only a very small fraction of ions is detected in one pass to the following analyzers, MS3 is usually the highest order achieved. This spatial arrangement of equipment, analyzer-modified-

analyser, corresponds to tandem MS in space, where ions are treated in different regions of space. Alternatively, tandem MS can be performed in time, with analyzers such as ion traps, orbitraps or FT-ICR, where the same analyzer performs different tasks successively[121] .

m/z modification can be achieved by various techniques, but the most common is Collision Induced (or Activated) Dissociation (CID or CAD), where precursor ions undergo collisional activation with neutral atoms or molecules (such as inert gases) in the gas phase. CID is an example of a post-source fragmentation, in which energy is added to the already vibrationally excited ions. An alternative to CID is ECD (Electron Capture Dissociation), in which multiply charged positive ions are submitted to a beam of low energy electrons producing radical cations. In opposition to post-source fragmentation, in-source fragmentation, ions already possess sufficient internal energy and fragment spontaneously within the mass spectrometer. Although usually this is an undesired effect, because it leads

to lower abundance of precursor ions, it may in some cases become useful[121,124].

Four general types of tandem MS scans are possible, and all may generate valuable information. In *product ion scan* analyses all the fragment ions are resulting from a single selected precursor ion (these are usually called *MS2 spectra*). Conversely, a *precursor ion scan* will identify all the precursors of a selected product ion; a *neutral loss scan* is

performed from a selected neutral fragment and will identify the fragmentations leading to the loss of that neutral fragment; these two techniques cannot be performed in time-based analyzers[121]

A particular application of neutral loss scans is in the identification of phase II conjugation metabolites that can be identified by specific neutral losses [125]. Neutral loss scans are widely used to detect phase II conjugation metabolites, such as glucuronides (loss of 176 Da) and sulfates (loss of 80 Da) as well as for the detection of glutathione adducts (loss of 129 Da). Scanning for neutral losses of 162 and 132 Da has also been used to separate flavonoids with hexose residues from those with pentose residues, respectively. More selective than these three techniques, *Selected reaction monitoring* (SRM) will analyze if a specific product ion comes from the fragmentation of a specific precursor ion; although more sensitive, it is much more specific.

1.12.2. Nuclear magnetic resonance spectroscopy

Nuclear Magnetic Resonance spectroscopy, hereafter simply designated by NMR, is one the most powerful research techniques used to investigate the structure and some properties of molecules. One of the main applications of NMR in flavonoid research is the structural elucidation of novel compounds, for which nothing is known; although NMR traditionally requires large amounts of sample, which is not easy to obtain when analyzing novel compounds, the technical developments in the last decade, both in NMR instrumentation, pulse programs and in

computing power, have allowed the complete assignment of all proton and carbon signals using amounts in the order of 1 mg[118].

The two most basic NMR experiments are the H^1 and the C^{13} NMR experiments, which are aimed at the determination of the resonance frequency of each H^1 or C^{13} nucleus in the molecule. H^1 NMR experiments register the chemical shifts (δ) and spin-spin couplings, the latter described by the coupling constants (*J*). This provides valuable information about the relative number of hydrogens and also their type, by comparison of the recorded chemical shifts with compiled data. This is particularly useful in establishing the aglycone type and the acyl groups attached to it, as well as in identifying the number and the anomeric configuration of the glycoside moieties attached to the aglycone. C^{13} NMR data is used to

complement H^1 NMR data, and is particularly useful at establishing the type of groups present in the samples by comparison with compiled data; however, it must be noted that C^{13} NMR is much less sensitive due to the abundance of C^{13} (1.1 %) when compared to H^1 (99.9%)[126].Together, these two 1D experiments are used primarily to identify aglycone types and substituent groups, but a definite structural elucidation involving accurate location of the various groups, requires various 2D experiments.

2D NMR experiments generate contour maps that show the correlations between different nuclei in the molecules, and can be either homo nuclear or hetero nuclear, depending on whether the interacting nuclei are of the same or different elements[126] .COSY (Correlation SpectroscopY) was one of the first multidimensional systems. COSY cross peaks are between protons that are coupled to each other, usually two bonds apart ($2J_{HH}$), but sometimes also three and four bonds apart ($3J_{HH}$ and $4J_{HH}$); the intensity of coupling affects the intensity of the peak. DQF-COSY (Double Quantum Filter COSY) is an improvement of the COSY experiment in which non-coupled proton signals, such as those from solvent, are eliminated as they may overlap signals from the analyte[126]

A further improvement is the TOCSY (TOtal Correlation Spectroscopy) experiment, which creates correlations between *all* protons in a given spin system, as long as there are couplings between every intervening protons; this is extremely useful to identify protons on sugar rings – every proton from one sugar ring will have a correlation with all other protons from the same ring but not with those of other rings. Magnetization is transferred over up to 5 or 6 bonds, and is interrupted by small or null H^1-H^1 couplings and hetero-atoms; also, the number of transfer steps can be adjusted by changing the spin-lock time[118]

A good reference for the TOCSY transfer in various sugars is Gheysen’s

Work[127] , Selective 1D TOCSY (also known as HOHAHA, homo nuclear Hartman-Hahn) is particularly useful in compounds with more than one sugar moiety, in which overlap occurs; in this experiment, one peak is selected and that magnetization is transferred stepwisely to the protons in the same spin system; instead of cross peaks, transfer is shown by increased multiplet intensity[118].

Heteronuclear 2D NMR experiments correlate nuclei of different elements. The most powerful techniques of all are undoubtedly the 2D proton–carbon experiments HMQC/HSQC (Heteronuclear Multiple Quantum Coherence/Hetero nuclear Single Quantum Coherence) and HMBC (Hetero nuclear Multiple Bond Correlation) as they provide an opportunity to dovetail proton and carbon NMR data directly.

HMQC and HSQC establish one bond correlations between the protons of a molecule and the carbons to which they are attached ($1J_{CH}$). Both these are much more sensitive than the correspondent 1D C^{13} experiments; while in 1D experiment the low abundance of the isotope leads to a low signal-to-noise ratio, in the hetero nuclear 2D experiment the initial magnetization occurs on the highly sensitive H^{1} nuclei and is then transferred to the C atoms that are connected to each proton. A similar C^{13} NMR experiment is H^{1}- C^{13} HMBC (Hetero nuclear Multiple Bond Correlation), in which long-range interactions (typically $2J_{CH}$ and $3J_{CH}$) are analyzed; HMBC is usually more sensitive to 3-bond correlations than to 2-bond correlations, but this depends on the overall

signal-to-noise ratios and on the adjustable parameters of each experiment. A newer experiment, 2*J*CH,3*J*CH-HMBC, has been designed to differentiate these two types of correlations[118,126,128].

HMBC application to flavonoids usually addresses assignment on non-protonated C atoms, from both the aglycones and acyl groups. Unlike TOCSY, HMBC transfer is not stopped by hetero atoms, and so it can also be used to determine the linkage points of hetero atom containing groups such as sugar residues. HMBC is also useful to distinguish some classes of flavonoids, as flavones from aurones, which have similar H^{1} and C^{13} NMR spectra but very different HMBC spectra. Currently, only enhanced variants of the HSQC and HMBC experiments, namely gradient enhanced (*ge*) ones, are used, due to their higher sensitivity and capacity. There have been used to establish strong intra molecular H bonding between the 4-oxo and 5-hydroxy groups in flavonoids[129,130.]

Further developments in NMR experiments, using new 2D and 3D techniques, have been developed in recent years, and are starting to be used for flavonoid analysis. In particular, 2D and 3D HSQC-TOCSY experiments are capable of assigning all C^{13} signals of individual glycosides in polyglycosylated flavonoids[118] . While the above mentioned 2D techniques are useful to establish the connectivities between atoms through bonds, the Nuclear Overhauser Effect (NOE), which can be summarized as “A change in the intensity of an NMR signal from a nucleus, observed when a neighboring nucleus is

saturated", is useful at establishing non-bonded connectivities, or connectivities through space. The crosspeaks in a H^1-H^1 NOESY (NOE SpectroscopY) spectrum correspond to correlations between protons that are close to each other in space (up to 4 Å) but not necessarily connected through bonds; these correlations may arise from both intramolecular and intermolecular proton interactions, and has been successfully used to establish rotational conformers and restrictions, establish intermolecular associations and even solve protein ligand and DNA-ligand structures. A 2D NOE experiment, ROESY (Rotating Overhauser Effect SpectroscopY), has been used in flavonoid research mainly to establish the stereochemistry of various flavonoids[118,126]

X-ray crystallography depends on the ability to obtain flavonoid crystals, which has only been achieved for a small number of flavonoids; in alternative, the H^1 CP-MAS (Cross Polarization Magic Angle Spinning) NMR techniques have been used to elucidate the solid state conformation of flavonoids, either pure or, for example, in tissues, providing enough sample is available (typically in the 10 mg scale). In particular, such an experiment had provided information on the planarity of the flavonoid rings, on the intramolecular H bonding between the 4-oxo group and the 3- and 5-hydroxy groups, and on intermolecular association[118,131].

1.12.3- UV-Vis spectrophotometry

Ultraviolet and visible spectroscopy was one of the earliest techniques routinely used for flavonoid analysis due to the existence of two characteristic UV/Vis bands in flavonoids, band I in the 300 to 550 nm range, arising from the B ring, and band II in the 240 to 285 nm range, arising from the A ring. For examples, while the band I of flavones and flavonols lies in the 240 – 285 nm range, that of flavanone (no C ring unstauration) lies in the 270 – 295 nm range; conversely, the band II of flavones and flavanones (no 3-OH group) lies around 303 –304 nm, and that of 3-hydroxylated flavone is centred around 352 nm.

Shift reagents, such as sodium methoxide and aluminium chrolide, lead to shifts in the maximum wavelength of these bands due to methoxide-induced deprotonation of OH groups or Al_3+ complexation by OH groups, were also routinely used to study flavonoid structure. Nowadays these techniques are not routinely used but still continue to be applied in some cases, in particular to HPLC eluates - a hyphenated LC-UV-MS has been developed using post-column UV shift reagents for the flavonoid analysis of crude extracts.

UV/Vis spectrophotometry is still widely used to study anthocyanidins, which change their form and color depending on pH, concentration, metal ions and copigmentation[132]. This multistate behaviour has been used to derive molecular machines based on flavonoids, particulary flavylium containing ones like anthocyanins[133,134]

1.12.4 -Other techniques

MS, NMR and UV/Vis are the most commonly used techniques to elucidate the structure of flavonoids. Three other techniques are also used: X-ray crystallography, although with the potential to solve complete structures, is hampered by the difficulty to obtain good crystals, circular dichroism and vibrational spectroscopies are used to solve specific structural details.

X-ray crystallography is able to detect the arrangement of atoms within a crystal by the atom-induced diffraction of X-rays. Many materials form crystals, such as salts, metals and organic and biological molecules, in particular proteins. Flavonoids, however, only form crystals in sporadic conditions, and the number of reported flavonoid crystals is very low[118].

Nevertheless, traditional X-ray crystallography has been used to identify the intermolecular π-π interactions that guide the stacking of parallel aglycones to form supramolecular layers, and to identify aminoacyl residues involved in the formation of protein-flavonoid complexes, which are critical to the circulation of flavonoids in mammals[135] this is one of the strongest applications of X-ray crystallography to the flavonoid area.

More recently, X-ray powder diffraction has been used, either alone or in association with solid-state NMR, to obtain structures of flavonoids, in particular of catechins[136].

Circular dichroism (CD) is a spectroscopic technique that allows the analysis of the differential absorption of left and right circularly polarized light. The major advantage of CD over optical rotation measurements is that CD absorption is confined to the narrow absorption range of each individual chromophore, and so it can be used to determine the contribution of individual chromophores and to access their possible substitution patterns[137].

CD is also routinely used to study the interaction of many flavonoids with biomolecules, providing valuable information on biomolecule-drug interaction, such as DNA binding of quercetin[138]

Vibrational spectroscopy, in its infra-red and Raman variants, is a spectroscopic technique that analyses the vibrational modes of molecules and molecular groups, allowing bond characterization, and, by comparison with known tabulated data, identification of functional groups; in the case of flavonoids, vibrational spectroscopy has been systematically used to study hydroxyl and carbonyl groups, but more recent technical developments have allowed its application to a broader set of research goals. Raman spectra are much less complex than the IR spectra of the same molecules, and for that reason Raman spectroscopy has been gradually taking over IR spectroscopy, although it is common to use both techniques as complement of each other. Vibrational spectroscopy is seldom used alone, and most studies are accompanied by

other spectroscopic approaches and/or quantum chemical computations[139].

Both these spectroscopies are routinely applied to study the effects of substituents on the geometry of the molecule, in particular of dihedral angles, and also on the analysis of intramolecular and intermolecular H bonding, either to other flavonoid molecules or to solvent molecules. Similarly, metal complexation by flavonoids is also routinely assessed by vibrational spectroscopy[140].

1.13- *Borassus aethiopium*

Borassus aethiopium	
Scientific classification	
Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Monocots
(unranked):	Commelinids
Genus:	*Borassus*
Species:	***Aethiopium***
Binomial name	
Borassus aethiopium Mart.	

Borassus aethiopium is a species of *Borassus* palm from Africa. In English it is variously referred to as African fan palm, African palmyra palm, deleb palm, ron palm, toddy palm, black rhun palm, ronier palm (from the French) and other names. It also has names in African

languages. The tree has many uses: the fruit are edible, as are the tender roots produced by the young plant fibres can be obtained from the leaves; and the wood (which is reputed to be termite-proof) can be used in construction. There are at least two varieties of this species: var. *bagamojensis* and var. *senegalensis*. They grow swelling, solitary trunks to 25 metres (82 ft) in height and 1 metre (3 ft 3 in) in diameter at the base. The green leaves — 3 metres (9.8 ft) wide — are carried on petioles — 2 metres (6 ft 7 in) long — which are armed with spines. The crownshaft is spherical to 7 metres (23 ft) wide, the leaves are round with stiff leaflets, segmented a third or half-way to the petiole. In male plants the flower is small and inconspicuous; females grow larger, 2 centimetres (0.79 in) flowers which produce yellow to brown fruit resembling the coconut containing up to 3 seeds[141].

1.14. **Annona squamosa**

Annona squamosa is a small, well-branched tree or shrub from the family Annonaceae that bears edible fruits called sugar-apples. It tolerates a tropical lowland climate better than its relatives *Annona*

reticulata and *Annona cherimola* (whose fruits often share the same name) helping make it the most widely cultivated of these species[142].

Annona squamosa is a small, semi-(or late) deciduous, much branched shrub or small tree 3 metres (9.8 ft) to 8 metres (26 ft) tall very similar to soursop (*Annona muricata*) with a broad, open crown or irregularly spreading branches and a short trunk short, not buttressed at base. The fruit of *A. squamosa* (sugar-apple) has delicious whitish pulp, and is popular in tropical markets[143].

Annona squamosa is native to the tropical Americas and West Indies, but the exact origin is unknown. It is now the most widely cultivated of all the species of *Annona*, being grown for its fruit throughout the tropics and warmer subtropics, such as Indonesia, Thailand, and Taiwan; it was introduced to southern Asia before 1590. It is naturalized as far north as southern Florida in the United States and as south as Bahia in Brazil, and is an invasive species in some areas[144].

1.15-Aim of this study

This study was aimed to:

- To Extract the flavonoids from the *Borassus aethiopum& Annona squamosa*
- To Isolate the flavonoids by chromatographic techniques.
- To Conduct spectroscopic studies on the isolated flavonoids.
- To evaluate the some biological activites of purified methanolic fraction extract of *Borassus aethiopum& Annona squamosa*.

2.Materials and Methods

2.1- Materials

2.1.1- Plant material

The leaves of *Annona squamosa* and stem bark of *Borassus aethiopium* were collected in April 2014 and June 2015 from Nyala (western Sudan) . The plant was identified and kindly authenticated by the Dept. of Botany , University of Khartoum.

2.1.2- Solvents

All solvents used are of analytical grade. Methanol HPLC grade is used for spectroscopic purposes (BDH , England).

2.1.3- Chromatographic materials

- Sheets of Whatman paper (No.1 and No.3 mm-46x57cm) from Whatman Ltd. Kent, England.

- Glass jars, 10x20x24 cm, fitted with covers, were used for chromatographic fractionation.

2.1.4- Equipments

The ultraviolet lamp used in visualizing TLC plates and paper chromatography was a multiband UV λmax (254 / 365 nm) portable ultaviolet, a product of Hanovia lamps (6 watt S/Y and L/W). Ultraviolet absorption spectra were obtained in spectroscopic methanol on UV - Visible Spectrophotometer(Shimadzu).

The electron impact ionization (EIMS) mass spectra were obtained on a solid probe using Shimadzu QP-class-500. ^{1}HNMR spectra were obtained on a Bruker AM 500 spectrophotometer (Germany) operating at 500 MHz in spectroscopic grade DMSO-d_6. The chemical shifts values are expressed in δ (ppm) units using (TMS) as an internal standard and the coupling constants (J) are expressed in Hertz (Hz).

2.2- Methods

2.2.1- Preparation of reagents for phytochemical screening

- Flavonoids test reagents

a- Aluminium chloride solution

(1g) of $AlCl_3$ was dissolved in 100 ml methanol.

b- Potassium hydroxide solution

(1g) of KOH was dissolved in 100 ml water.

c- Ferric chloride solution

(1g) of $FeCl_3$ was dissolved in 100 ml methanol.

- Alkaloids test reagents

-Mayer's reagent

(1.358 g) of $HgCl_2$ was dissolved in 60 ml of water and poured into a solution of (5 g) of KI in (10 ml) of H_2O,then sufficient water was added to 100 ml.

- Wagner's reagent

(2 g) of iodine and (6 g) of KI were dissolved in 100 ml of water.

-Glycosides test reagents

-Molisch reagent

(2 g) α-naphthol dissolved in 20 ml EtOH 96%

2.2.2- Shift Reagents

The diagnostic reagents used for the UV spectral measurements of the isolated flavonoids were prepared as follows:

-Sodium methoxide (NaOMe)

Freshly cut metallic sodium(2.5 gm.) was dissolved in100 ml spectroscopic methanol.

- Aluminum chloride

Anhydrous $AlCl_3$(5 gm) was cautiously dissolved in100 ml spectroscopic methanol and filtration was carried out after about 24 hours.

-Hydrochloric acid

Fifty ml. concentrated HC1 were mixed with 100 ml. distilled water.

- Sodium acetate

(NaOAc) Anhydrous sodium acetate was melted and allowed to stand for about 10 minutes. The material was then powdered and stored in a dry bottle.

- Boric acid

Anhydrous powdered reagent grade H_3BO_3 was used.

2.2.3- Stepwise procedure for use of shift reagents for UV

- The UV spectrum of the compound in methanol was first recorded.

- 3 drops of NaOMe reagent were added to the sample and the NaOMe spectrum was recorded, and after 8 minutes the NaOMe spectrum was re-recorded.

- 6 drops of $AlCl_3$ reagent were added to the fresh sample and the $AlCl_3$ spectrum was recorded, 3 drops of HCl were added and after mixing , the $AlCl_3$/ HCl spectrum was recorded.

- Powdered NaOAC was then added to the fresh sample, the mixture was shaked and the NaOAC spectrum was recorded. NaOAC/ H_3BO_3 spectrum was then recorded after adding H_3BO_3.

2.2.4- Preparation of plant extract for phytochemical screening

(100g) of powdered shade-dried leaves of *Annona squamosa*(or stem bark of *Borassus aethiopium*) were extracted with 80% methanol (soxhlet) for 6 hours. The cooled solution was filtered and evaporated to dryness. This prepared extract(PE) was subjected to preliminary phytochemical screening for the presence of flavonoids, saponins, tannins, alkaloids, carbohydrates and/or glycoside, and triterpenes and/or sterols.

-Test for saponins (Froth test)

The dried extract was dissolved in water, transferred into a test tube and shakend vigorously, then it was left to stand for 10 minutes, when a thick persistent froth appears and persists for one hour saponins are present.

-Test for alkaloids

(50 mg) of extract was dissolved in (50 ml) of methanol in a water bath for 20 minutes, the extract was then filtered off and allowed to cool. Two ml of the extract was poured into test tubes. Dragendorff's or Mayer's reagent was added to the tube and the presence or absence of colours or any precipitates was noted.

- Test for tannins

(50 mg) of extract was dissolved in (50 ml) of water .The aqueous extract was then treated with a 15% ferric chloride solution. A blue color indicates condensed tannins, a green color indicates hydrolysable tannins.

- Test for glycosides (Molisch's test)

(1 ml) of the extract solution was pipetted into a test tube, 3 drops of Molisch reagent were added . After mixing, (1ml) of concentrated sulfuric acid was added to the wall of test tube. A positive test for carbohydrates is indicated by a violet ring forming at the interface between the denser sulfuric acid and the less dense test solution above.

-Test for sterols and triterpenes (Liebermann-Burchard test)

(1 ml) of glacial acetic acid was added to (1 ml)chloroform and cooled to 0°C, then one drop of concentrated sulphuric acid was added to the cooled mixture followed by the extract.

- Test for flavonoids

(50 mg) of extract was defatted with petroleum ether and the residue was dissolved in(30ml) 80% methanol and filtered. The filtrate was used for the following tests:

(1) To (30 ml) of the filtrate few drops of methanolic aluminium chloride were added . A dark yellow colour was observed .

(2) To (30 ml) of the filtrate few drops of potassium hydroxide solution were added . A dark yellow colour was observed .

(3) To (30 ml) of the filtrate few drops of ferric chloride solution were added . A blue coloration was observed .

2.2.5-Isolation of flavonoids

Powdered shade- dried stem barks of *Borassus aethiopium* (or leaves of *Annona squamosa*) were macerated with 95% ethanol at room temperature for 48 hours. The crude extract(4g) was mounted on top of a silica gel(400g) column and then eluted with methanol: ethyl acetate in order of increasing polarity starting with 4:1; 1:1(ethyl acetate:methanol) and ending with methanol. The methanol fraction was rich in phenolics . It was applied on Whatman paper (No. 3 mm – 46x 57cm) as narrow strips. The bands were irrigated with BAW (n-butanol- acetic acid-water; 4:2:5;v:v:v; upper layer). The developed chromatograms were air-dried and examined under both visible and UV light (ʎ 366,245nm).. The equivalent bands from each paper were then cut out, combined and cut into small strips and slurred with methanol. After several hours of

contact, with occasional shaking, the liquid was evaporated *in vacuo* to dryness. In this way compounds II and III were isolated from stem bark of *Borassus aethiopium* in chromatographically pure form. Compound IV was isolated from leaves of *Annona squamosa* when a ration of BAW(4:1:5;v:v:v) was used.Compond I was isolated from the methanolic fraction when a Sephadex column was eluted with methanol:water(1:1)-fractions 5-12.

2.2.6- Antimicrobial assay

The isolated flavonoids were screened for their antimicrobial activity against four human pathogenic bacterial strains: Gram-positive (*Staphylococus aureus* and *Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and fungal species **(***Aspergillus niger*, *Candida albicans*). The cup plate agar diffusion assay was used.

2.2.6.1- Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline solution to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.2.6.2- Preparation of fungal suspension

The fungal cultures were maintained on dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100ml of sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.6.3- Testing of antibacterial susceptibility

The cup plate agar diffusion assay was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS,

1999). Bacterial suspension was diluted with sterile physiological solution to 10^8cfu/ ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on surface of MHA and the inoculum was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extract.The inoculated plates were incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured in duplicates and averaged.

2.2.6.4-Testing of antifungal susceptibility

The above mentioned method was adopted for antifungal activity, but instead of agar , dextrose agar was used. Samples were used here by the same concentrations used above.

3-Results and Discussion

3.1-Phytochemical screening

The leaves of *Annona squamosa* and stem bark of *Borassus aethiopium* were screened for secondary metabolites and the results are depicted in Table 3.1.

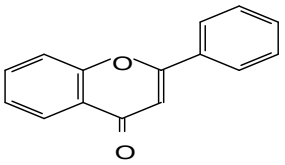
Table (3.1): Phytochemical screening of *Anona squamosa* and *Borassus aethiopium*

Constituents	*Annona squamosa*	*Borassus aethiopium*
Alkaloids	**+ve**	**+ve**
Saponins	**+ve**	**-ve**
Glycosides	**+ve**	**+ve**
Steroids and/or triterpenes	**-ve**	**+ve**
Flavonoids	**+ve**	**+ve**
Tannins	**-ve**	**+ve**

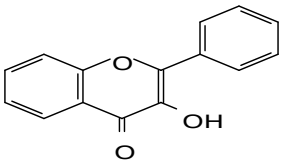
3.2- Identification of compound I

Compound I was isolated as yellow powder from the stem bark of *Borassus aethiopium*. The structure was partially elucidated via a combination of spectral techniques(UV,^{1}HNMR and MS).

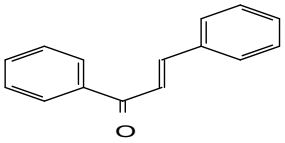
The UV spectrum of flavonoids can differentiate between those flavonoids with unsaturation at 2 and 3 positions(flavones,flavonols,chalcones and aurones) and those classes lacking such unsaturation like flavones,dihydrochalcones,dihydroflavonols and isoflavones[19].



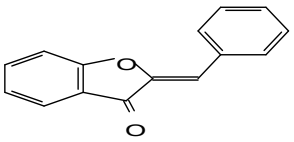
Flavone



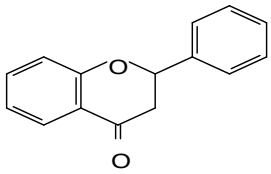
Flavonol



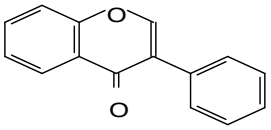
Chalcone



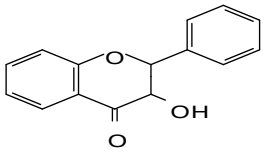
Aurone



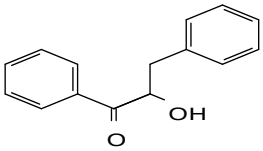
Flavanone



Isoflavone

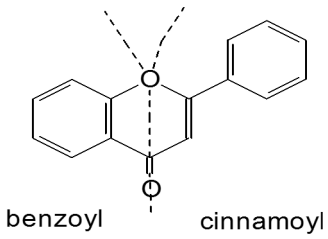


Dihydroflavonol



Dihydrochalcone

Flavonoids with conjugation between A and B rings show two absoption bands in the UV spectrum : band I (due to cinnamoyl chromophore) and band II (due to benzoyl chromophore) .



The UV spectrum of compound I gave λ_{max} (MeOH) 206nm (Fig. 1) a pattern which is characteristic of dihydrochalcones.

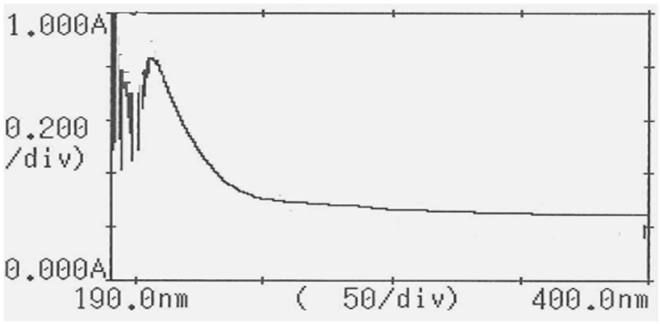


Fig.1: UV spectrum of compound I

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 α-OH and 4 –OH groups of dihydrochalcones . In both cases it induces a bathochromic shift , but with decreased intensity in case of α-OH function[19]. Addition of sodium methoxide to a methanolic solution of compound I, induced a 10nm bathochromic shift without decrease in intensity indicating a 4-OH function(Fig.2)

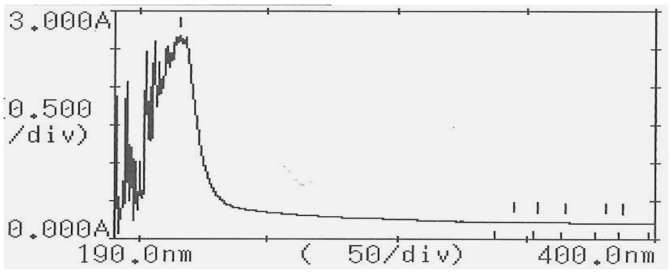


Fig.2: Sodium methoxide spectrum of compound I

The sodium acetate(Fig.3) gave a 12 nm bathochromic shift with increase in intensity diagnostic of a 4- and a 4`-OH functions of dihydrochalcones[13,19.]

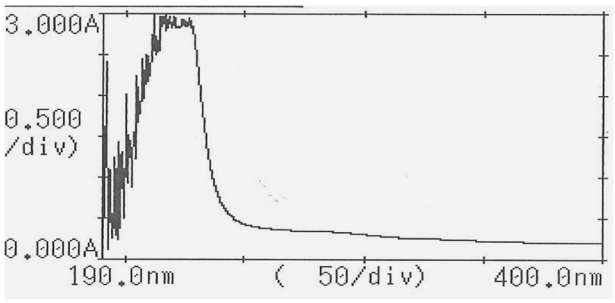


Fig.3: The sodium acetate spectrum of compound I

The aluminium chloride spectrum(Fig.4) did not reveal any detectable bathochromic shift indicating absence of α-OH , 2`-OH functions as well as catechol systems[13,19].

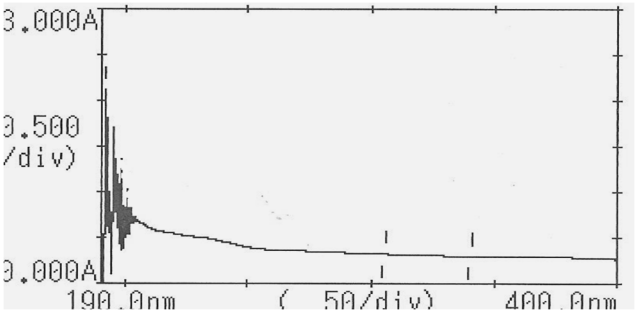


Fig.4: Aluminium chloride spectrum of compound I

Aluminum chloride is a useful diagnostic tool for α- and 2`-OH groups of dihydrochalcones as well as catechol moieties .Catechols form acid – labile complexes with aluminium chloride , while the α-OH and 2`-OH afford acid – stable complexes[13,19].

The ^{1}H NMR spectrum (Fig.5) showed amultiplet at δ 0.95-1.60(10H) assigned for two methyl functions and two methylene moieties . The

resonance at δ3.80(6H) accounts for two methoxyl functions.The multiplet at δ6.40-7.00 ppm was assigned for the aromatic protons.

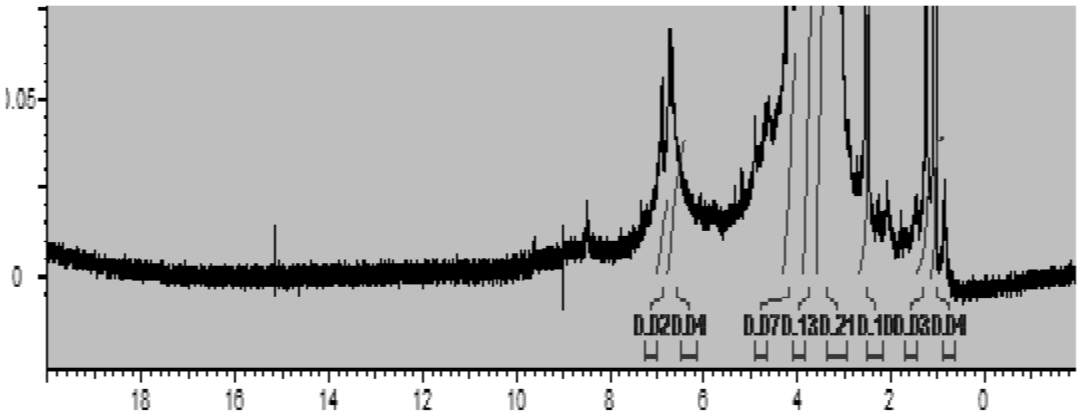


Fig.5: ^{1}H NMR spectrum of compound I

The mass spectrum(Fig.6) gave m/z 332 for(M^{+}+ $2H^{+}$). Other important fragments resulting from intact A and B rings appeared at m/z182 and m/z148 respectively (Scheme I) . This pattern of fission suggests two methoxyl functions in ring A and two methyls in ring B.

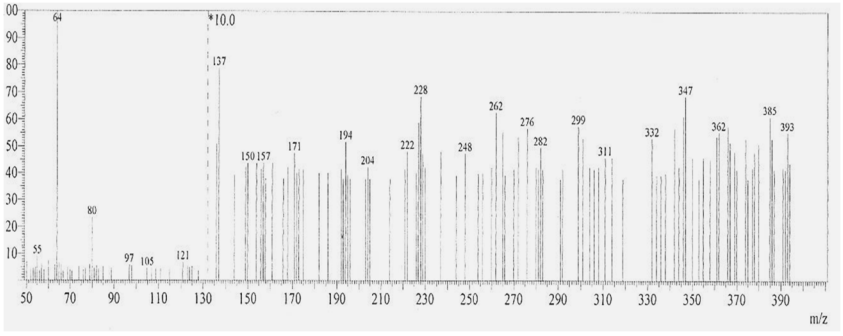
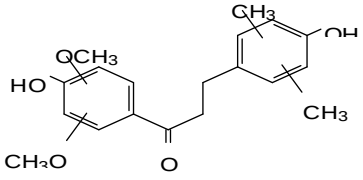
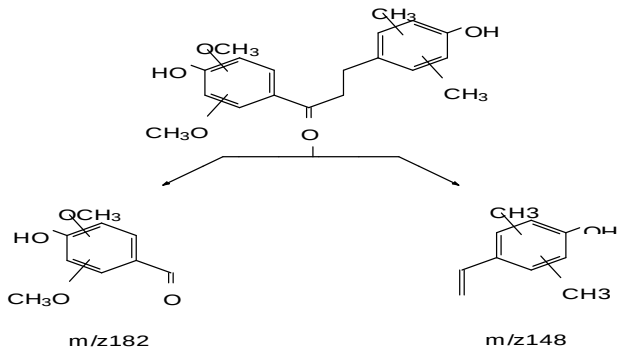


Fig.6: Mass spectrum of compound I

On the basis of the above cumulative data the following partial structure was proposed for the aglycone of compound I:



I



Scheme I : Fragmentation pattern of compound I

3.3- Characterization of compound II

Compound II was isolated as yellow powder from stem bark of *ssus aethiopium* . The UV spectrum (Fig.7) gave λ_{max}279nm. Such absorption is usually given by flavanones, dihydroflavonols, dihydrochalcones and isoflavones[13,19]. However,the sodium methoxide spectrum(Fig.8) showed a bathochromic shift without decrease intensity indicating a 4`- OH.

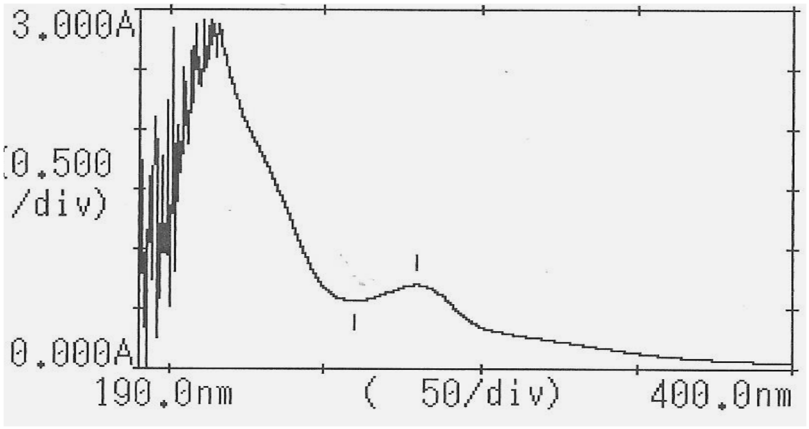


Fig.7: UV spectrum of compound II

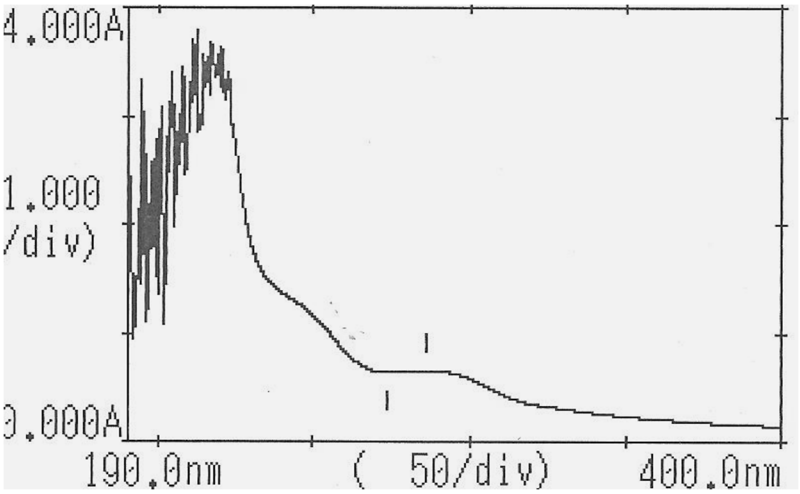


Fig.8: Sodium methoxide spectrum of compound II

The sodium acetate spectrum(Fig.9) did not show a bathocromic shift diagnostic of a 7-OH function. Also no bathochromic shifts were observed in the aluminium chloride spectrum (Fig.10) and the boric acid spectrum (Fig.11). This indicates absence of 3-, 5- OH functions as well as catechol systems[13,19].

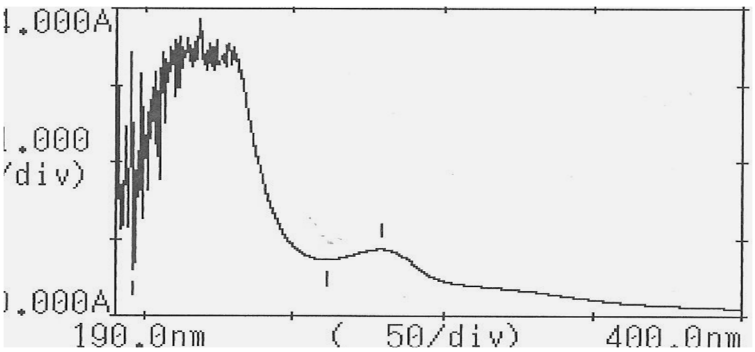


Fig.9: Sodium acetate spectrum of compound II

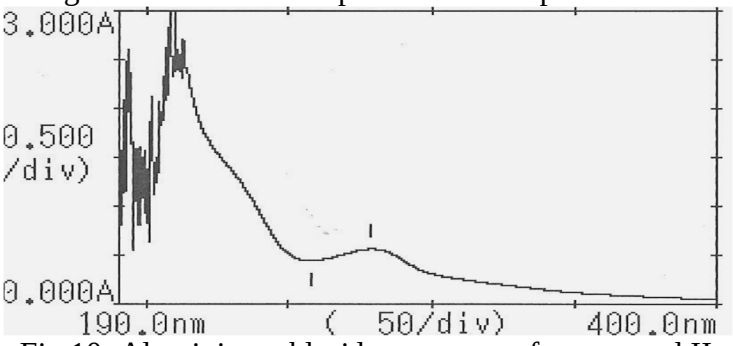


Fig.10: Aluminium chloride spectrum of compound II

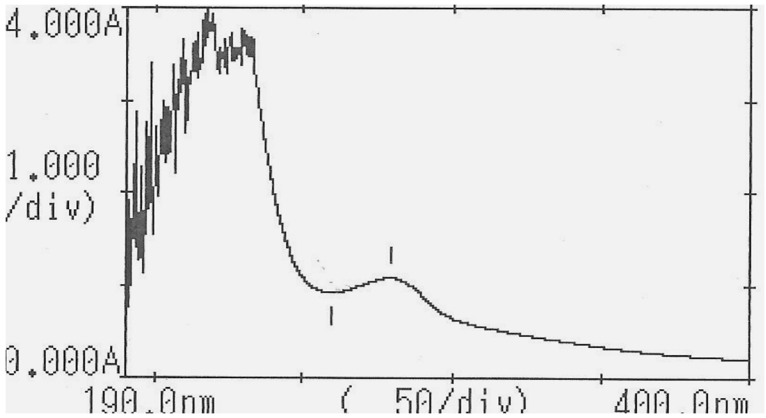


Fig.11: Boric acid spectrum of compound II

The ^{1}H NMR spectrum (Fig.12) showed δ0.90(3H) and δ1.60 due to two methyl groups.The multiplet at δ3.00-4.40 was assigned for a sugar moiety , while the resonance at δ3.80 account for a methoxyl group. The signal at δ2.40 was attributed to C_2-H,while the resonance at δ5.40 accounts for C_3- H. The resonances at δ6.60(2H) assigned to C_6- and C_8 – protons[13,19]. The resonance at δ7.80 and δ8.40 ppm are due to aromatic protons.

On the basis of the retro Diels-Alder fission (Scheme II) one methyl group was assigned for ring A , while two methyls functions were cited at the B ring.This is mainly due to the appearance of fragments at m/z134 and m/z176 resulting from intact A and B rings respectively.The mass spectrum(Fig.13) gave m/z 312 due to M^{+}. The substitution pattern of the B ring was mainly imposed by the appearance of a B ring signal for two magnetically equivalent protons at δ8.40.The $C_{2`}$ and $C_{6`}$ protons usually resonates at such low field due to the deshielding influence of the heterocyclic C ring.The A ring methyl function was assigned position 5 . This is due to the absence of a low

field signal (around 8ppm) characteristic of C_5 proton. Such proton resonates at low field due to the deshielding influence of the 4-keto function.

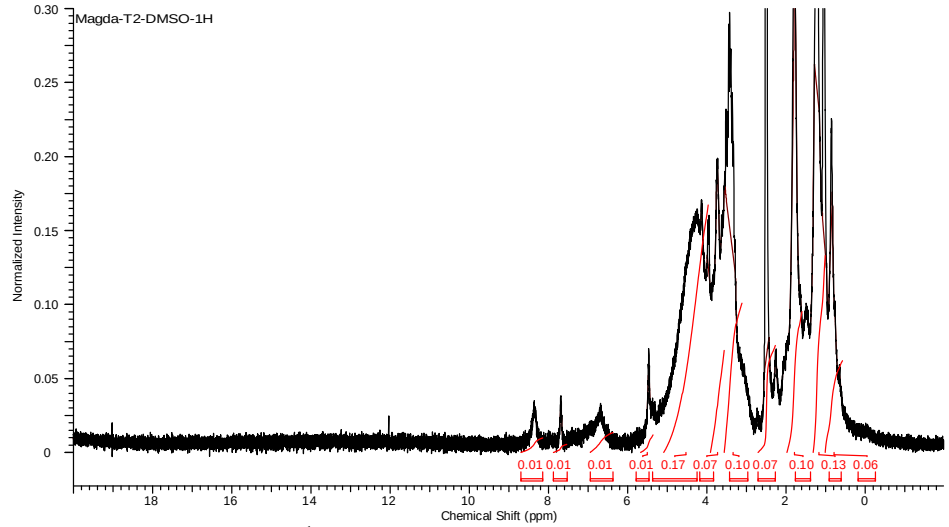


Fig.12: ^{1}H NMR spectrum of compound II

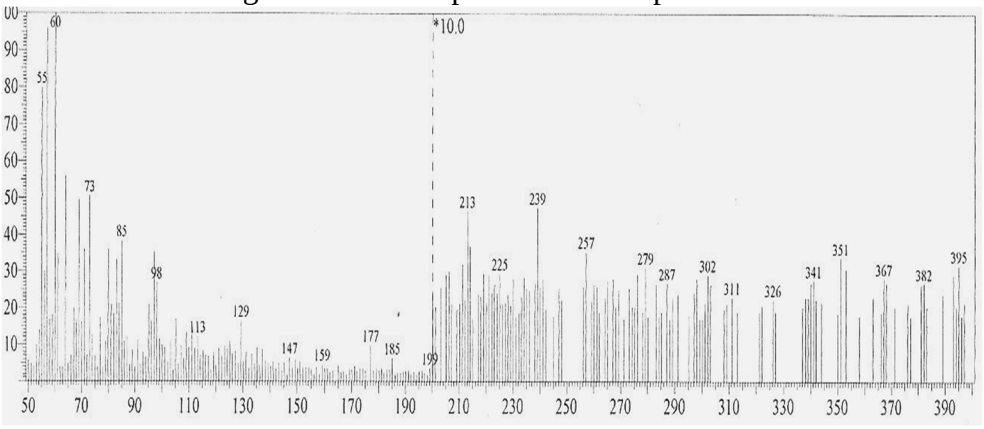
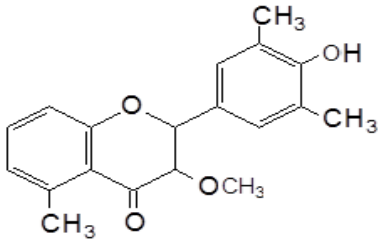
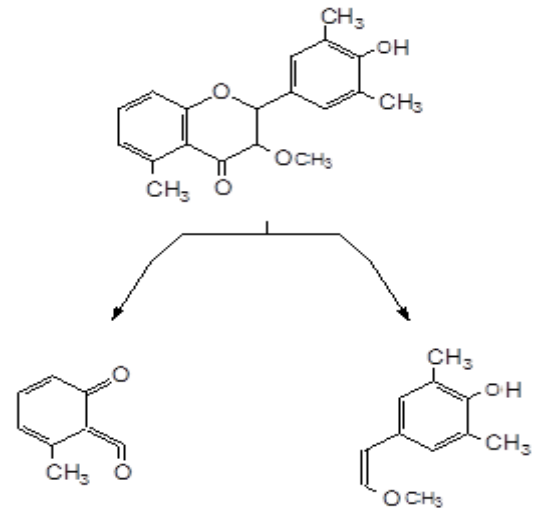


Fig.13: Mass spectrum of compound II

On the basis of the above spectral data , the following structure was proposed for compound II:



Compound II



m/z134 m/z178

Scheme II : Retro Diels-Alder fission of compound II

3.4- Caracterization of compound III

Compound III was isolated from the heartwood of *Borassus aethiopium* as yellow amorphous powder. In the UV compound III absorbs(Fig.14) at λ_{max}217,280. This absorption is characteristic of dihydrochalcones[13,19].The sodium methoxide spectrum (Fig.15) gave a 12 nm bathochromic shift without decrease in intensity indicating a 4-OH function.

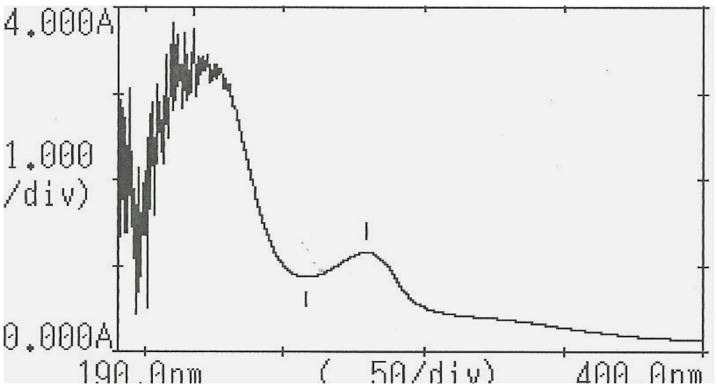


Fig.14: UV spectrum of compound III

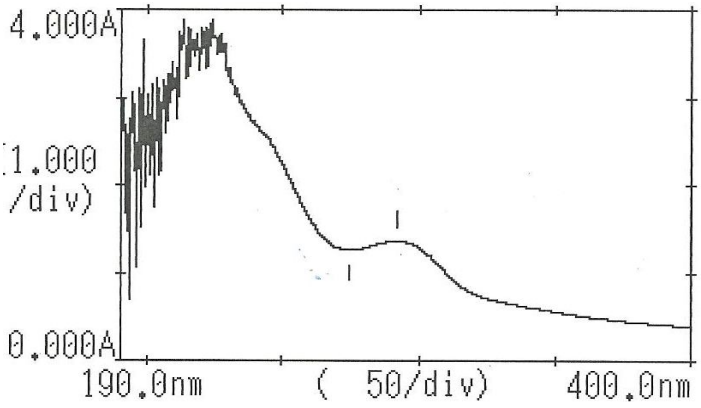


Fig.15: Sodium methoxide spectrum of compound III

Neither the aluminium chloride spectrum(Fig.16) nor the boric acid spectrum(Fig.17) revealed bathochromic shifts. This suggests absence of a 3-, 5-OH functions as well as catechol moieties[19].

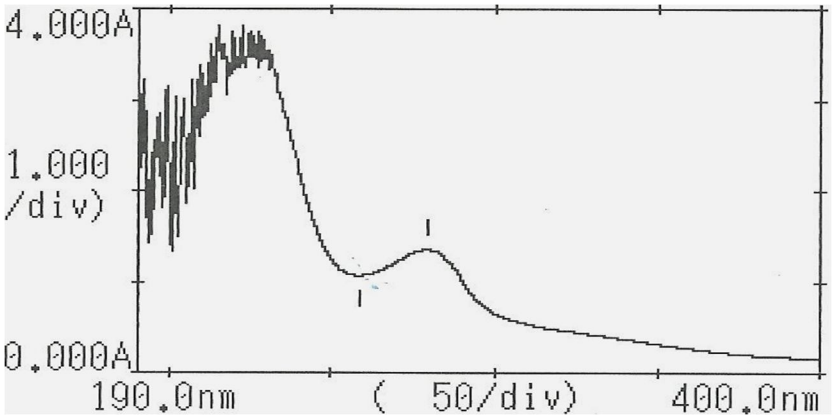


Fig.16 : Aluminium chloride spectrum of compound III

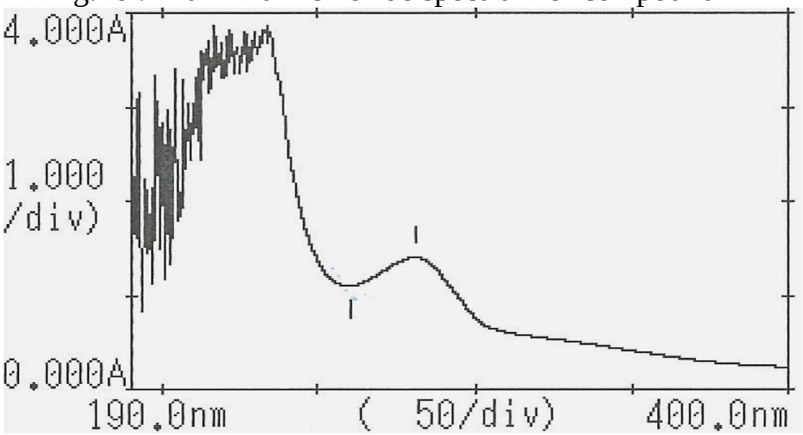


Fig.17 : Boric acid spectrum of compound III

The ^{1}H NMR spectrum (Fig.18) showed δ1.00(3H) due to a methyl group.The signal at δ1.6098(4H) accounts for two methylene protons. The multiplet at δ3.00-4.40 is due to sugar protons.The C_6- and C_8-protons resonate at δ 6.60 and 6.90ppm respectively.The signals at δ7.60 and δ8.40 account for the aromatic protons. The mass spectrum (Fig.19) gave m/z239 for the aglycone. Fragments >239 originate from glycoside.

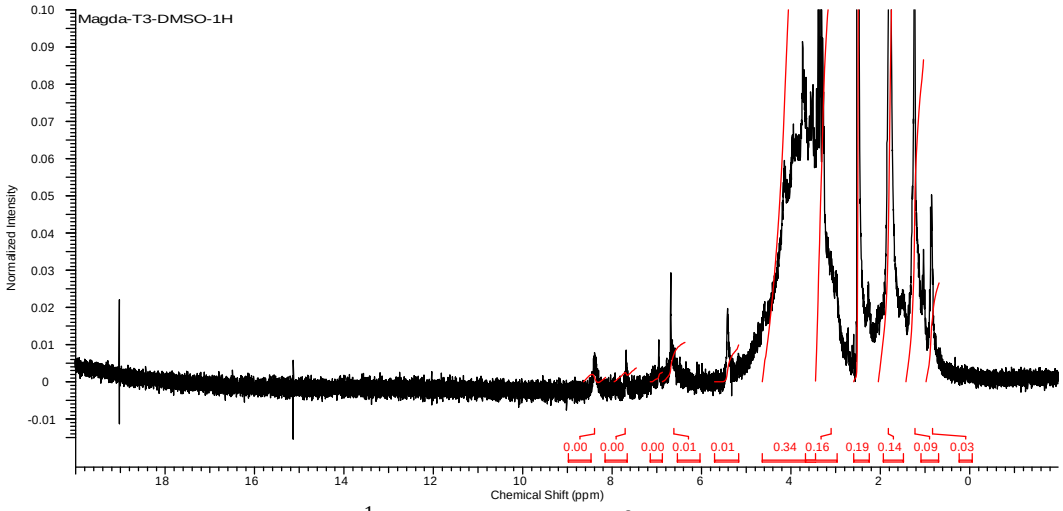


Fig.18 : ^{1}HNMR spectrum of compound III

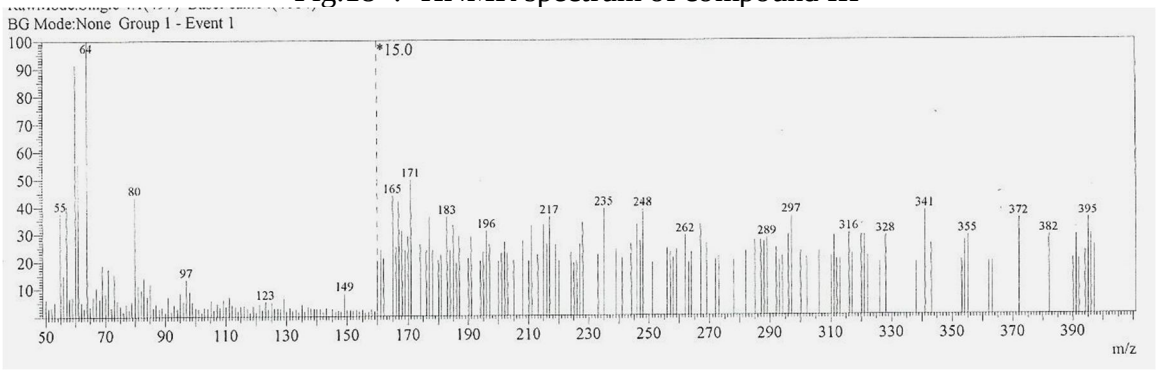
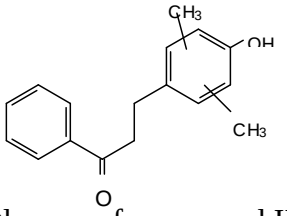
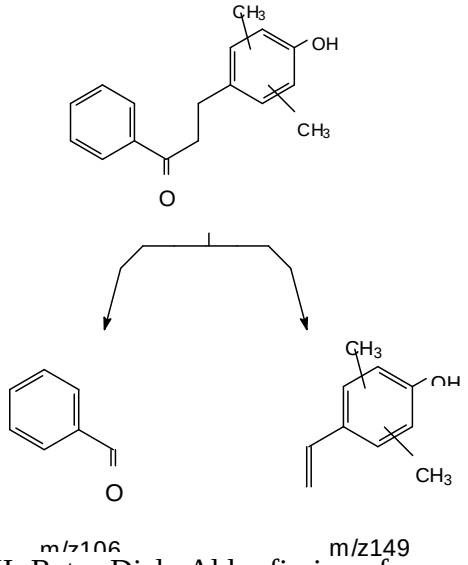


Fig.19: Mass spectrum of compound III

On the basis of the above argument , the following tentative structure was proposed for the aglycone of compound III:



Aglycone of compound III



Scheme II: Retro Diels-Alder fission of compound III

3.5-Characterization of compound IV

Compound IV was isolated from the leaves of *Annona squamosa* as yellow solid. In the UV compound IV absorbs(Fig.20) at λ_{max}222,278nm. The strong absorption at λ_{max}222 nm and the peak at 278nm beside the inflection which appeared above 300nm indicate a flavanone skeleton.

The sodium methoxide spectrum (Fig.21) did not reveal any bathochromic shift indicating absence of 3- and 4`-OH functions.

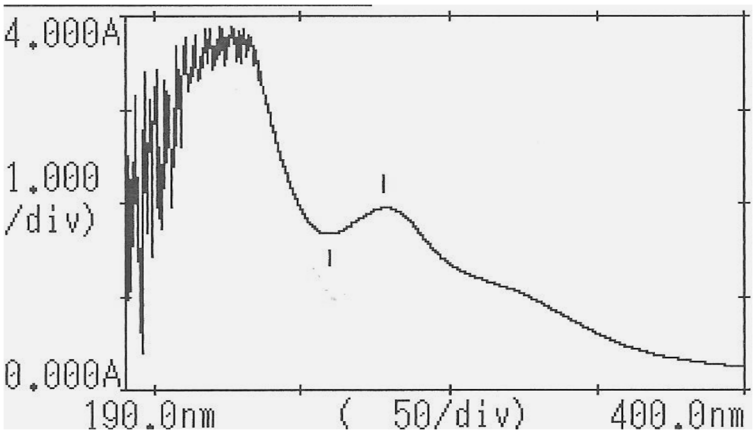


Fig.20 : UV spectrum of compound IV

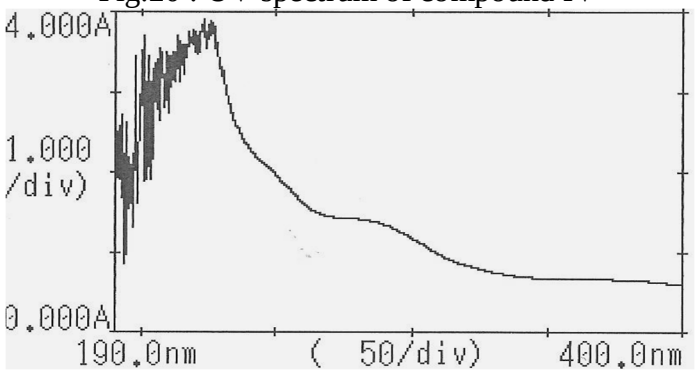


Fig. 21 : Sodium methoxide spectrum of compound IV

The sodium acetate spectrum (Fig.22) gave a bathochromic shift diagnostic of a 7-OH function[13,19]. The aluminium chloride and boric acid spectra (Figures 23,24) did not reveal any bathochromic shifts. This suggests absence of 3- , 5-OH functions and catechol systems(aluminium chloride and boric acid spectra).

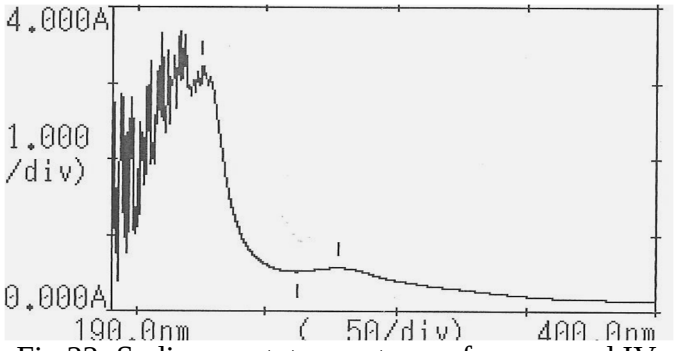


Fig.22: Sodium acetate spectrum of compound IV

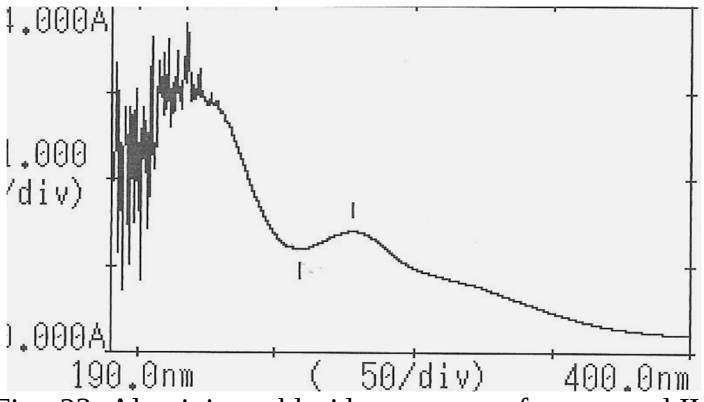


Fig. 23: Aluminium chloride spectrum of compound IV

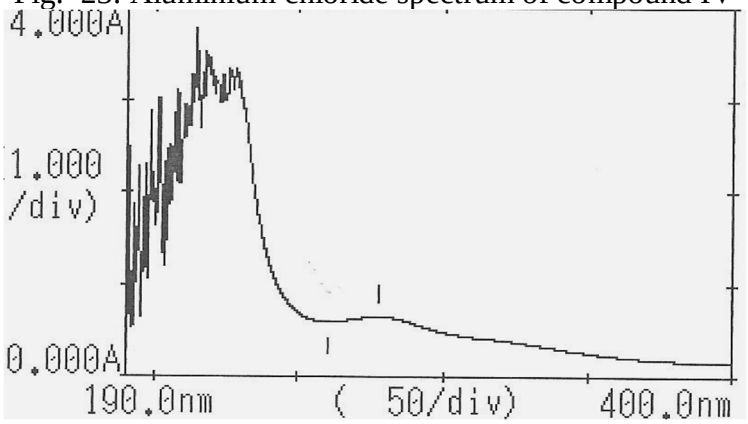


Fig. 24 : Boric acid spectrum of compound IV

The ^{1}H NMR spectrum (Fig.25) showed δ1.00(3H), δ1.60(3H) due to two methyl groups.The signal at δ1.60(2H) accounts for methylene protons. The multiplet at δ3.00-4.40 is due to sugar protons, while the two multiplets near δ 2.80ppm and δ 5.2ppm account for the C_2- and C_3 – protons. Such multiplets are due to mutual spin-spin splitting between C_2- H and the magnetically unequivalent C_3 – protons. The multiplet at δ 6.20-7.60ppm is due to A ring protons , while the resonance at δ8.40 is due to B ring protons. The mass spectrum (Fig.26) gave m/z298 for the aglycone. Fragments > m/z298 originate from glycoside.

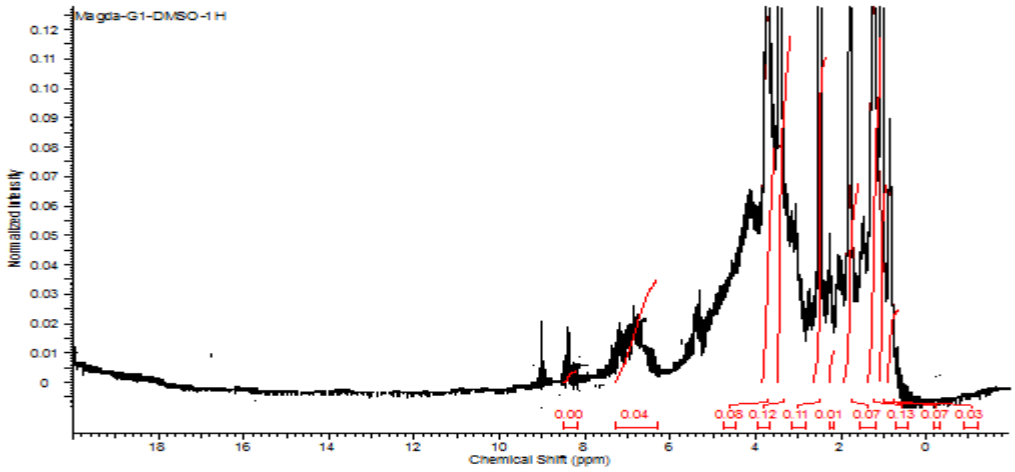


Fig. 25 : ^{1}HNMR spectrum of compound IV

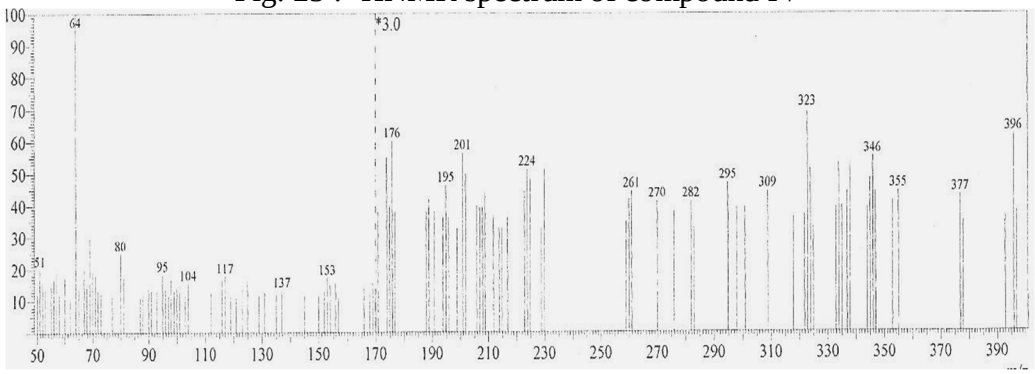
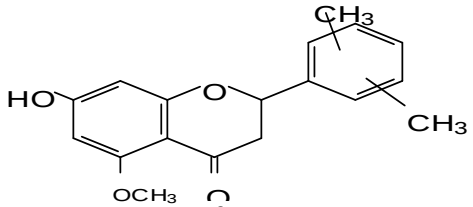
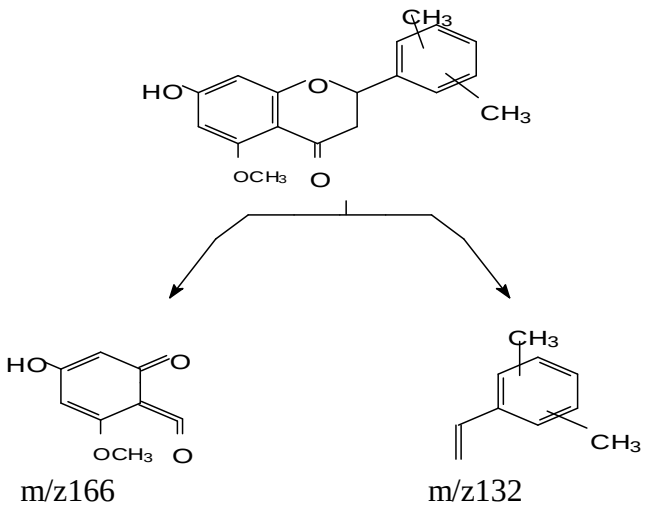


Fig. 26: Mas spectrum of compound IV

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



Aglycone of compound IV



Scheme IV : Retro Diels-Alder fission of compound IV

3.6-Antimicrobial activity

In cup plate agar diffusion assay , the methanolic extracts of *Borassus aethiopium* stem bark and the leaves of *Annona squamosa* were screened for antimicrobial activity against six standard human pathogens. The average of the diameters of the growth of inhibition zones are depicted in Tables (3.2 and 3.3) .The results were interpreted in commonly used terms (<9mm: inative;9-12mm:partially active; 13-18mm: active; >18mm:very active) .Tables (3.4) and (3.5) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table 3.2: Antimicrobial Activity of *Borassus aethiopium*. stem bark

Concentration		Antibacterial activity				Antifungal activity	
		Gram-positive		Gram -negative			
mg/ml	Control Methanol	*Bs.*	*Sa.*	*Ec.*	*Pa.*	*Ca.*	*An.*
100	0.0	20	18	19	18	15	18
50	0.0	20	17	17	20	14	13
25	0.0	19	15	18	20	13	12

Table 3.3: Antimicrobial Activity of the leaves of *Annona squamosa*

Concentration		Antibacterial activity				Antifungal activity	
		Gram-positive		Gram -negative			
mg/ml	Control Methanol	*Bs.*	*Sa.*	*Ec.*	*Pa.*	*Ca.*	*An.*
100	0.0	16	15	16	15	14	12
50	0.0	-	-	-	-	-	-
25	0.0	-	-	-	-	-	-

Table 3.4 : Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 3.5 : Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	An	Ca
Clotrimazole	30	22	38
	15	17	31
	7.5	16	29

Sa.: *Staphylococcus aureus*

Ec.: *Escherichia coli*

Pa.: *Pseudomonas aeruginosa*

An.: *Aspergillus niger*

Ca.: *Candida albicans*

Bs.: *Bacillus subtilis*

The methanolic extract of *Borassus aethiopium* stem bark showed significant antibacterial activity against test organisms specially at doses of 100 and 50mg. The extract also gave significant inhibition against the fungal species *Aspergillus niger* at100mg/ml(Table3.2).

The methanolic extract of leaves of *Annona squamosa* was active against all test organisms except the fungal species *Aspergillus niger* where it showed partial activity.

Conclusion

Phytochemical screening the leaves of *Annona squamosa* and the stem bark of *Borassus aethiopum* revealed the presence of flavonoids, tannins, alkaloids and glycosides in both species. Three components were isolated from *Borassus aethiopum* and one from leaves of *Annona squamosa* .The structures of the isolates were deduced on the basis of their spectral data.

The methanolic fractions of both species were evaluated ,*in vitro*, for their potential antimicrobial activity using the cup plate agar diffusion bioassay . Different antimicrobial responses were observed. The activity ranged from high to moderate.

Recommendations

i)A future ^{13}CNMR is recommended to lend additional evidence for the proposed partial structures.

ii) 2D NMR experiments are also needed for full elucidation of partial structures.

iii)The isolates may be screened for their antimicrobial, anti-inflammatory, antimalarial and other biological activities.