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EXTRACTION, ISOLATION AND CHEMICAL STRUCTURE ELUCIDATION OF DAIDZEIN FROM BARK OF *ACACIA ARABICA* (LAM.) WILLD OF BHOPAL, MADHYA PRADESH, INDIA

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ABSTRACT: Purpose: To extract, isolate and identify daidzein from the bark of *Acacia Arabica* (Lam.) Willd of Bhopal, Madhya Pradesh, India. **Methods:** Succesesive methanolic extract from the bark of *Acacia arabica* was subjected to column chromatography and fractionated by benzene, n-butanol, and acetone successively. Eluents having similar Rf values were pooled together. These pools were subjected to flavonoid test and only a single pool passed it which was again subjected to chromatographic separation for purification and crystallized powders was subjected to UV, FT-IR, EI-MS and ^{1}H-NMR for structure identification. **Results:** We have successfully isolated and identified daidzein by comparing with previously published literatures. **Conclusion:** From this study it can be concluded that the whole plant can be used as a source of daidzein.

INTRODUCTION: *Acacia nilotica* (L.) Del. syn. *Acacia arabica* (Lam.) Willd. (Mimosaceae). Commonly known as babul, kikar or Indian gum Arabic tree has been recognized worldwide as a multipurpose tree. It is widely distributed throughout arid and semi-arid zones of the world. *Acacia arabica* has been used as astringent, demulcent, nutritive, expectorant, styptic and tonic and have astringent, immunosuppressant, antibacterial, antitumor, antithrombotic, hypoglycemic activity.

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And has been proved as effective medicine in treatment of malaria; sore throat, toothache and hepatic diseases [1-5]

The plant *Acacia arabica* is the source of a diverse kind of chemical constituents. Phytochemical analysis of polyphenolic complex present in the bark of plant revealed the presence of phenols, flavones, tannins and glycoside. The total phenolic and tannin content in the dried bark found to be 9.86% and 13.40%, respectively; while the total flavonols and total *O*-dihydric phenols including chlorogenic acid were 0.0025% and 0.013% respectively[6]. The bark contain saponins[7, 8].

A number of secondary metabolites have been reported from Acacia species i.e. amines and alkaloids, cyanogenic glycosides, cyclitols, fatty

acids and seed oils, fluoroacetate, gums, nonprotein amino acids, terpenes (including essential oils, diterpenes, phytosterol and triterpene genins and saponins), hydrolyzable tannins, flavonoids and condensed tannins. The most evident and best known are polysaccharides (gums) and complex phenolic substances (condensed tannins)[9-12]. Phytochemical screening of the stem bark revealed that the plant contain terpenoids, alkaloids, saponins and glycosides [13-15]

Several researchers have shown that phytomolecules such as ellagic, gallic, epicatechin, rutin, myricetin, (+)-catechin, (−)-epicatechin, myricetin 3-glucopyranoside, kaempferol[16] umbeliiferone[17] myricetin 3-rhamnopyranoside, methyl 3,4,5 tri hydroxyl benzoate ($C_8H_8O_5$), p-coumaroyl glucoside ($C_{15}H_{19}O_7$), p-coumaroyl quinic acid ($C_{16}H_{18}O_8$), Ferulic acid ($C_{10}H_{10}O_4$), isoferulic acid, epi catecine-3-gallate ($C_{22}H_{18}O_{10}$), ascorbic acid , quercetine 3-O- (4'-O-acetyl)-rhamnopyranoside $C_{28}H_{30}O_{16}$), oleic acid ($C_{18}H_{34}O_2$), myristic acid (C14H28O2), Palmitic acid ($C_{16}H_{32}O_2$) and steroidal sapogenin aglycons[18, 19], stearic acid, kaempferol-3-glucoside, isoquercitrin, leucocyanidine[20] present in *Acacia* sp.

As mentioned earlier, previous studies have reported the presence of daidzein in the fruits[21] however in the present study an attempt has been made to isolate daidzein possibly for the first time from the bark.

EXPERIMENTAL:

Collection of plant material

Fresh stem bark of *Acacia arabica* were collected in the month of May 2010 from the local area near to TIT Group of institute, Hathaikheda Anand Nagar, Raisen Road, District Bhopal, Madhya Pradesh, India. The specimens were identified by Dr. Padma Shrivastava, Professor and Head of Post Graduate Department of Botany, Government Post Graduate College BHEL, Bhopal, Madhya Pradesh, India and voucher specimen (PGDB/BHEL/Ph.D/AM23) was deposited there for future reference.

Chemicals

Acetone, Pet. Ether, benzene, n-Butanol, Ethyl acetate, n-hexane, methanol, ethanol, chloroform, diethyl amine, Toluene was analytical grade and purchased from merck (Bhopal, India). Reverse osmosis Milli-Q water (18 MV) (Millipore, USA) was used for all solutions and dilutions. All other reagents were of the highest commercial grade available.

General experimental procedures[22]

UV spectra of the isolated compounds were recorded in methanol over a scanning range of 200-400 nm and λmax of compounds were determined. Spectra were recorded with a Systronics double beam-2203 UV-VIS spectrophotometer. EIMS (electron impact mass spectrum) in positive mode, were recorded on Bruker's aurora M90 (USA) instrument. The isolate was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 375-7500 cm^{-1} in FT-IR spectroscopy (Bruker FT-IR Spectrometer, USA). ^{1}HNMR spectra was recorded on a bruker biospin ^{1}HNMR spectrometer operating at 400 MH_Z for proton using tetramethylsilane (TMS) as internal standard. The solvents used were methanol and DMSO-d6. Chemical shifts ware shown in δ values (ppm) with TMS as an internal reference. Paper chromatography was carried out by Whattman 3 MM CHR. For column chromatography silica gel 60 (70-230 mesh, Merck, Darmstadt, Germany) was used. Thin layer chromatography (TLC) was performed using precoated TLC plates (Silica Gel G-60 F254, Merck, Germany).

Preparation of extracts

Plant samples were shade dried for 15 days and then pulverized to fine powders using mortar, pestle and grinder. 250gm of powder sample was successively extracted exhaustively by various solvents in increasing polarity (pet. ether, chloroform, ethyl acetate, acetone, methanol and water)[17]. Successive methanolic extract was then concentrated by distilling off the solvent and evaporated to dryness. The residue (8.76 g) stored at 4^{o}C for further analysis.

Phytochemical Screening for flavonoids

Sodium hydroxide test, lead ethanoate test and Shinoda's test were performed on all the prepared extracts for the presence of flavonoids with suitable solvents according to the standard procedures[23, 24].

Chromatographic characterization

Paper chromatography

Paper chromatography was carried out as per standard procedure. Different solvent extracts of the plant samples were applied on chromatography paper and chromatogram was developed in seven different solvents.

Column chromatography

4 gm of successive methanolic extract of the plant sample was adsorbed onto sufficient quantities of silica gel by triturating in a mortar under hood and left for about 9 hours to dry. Meanwhile, silica gel was suspended in water and stirred gently until the formation of slurry. This slurry was then poured carefully into previously cleaned, uprightly fitted; glass-wool sealed open glass column (2.5 cm inner diameter and 75 cm length) till it is about three-fourths filled, with tapping the walls with a cushioned rod in order to avoid air-bubbles. Solvent system having same proportion of n-butanol, acetic, acid, water was poured continuously into the column and allowed to drain until the silica particles pack. The quantity collected was poured back into the column. The silica gel adsorbed extract sample was sprinkled over the surface of the supernatant in the column using a fine spatula and allowed to settle on the surface of the stationary phase. A small quantity of sand sprinkled on the supernatant after adding the sample to prevent dissolution of the extracts into the mobile phase. Three mobile phases (benzene, n-butanol, and acetone) were successively passed through the column under the force of gravity and the tap at the bottom of the column was allowed to adjust the flow. Several fractions were eluted depending on the visible changes in the colorful bands running out of the column and collected in dry glass bottles.

TLC

1 cm was measured from the base of the TLC plate, marked with a pencil and labeled. Capillary tube was used to spot the plates with the column eluents. Small quantities of the eluents collected with capillary tube by dipping it in the solution. They were then used to spot the plates and put in a lidded tank containing the solvent system, Benzene: Acetone (9:1). The level of solvent system in the tank was about 1 cm beneath the origin. The solvent travelled up the plate by capillary action till it reached the solvent front, marked by a straight line across. The lid was lifted off and the plates ware dried before they were visualized by spraying with detection agents are ammonium vanadate solution and under UV light (365 nm). The retention factors were calculated by making use of the distance moved by the solvent and the distance moved by the component as follows.

RF = Distance travelled by the component/Distance travelled by the solvent front

RESULTS:

The preliminary phytochemical screening of succesive methanolic extract of bark of *Acacia arabica* showed the presence of flavonoids in the methanolic fraction after fractionation, as a red coloration, buff-colored precipitate and a colorless solution appeared in Shinoda's test, Lead ethanoate test and Sodium hydroxide test respectively and only this fraction was used for further characterization study.

Prior to the chromatographic separation, solvents with different polarities such as cyclohexane, benzene, chloroform, n-butanol, acetone, methanol and water were chosen to determine the appropriate solvent for separation of compounds by paper chromatography for the selected extract. Among these seven solvents, better separation of compounds with clear and distinct spots was achieved with benzene, n-butanol and acetone against the other four solvents which gave unclear spots with varying Rf values. Subsequently the extract was fractionated by column chromatography with benzene, n-butanol, and acetone successively. The column fractions were tested with TLC chromatogram and the Rf values were determined. Eluents showing similar pattern of TLC separation with respect to their Rf values were considered as one fraction alike and were pooled together. A total of 16 pools were made, from which a single pool was selected for further studies as the eluents in this pool showed positive inferences when subjected to the flavonoid test i.e., they shown yellow fluorescence in UV light after spraying with an ammonium vanadate solution. This fraction was again chromatographed under the same conditions and finally purified (70 ml) by crystallization to yield 70 mg of Yellowish red amorphous powder.

The chemical structure identification was carried out by FT-IR, EI-MS and ^{1}H-NMR as follows.

M.P.: 318°C.

UV (MeOH) λ max: 255 and 300

IR (KBr) νmax cm^{-1}: 3146.17, 2901.29, 2663.44, 2338.41, 1741.35, 1504.69, 1340.92, 1220.38, 1102.55, 854.74, 739.33, 660.09 **(Fig. 1).**

1H NMR (400 Hz, DMSO-d6) δ: (DMSO-d6, 300MHz): 3.88 (3H, s), 6.80 (2H, d, J¼8:0Hz), 7.06 (1H, s), 7.39 (2H, d, J¼8:4Hz), 8.31(1H, s), 9.46 (1H, br), ca.9.6 (2H) **(Fig. 2).**

EI/MS m/z: 255 and 162 [M+1], 26.0220, 28.1746, 42.5100, 93.2951, 162.0501, 179.082, 213.1429, 227.1159, 229.1746, 255.2241 **(Fig. 3).**

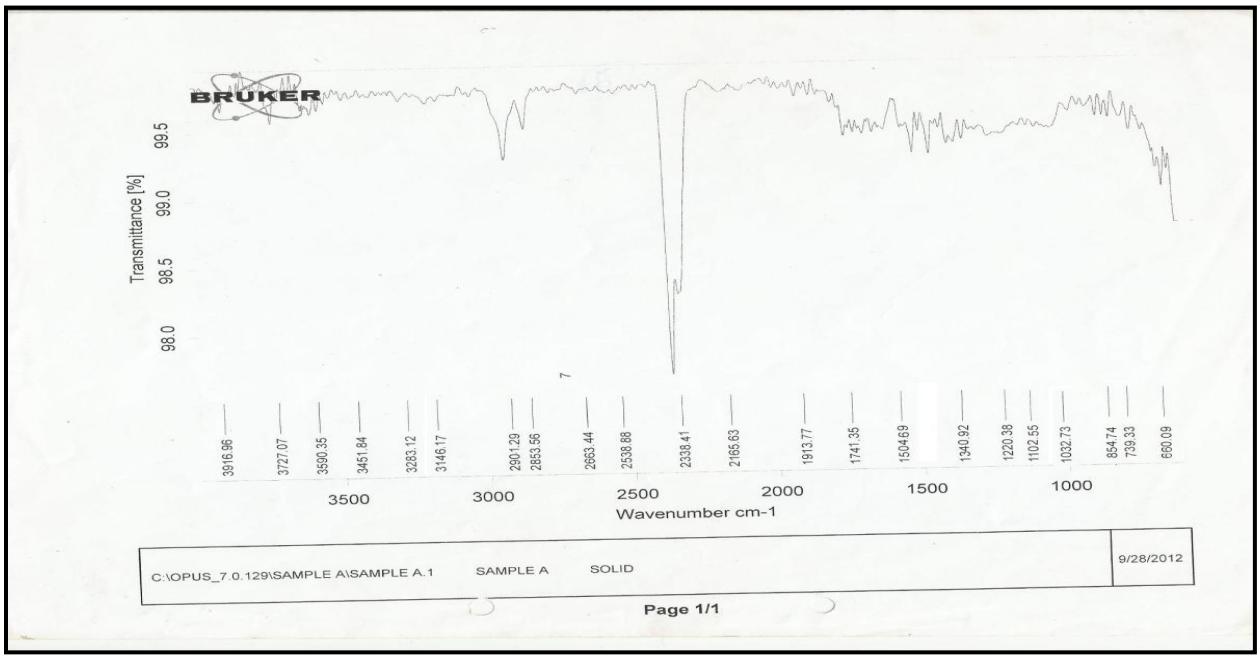


FIG. 1: FTIR SPECTRUM OF FLAVONOID COMPOUND

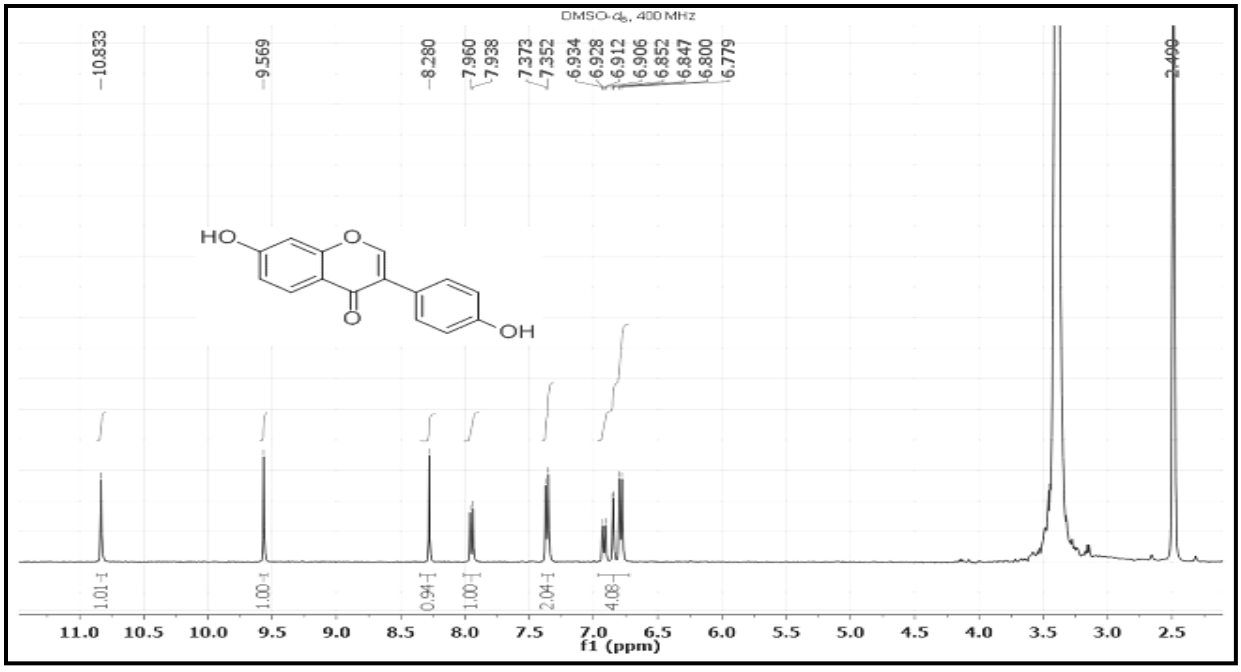


FIG. 2: ^{1}H NMR (400 MHZ) SPECTRUM OF COMPOUND IN DMSO-D_6

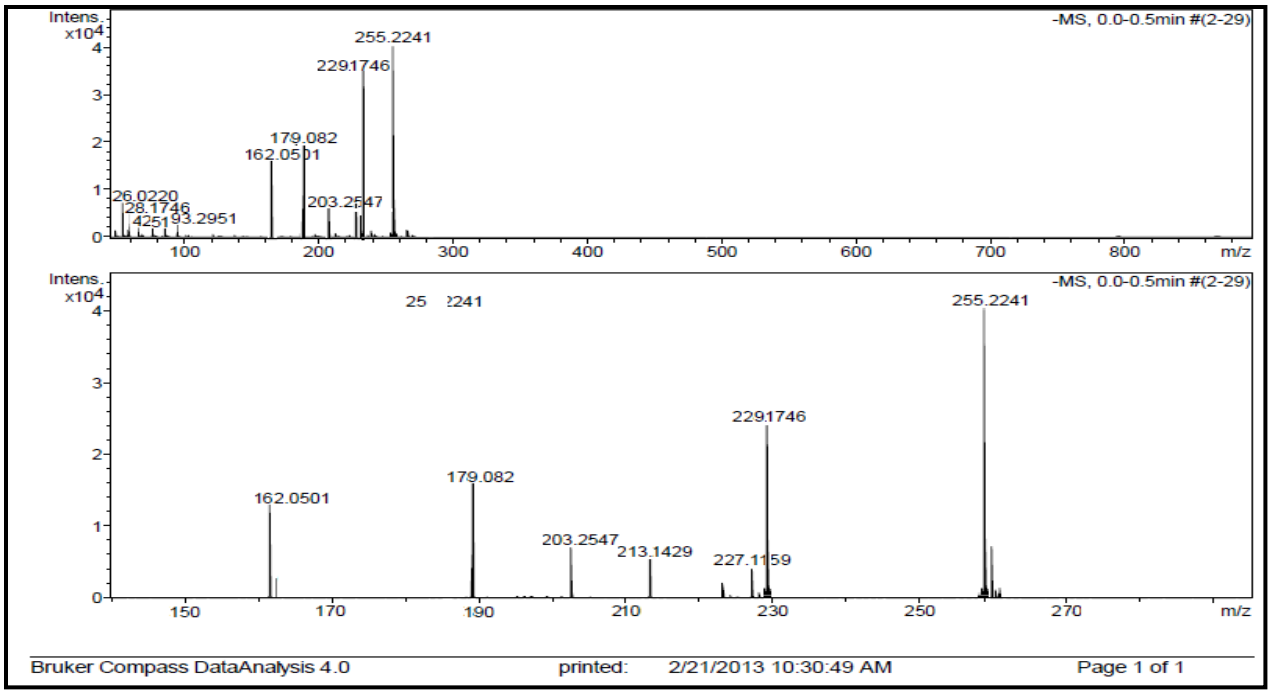


FIG. 3: EI-MS OF FLAVONOID COMPOUND

DISCUSSION: Different non polar and polar solvents such as cyclohexane, benzene, chloroform, n-butanol, acetone, methanol and water in different combinations were used to carry out TLC. Except Benzene: Acetone (9:1), no other solvent system gave successful results in chromatographic separation of the components. The successful separation of active constituents by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coefficient for each target compound[25]. TLC chromatogram of a specific pool of column chromatography eluted plant extracts showed the presence of flavonids because they have shown yellow fluorescence in UV light at 365 nm after spraying with ammonium vanadate solution. There after the re-chromatogarphed and crystallized flavonoid fraction was used for the following studies for structure elucidation.

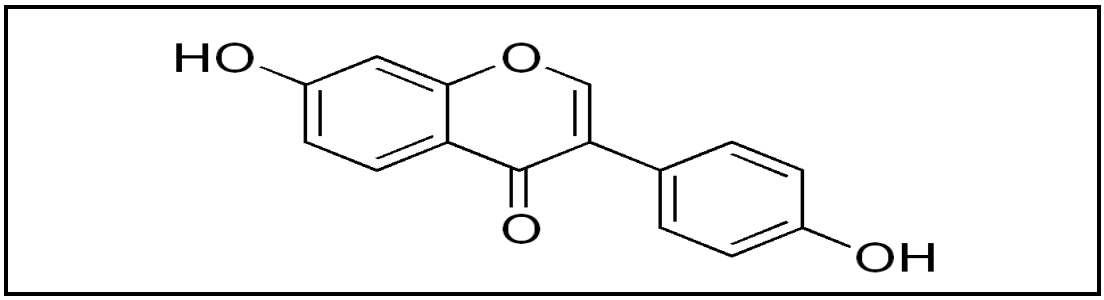
Isolated compound was identified by various analytical methods and chemical test. Compound was obtained as yellowish colored crystalline solid with melting point of 315-320^{o}C

The UV spectrum of compound in methanol exhibited the absorption maxima at 255 nm and 300 nm which are characteristics for an isoflavone structure.The FT - IR spectra interprets the compound having phenolic group, alkyl side chain with isopropyl group may be a phenolic compound (e.g. ferulic acid, caffeic acid), flavanone, flavone (chrisyn, quercetin) which can be confirmed by further spectral analysis.

The spectral analysis of the flavonoid fraction was carried out by comparing with previous published literatures[21] following remarks were manifested. The absorbance bands at 3146.17 cm^{-1}, 2901.29 cm^{-1}, and 2663.44 cm^{-1} possibly due to the presence of –OH stretch of phenols or alcohols, C–H stretching vibration of alkenes and alkanes respectively. 2338.41 cm^{-1} is a carbonyl range give idea about O distribution. The strong stretching band at 1741.35 cm^{-1} indicates the presence of carbonyl group (C=O) and 1504.69 cm^{-1} showed O-H peak for carboxylic acid in plane banding. also other inferences in favor of the aromatic structure we had drawn from the band at 1340.92 cm^{-1}, 854.74 cm^{-1} and 739.33 cm^{-1} which were represented as C–H deformation in methyl moiety of aromatic nucleus, C–H deformation in aromatic ring and for the presence of a di-substituted (meta) benzene ring respectively.

A strong broad band appeared at 1220.38cm^{-1} represents the characteristic band for C–O stretch of phenol. A band at 1102.55cm^{-1} can be interpreted for alkyl C–O stretch of ethers. Mild band at 660.09 cm^{-1} can be identified for C–H out of plane deformation in alkene **(Table: 1).**

This study suggest the following possible structures-



Or

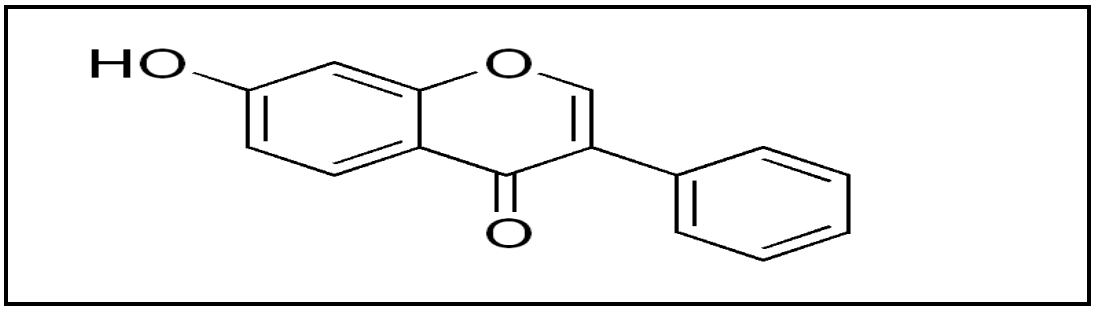
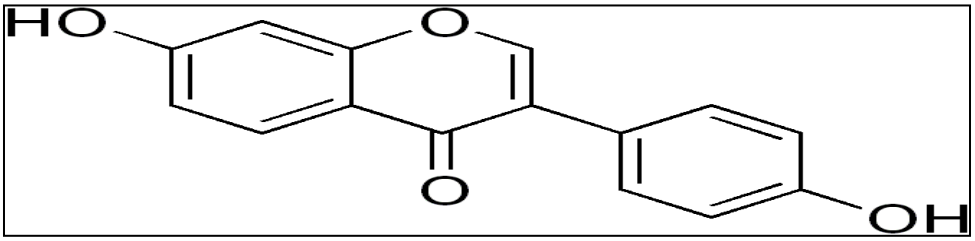


TABLE 1: INTERPRETATION FROM FTIR INTERPRETATIONS OF FLAVONOID COMPOUND

Peak at	Name of Peak	Possible Groups	Intensity
2338.41	Carbonyl range	O-disubstituted	m-s
3146.17	=C-H	alkenes and aromatics	m
2901.29	C-H	alkanes	m-s
2663.44	C-H	alkanes	m-s
2663.44	C-H	alkanes	m-s
2663.44	C-H	alkanes	m-s
1741.35	C=O	carboxylic acids	s
1504.69	O-H	carboxylic acid (in plane bending)	
1340.92	C-H	alkanes, isopropyl group (2 peaks)	
1220.38	sp2 C-O (stretch)	Phenol, usually 1230 (may be split)	s
1102.55	sp3 C - O	Ester (alkyl C-O)	mod
	C-O-C	ethers	vs-s
854.74	Ar	H 2 adjacent hydrogens	
739.33	Ar	H 4 or 5 adjacent hydrogens	Ar
660.09	=C H	bend Cis alkene	s =C

Position of Phenolic hydroxyl group may change, and is confirmed by ^{1}H NMR.

^{1}H NMR study suggest the presence of two phenyl ring with phenolic hydroxyl group (broad peak), one hydroxyl group is at para position to the keto group and one hydroxyl group is at p position to the ester group and by combining with IR spectra the most probable structure is –



(Molecular Weight: 254)

TABLE: 2 INTERPRETATIONS FROM EI-MS OF FLAVONOID COMPOUND

S.No.	Peak/M.W.	Name	Remark	M+1 peaks
1.	26	C(2)H(2)		26.0220
2.	28	Carbonmonooxide		28.1722
3.	42	CH(2)=C=O		42
4.	273	C(14)H(9)0(6)	A,B and C excluding methoxy group with carbon at A	274.2056
5.	274	C(14)H(10)0(6)	A,B and C excluding hydroxyl group with carbon and CH at C	275.2146
6.	285	C(15)H(9)0(6)	At A,B and C excluding methoxy at C nucleus	286.2187
7.	288	C(15)H(12)0(6)	At A,B and C excluding CO at C	289.2551
8.	290	C(14)H(10)O(7)	At A,B and open C nucleus without C(2)H(2)	291.2246
9.	299	C(16)H(11)0(6)	At A,B and C excluding para hydroxy at C nucleus	291.2246
10.	299	C(16)H(11)0(6)	At A,B and C excluding hydroxy at B nucleus	299.1450
11.	299	C(16)H(11)0(6)	At A,B and C excluding ortho hydroxy at C nucleus	299.2501
12.	299	C(16)H(11)0(6)	At A,B and C excluding hydroxy at A	302.1915
13.	301	C(15)H(9)0(7)	At A,B and C excluding para hydroxy at C	302.1915
14.	316	C(16)H(12)O(7)	Molecular Ion	317.1761

This structure is of Diadzein and can be confirmed by Mass Spectroscopy according to fragmentation pattern and molecular weight by using molecular ion peak.

Fragmentation pattern is similar to Flavones and Isoflavones and molecular wt can be confirmed by first molecular ion peak. The peak results are in M+1 form, the exact peaks will be as the followings- molecular ion peak (255) will be at 254 of $C_{(15)}H_{(10)}0_{(4)}$ and the main fragment ion peak (162) will be at 161of $C_{(9)}H_{(5)}0_{(3)}$. After comparing all data with the spectra library of flavonoids, daidzein and published literature of daidzein[21], it was confirmed as daidzein (Table: 2).

CONCLUSION: We have successfully isolated daidzein from the bark of plant of *Acacia arabica* L., which was previously been reported from the fruits[21]. Therefore, from this study it can be concluded that the bark can be used as a source of daidzein which has several bio-active characteristics, out of which antioxidant activities and its related activities are predominant.

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