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Research Article

ISOLATION AND CHARACTERIZATION OF A FLAVONOID FROM ETHANOLIC EXTRACT OF *ALTERNANTHERA SESSILIS* LINN.

Mrinmay Das*[1], Ashok Kumar D[2], Jyotirmoy Deb[3]and Durga Srinivasa Rao[3]

[1] PRIST University, Vallam, Thanjavur, Tamilnadu-613403

[2] Department of Pharmacy, Pratistha Institute of Pharmaceutical Sciences, Suryapet Dist, Andhra Pradesh-508214.

[3] Department of Pharmacy, S. Chaavan College of Pharmacy, Jangalakandriga (Vi), Nellore Dist, Andhra Pradesh-524346.

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ABSTRACT

This study was performed to isolate and characterize the flavonoid present in *Alternanthera sessilis* Linn. Direct soxhlet extraction process was adopted for extraction by using 95% ethanol and vacuum evaporator was used for drying the extract. The concentrated ethanolic fraction was subjected to thin layer chromatography and column chromatography for isolation. The isolated compound was identified as a flavonoid by confirming the standard flavonoid tests: viz. Shinoda's test. The R_f value of isolated flavonoid was calculate and different physical tests were also performed to find out its physical characteristics. Characterization of isolated flavonoid was done by FTIR, ^{1}H NMR and MASS. The IR spectrum indicated the presence of hydroxyl and carbonyl functions. ^{13}C NMR signal indicated the presence of hydroxyl group and unsaturated keto function and four methoxy group at different position of flavones skeleton. The ^{1}H-NMR further showed the presence of three hydroxyl, four methoxy groups and three methine groups. On the basis of chemical and spectral analysis the structure was elucidated as 3´, 3, 6, 7-tetramethoxy - 4´, 5, 8-trihydroxy flavones.

Keywords: Flavonoids, TLC, Column Chromatography, FTIR, NMR, MASS.

INTRODUCTION:

The bioactive compounds are mostly plant secondary metabolites, which become medicine after processing to pure compounds; some are very useful dietary supplements, and many useful commercial products. Further modification of the active compounds lead to enhance the biological profiles and a large number of such compounds which are approved or undergoing clinical trials for clinical uses against different diseases like pulmonary diseases, cancer, HIV/AIDS, malaria, Alzheimer's and other diseases [1, 2]. Crude herbs are used as drugs in different country of the world and therefore it take a basic part of many traditional medicines worldwide. In Asia, traditional Chinese medicine (TCM), Korean Chinese medicine, Japanese Chinese medicine (kampo), ayurvedic medicine (India) and jamu (Indonesia), phytotherapy and homeopathy in Europe, alternative medicines are typically named when herbal therapies use with various other traditional remedies in America. Integrative medicine came into being when the alternative medicine, mainly the aforementioned traditional and folk medicines used worldwide, with conventional medicine (Western medicine). In recent years, the popularity of complementary medicine has increased.

Flavonoids are secondary metabolites Characterized by flavan nucleus [3] and a C_6-C_3-C_6 carbon skeleton. These are group of structurally related compounds with a chromane - type Skeleton having phenyl substituent in C_2 - C_3 position. The flavonoids belong to one of the most bioactive compounds which naturally exist in the plant kingdom. Till now, over 8000 varieties of flavonoids have been identified [4]. Different naturally occurring flavonoids have been described and sub-categorized into flavones, flavans, flavanones, isoflavonoids, chalcones, aurones and anthocyanidines. These flavonoids have

*Corresponding author: Mrinmay Das | E-mail:

remarkable biological activities, including inhibitory effects on enzymes, modulatory effect on some cell types and protection against allergies, antiviral, anti-malarial, anti inflammatory and anti-carcinogenic properties. A number of flavones, flavonols, flavanones, and isoflavones, as well as some of their methoxy, isoprenyl, and acylated derivatives, show antibacterial activity [5]. Flavonoids are major components of medicinal plants and have been used in traditional medicine around the world.

Flavonoids are phenolic compounds which are widely distributed in plants, and have been reported to exert multiple biological effects, including antioxidant, free radicals scavenging abilities anti- inflammatory and anti-carcinogenic activity [6-8].

The process of separation of the individual components of a mixture based on their relative affinities towards stationary and mobile phases is called as chromatography. The identification, separation and purification of plant constituents are mainly carried out using one or a combination of chromatographic techniques.

The IR region is divided into three regions: the near, mid, and far IR. Infrared radiation is absorbed by organic molecules and converted into energy of molecular vibration. The wave numbers (sometimes referred to as frequencies) at which an organic molecule absorbs radiation give information on functional groups present in the molecule [9].

Nuclear magnetic resonance, induces changes in the magnetic properties of certain atomic nuclei, notably that of hydrogen. NMR spectroscopy is used to investigate the properties of organic molecules and provide detailed information about the structure, dynamics, reaction state and chemical environment of molecules [10].

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances [11].

Alternanthera sessilis Linn. (Amaranthaceae) is an annual or perennial prostate herb with several spreading branches, bearing short petioled simple leaves and small white flowers, found throughout the hotter part of India, ascending to an altitude of 1200m [12]. The plant spreads by seeds, which are wind and water-dispersed and by rooting at stem nodes. Young shoots and leaves are eaten as a vegetable in Southeast Asia [13]. It is a weed of rice throughout tropical regions and of other cereal crops, sugarcane and bananas. Although it is a weed, it has many utilities. The leaves were used in eye diseases, cuts, wounds and antidote to snake bite; skin diseases [14].

It is also reported about the wound healing property of *Alternanthera sessilis* Linn. [15]. The degenerative and necrotic changes in the liver and kidney in Swiss mice, caused by oral administration of water extract of *A. sessilis* in high doses through histopathological test were revealed [16].

MATERIALS AND METHODS:

Plant material

The plant was identified by the Botanist of VR College, Nellore, Andhra Pradesh. After authentication the fresh aerial parts were collected from rural belt of Jangalakandriga village, Nellore, Andhra Pradesh. The plants were washed properly, shade dried and then milled to coarse powder by a mechanical grinder. The crude powder drug was kept in air tight container for further use.

Preparation of extract

The powdered plant material was defatted with petroleum ether (60-80°C) and then extracted with 95% ethanol using Soxhlet apparatus. The solvent was removed under reduced pressure, which gave a greenish-black coloured sticky residue (yield- 14.8% w/w on dried material basis). Preliminary phytochemical screening [17] of the extract gave positive tests for presence of alkaloids, flavonoids, triterpenoids, glycosides, tannins, amino acids and saponins.

Identification of phytoconstituents by TLC

In thin layer chromatography technique the extract was dissolved in solvent and mixed thoroughly. The mixture was then used for spotting in the TLC plates. The plates are prepared

by using adsorbent like Silica gel G. A fine capillary had been used for spotting. The spot was done on the TLC plate near about 1cm above from the bottom of the plate. The plate was then dried and kept in developing chamber containing suitable solvent systems. After a proper running period the plate(s) were removed and dried in the air and spraying reagent was used to locate the spot. The R_f value was calculated. Different solvents were used in different ratios and TLC had been carried out to confirm the presence of different mixtures of phytoconstituents in the extract [18-20].

$$R_f\ value = \frac{Distance\ travelled\ by\ solute}{Distance\ travelled\ by\ solvent\ front}$$

Separation of Phytoconstituents by Column Chromatography

In this technique the stationary phase is solid and the mobile phase is liquid. The separation takes place when the component of two or more compound mixture is more strongly adsorbed than the other by the solid stationary phase. The isolation of active constituent was performed by using the Column chromatography technique [21].

The adsorbent was dissolved in chloroform to make slurry poured in to a column up to ¾th level. The solvent were continuously run to get proper packing. Then the sample was packed as slurry with the same solvent [22]. Mobile phase was poured on the column bed to make the column to settle properly. Then sample was mixed with chloroform and poured in to the column. Different solvent systems of n-Hexane, Benzene, Chloroform and Ethanol in different ratios were used for the elution of phytoconstituents. The fractions of 200 ml were collected each time. Detection of the component was done by monitoring each fraction by TLC. The fraction details are tabulated in Table 1.

Confirmation of constituents by using Thin Layer Chromatography

The fractions were collected and the residue of fraction was obtained each time by evaporating the solvent by using rotary vacuum evaporator and tested for the components by using Thin Layer Chromatography. TLC spot was identified by spraying 5% w/v alcoholic solution of H_2SO_4 as a spraying reagent. The sprayed plates were heated at 100°C for 5-10 min and the numbers of constituents present in the each fraction were found.

Isolation of phytoconstituents from EEAS:

The Chloroform-Ethanol 60:40 ratios gives the fractions 102 to 107, were found to be similar and showed a single spot. Thus they were mixed and recrystallized from ethanol as colourless powder which shown a melting point of 178-180^{0}C (104 mg). It light dark green colour with ferric chloride, pink colour in Shinoda's test suggesting that it was a flavone. The isolated compound was designated as **EEAS-I**. The physical characteristic of the compound was tabulated in Table 2. The TLC solvent system and R_f value of the isolated compound EEAS-I is given in the Table 3.

CHARACTERIZATION OF ISOLATED COMPOUND:

IR spectrum of EEAS-I

The IR region is divided into three regions: the near, mid, and far IR. The mid IR region is of greatest practical use to the organic chemist. This is the region of wavelengths between 3 x 10^{-4} and 3 x 10^{-3} cm. In wave numbers, the mid IR range is 4000–400 cm^{-1}. Infrared radiation is absorbed by organic molecules and converted into energy of molecular vibration.

The IR spectrum of isolated compound EEAS-1 had shown absorption bands at 3608.9 to 3315.7 (O-H, free hydroxyl group), 2953.1 (Cyclic C-H, stretching), 2866.3 (Ali- C-H, stretching), 1660.7 (C=O, stretching), 1500.0-1400.3 (C-C, ring stretching), 1284.6-1193.8 (C-C, ring stretching), 1114.8-997.2 (O-H, out of plane bend).The FT-IR spectrum of EEAS-I had shown in Fig: 1. The spectral data of the compound EEAS-I and their functional group assignments were tabulated in Table 4.

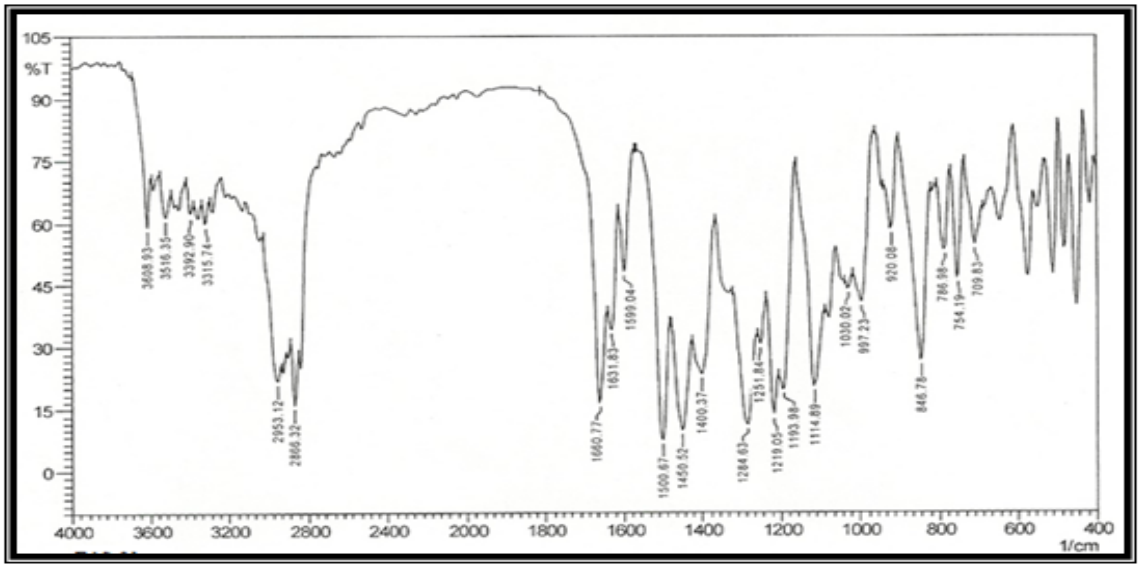

Fig 1: FT-IR Spectrum of Isolated Compound EEAS-I

Nuclear Magnetic Resonance Study

Nuclear magnetic resonance, induces changes in the magnetic properties of certain atomic nuclei, notably that of hydrogen. Hydrogen atoms in different environments can be detected, counted and analyzed for structure determination.

^{13}C NMR spectroscopy is the most powerful and in dispensable technique provide information about intricate nature of the carbon skeleton of a compound such as, the total number of carbon, number of oxygenated carbons and the number of carbon present in the sugar moiety and exhibits carbon resonance signal extending over 200 ppm.

^{1}H-NMR spectrum of EEAS-I

The ^{1}H-NMR spectrum of the isolated compound EEAS-I had displayed the characteristic signals at δ_H 7.80 (H-2′, s), 7.30 (H-5′, d), 6.67 (OH-4′, s), 5.89 (OH- 5, s), 4.29 (OH-8, s), 4.17 (OCH_3-3, s), 3.85 (OCH_3-3′, s), 3.14 (OCH_3-6, s), 2.73 (OCH_3-7, d). The ^{1}H-NMR spectrum of EEAS-I had shown in Fig 2 and data were tabulated in Table 5.

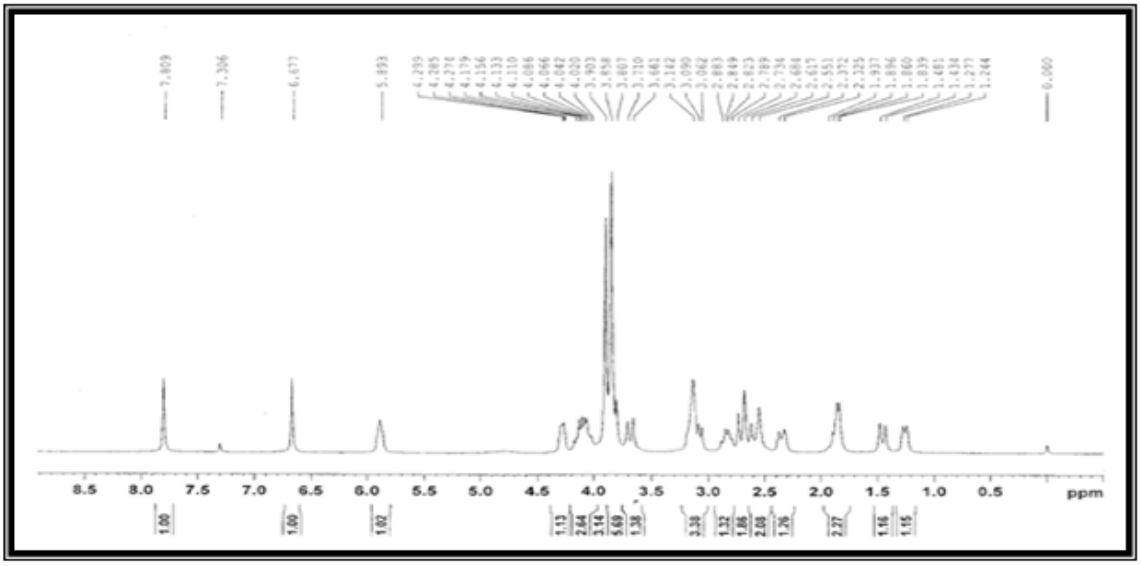

Fig 2: ^{1}H-NMR spectrum of isolated compound EEAS-I

^{13}C NMR spectrum of EEAS-I

The ^{13}C-NMR spectrum of isolated compound EEAS-I had shown the characteristic signals at δ_H 2-77.37, 3-126.99, 4-123.17, 5-135.55, 6-140.11, 7-145.84, 8-105.20, 3´-148.77, 1´-100.62, 2´-77.00, 5´-76.57, 6´-64.21.The carbon signals indicated the presence of carbons due to the flavones skeleton. The hydroxylated C-2, C-3, C-4, C-5, C-6, C-7 and C-8 resonate at δ ppm. The ^{13}C-NMR spectrum had shown in Fig: 3 and the spectral data of EEAS-I and corresponding signal assignments were tabulated in Table 6.

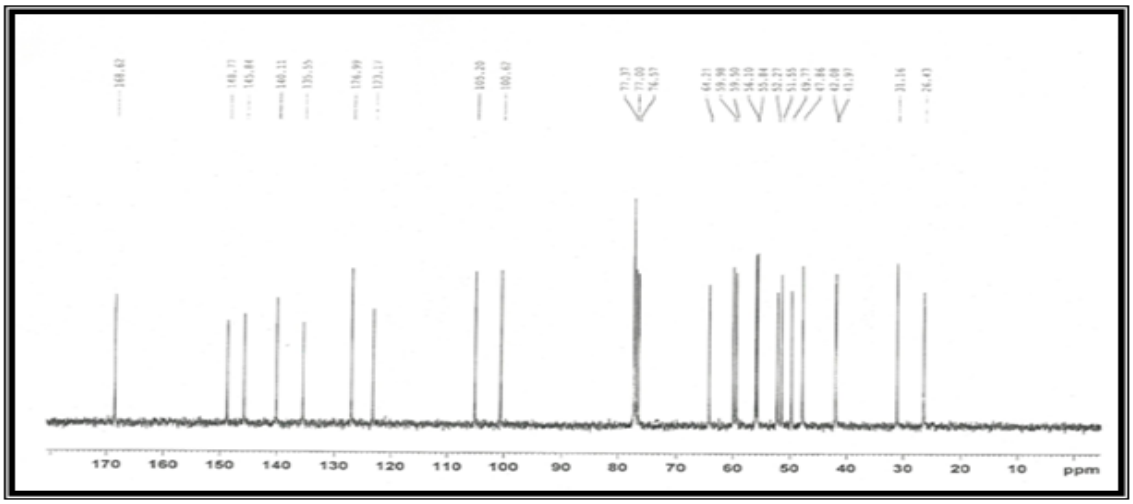

Fig 3: ^{13}C-NMR spectrum of isolated compound EEAS-I

MASS spectrum of EEAS-I

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

The mass data of isolated compound EEAS-I had shown the m/z = 390 indicative of **$C_{19}H_{18}O_9$,** m/z = 358 indicative of **$C_{18}H_{14}O_8$,** m/z = 334 indicative of **$C_{16}H_{14}O_8$,** m/z = 304 indicative of **$C_{15}H_{12}O_7$,** m/z = 212 indicative of **$C_9H_8O_6$,** m/z = 198 indicative of **$C_5H_6O_2$,** m/z = 42 indicative of **C_2H_2O**. The mass data were in decreased sequence due to the absence of different parts on the compounds. The MASS spectrum of isolated compound EEAS-I had shown in Fig. 4 and the EI-MS spectrum of EEAS-I exhibited the molecular ion peak at *m/z* 390.

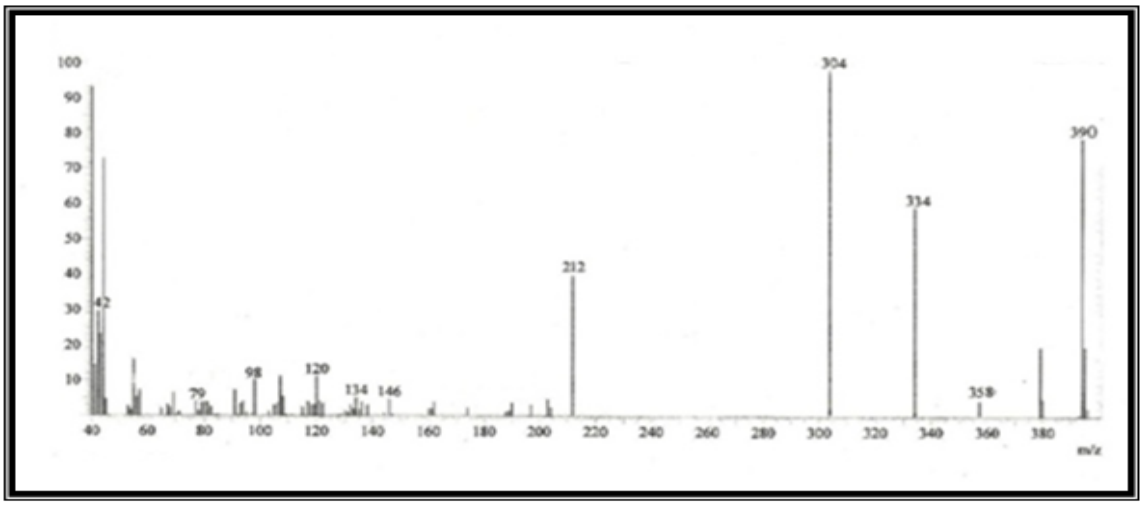

Fig 4: MASS spectrum of isolated compound EEAS-I

Structure of Isolated Compound

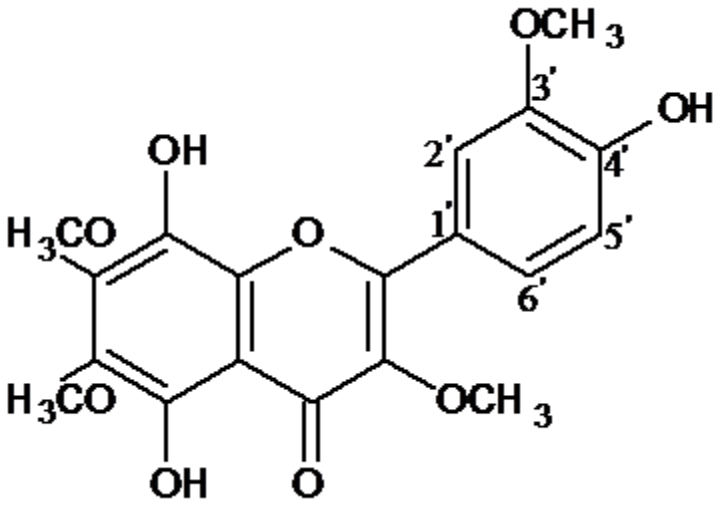

Fig: 5: 3´, 3, 6, 7-tetramethoxy - 4´, 5, 8-trihydroxy flavones

RESULT:

Table: 1 Chromatographic fractions of ethanolic extract of *Alternanthera sessilis* Linn.

Sl. No.	Elution Composition	Fractions	Compounds
1.	n-Hexane	1-5	Oily
2.	n-Hexane: Benzene (95:5)	6-11	Waxy
3.	n-Hexane: Benzene (90:10)	12-20	Waxy
4.	n-Hexane: Benzene (50:50)	21-28	Waxy
5.	n-Hexane: Benzene (40:60)	29-39	Waxy
6.	Benzene	40-45	Intractable gum
7.	Benzene: Chloroform (95:5)	46-49	Intractable gum
8.	Benzene: Chloroform (85:15)	50-54	Intractable gum
9.	Benzene: Chloroform (65:35)	61-66	Intractable gum
10.	Benzene: Chloroform (50:50)	67-72	Intractable gum
11.	Benzene: Chloroform (30:70)	73-80	Intractable gum
12.	Chloroform	81-85	Intractable gum
13.	Chloroform: ethanol (97:3)	86-89	Intractable gum
14.	Chloroform: Ethanol (95:5)	90-96	Intractable gum
15.	Chloroform: Ethanol (90:10)	97-101	Intractable gum
16.	Chloroform: Ethanol (60:40)	102-107	**EEAS-I**
17.	Chloroform: Ethanol (50:50)	108-116	Intractable gum
18.	Chloroform: Ethanol (20:80)	117-122	Intractable gum
19.	Ethanol	123-127	Intractable gum

Table 2: Properties of isolated compound EEAS-I

Sl. No.	Property	Observation
1.	Appearance	Colourless Powder
2.	Melting Point	178 – 180^{0}C
3.	Solubility	Ethanol, Methanol, Chloroform

Table: 3 R_f value of the isolated compound EEAS-I

Sl. No.	TLC Solvent System	R_f Value
1.	n-Hexane : Diethyl ether = (1 : 1)	0.47

Table 4: FT-IR spectral data of compound EEAS-I

Sl. No.	Wave Number (cm^{-1})	Type of Vibration	Functional group assigned
1.	3608.9 – 3315.7	O - H	Free hydroxyl group
2.	2953.1	Cyclic C - H, str	Aromatic Hydrocarbon
3.	2866.3	C - H, str	Aliphatic Hydrocarbon
4.	1660.7	C = O, str	Ketone
5.	1500.0 – 1400.3	C = C, ring stretch	Aromatic Nuclei
6.	1248.6 – 1193.8	C – C, str	Aliphatic Hydrocarbon

Table 5: ^{1}H-NMR spectral data of compound EEAS-I

Sl. No.	Chemical shift value (δ ppm)	Signal Assignment - H
1.	7.80	2′ - H, s
2.	7.30	5′ - H, d
3.	6.67	4′ - OH, s
4.	5.89	5 - OH, S
5.	4.29	8 - OH, s
6.	4.17	3 - OCH_3, s
7.	3.85	3′ - OCH_3, s
8.	3.14	6 - OCH_3, S
9.	2.37	7 - OCH_3, d

Table 6: ^{13}C-NMR spectral data of isolated compound EEAS-I

Sl. No.	Chemical shift value (δ ppm)	Signal Assignment - C
1.	77.37	C-2
2.	126.99	C-3
3.	123.17	C-4
4.	135.55	C-5
5.	140.11	C-6
6.	145.84	C-7
7.	105.20	C-8
8.	148.77	C-3´
9.	100.62	C-1´
10.	77.00	C-2´
11.	76.57	C-5´
12.	64.21	C-6´

DISCUSSION:

Thin layer chromatography was the first attempt taken to find out the presence phytoconstituents presents in the extracts. TLC was performed by using stationary phase as silica gel G and mobile phase as n-hexane : diethyl ether in the ratio of 1: 1 for ethanol extract of *Alternanthera sessilis* Linn. The TLC plate showed mixture of compounds with yellow and brownish yellow colour spots. This was separated by column chromatography.

The ethanolic extract of *Alternanthera sessilis* Linn. was packed on column chromatography with silica gel G 60-120 mesh size and mobile phase was eluted as per increasing polarity. The fractions were collected and tested for the components by using Thin Layer Chromatography. TLC spot was identified by spraying 5% w/v alcoholic solution of H_2SO_4 as a spraying reagent. The sprayed plates were heated at 100°C for 5-10 min and the numbers of constituents present in the each fraction were found. The Chloroform-Ethanol 60:40 ratios gives the fractions 102 to 107, were found to be similar and showed a single spot. Thus they were mixed and recrystallized from ethanol as colourless powder which shown a melting point of 178-180^{0}C (104 mg). It light dark green colour with ferric chloride, pink colour in Shinoda's test suggesting that it was a flavone. The isolated compound designated as **EEAS-I**.

The isolated compound EEAS-I had shown the positive response to the Shinoda's test for flavonoids. The structures of isolated compound EEAS-I was elucidated by IR, NMR and Mass spectroscopy.

The Compound EEAS-I was isolated and its molecular formula was determined as $C_{19}H_{18}O_9$ (m/z = 390 (100) [M^+]). The structures of the flavone were identified on the basis of extensive spectroscopic data analysis and by comparison of their spectral data with those reported in the literature. The IR spectrum indicated the presence of hydroxyl (3608.9 cm^{-1}) and carbonyl functions (1660.7 cm^{-1}). The occurrence of a flavone skeleton in the molecule could be easily deduced from the ^{1}H-NMR and ^{13}C-NMR spectrums. From the above mentioned data of ^{13}C NMR signal indicated the presence of hydroxyl group at C-5, C-8 and C-4´and unsaturated keto function. The ^{13}C NMR signal also reported the presence of four methoxy group at different position of flavones skeleton. The ^{13}C-NMR signal of different location of carbon for functional group was confirmed by the signal of were confirmed spectra of ^{1}H-NMR. The ^{1}H-NMR further showed the presence of three hydroxyl, four methoxy groups and three methine groups. The compound characterized as **3´, 3, 6, 7-tetramethoxy - 4´, 5, 8-trihydroxy flavones.**

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