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ISOLATION, IDENTIFICATION, ANTI-OXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF FLOWER EXTRACTS OF *SIDA CORDIFOLIA*

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ABSTRACT

Objectives: *A flavonoid was isolated and biological activities of flower extracts of Sida Cordifolia.* ***Methods:*** *From the ethyl acetate extract flavonoid pigment was isolated in the form of yellowish needles on the paper chromatogram and further UV, IR, and NMR spectroscopic analysis revealed the structure of the flavonoid to be Kaempferol-3-O-β -D-glucoside (astragalin). This is first report for the isolation of astragalin from flowers of Sida Cordifolia may be used as a possible source material for natural health supplements in the future.* ***Results:*** *Antioxidant activity of petroleum ether, ethyl acetate and methanol extracts from the flower parts of S. Cordifolia was evaluated using DPPH as a stable free radical and observed that maximum radial scavenging activity (IC_{50} value of 37.74 µM) was concentrated in ethyl acetate extract and compared to L-ascorbic acid (IC_{50} value of 36.89 µM). In vivo, the model of oedema induced by carrageenan to causes inflammation was used to study the anti-inflammatory activity of the above three extracts. These extracts at dose of 200, 300 mg/kg produced significant inhibition of oedema and pain induced by above mentioned methods.* ***Conclusion:*** *The results obtained suggesting that the presence of astragalin in the ethyl acetate extract possesses significant antioxidant and anti-inflammatory activities and justify the contribution of the flowers of S. Cordifolia in traditional medicine.*

Keywords*: Sida Cordifolia, Kaempferol-3-O-β -D-glucoside (astragalin), Antioxidant activity, Anti-inflammatory activity.*

INTRODUCTION

Plants are storehouses of a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids that have demonstrated their antimicrobial properties *in vitro* [1]. Demand for medicinal plants is increasing both in developing and developed countries. It is estimated that world market for plant derived drugs may account for about Rs.2, 00,000 crores. Presently, Indian contribution is less than Rs.2, 000 crores [2]. Phytochemistry is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their bio-synthesis, turnover and metabolism, their natural distribution and their biochemical function [3]. Phenolic are defined as a class of polyphenols which are important secondary metabolites present in plants [4] and are also responsible for their antioxidant action and various beneficial effects in a multitude of diseases [5, 6]. Recently certain polyphenolic and bioflavonoid have been found to be the potential source of reverse transcription inhibitors [7]. Flavonoids have been reported to exert wide range of biological activities, these include: anti-inflammatory, antibacterial, antiviral, and anti-allergic [8, 9], cytotoxic antitumor, neurodegenerative diseases [10]. Flavonoids are powerful antioxidants against free radicals and are described as free-radical scavengers [11]. This activity is attributed to their hydrogen-donating ability. Various flavonoids have also been shown to have anti-inflammatory activity by inhibiting cycloxygenase-2 (COX2) and inducible nitric oxide syntheses [12], which is related to antioxidant activity.

Sida cordifolia (Malvaceae) widely distributed along with other species are common throughout the tropical and sub tropical plains all over India and Srilanka, growing wild along the roadside. It is also known as the "Bala" in Hindi and Sanskrit [13]. This plant has great potential to develop the Ayurvedic, modern medicine and athletic supplements by pharmaceutical industries. It is useful in blood, throat, urinary system related troubles, piles, phthisis; insanity etc. [14]. The present work highlights the contribution of *Sida cordifolia* in modern system of herbal medicine for new drug development. Due to pronounced therapeutic uses of *Sida Cordifolia* species in medicine, a lot of work has been carried out on the pharmacological evaluation of crude extracts and their isolates. The various kinds of biological activities have generated interest in the chemistry of this genus, which resulted in isolation and characterization of various classes of chemical constituent from flower extracts.

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MATERIALS AND METHODS

Plant materials:

Sida Cordifolia was used as the test plant, which was collected from Kumbakonam surrounding areas, Thanjavur District, Tamilnadu, India in the month of October – November and verified by Dr. N. Ramakrishnan, Head and Associate Professor. Voucher specimen of the plant (No. GACBOT-336) was deposited at the Herbarium of Department of Botany, Government Arts College (Autonomous), Kumbakonam, Bharathidasan University, India.

Extraction and isolation:

The isolation includes the plant collection, extraction, Chromatographic separation etc. The characterization involves the study of qualitative chemical analysis in order to know the purity of the compound. The flowers of the plant were taken for the present study. About 1.5 kg of plant material was collected, and shade dried under controlled condition. The dried plant material was extracted (refluxed) with methanol (95%) under reflux for 5 days, filtered and re-extracted the same way. The various extracts were combined and concentrated to dryness under reduced pressure in a rotary evaporator, yielding 34.7 g crude sample. After the filtrate was washed with ether several times, the residual aqueous solution was extracted with ethyl acetate repeatedly until the ethyl acetate layer became colorless. After the yellowish ethyl acetate extract was dried with anhydrous sodium sulfate, ethyl acetate was distilled off under reduced pressure. The residue was dissolved in a small quantity of hot water and the resulting solution covered with toluene was allowed to keep in ice box until the pigment crystallized.

Identification of compound:

The flower extract of *Sida cordifolia* was subjected to chromatographic separation analysis (paper chromatography) to isolate flavonoids. The results of the paper chromatography showed that this crude pigment consisted of flavonoid pigment, therefore the recrystallization from the boiling water was repeated, and slightly soluble crystals was obtained. Based on the R_f - value, number of fractions were obtained and the one with good resolution was visualized under ultraviolet (UV) light and also IR spectrum indicating that it was a pure compound, kaempferol 3-glucoside, (astragalin) was selected (Table 1). Lambda 35 model, data interval 10000 nm and scan speed 480.00 nm / min was used with UV detection. The infra-red (IR) was recorded on the Perkin Elmer-Universal ATR sampling Accessory, FT-IR spectrometer. Supporting evidence for the structure of the compound is provided by the ^{1}H (CDCl3, 500 MHz) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectra were recorded on a Bruker AMX 500 NMR spectrometer. Chemical shifts were reference to the respective residual solvent peaks and the values were recorded in δ.

DPPH free radical scavenging test:

The DPPH free radical is a stable free radical, which has widely accepted as a tool for estimating free radical-scavenging activities of antioxidants [15]. Hydrogen or electron donation capacities of the compounds have measured from the bleaching of the purple-colored methanol solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH). This spectrophotometer test uses the stable radical DPPH as a reagent. The sample solution of material (50 µL) at four concentrations (0.5, 1.0, 1.5 and 2.0 µg / mL) has mixed with freshly prepared methanolic solution of DPPH (634 µM) and allowed to stand for 30 min at room temperature. The absorbance has measured at 515 nm using a spectrophotometer and inhibits free radical DPPH in percent (%) has calculated using the formula below: The percent of inhibition of DPPH reduction (decolourization)

$$\% \text{ of inhibition} = \frac{A_0 - A\text{sample}}{A_0} \times 100$$

Anti-inflammatory activity by carrageenan induced paw edema:

The anti-inflammatory activity of the test compounds has evaluated in male albino rats employing the method [16]. Animals have fasted overnight and have divided into control, standard and different test groups each consisting of six animals. The different test concentration at the dose of 300 mg / kg of chloroform, ethyl acetate and methanolic extract and 100 mg / kg Diclofenac sodium has administrated to the animals by oral route. Control group animals have received 1% DMSO at the dose of 10 ml / kg body weight. They housed in cages and kept under standard conditions at 26 ± 2°C and relative humidity 60 - 65 % and 12 h light and 14 h dark cycles each day for one week before and during the experiments. The acute inflammation has induced by the sub-plantar administration of 0.1 ml of 1% carrageenan in the right paw. Paw volume has measured by using digital plethysmometer (Ugo Basile-Italy) before administration of carrageenan and after 1, 2 and 3 hrs intervals. The efficacy of different drug has tested on its ability to inhibit paw edema compared to control group.

Volume of edema = Final Paw Volume - Initial Paw Volume

RESULTS AND DISCUSSION

Compound **1** was obtained in the form of a brown powder (37 mg) has shown chromatographic properties (dark purple spot on paper chromatograms under UV light turning orange when fumed with NH_3 vapor for flavonoids and giving a green color on spraying with $FeCl_3$). Its melting points were compared using pure chemical (95%) purchased from Sigma Aldrich Co (India) as external standard and found to have the same values. The spectral data's (UV, IR and NMR) of the compound revealed that the compound **1** ($C_{21}H_{20}O_{11}$, m.p. 178-180ºC) as follows: As mentioned above, the UV spectrum indicated the presence of a flavone or a flavonol. Observation of the UV spectra with the use of shift reagents enabled determination of the flavonoid substitution scheme [17]. A shift of + 45 nm of Band I was observed after addition of $AlCl_3$, indicating the presence of a hydroxyl group in position C-5. Addition of the strong base NaOMe caused a shift of + 50 nm, showing the presence of a hydroxyl in C-4', and the appearance of a third band at 325 nm indicated the presence of a 7-OH. (18). The IR (KBr) ν cm^{-1} spectrum indicated presence of phenolic groups (3370 cm-^{1}), 3020 (aromatic–H stretching); 2992 (=C–H stretching); 2850–2925 (C–H stretching of glycoside moiety); 1730 (>C=O stretching); 1460 (C=C aromatic stretching) 1020 -1220 (C–O stretching).

The ^{1}H-NMR spectrums of compound **1** indicated the presence of four olefin methine proton signals at δ_H 8.16 (1H, d, *J* = 8.8 Hz, H-2'), 8.11 (1H, d, *J* = 8.8 Hz, H-6') and 6.86 (1H, d, *J* = 8.8 Hz, H-3'), 6.89 (1H, d, *J* = 8.8 Hz, 5') due to a *para* substituted benzene ring and two additional olefin methine proton signals at δ_H 6.19 (1H, d, *J* = 1.5 Hz, H-6) and 6.37 (1H, d, *J* = 1.5 Hz, H-8). One anomeric proton signal was assigned to the anomeric proton of a β-glucose at δ 5.25 (1H, *d*, *J*= 7.2 Hz, Glc-1") and the carbon signal at δ 105.2 (Glc-1"), four oxygenated methine proton signals and one oxygenated methylene proton signal between at δ 3.2-3.7(Glc-2-6") were observed. Consistently, the^{13}C-NMR spectrum revealed 21 carbon signals depicted the presence of one methylene, eleven methine and nine quaternary carbons. In the downfield conjugated ketone signal at δ_C 178.4 (C-4), six oxygenated olefin quaternary carbon signals at δ_C 159.2 (C-2), 136.4 (C-3), 163.6 (C-5), 166.2 (C-7), 157.6 (C-9), 162.02 (C-4'), and, two olefin quaternary carbon signals at δ_C 122.6 (C-1') and 109.94 (C-10), and six olefin methine carbon signals at δ_C 133.1 (C-2'), 132.8 (C- 6'), 116.12 (C-3'), 115.9 (C- 5'), 101.12 (C-6), and 94.62 (C-8) were observed . The chemical shifts of the glycosidic carbon signals were shown at δ_C 77.8 (Glc-2"), 74.9 (Glc-3"), 71.7 (Glc-4"), 78.2 (Glc-5"), and 62.12 (Glc-6"). Accordingly, the structure of Compound **1** was therefore identified as 3-*O*-β-glucopyranosyl - 4', 5, 7- trihydroxyflavone, or kaempferol 3-glucoside, also called astragalin (Figure 1) by comparison with spectral data in the literature [17, 19].

Anti-oxidant activity:

Exogenous chemical and endogenous metabolic processes in the human body or in the digestive system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage [20]. DPPH reaction has been widely used to test the ability of compounds to act as free-radical scavengers or hydrogen donors and to evaluate the antioxidant activity of foods and plant extracts [15]. The DPPH radical is a stable nitrogen-derived organic free radical, which can be reduced to a nonradical form (DPPH-H) by accepting an electron or hydrogen in the presence of a hydrogen-donating antioxidant. The anti-oxidant activity of *Sida cordifolia* flowers extract in solvents of varying polarity has measured with hydrogen granting or radical scavenging ability, using the stable radical, DPPH. The method has based on reduce alcoholic DPPH solutions in the presence of a hydrogen granting anti-oxidant. Table 3 illustrates the concentration of DPPH radical due to the scavenging ability of the petroleum ether, ethyl acetate and methanol extracts and standard L-ascorbic acid were presented, shown to be statistically significant at the various concentrations. The free radical scavenging activity of the ethyl acetate extract of *Sida cordifolia* showed the highest scavenging activity 43.33 ± 1.23, 79.65 ± 1.32, 90.43 ± 1.39, 96.21 ± 1.32 at 0.5, 1.0, 1.5 and 2.0 µg / mL respectively), followed by petroleum ether extract (34.23 ± 1.13, 46.48 ± 1.22, 61.72 ± 1.45 and 82.29 ± 1.98) and methanol extract showed least radical scavenging ability with % inhibition and 24.27 ± 1.18, 41.32 ± 1.32, 65.12 ± 1.29, 77.18 ± 1.26 at 0.5, 1.0, 1.5 and 2.0 µg / mL respectively. The results of the free radical scavenging activity of *Sida cordifolia* assessed by DPPH test and amount of the sample needed for 50% inhibition of free radical activity, IC_{50} values has summarized in Table 3. Lower IC_{50} value suggests higher anti-oxidant activity. The highest antioxidant activity was observed by petroleum ether and ethyl acetate, (IC_{50} value of 41.97 and 37.74 μM) corresponding kaempferol and kaempferol glycoside present in these extracts, more over which can be accredited to an additional hydroxyl groups presence of kaempferol glycoside has comparable with standard anti-oxidant of L-ascorbic acid IC50: (36.89 μM) respectively.

Anti-inflammatory activity studies:

To evaluate the anti-inflammatory activity of petroleum ether, ethyl acetate and methanol extract of *Sida cordifolia* flowers. Carrageenan-induced paw edema was tested on male albino rats weighing (200-250 g) is a well established animal model to assess the anti-inflammatory effect of natural products as well as synthetic chemical compounds [16]. Carrageenan induced rat paw edema shows a biphasic effect. The first phase is due to release of histamine and serotonin (5-HT) (0 - 2 h), plateau phase is maintained by kinins

like substance (3 h) and second accelerating phase of swelling is attributed to PG release (4 h) [21]. Carrageenan (2%) was injected into five groups (six animals each). Group I served as control, group II petroleum ether extract 300 mg, group III ethyl acetate extract 300 mg, group IV methanol extract 300 mg, group V standard diclofenac sodium 100 mg. Carrageenan induced edema is a biphasic response. The first phase was mediated through the release of histamine, serotonin, and kinins whereas the second phase was related to the release of prostaglandin and slow reacting substances which peak at 4h [22]. All tested groups decreased the thickness of edema of the hind paw compared to the control group as showed in Table 4. The percentage decrease in the paw volume in the group of animals treated with *Sida cordifolia* methanolic extract 300 mg was 1.98 ± 0.16 and for the petroleum ether (300 mg) was 1.54 ± 0.18 and ethyl acetate (300 mg/kg) was 1.12 ± 0.20 at 4 h. It shows the plant extract has significant anti-inflammatory effect and the results have compared with diclofenac sodium (100 mg/kg) was used as standard reference and it has significantly reduced paw oedema volume which was found to be a time dependent effect. Diclofenac showed maximum response at 4th hour which was found to be 0.98 ± 0.21. The group II & III showed pronounced anti-inflammatory effects after three hours of injection. Group IV has shown to inhibit the induced inflammatory response to carrageenan to a lesser extent than petroleum ether and ethyl acetate fractions. These results may suggest that the plant extracts exert significant anti-inflammatory activity, especially in the acute inflammatory response. On the other hand, the anti-inflammatory and analgesic activities of many plant extracts have been attributed to their high flavonoid content [23]. On the basis of these results, it can be concluded that the different extracts of *Sida cordifolia* possess anti-inflammatory activity and this may be due to any one or a combination of their phytochemical constituents.

Table No. 1: R_f (X100) values of the constituents of the flowers of *Sida Cordifolia* (Whatman No.1, Ascending, 30 ± 2°C)

Compounds	*Developing Solvents			
	a	B	c	D
[1]Aglycone	06	20	44	83
Kaempferol (authentic)	05	19	42	83
[2]Glycoside	44	67	76	72
Glycoside (authentic)	43	68	77	70

Compounds: *[1]Aglycone - kaempferol; [2]Glycoside - kaempferol 3-glucoside (astragalin)*
****Solvent key:*** *a - 15% aqueous acetic acid; b - 30% aqueous acetic acid; c - 60% aqueous acetic acid; d- n-Butanol (B): Acetic acid (A): Water (W) = 4:1:5 BAW (upper phase),*

Table No. 2: ^{13}C NMR and ^{1}H NMR spectroscopic data for astragalin isolated from *Sida cordifolia*

^{13}C NMR (500 MHz, δ in ppm)		^{1}H NMR (500 MHz, δ in ppm)	
Carbon	compound	Proton	δ_H
2	159.2	-	
3	136.4	-	
4	178.4	-	
5	163.6	-	
6	101.12	6	6.29 (1H, d, *J*=,1.5 Hz)
7	166.2		
8	94.62	8	6.37 (1H, *d*, *J*=1.5 Hz)
9	157.6		
10	104.94		
1′	122. 6		
2′	133.1	2′	8.16 (1H, *d*, *J*=8.8 Hz)
3′	116.12	3′	6.86 (1H, *d*, *J*=8.8 Hz)
4′	162.02		
5′	115.9	5′	6.89 (1H, *d*, *J*=8.8 Hz)
6′	132.18	6′	8.11 (1H, *d*, *J*= 8.8 Hz)
Glc 1″	105.2	1″	5.25 (1H, *d*, *J*= 7.2 Hz)
2″	77.8	2″- 6″	3.2-3.7(*m*)
3″	74.9		
4″	71.7		
5″	78.2		
6″	62.12		

Table No. 3: DPPH free radical scavenging activity of the flower extracts of *Sida cordifolia* extracts

Concentration (mg/ml)	IC50 (µg/mL^{-1})				
Samples	0.5	1.0	1.5	2.0	IC50 (µM)
	Radical scavenging effect (%)				
T. procumbens Petroleum ether extract	34.23 ± 1.13	46.48 ± 1.22	61.72 ± 1.45	82.29 ± 1.98	41.97
T. procumbens Ethyl acetate extract	43.33 ± 1.23	79.65 ± 1.32	90.43 ± 1.39	96.21 ± 1.32	37.74
T. procumbens Methanol extract	24.27 ± 1.18	41.32 ± 1.32	65.12 ± 1.29	77.18 ± 1.26	52.23
L-ascorbic acid	48.32 ±1.14	79.47 ± 1.47	91.33 ± 1.35	97.78 ± 1.28	36.89

Each value is expressed as the mean ± SD (n=3). The IC50 value was defined as the necessary concentration at which the radicals generated by the reaction systems were scavenged by 50%.

Table No. 4: Determination of paw volume of rats for *Sida cordifolia* extracts

Groups	Initial paw volume	Paw volume at different time interval (in ml)			
		1h	2h	3h	4h
Control (1% DMSO)	1.05 ± 0.12	1.46 ± 0.16	1.67 ± 0.13	1.94 ± 0.19	2.02 ± 0.14
Petroleum ether extract (200 mg/kg)	1.17 ± 0.24	1.45 ± 0.16	1.71 ± 0.23	2.14 ± 0.25	2.02 ± 0.15
(300 mg/kg)	1.18 ± 0.21	1.36 ± 0.18	1.80 ± 0.25	1.97 ± 0.24	1.54 ± 0.18

Ethyl acetate extract (200 mg/kg)	1.27 ± 0.26	1.38 ± 0.16	1.77 ± 0.27	1.80 ± 0.26	1.63 ± 0.19
(300 mg/kg)	1.18 ± 0.27	1.30 ± 0.18	1.48 ± 0.28	1.38 ± 0.27	1.12 ± 0.20
Methanol extract (200 mg/kg)	1.16 ± 0.19	1.38 ± 0.15	1.89 ± 0.21	2.26 ± 0.23	2.12 ± 0.17
(300 mg/kg)	1.18 ± 0.16	1.62 ± 0.17	1.81 ± 0.24	2.06 ± 0.26	1.98 ± 0.16
Diclofenac Sodium (100 mg/kg)	1.09 ± 0.26	1.26±0.18	1.34±0.27	1.21 ± 0.23	0.98 ± 0.21

Values are expressed in Mean ± SD (n=6); There is no significant difference between standard and test drug at P< 0.05 significant level.

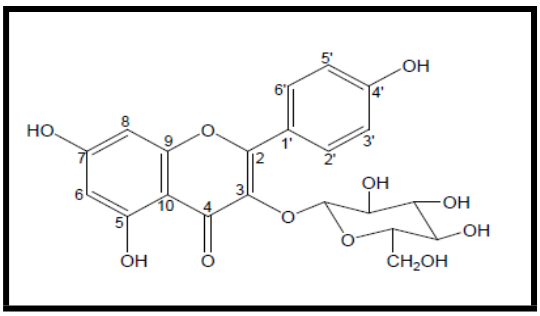

Fig. 1: Chemical structure of kaempferol 3-*O*-β-glucoside (astragalin)

CONCLUSION

The present investigation Physico-chemical studies will serve as standard reference for identification of kaempferol 3-glucoside (astragalin) from ethyl acetate extract of *Sida cordifolia,* which may account for some of the medical claims attributed to this plant and can be used as a source of antioxidant and anti-inflammatory for pharmacological prepared. The results of the present work clearly demonstrate the significant antioxidant and anti-inflammatory of the different extracts from the flower part of *Sida cordifolia*. The antioxidant and anti-inflammatory effects of the extracts may be ascribed to their flavonoid, alkaloids, saponins, and tannin contents.

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