IJP (2021), Vol. 8, Issue 2 **(Research Article)**

Received on 15 December 2020; received in revised form, 20 February 2021; accepted, 25 February 2021; published 28 February 2021

ISOLATION AND CHARACTERIZATION OF ACTIVE CONSTITUENTS FROM PLANT *PISONIA ACULEATA* LINN BY SPECTRAL ANALYSIS

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Keywords:

MPA, Rf values, UV, ^{1}H-NMR, 13C-NMR, EIMS l

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ABSTRACT: Botanicals and herbal preparations for medicinal usage contain various types of bioactive compounds. The focus of this paper is on the analysis of bioactive compounds present in the *Pisonea aculeata* Linn. having anticancer activity involving the applications of chromatographic techniques such as TLC and column chromatography, Spectrophotometric techniques such as UV, ^{1}H-NMR, ^{13}C-NMR, and EIMS spectral studies. The present investigation was carried out by fractionating the Methanolic extract of *Pisonia aculeata* (MPA) using benzene, diethyl ether, and ethyl acetate. Ethyl acetate fraction on concentration subjected to separation and purification on column chromatography. Fractions were collected & homogeneity was examined on TLC. A Fraction 77-90 on concentration yielded a pure yellowish homogeneous solid and gave dark green coloration with neutral ferric chloride and violet coloration with Molish's reagent. In order to find out the nature of the glycoside, compound I was subjected to acid hydrolysis. The basic flavonoid structure of the compound studied by recording UV spectrophotometer showed an intense UV maxima at 273 nm (band II) and 329 nm (band I), indicating the flavone nature of it. The ^{1}H-NMR spectrum of compound I was recorded using AMX 400 (400 MHz) spectrometer using DMSO-d6 as the solvent showed a pair of doublets in the aromatic region at d 7.89 ppm and d 6.93 ppm. Electron Impact – Mass Spectrometry (EI-MS) of the compound exhibited M+ at m/z 330. Thus based on the R_f values, UV, ^{1}H-NMR, ^{13}C-NMR, and EIMS spectral studies, the structure of the compound has identified as 5, 4¢-Dihydroxy 6,8-dimethoxy 7-O-rhamnosyl flavones.

INTRODUCTION: *Pisonia aculeata* Linn. (Nyctaginaceae) is a large scandent shrub, which holds an important place in folklore medicine. It is extensively used by native medical practitioners and tribes for treating swelling, rheumatic pains, jaundice, and tumors.

QUICK RESPONSE CODE	DOI: 10.13040/IJPSR.0975-8232.IJP.8(2).82-88
	The article can be accessed online on www.ijpjournal.com
DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.IJP.8(2).82-88	

Preliminary phytochemical screening of the extract showed the presence of alkaloids, triterpenes, phenolic compounds, flavonoids, and glycosides.

However, no studies to date have been able to demonstrate the isolation and characterization of active constituents. *Pisonia aculeata* was fractionated using benzene, diethyl ether, and ethyl acetate. Ethyl acetate fraction on concentration yielded a yellow solid, which was non-homogenous in TLC. Hence subjected to separation and purification on column chromatography in that silica gel column (60-120 mesh, 300 gm, 100 ´ 5 cm) solvents of increasing polarity. Fractions were

collected & homogeneity was examined on TLC. A Fraction 77-90 on concentration yielded a pure yellowish homogeneous solid and was designated as compound I.

It gave dark green coloration with neutral ferric chloride and violet coloration with Molish's reagent. In order to find out the nature of the glycoside, compound I was subjected to acid hydrolysis. The basic flavonoid structure of compound I and position of attachment of hydroxyl and other substituents were conveniently studied by recording UV spectrophotometer (Shimadzu 1601) in MeOH as well as in various Shift reagents. And the l max values were determined.

The compound was crystallized from alcohol as a pale yellow amorphous powder. It showed intense UV maxima at 273 nm (band II) and 329 nm (band I), indicating the flavone nature of it. Band I underwent a significant bathochromic shift of +60 nm on addition of NaOMe which suggested the presence of free 4¢-OH group in ring B. Absence of characteristic bathochromic shift (5-20 nm) on addition of NaOAc suggested that C-7 was not free. The absence of characteristic bathochromic shift on addition of NaOAc/H_3BO_3 indicated the absence of O-dihydroxy substituent in ring B. A consistent bathochromic shift of band II (14 nm) with $AlCl_3$/ HCl indicated the presence of hydroxyl substituent at C-5 along with oxygen at C-6 position.

The ^{1}H-NMR spectrum of compound I was recorded using AMX 400 (400 MHz) spectrometer (Plate 1) using DMSO-d6 as the solvent, and a complete assignment of protons were determined. A 13C-NMR spectrum of compound I was recorded using AMX 400 (100 MHz) spectrometer (Plate 2) using DMSO-d6 as the solvent, and the complete assignment of carbon was determined. In the ^{1}H-NMR spectrum of the compound, I showed a pair of doublets in the aromatic region at d 7.89 ppm and d 6.93 ppm each integrating two protons indicated the presence of two A2 B2 pattern due to protons a C-3¢, C-5¢and C-2¢, C-6¢ respectively of ring B of flavone. This was supported by the UV shift experiments and ^{13}C-NMR values.

Violet colouration of the compound with Molisch's reagent indicated the presence of glycoside moiety. The position of glycosylation at C-7 as indicated by UV studies was confirmed by the presence of anomeric proton signal displayed at 5.15 ppm [For C-3 anomeric proton appears at d 5.8 ppm]. Rhamnosyl nature of the sugar and its attachment to C-7 carbon was confirmed by acid hydrolysis and ^{1}H-NMR studies. The ^{1}H-NMR spectrum also showed one singlet at d 6.2 ppm corresponding to C-3 proton of the flavone skeleton, which is also supported by the ^{13}C-NMR signals at d 164. 9 (C-2), 102.3 (C-3), and a quaternary signal at 180.9 (C-4). The absence of other characteristic signals in the aromatic region of the ^{1}H-NMR spectrum suggested that all the carbon atoms of ring A are substituted.

The 5-hydroxy and C-6, C-7, and C-8 substitution of ring A is further supported by the 13C-NMR values at d 157.6 (C-5), 161.1(C-7), 131.5(C-6), and 128.6 ppm (C-8). The absence of signals for H-6 and H-8 in ^{1}H-NMR, the downfield shift of C-6 and C-8 in ^{13}C-NMR4 and the appearance of two methoxyl signals at d 60.1 and d 56.1 ppm suggested the possibility of substitution of C-6 and C-8 by methoxyl groups.

Electron Impact – Mass Spectrometry (EI-MS) of the compound exhibited M+ at m/z 330. Fragment ion at m/z 212 and m/z 118 consistent with retero-Diel's Alder fragmentation. Fragment ion at m/z 118 confirmed the presence of C-4¢ hydroxyl group in ring B. Thus based on the R_f values, UV, ^{1}H-NMR, 13C-NMR, and EIMS spectral studies, the structure of compound has identified as 5, 4¢-Dihydroxy 6,8-dimethoxy 7-O-rhamnosyl flavones.

MATERIALS AND METHODS:

Isolation of Compound: The concentrate of the alcohol extract of *Pisonia aculeata* was fractionated using benzene (3 × 300 ml), diethyl ether (3 × 300 ml), and ethyl acetate (4 × 300 ml). The ethyl acetate fraction on concentration yielded a yellow solid, which was non-homogenous in TLC and hence was further subjected to separation and purification on column chromatography.

Column Chromatographic Analysis: The residue obtained from the ethyl acetate fraction (15 g) of *Pisonia aculeata* was chromatographed in silica gel column (60-120 mesh, 300 gm, 100 × 5 cm) using gradient elution with the solvents of increasing polarity. Fractions of 100 ml were collected each time, and the homogeneity was examined on TLC with suitable solvents. The details of the

fractionations and their characteristics are given in **Table 1**. Fractions 1-5, 6-10, 11-48, 49-64 and 65-76 each of which on concentration yielded residues with varying intensities of yellow colour. These were tested individually by TLC, and further purification was not carried out because of the paucity of the samples. Fractions 77-90 on concentration yielded a pure yellowish homogeneous solid and was designated as compound I. It gave dark green colouration with neutral ferric chloride and violet colouration with Molish's reagent. The R_f values of compound I in various solvent systems are given in **Table 2**.

Acid Hydrolysis of Compound I: In order to find out the nature of the glycoside, compound I was subjected to acid hydrolysis. To a solution of the glycoside (10 mg) in hot methanol (10 ml), an equal volume of H_2SO_4 (7%) was added, and the mixture was gently refluxed at 100 ºC for 2 h. The excess of alcohol was distilled off in vacuo, and the resulting aqueous solution was partitioned with ether to separate the ether soluble aglycone and the aqueous sugar.

Identification of the Sugar: The aqueous layer was treated with $BaCO_3$ to remove excess sulphuric acid, and the barium sulphate formed was filtered off using Whatman no. 42 filter paper, and the filtrate (sugar portion) was concentrated. The concentrate was analysed by Paper chromatography (PC) with various authentic sugar samples on a Whatman no.1 filter paper strip and identified using Aniline hydrogen phthalate spray reagent (prepared by dissolving 9.2 ml of aniline and 16 g of phthalic acid in 490 ml of n-butanol, 490 ml of ether and 20 ml of water). The various solvent systems used for PC and Rf values of the identified sugar in these solvent systems are presented in **Table 3**.

UV Spectral Characteristics of Compound I: Basic flavonoid structure of compound I and position of attachment of hydroxy and other substituents were conveniently studied by recording UV spectrophotometer (Shimadzu 1601) in MeOH as well as in various Shift reagents. The λ max values are given in **Table 4**.

^{1}H-NMR Spectral data of compound I: The ^{1}H-NMR spectrum of compound I was recorded using AMX 400 (400 MHz) spectrometer (Plate 1) using DMSO-d6 as the solvent and complete assignment of protons are shown in **Table 5**.

^{13}C-NMR Spectral Data of Compound I: ^{13}C-NMR spectrum of compound I was recorded using AMX 400 (100 MHz) spectrometer (Plate 2) using DMSO-d6 as the solvent, and the complete assignment of carbon are given in **Table 6**.

EI-MS Study of Compound I: EI-MS spectrum (Fenniganmat 8230, 70 eV) of compound I (Plate 3) was taken, and it gave various fragments at m/z: 330, 213, and 118. The compound was crystallized from alcohol as a pale yellow amorphous powder. It showed intense UV maxima at 273 nm (band II) and 329 nm (band I), indicating the flavone nature of it. Band I underwent a significant bathochromic shift of + 60 nm on addition of NaOMe which suggested the presence of free 4¢-OH group in ring B. Absence of characteristic bathochromic shift (5-20 nm) on addition of NaOAc suggested that C-7 was not free.

The absence of characteristic bathochromic shift on the addition of NaOAc/H_3BO_3 indicated the absence of O-dihydroxy substituent in ring B. A consistent bathochromic shift of band II (14 nm) with $AlCl_3$/ HCl indicated the presence of hydroxyl substituent at C-5 along with oxygen at the C-6 position (Mabry TJ, *et al.,* 1970). In the 1H-NMR spectrum of a compound, I showed a pair of doublets in the aromatic region at d 7.89 ppm and d 6.93 ppm each integrating two protons indicated the presence of two A2 B2 pattern due to protons a C-3¢, C-5¢ and C-2¢, C-6¢ respectively of ring B of flavone. This was supported by the UV shift experiments and ^{13}C-NMR values (Faini FA, *et al.,* 1982). Violet colouration of a compound with Molisch's reagent indicated the presence of glycoside moiety. The position of glycosylation at C-7 as indicated by UV studies was confirmed by the presence of anomeric proton signal displayed at 5.15 ppm [For C-3 anomeric proton appears at d 5.8 ppm] (Faini FA, *et al.,* 1982) **Fig. 2**. Rhamnosyl nature of the sugar and its attachment to C-7 carbon was confirmed by acid hydrolysis and ^{1}H-NMR studies.

The ^{1}H-NMR spectrum also showed one singlet at d 6.2 ppm corresponding to C-3 proton of the flavone skeleton, which is also supported by the

^{13}C-NMR signals at d 164.9 (C-2), 102.3 (C-3), and a quaternary signal at 180.9 (C-4) (Agarwal PK, 1989). The absence of other characteristic signals in the aromatic region of the ^{1}H-NMR spectrum suggested that all the carbon atoms of ring A are substituted. The 5-hydroxy and C-6, C-7 and C-8 substitution of ring A is further supported by the ^{13}C-NMR values at d 157.6 (C-5), 161.1(C-7), 131.5(C-6) and 128.6 ppm (C-8) (Agarwal PK, 1989). The absence of signals for H-6 and H-8 in ^{1}H-NMR, the downfield shift of C-6 and C-8 in ^{13}C-NMR4 and the appearance of two methoxyl signals at d 60.1 and d 56.1 ppm suggested the possibility of substitution of C-6 and C-8 by methoxyl groups.

EI-MS of the compound exhibited M+ m/z 330 and fragment ion at m/z 212 and m/z 118 consistent with retero-Diel's Alder fragmentation, and a fragment ion at m/z 118 confirmed the presence of C-4¢ hydroxyl group in ring B. Thus based on the R_f values, UV, 1H-NMR, ^{13}C-NMR, and EIMS spectral studies, the structure of the compound has identified as 5, 4¢-dihydroxy 6, 8-dimethoxy flavone skeleton **Table 1-6, Fig. 1-4.**

RESULTS:

TABLE 1: CHROMATOGRAPHIC FRACTIONS OF ETHYL ACETATE CONCENTRATE OF MPA

Fractions Collected	% Eluent Composition	Remarks
1-5	100 Petroleum ether	Yellow waxy substance
6-10	90/10 Pet. Ether/benzene	Yellow waxy substance
11-48	80/20 to 10/90 Pet. Ether/benzene	Pale yellow solid
49-64	100 benzene	Pale yellow solid
65-76	90/5 benzene /EtOAc	Yellow solid
77-90	80/20 benzene /EtOAc	Yellow solid
91-104	70/30 benzene /EtOAc	Yellow-brown solid

TABLE 2: RF 100 VALUES OF COMPOUND IN PAPER CHROMATOGRAPHY

Compound I	Solvent System						
	15% HOAc	30% HOAc	50% HOAc	60% HOAc	BAW*	Forestal#	PhOH+
	11.26	31.34	78.76	83.60	20.83	93.84	34.42

*BAW – n- butanol: acetic acid: water (4:1:5) m#Forestal- Acetic acid: Conc HCl: H2O (30:3:10) +PhOH- Phenol saturated with water (3:1)

TABLE 3: RF 100 VALUES OF SUGAR OF COMPOUND

Sugar	Developing Solvents			
	BAW*	PhOH+	Forestal#	EtOAc: Pyridine: H_2O 10:4:3
Sugar from compound I	38	55	58	54
Authentic Rhamnose	37	55	59	55

* BAW – n- butanol: acetic acid: water (4:1:5) + PhOH- Phenol saturated with water (3:1) #Forestal- Acetic acid: Conc HCl: H_2O (30:3:10)

TABLE 4: L_{MAX} VALUES OF COMPOUND

Solvent / Shift Reagents	λ_{max} (nm)
MeOH	273, 329
+NaOMe	273, 340, 389
+NaOAc	273, 313
+NaOAc +H_3BO_3	272, 329
+$AlCl_3$	270, 348
+$AlCl_3$ + HCl	270, 300, 315, 347

TABLE 5: ^{1}H-NMR DATA OF COMPOUND IN DMSO-D6

δ H (ppm)	Signal Assignment
12.95	1 H, s, 5-OH
7.89	2H, d, (J=8.7 Hz), H-2′ 6′
6.93	2H, d, (J=8.7 Hz), H-3′ 5′
6.38	1H, s, H-3
5.15	1H, d, (J=2 Hz), H-1 of rhamnose
3.9	3H, s, OCH_3 group
3.8	3H, s, OCH_3 group
3.0 - 3.75	Sugar protons
1.2	3H, d, J=6 Hz, H-6″

TABLE 6: 13C-NMR DATA OF COMPOUND IN DMSO - D6

C	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
Ppm	164.9	102.3	180.9	157.6	131.5	161.1	128.6	148.0	105.5

C	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
Ppm	120.0	127.9	115.9	160.5	115.9	127.9

C	C-1″	C-2″	C-3″	C-4″	C-5″	C-6″	OCH_3	OCH_3
Ppm	98.4	70.1	69.9	71.8	69.9	18.7	60.1	56.1

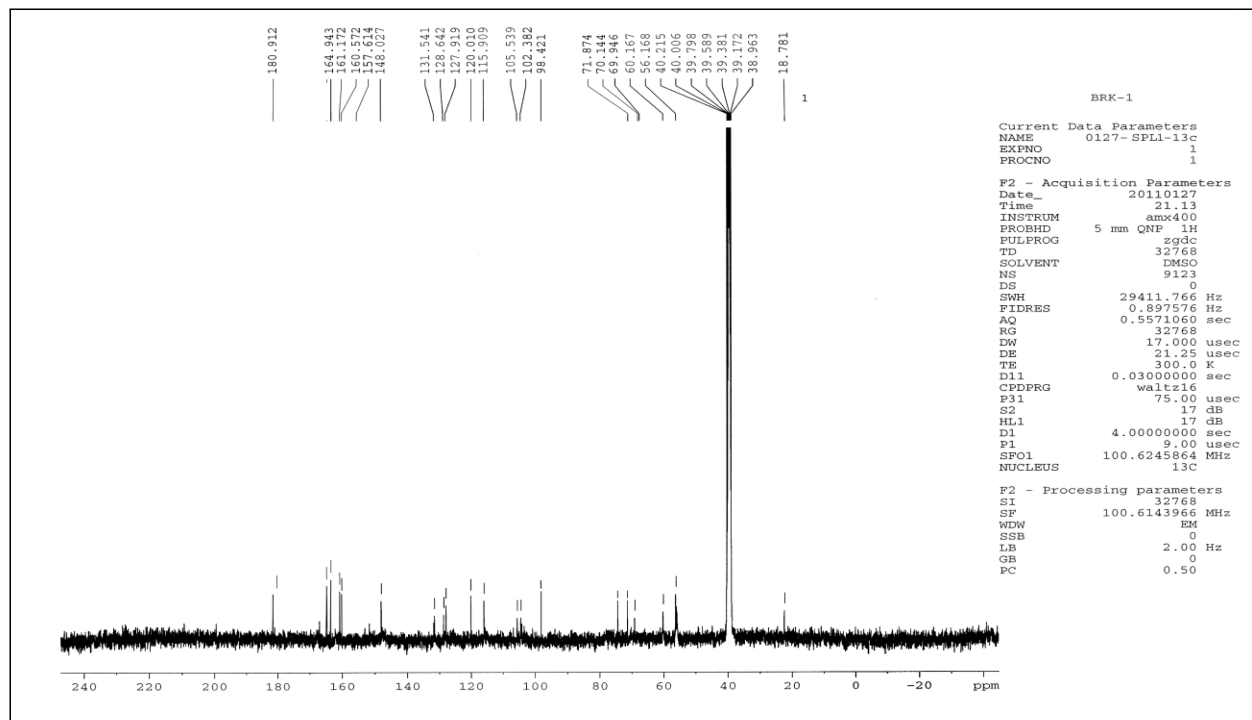

FIG. 1: BRK C1

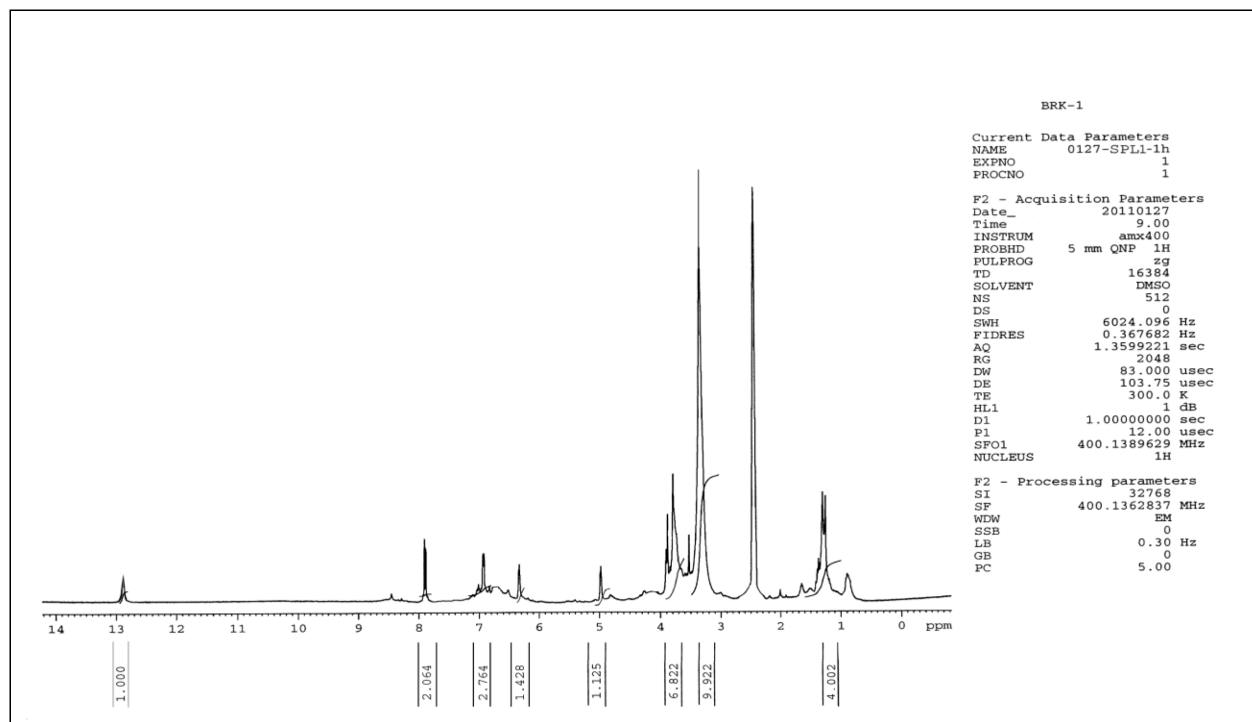

FIG. 2: BRK P1

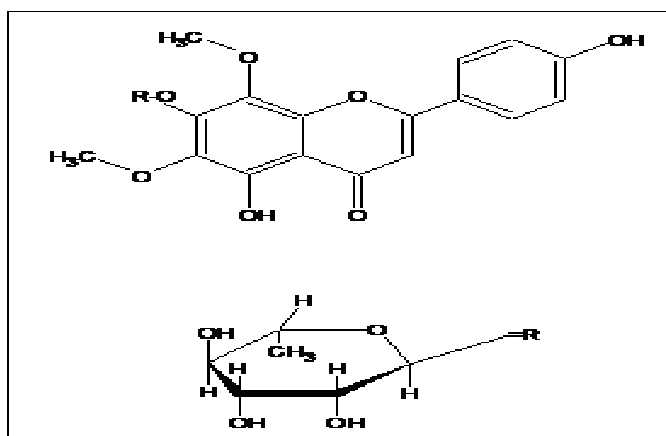

FIG. 3: 5, 4¢-DIHYDROXY 6, 8-DIMETHOXY 7-O-RHAMNOSYL FLAVONE

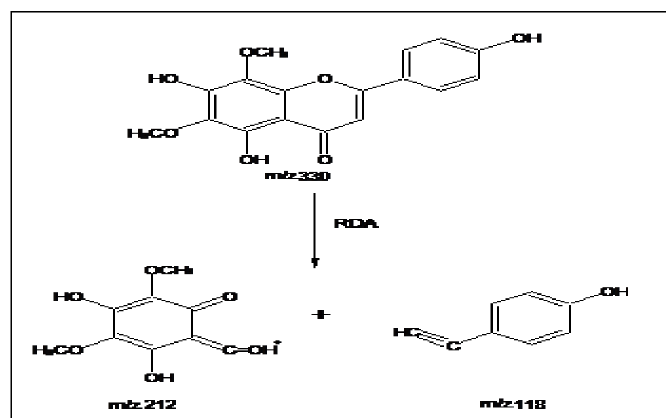

FIG. 4: EI-MS FRAGMENTATION

DISCUSSION: The focus of this paper is on the analysis of bioactive compounds present in the *Pisonea aculeata* Linn. Having anticancer activity involving the applications of chromatographic techniques such as TLC and column chromatography, Spectrophotometric techniques such as UV, 1H-NMR, ^{13}C-NMR and EIMS spectral studies. The present investigation was carried out by fractionating the Methanolic extract of *Pisonia aculeata* (MPA) using benzene, diethyl ether, and ethyl acetate.

Ethyl acetate fraction on concentration subjected to separation and purification on column chromato-graphy. Fractions were collected & homogeneity was examined on TLC. A Fraction 77-90 on concentration yielded a pure yellowish homogeneous solid and gave dark green coloration with neutral ferric chloride and violet coloration with Molish's reagent. In order to find out the nature of the glycoside, compound I was subjected to acid hydrolysis. The basic flavonoid structure of compound studied by recording UV spectro-photometer showed an intense UV maxima at 273 nm (band II) and 329 nm (band I), indicating the flavone nature of it. The 1H-NMR spectrum of compound I was recorded using AMX 400 (400 MHz) spectrometer using DMSO-d6 as the solvent showed a pair of doublets in the aromatic region at d 7.89 ppm and d 6.93 ppm. Electron Impact – Mass Spectrometry (EI-MS) of the compound exhibited M+ at m/z 330.

CONCLUSION: In the present study, based on the R_f values, UV, ^{1}H-NMR, ^{13}C-NMR, and EIMS spectral studies, the structure of the compound has been identified as 5, 4¢-Dihydroxy 6, 8-dimethoxy 7-O-rhamnosyl flavones from leaves of *P. aculeate*. The fact that these are the active compounds responsible for anticancer activity is evident from previous cytotoxic studies of the extract. These isolated compounds could also be active as an anticancer drug by studying the different human cancer cell lines.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: Nil

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How to cite this article:
Ghode SP, Ghode PD, Chatur VM and Kolhe R: Isolation and characterization of active constituents from plant *Pisonia aculeata* linn by spectral analysis. Int J Pharmacognosy 2021; 8(2): 82-88. doi link: http://dx.doi.org/10.13040/IJPSR.0975-8232.IJP.8(2).82-88.

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