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Phytochemical Screening of *Sida spinosa* Linn.(Malvaceae)

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Abstract : To investigate the phytochemical screening (group determination) of the plant Sida spinosa Linn. Preliminary phytochemical analysis for alkaloids, flavonoids, glycosides, phenols, resins, saponins, steroids, tannins, terpenoids were made by following standard procedures. The extracts were subjected to qualitative tests for the identification of the phytoconstituents present in it viz. alkaloids, carbhohydrates, glycosides, phytosterols, fixed oils & fats, phenolic compounds & tannins, proteins & free amino acids, gums & mucilages, flavonoids, lignins and saponins. From the identification test, melting poit studies and spectral analysis it is concluded that the isolated compound SS1 may be a Phenolic / Steroidal compound. The diversity of phytochemicals found present suggests that Sida Spinosa Linn. could serve as a source of useful drugs.
Key words : Phytochemical Screening, *Sida spinosa* Linn., Malvaceae, Sida species.

Introduction:

Medicinal plants are part and parcel of human society to combat diseases, from the dawn of civilization.[1] There exists a plethora of knowledge, information and benefits of herbal drugs in our ancient literature of Ayurvedic (Traditional Indian Medicine), Siddha, Unani and Chinese medicine. According to the World Health Organization, 2003 about 80% of the population of developing countries being unable to afford pharmaceutical drugs rely on traditional medicines, mainly plant based, to sustain their primary health care needs.[2] Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological and medicinal activities, higher safety margins and lesser costs.[3-5]. Since the time immemorial our traditional system of medicine and folklore claiming that medicinal plants as a whole or their parts are being used in all types of diseases successfully[6]. About 65% of world populations have access to local medicinal plant knowledge system[7]. Traditional systems of medicine are popular in developing countries and upto 80% of population relies on traditional medicines or folk remedies for their primary health care needs[8]. India has about 45000 plant species and among them, several thousands have been claimed to posses medicinal properties[9,10]. Phytochemical (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans[11]. The quantity and quality of phytochemicals present in plant parts may differ from one part to another. In fact, there is lack of information on the distribution of the biological activity in different plant parts essentially related to the difference in distribution of active compounds (or active principles) which are more frequent in some plant parts than in others[12]. Phytochemicals have been recognized as the basis for traditional herbal medicine practiced in the past and currently en vogue in parts of the world[13]. In the search for phytochemicals that may be of benefit to the pharmaceutical industry, researchers sometimes follow leads provided by local healers in a region[14]. Following such leads, plant parts are usually screened for phytochemicals that may be present. The presence of a phytochemical of interest may lead to its further isolation, purification and characterization. Then it can be used as the basis for a new pharmaceutical product. Successful determination of

biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure [15]. This therefore underscores the need to try as much solvents as possible in screening plant parts for phytochemicals.

Materials and Methods:

Sample Collection:

Whole plant parts of Sida spinosa Linn. were collected in a separate sterile polythene bags from the area in and around Thirunelveli (dist), Tamilnadu. Collected plant parts were examined and identified with the help of regional floras.

Preparation of Solvent Extracts

The cleaned, healthy plant materials are cut in to small sections and dried under shade for three to four weeks. The dried material was ground into fine powder in an electric grinder. Powder so obtained was stored in desiccators setup and used for extraction. Powder and it was extracted in soxhlet apparatus using various solvents according to their polarity. Petroleum ether extract, Chloroform extract, Acetone extract, Ethanolic extract, Aqueous extract.

Phytochemical Evaluation:

The extracts were subjected to qualitative tests for the identification of the phytoconstituents present in it viz. alkaloids, carbhohydrates, glycosides, phytosterols, fixed oils & fats, phenolic compounds & tannins, proteins & free amino acids, gums & mucilages, flavonoids, lignins and saponins.[16]

Table – 1 Data Showing the Preliminary Phytochemical Screening of *Sida spinosa* Linn.

Phytoconstituents	Petroleum ether extract	Chloroform extract	Acetone extract	Ethanolic extract	Aqueous extract
Alkaloids	(-)	(+)	(+)	(-)	(-)
Carbohydrates	(-)	(+)	(+)	(+)	(-)
Glycosides	(-)	(-)	(-)	(+)	(-)
Flavonoids	(-)	(-)	(-)	(+)	(-)
Phytosterols	(+)	(+)	(-)	(+)	(-)
Fixed oils and Fats	(+)	(-)	(-)	(-)	(-)
Saponins	(-)	(-)	(+)	(+)	(+)
Phenolic compounds and Tannins	(+)	(+)	(+)	(+)	(+)
Lignins	(+)	(+)	(+)	(-)	(+)
Proteins and Free Amino acids	(-)	(+)	(-)	(-)	(-)
Gums and Mucilage	(+)	(-)	(-)	(+)	(+)

(+) Presence **(-) Absence**

Separation and Isolation of Plant Constituents by Chromatographic Methods

The various methods of separating and isolating the plant constituents, the chromatographic procedure originated by Tswett is one of the most useful technique for general application. All finely divided solids have the power to adsorb other substance on their surface to a greater or lesser extent, similarly, all substance are capable of being adsorbed, some much more readily than others. This phenomenon of selective adsorption is the fundamental principle of chromatography[17,18]. In the present study Thin Layer Chromatography (TLC) and Column Chromatography methods were used.

Table – 2 Thin Layer Chromatography of Ethanolic Extract of *Sida spinosa* Linn.

S.No	Mobile Phase	Detecting Agent	No. of Spots	R_f Value
1	Benzene : Ethanol (9 : 1)	Dragendroff reagent	3	0.57 0.67 0.72
2	Toluene : Ethyl acetate (7 : 3)	Anisaldehyde sulphuric acid	1	0.61
3	Petroleum ether : Ethyl acetate (2 : 1)	Iodine vapour	2	0.75 0.71
4	Benzene : Ethyl acetate (9 : 1)	Vanillin in sulphuric acid	3	0.54 0.66 0.77
5	Chloroform	Vanillin in sulphuric acid	1	0.65

An yellow crystalline compound was obtained by column chromatography in the fractions of Ethanolic extract (Acetone : Ethyl acetate 50 : 50) and was named SS1.

Description of the Isolated Compound SS1

Nature :Crystalline
Colour :Yellow
Taste :Bitter
Solubility :Soluble in Methanol and Ethyl acetate
Melting Point : 155° - 160°c
TLC :Solvent System [Chloroform: Ethyl Acetate: Methanol: Water (15 : 40 : 22 : 5.0)]
Identification Test: Positive for Libermann's Burchard Test (Phytosterol)

Characterisation of Isolated Plant Constituents

Spectroscopy [19, 20, 21]

Spectroscopy is the measurement and interpretation of Electro Magnetic Radiation **(EMR)** absorbed or emitted when the molecules or atoms or ions of a sample move from one energy state to another energy state.

The arrangements of all types of electromagnetic radiations in order of their increasing wave lengths or decreasing frequencies is known as complete electromagnetic spectrum. Visible and Ultra Violet radiations cover the wavelength range from 200 – 800nm.The absorption of radiation in this region cause the excitation of π electron conjugated or an unconjugated system. In case of a conjugated system, the separation between the ground state and the excited energy level will be less and hence absorption occours at a longer wavelength. Also carbonyl group of an aldehyde or a ketone absorbs at some characteristic wavelengths. Thus an ultraviolet or visible spectrum is quite useful for the detection of conjugation, carbonyl group etc. and may not provide any information about the remaining part of the molecule.

The different types of EMR are visible radiation, UV radiation, IR radiation, Microwaves, Radio waves, X rays, Gamma rays, or Cosmic rays. as these radiations have different wavelength or frequency or energy.

Infra Red Spectroscopy (IR)

Infra Red spectrum is an important record which gives sufficient information about the structure of a compound. The absorption of Infra Red radiation causes the various bands in a molecule to stretch and bend with respective one another. The most important region for an organic chemist is 2.5 μ to 15 μ in which molecular vibrations can be detected and measured in an Infra Red region.

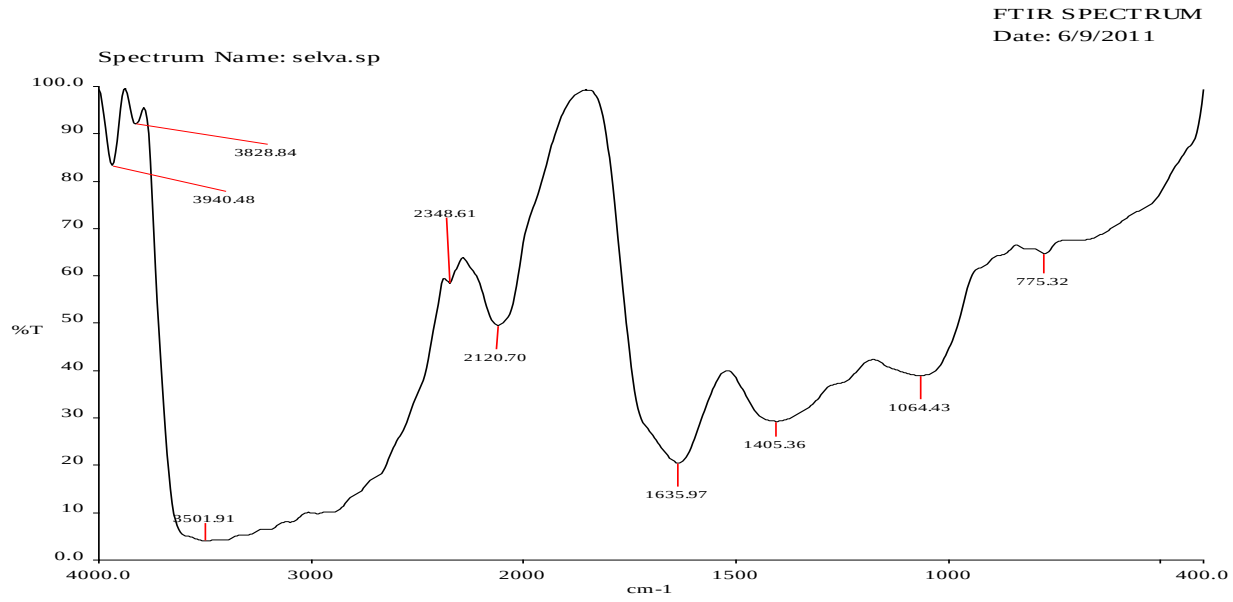
Infra Red spectroscopy offers the possibility to measure different types of atomic bond vibrations at different frequencies. Especially in organic chemistry the analysis of IR absorption spectra shows what type of bonds is present in the sample. It is also an important method for analyzing polymers and constituents like filters, pigments and plasticizers. Identification of functional group and structure elucidation, the entire IR region is divided into

Group frequency region : 4000 – 1500 cm^{-1}
Finger print region : 1500 – 400 cm^{-1}

In the frequency region, the peaks corresponding to different functional groups can be observed. (eg) Amino group, Alcoholic group etc. The crystals obtained from ethanolic extract of *Sida spinosa* were subjected to Infrared Spectroscopy and the spectrum is shown in **Fig – 1.**

IR Spectra of the crystalline compound SS1 showed characteristic absorption (in cm^{-1}) at

- 3828 – 3940 - O – H Stretching
- 3501 - N – H Stretching
- 3000 - O – H Stretching
- 2348.61 - COOH and Enols
- 2120.70 - C ≡ N Nitrites
- 1635.97 - Primary amines
- 1405 - O – H Bending
- 1064 - C – O Stretching
- 775.32 - N – H Wagging



selva.pk

selva.sp 3601 4000.00 400.00 4.01 100.00 4.00 %T 5 1.00

REF 4000 99.48 2000 68.20 600
3940.48 83.26 3828.84 92.06 3501.91 4.01 2348.61 58.45 2120.70 49.54
1635.97 20.44 1405.36 29.27 1064.43 39.01 775.32 64.76

Fig – 1 IR Spectrum of SS1

High Performance Thin Layer Chroı

The High Performance Thin Layar Chromatography is a method of separation in which the stationary phase is contained in a column, one end of which is attached to a source of pressurized liquid eluent (mobile phase).

Instrument: Developed by Kirkland and Huber
Pressure : $2.07 * 10^7$ Nm^{-2} (3000 psi)
Diameter of column : 1 - 3 mm

High Performance Thin Layer Chromatography finds many applications, including

- Radiochemical purity of radiopharmaceuticals
- Determination of the pigments in plant.
- Detection of pesticides or insecticides in food
- Analyzing the dye composition of fibres in forensics
- Identifying compounds present in a given substance
- Monitoring organic reactions

The crystals obtained from ethanolic extracts of *Sida spinosa* were subjected to HPTLC in different concentrations, and the HPTLC profile are shown in **Fig**. **23 - 25** and **Table 10 – 12**

Chromatographic Condition for HPTLC Finger Print

Sample Name : SS1
Solubility : Ethanol
Stationary phase : Silica gel 60 F 254
Mobile phase : Chloroform: Ethyl Acetate: Methanol: Water (15 : 40 : 22 : 5.0)
Scanning wavelength : 298 nm
Sample concentration : 20 mg/ml
Applied volume : 5.0, 10 & 20 µl
Development mode : Ascending mode

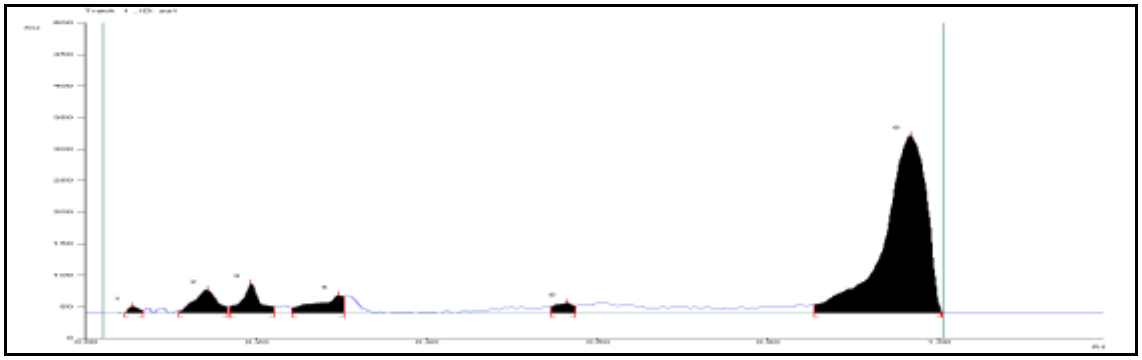


Fig – 2 HPTLC PROFILE OF SS1, 5.0 µl Concentration of SS1

Table – 3 HPTLC Profile of SS1 (5 µl)

Peak	Start R_f	Start Height	Maximum R_f	Maximum Height	Maximum %	End R_f	End Height	Area	Area %
1	0.05	0.2	0.05	10.3	2.46	0.07	3.9	86.2	0.75
2	0.11	3.3	0.14	36.7	8.79	0.17	9.8	664.0	5.75
3	0.17	10.2	0.19	45.9	10.99	0.22	10.2	635.7	5.51
4	0.24	8.2	0.30	27.9	6.69	0.30	26.8	611.5	5.30
5	0.55	9.7	0.56	15.7	3.76	0.58	11.3	235.2	2.04
6	0.85	13.3	0.97	281.2	67.31	1.00	3.8	9311.9	80.66

Fig – 3 HPTLC PROFILE OF SS1 10.0 µl Concentration of SS1

Table – 4 HPTLC Profile of SS1 (10 µl)

Peak	Start R_f	Start Height	Maximum R_f	Maximum Height	Maximum %	End R_f	End Height	Area	Area %
1	0.09	6.6	0.13	49.5	8.17	0.14	45.5	1019.9	5.77
2	0.15	45.6	0.16	66.1	10.91	0.20	39.8	1938.8	10.98
3	0.22	42.4	0.24	50.1	8.27	0.25	48.9	1093.0	6.19
4	0.26	49.8	0.28	71.2	11.75	0.34	2.20	1654.6	9.37
5	0.47	8.70	0.50	15.2	2.51	0.51	13.9	306.4	1.74
6	0.51	14.0	0.53	21.8	3.59	0.56	15.6	533.3	3.02
7	0.56	17.0	0.59	27.0	4.45	0.61	20.2	607.7	3.44
8	0.85	20.7	0.97	304.9	50.34	1.00	1.10	10498.1	59.48

Fig – 4 HPTLC PROFILE OF SS1, 20.0 µl Concentration of SS1

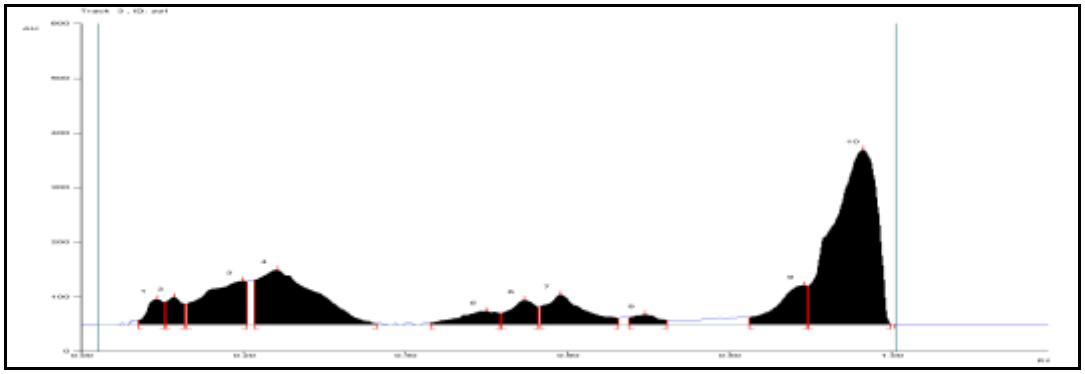


Fig – 4 HPTLC PROFILE OF SS1, 20.0 µl Concentration of SS1

Table – 5 HPTLC Profile of SS1 (20 µl)

Peak	Start R_f	Start Height	Maximum R_f	Maximum Height	Maximum %	End R_f	End Height	Area	Area %
1	0.07	8.00	0.09	46.0	5.70	0.10	40.9	619.5	2.37
2	0.10	41.4	0.11	49.8	6.18	0.13	37.5	655.2	2.51
3	0.13	37.7	0.20	79.6	9.87	0.20	78.7	2801.5	10.73
4	0.21	81.3	0.24	99.5	12.35	0.37	1.40	4857.9	18.61
5	0.43	2.90	0.50	24.2	3.00	0.52	20.0	768.0	2.94
6	0.52	20.1	0.55	44.2	5.49	0.56	32.3	942.9	3.61
7	0.57	32.7	0.59	54.0	6.70	0.66	11.2	1721.2	6.59
8	0.68	12.9	0.70	17.6	2.19	0.72	7.80	392.2	1.50

9	0.82	12.5	0.89	71.4	8.86	0.90	70.7	1713.7	6.56
10	0.90	70.0	0.97	319.5	39.66	1.00	0.40	11635.7	44.57

Gas Chromatography – Mass spectroscopy (GC–MS)

In the simplest terms the GC-MS instrument represents a device that separates chemical mixtures (the GC component) and a very sensitive detector (the MS component) with a data collector (the computer component).

Once the sample solution is introduced into the GC inlet it is vaporized immediately because of the high temperature (250 degrees C) and swept onto the column by the carrier gas (usually Helium).

The sample flows through the column experiencing the normal separation processes. As the various sample components emerge from the column opening, they flow into the capillary column interface. This device is the connection between the GC column and the MS. Some interfaces are separators and concentrate the sample via removal of the helium carrier.

The sample then enters the ionization chamber. Two potential methods exist for ion production. The most frequently used method in the Toxicology lab is electron impact (EI). The occasionally used alternative is chemical ionization (CI). For electron impact ionization a collimated beam of electrons impact the sample molecules causing the loss of an electron from the molecule. A molecule with one electron missing is represented by M+ and is called the molecular ion (or parent ion). When the resulting peak from this ion is seen in a mass spectrum, it gives the molecular weight of the compound. Chemical ionization begins with ionization of methane (or other gas), creating a radical which in turn will impact the sample molecule to produce M.H+ molecular ions.

Some of the molecular ions fragment into smaller daughter ions and neutral fragments. Both positive and negative ions are formed but only positively charged species will be detected.

Less fragmentation occurs with CI than with EI, hence CI yields less information about the detailed structure of a molecule, but does yield the molecular ion; sometimes the molecular ion cannot be detected by the EI method, hence the two methods complement one another. Once ionized, a small positive potential is used to repel the positive ions out of the ionization chamber.

The next component is a mass analyzer (filter), which separates the positively charged particles according to their mass. Several types of separating techniques exist; quadrupole filters, ion traps, magnetic deflection, time-of-flight, radio frequency, cyclotron resonance and focusing to name a few. The most common are quadrupoles and ion traps.

After the ions are separated according to their masses, they enter a detector and then on to an amplifier to boost the signal. The detector sends information to the computer which acts as a "clearing house". It records all the data produced, converts the electrical impulses into visual displays and hard copy displays. The computer also drives the mass spectrometer.

Identification of a compound based on it's mass spectrum relies on the fact that every compound has a unique fragmentation pattern. Even isomers can be differentiated by the experienced operator. Generally, more information is generated than could possibly be used. A library of known mass spectra which may be several thousand compounds in size is stored on the computer and may be searched using computer algorithms to assist the analyst in identifying the unknown.

It is important to incorporate all other available structural information (chemical, spectral, sample history) into the interpretation wherever appropriate.

The ultimate goal is accurate identification of a compound, which can be facilitated by the utilization of the GC-MS.

Instrument Details

Make	:	Perkin Elmer Clarus 500
Column Type	:	Capillary Column Elite-5 (5% Phenyl 95% dimethylpolysiloxane)
Column Length	:	30m
Column id	:	250µm
GC Conditions		
Oven Program	:	50ºC @ 6ºC to 150ºC (2min) @ 6ºC to 290ºC (5min)
Injector temp.	:	290ºC
Carrier gas	:	He @ 1ml/min
Split ratio 1	:	10
MS Conditions		
Mass Range	:	40 - 600 amu
Electron energy	:	70 ev
Source and Inlet line		
Temperature	:	200ºC
Scan mode	:	Full Scan
Library used	:	NIST 2005
Sample injected	:	1µl

Chromatogram:

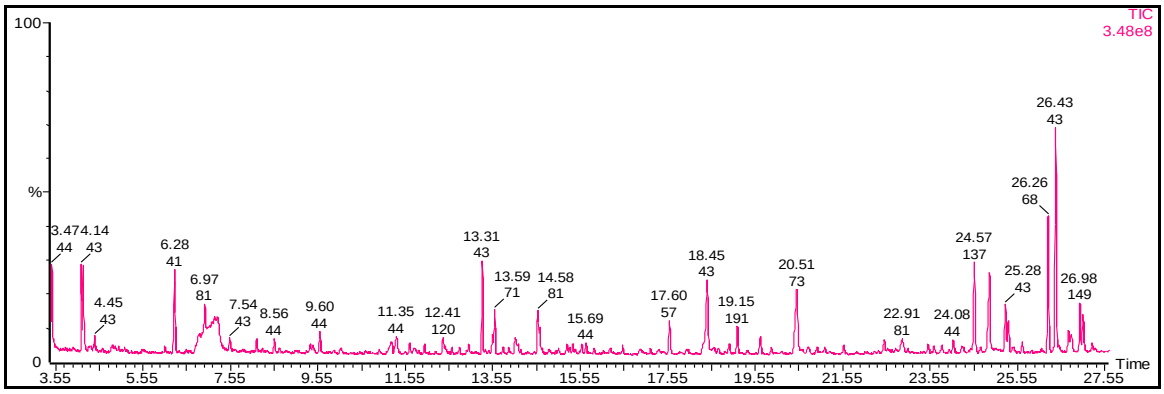


Fig – 5 Chromatogram of SS1 (a)

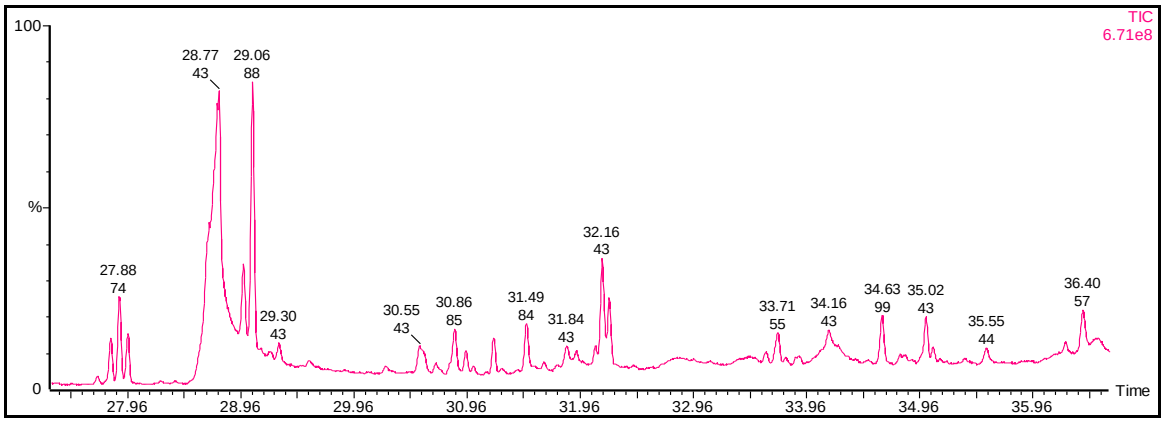
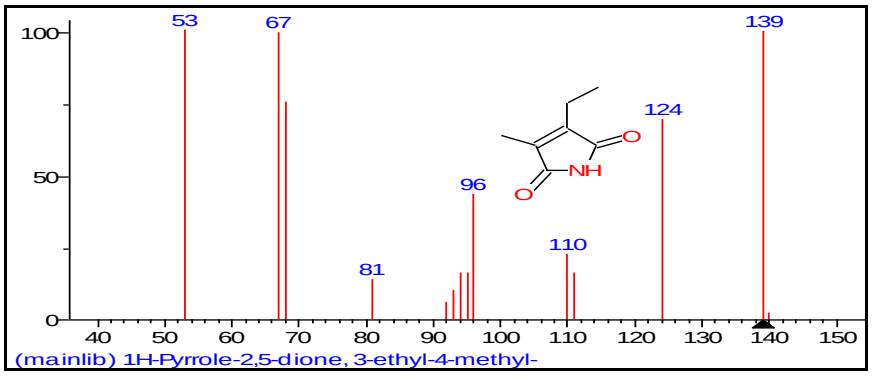
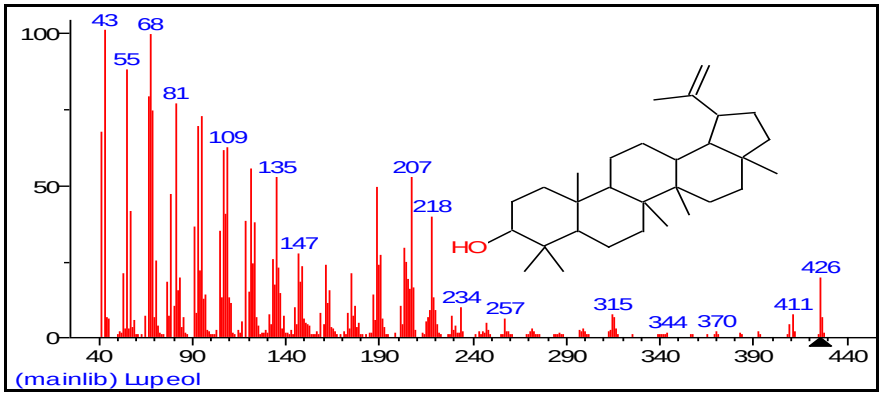


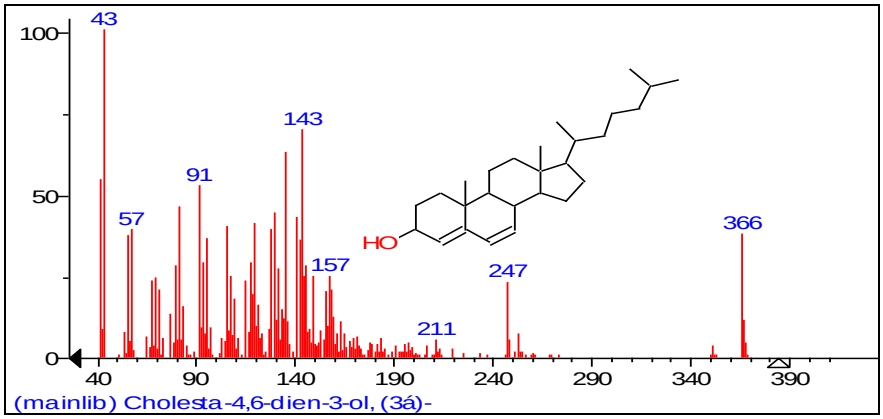
Fig – 6 Chromatogram of SS1 (b)



Formula - $C_7H_9NO_2$
Molecular Weight - 139
Fig – 7 GC - MS Spectra of Isolated Compound SS1 (a)



Formula - $C_{30}H_{50}O$
Molecular Weight - 426
Fig – 8 GC - MS Spectra of Isolated Compound SS1 (b)



Formula - $C_{27}H_{44}O$
Molecular Weight - 384

Fig – 9 GC - MS Spectra of Isolated Compound SS1 (c)

Results:

All the extracts were subjected to preliminary phytochemical investigation. More number of phytoconstituents like alkaloids, flavonoids, phytosterols were found to be present in Ehanolic extract. Data Showing the Preliminary Phytochemical Screening of *Sida spinosa* Linn Ethanolic extract **Table – 1**. Ethanolic

extract was subjected to Thin Layer Chromatography (TLC) for alkaloids, glycosides, flavonoids, steroids and essential oil. The number of spots with its R_f values are shown in **Table – 2.**

From the results obtained in Thin Layer Chromatography (TLC), it was observed that more prominent spots was observed for the Ethanolic extract of *Sida spinosa* and were subjected to Column Chromatography. An yellow crystalline compound was obtained by column chromatography in the fractions of Ethanolic extract (Acetone : Ethylacetate 50 : 50) and was named SS1. The isolated compound SS1 was crystalline in nature, yellow in colour, odourless and bitter in taste with melting point (155^0 - 160^0 C), Soluble in Methanol and Ethyl acetate. The isolated compound SS1 subjected to FTIR studies for the identification of characteristic functional groups present in the compound. In this studies it showed Phenolic (3828 – 3940 cm^{-1}), Nitro (3501cm^{-1}), Carbonyl (1064cm^{-1}) Aromatic (1405 cm^{-1} O-H Bending, 3000 cm^{-1} O-H Stretching) **(Fig - 1).** The isolated compound SS1 subjected to HPTLC studies and the results are shown in **Table – 3,4,5. Fig – 2,3,4.** To characterize the isolated compound SS1 it was also subjected to GC – MS studies and compared with NIST 2005 library, structures of these constituents are shown in **Fig – 6 - 9.** Molecular weight 139, formula – $C_7H_9NO_2$, (1H – Pyrrole - 2, 5 - dione) it is a nitrogen containing compound. Molecular weight 426, formula– $C_{30}H_{50}O$ (Lupeol) it is a characteristic cyclic nucleus in Steroidal structure. Molecular weight 384, formula – $C_{27}H_{44}O$ (Cholesta - 4, 6 – dien - 3ol) it is poly phenolic compound and commonly known as Steroidal compounds. Based on the above spectral data, the isolated compound may be constitute a 1H-pyrrole ring (Phenolic), and Lupeol (Steroidal) nucleus, Cholesta (Steroidal) nucleus, FTIR evidenced the presence of phenolic group (3828 – 3940 cm^{-1} O - H stretching). From the identification test, melting poit studies and spectral analysis it is concluded that the isolated compound SS1 may be a Phenolic/ Steroidal compound.

Conclusion:

Phytochemicals found present in Whole plant of extracts of *Sida spinosa* Linn. indicates their potential as a source of principles that may supply novel medicines. Furthermore, isolation purification and characterization of the phytochemicals found present will make interesting studies.

Reference:

1. K. Biswas, I. Chattopadhyay, R.K. Banerjee and U. Bandyopadhyay , Biological activities and medicinal properties of neem (Azadirachta indica)" Currnt Sci,vol.82, no.11,pp.1336-1345,2002.
2. B.R. Goyal, R.K. Goyal and A.A. Mehta, "Phyto-Pharmacognosy of Archyranthes aspera: A Review" Pharmacog Rev, vol.1,no.1,2008.
3. G.M. Cragg, D.J. Newman and K.M. Snader, "Natural products in drug discovery and development" J Nat Prod, vol.60, pp.52-60,1997.
4. T.V. Padma , " India Ayurveda", Nature, pp.436-486, 2005.
5. G. Chaudhary ,S. Goyal and P. Poonia, "Lawsonia inermis Linnaeus: A Phytopharmacological Review"International Journal of
6. Pharmaceutical Sciences and Drug Research,vol.2, no.2, pp.91-98,2010.
7. Y. Shivhare, P. Singh and S. Singh. " Ethanobotanical and Ethano pharma cological profile of Abutilon indicum Linn, A Review", Der Pharmacia Sinica, vol. 1, pp.40-45,2010.
8. S. Srivastava, P. Singh , G. Mishra , K.K. Jha and R.L. Khosa, "Costus speciosus (Keukand): A review", Der Pharmacia Sinica, vol.2, no.1,pp. 118-128, 2011.
9. V.K. Garg and S.K. Paliwal, "Anticonvulsant activity of ethanolic extract of Cynodon dactylon" Der Pharmacia Sinica, vol. 2,no. 2, pp. 86-90, 2011.
10. R. Vadivelan , M. Dipanjan, P. Umasankar , S.P. Dhanabal , M.N. Satishkumar , S. Antony and K. Elango, " Hypoglycemic, antioxidant and hypolipidemic activity of Asparagus racemosus on streptozotocin-induced diabetic in rats" Advances in applied science research, vol. 2, no.3,pp. 179-185,2011.
11. Y.C.K. Reddy, L. Sandya, D. Sandeep, K.R. Salomi,S. Nagarjuna and Y. P. Reddy, "Evaluation of diuretic activity of aqueous and ethanolic extracts of Lawsonia inermis leaves in rats",Asian Journal of Plant Science and Research,vol. 1, no.3, pp.28-33, 2011.

12. C.M. Hasler and J.B. Blumberg, "Symposium on Phytochemicals: Biochemistry and Physiology", Journal of Nutrition,vol.129,pp.756S-757S, 1999.
13. M. Lahlou, Phytother. Res., 2004, 18, 435-445.
14. T. P. Lalitha, P. Jayanthi, Asian J. Plant Sci. Res., 2012, 2(2), 115-122.
15. K. Das, R. K. S. Tiwari, D. K. Shrivastava, J. Med. Plant Res., 2010, 4(2), 104-111.
16. 15.P. Tiwari, B. Kumar, M. Kaur, G. Kaur, H. Kaur, Int. Pharm. Sciencia, 2011, 1, 98-106
17. Kokate, C. K., (2001). "Practical Pharmacognosy," Vallabh Prakashan, 4th ed, P. 123 – 124.
18. Egonsthal., (2005). "Thin Layer Chromatography," 2nd ed, Springer, Delhi P. 105
19. Markham, K.R., (1982). "Technique of flavonoid identification," Academic press, London. P. 320
20. Sharma, Y.R., Vig, O.P., (1980). "The Text Book of Organic Spectroscopy," Principles and Applications Chand and Company Ltd. 1st ed, P. 24 - 40.
21. Silverstein., (2004). "Spectrometric Identification of organic compounds, 6th ed. John Wiley & Sons, Inc. P. 102 – 113.
22. Beckett, A. H., and Stanlake, J. B., (2005). "Practical Pharmaceutical Chemistry," 4th ed, V. 2: CBS Publishers, Delhi., P. 6 - 91.

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