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ORIGINAL ARTICLE

Bioactive Flavonids of *Spinifex littoreus* (Burm. f.) Merr - Investigation By Elisa Method

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Spinifex littoreus Burm.f.Merr. Coastal shrubs used for the treatment of a wide variety of diseases such as galactogenic, blistering agent, antihelmenthic, antipyretic, antidiruetic, anti – haemorrhages, antileukaemias and anti-inflammatory. It is rich in flavonoids and known to possess various biological activities. Fresh leaves of Spinifex littoreus have been examined for their bioactive flavonoids. The flavonol Kaempferol, flavonol glycoside astragalin have been identified. The structure of isolated polyphenolic compounds were characterised with the help of physical methods like UV, ^{1}H NMR, ^{13}C NMR, Chemical reactions, Chromatographic techniques and hydrolytic studies The *in-vitro* studies showed that the isolated flavonol glycosides have been found to contain ample anti-oxidant property with the help of ELISA method.

Key words: Astragalin, Kaempferol, Poaceae family

Spinifex littoreus Burm. f. Merr. (Syn.) *Spinifex squarrosus L; Stipa spinifex L* belongs to the family poaceae commonly called as Ravana – moustache. It is distributed throughout Fuijian, Guangdong, Guangri, Hainan – Taiwan, Cambodia, India, Indonesia, Malaysia, Myanmar, Philippines, Srilanka, Thailand and Vietnam (Wu & Raven 2006). It is used as galactogenic, blistering agent, antihelmenthic, antipyretic, antidiruetic, anti – haemorrhages, antileukaemias and anti-inflammatory (Muthukumaran *et al.*, 2012). Leaves are found to contain analgesic activity (Yogamoorthi & Priya 2006) and antimicrobial activity (Thirunavukkarasu *et al.*, 2010).

A biological system of antioxidant may preserve the cells from impairment caused by unstable molecules and the free radicals. Antioxidants terminate chain reactions (present in cell molecules) by removing free radical intermediates and inhibit other oxidation reactions being observed by them. They are believed to play a role in hindering the development of such chronic diseases as cancer, heart disease, stroke, arthritis and cataracts (Vinegar *et al.*, 1969).

MATERIALS AND METHODS

Plant extraction and fractionation

Air dried leaves (800g) of *S. littoreus* collected from sea shore of chandrabady in Nagapattinam district were extracted with 85% EtOH (4x 500 ml) under reflux. The specimen for the *S. littoreus* is kept at Rapinat Herbarium and Centre for Molecular Systematics, St.Josheph's college (Campus), Tiruchirappalli- 620 002, the specimen number being SA 011.

The alc. extract was concentrated *in vacuo* and the aq. concentrate successively fractionated with benzene (3 x 250 ml), peroxide free Et_2O (3x 250 ml) and EtOAc (4 x 250 ml).

The benzene and Et_2O fractions did not yield any isolable material.

Physical parameters

The EtOAc fraction was concentrated in vacuo and left in ice – chest for a day when a yellow solid was separated which was filtered and studied. When recrystallized from MeOH, it came out as long yellow needles, m.p. 176 – 78^0 C. It was freely soluble in aq. NaOH, hot water, EtOH and EtOAc, but insoluble in Et_2O, Me_2CO and $CHCl_3$.

Chemical reactions

It gave a greenish – brown colour with alc. Fe^{3+}, an intense yellow colour with NaOH, red colour with Mg – HCl and yellow precipitate with aq.lead acetate. It appeared dark purple under UV, which turned yellow on fuming with NH_3. It answered Wilson's boric acid (Wilson, 1939), Gibb's, Shinoda (Shinoda, 1928) and Molisch's tests but did not respond to the Horhamer – Hansel test (Horhammer & Hansel, 1955).

Spectral data

UV values of isolated compound had λ_{max}^{MeOH}nm 264, 301sh, 350; +NaOMe 273, 324, 398; +$AlCl_3$ with and without HCl 275, 304, 353, 397sh; +NaOAc 269, 305, 317sh, 353; +NaOAc/H_3BO_3 265, 301sh and 351.

Chromatographic data

Sheets of 20×45cm Whatman no 1 were used. The papers were run in glass cylinders by the ascending technique, usually to a height of 22-30cm at 25^{o}C. The solvents used were H_20, 5% HOAC, 15% aq. HOAC, 30% aq. HOAC, 60% aq. HOAC, n-BuOH:AOAC:H_2O (4:1:5 upperphase), phenol saturated with water, BuOH:HOAC:H_2O (3:1:1), Forestal (HOAC:Conc.Hcl: H_2O 30:3:10), formic acid (EtOH:ACOOH:H_2O 10:2:3). After through drying the chromatograms were dipped quickly into a saturated solution of ninhydrin. Rf values are varied greatly with the amount of flavonoid present and adsorption occurred at the origin of the chromatogram. It had Rf values as depicted in Table (I). The glycoside was identified as astragalin and that was confirmed by co- pc with an authentic sample of astragalin isolated from *Cestrum noctunum* (Barnabas, 1986).

Hydrolysis of the glycoside

The glycoside (0.05 g) dissolved in hot. aq. MeOH (2 ml, 50%) was hydrolysed with H_2SO_4 (5%) at 100^0 C for about 2h, and the hydrolytic products identified as described below.

Identification of aglycone (flavonol:Kaempferol)

The aglycone on recrystallisation from MeOH

afforded a yellow crystalline solid, m.p. 278 – 80 ^{0}C, which was identified as kaempferol by colour reactions, behaviour under UV. It was soluble in organic solvents but insoluble in water. It developed a reddish orange colour with Mg – HCl and yellow colour with NaOH. It appeared pale yellow under UV with or without NH3. It responded to Wilson's boric acid, Horhammer Hansel and Gibb's tests but did not answer the Molisch's test. UV values, λ_{max}^{MeOH} nm 253sh, 266, 294sh, 322sh, 367; +NaOMe 278, 316, 416(dec.); (+$AlCl_3$) 260sh, 268, 303sh, 350, 424; (+$AlCl_3$ - HCl) 256sh, 269, 303sh, 348, 424; +NaOAc, 274, 303, 387, and +(NaOAc +H3BO3) 267, 297sh, 320sh, 372. It has Rf values as depicted in Table (2). The structure has been ascertained by comparing the sample with an authentic sample isolated from *Bauhunia acuminata* (Barnabas & Nagarajan, 1979)

Identification of sugar

The aq. hydrolysate after the removal of the aglycone was neutralized with $BaCO_3$ and filtered. The concentrated filtrate on PC gave Rf values corresponding to those of glucose. The identity of the sugar was confirmed by direct comparison with and authentic sample of glucose.

DPPH Elisa method:

The antioxidant activity of the plant extract was estimated using a slight modification of the DPPH radical scavenging protocol given by Chang *et al.* (2001) DPPH free radical scavenging is one of the generally accepted mechanisms against lipid oxidation. The difference between DPPH free radical binding method and the other method is the short run time allowing rapid determination of the radical scavenging. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. The antioxidant activity of plant extracts were calculated according to the percentage inhibition in DPPH assay. It was optimized to be able to use micro titer plates, a multichannel pipet, and an ELISA reader, which makes it possible to analyze large numbers of samples in a run. With this method it was possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. As the result of the colour changing from purple to yellow the absorbance is decreased when the DPPH radical is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule (Matthäus, 2002). For a typical reaction, 2 ml of 100 µM DPPH solution in ethanol was mixed with 2 ml of 100 µg/ml of plant extract. The effective test concentrations of DPPH and the extract were therefore 50 µM and 50 µg/ml, respectively. The reaction mixture was shaken vigorously and allowed to stand at room temperature and incubated in the dark for 15 min and thereafter the optical density was recorded at 517nm against the blank. For the control, 2 ml of DPPH solution in ethanol was mixed with 2 ml of ethanol, and the optical density of the solution was recorded after 15 min. The assay was carried out in triplicate. Then the absorbance was measured at 517 nm in an ELISA reader. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity (Oktay *et al.*, 2003). The decrease in optical density of DPPH on the addition of test samples in relation to the control was used to calculate the antioxidant activity, as percentage inhibition (%IP) of DPPH radical:

$$\text{Radical scavenging (\%)} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

(Galani *et al.*, 2010)

The polyphenolic compounds and their percentage of inhibition of oxidation are listed in table 4.

RESULTS AND DISCUSSION

Characterisation of flavonoids from the active fraction of EtOAc fraction

The leaves of *S. littoreus* have been found to contain astragalin.

The band I absorption of the glycoside is at 350nm, which is an indicative of a flavonol skeleton. A comparison of band I absorption of the glycoside and the aglycone reveals that there may be 3 –glycosylation in the flavonol. A bathochromic shift of 48nm (band I) ascertained the presence of a free – OH at C- 4'. The $AlCl_3$ spectra, with and without HCl showed four absorption peaks to reveal the presence of a 5 –OH group. They were absorption peaks by the bathochromic shift of 47nm on the addition of NaOAc. The H_3BO_3

spectrum is exactly same as that of MeOH indicating the absence of catechol type of substitution in B – ring.

In the ^{1}H – NMR spectrum (400 MHz, DMSO – d6, TMS), the A- ring protons at C- 6 and C-8 appear separately as doublets at δ6.21 ppm (J= 1.7Hz) and δ6.21 ppm (J = 1.6 Hz) respectively. The 5 – OH proton resonates at δ12.6ppm. In the B – ring, the protons at C-2', 6' and C-3' and 5' are due to the free rotation of phenyl ring appear as two pairs of ortho coupled doublets at δ6.88ppm (J = 8.7Hz) and δ8.03ppm (J =8.8 Hz). The H -1" signal of the glucose moiety appears at δ5.46ppm (J = 7.2 Hz) found downfield from the bulk of the sugar protons. The remaining glycosyl protons appear in the range δ3.1 to δ3.7 ppm. The β – linkage of the glucose to 3 –OH is evident from the large coupling constant δ7.2 Hz of H – 1".

Comparing ^{13}C- NMR (100MHz, DMSO – d6, TMS) spectrum of the glycoside with that of the aglycone, the carbonyl carbon at C–4 of the glycoside appears at 1.59 ppm downfield than the aglycone. Due to glycosylation at C-3, the C-2 signal is shifted to 10.5 ppm downfield. The C-1" of the sugar appears at δ100.7 ppm and the rest of the sugar protons appear in the range δ 60.9 and δ77.2 ppm.

A complete assignment of the ^{13}C – NMR spectrum of the flavonol glycoside is available in Table (3). Based on these observations, the glycoside obtained from EtOAc fraction of S.littoreus could be confirmed as astragalin(kaempferol – 3- O- glucoside).

Investigation of antioxidant property

DPPH is one of the compounds that have a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi *et al.*, 1998). It is well accepted that the DPPH radical-scavenging by antioxidants is attributable to their hydrogen-donating ability (Chen & Ho, 1995; Wu *et al.*, 2008). In this current study, the DPPH radical scavenging method was used to evaluate the antioxidant capacity of the yellow pigments isolated from the leaves, because the use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers (Nickavar *et al.*, 2007).

The antioxidant activities of the isolated polyphenolic compound was tested and assessed on the basis of radical scavenging effect of the stable DPPH free radical. The scavenging capacities of phenolic compound were compared with that of ascorbic acid 100 µg/ml. These results imply that there are abundant antioxidative phytochemicals present in the plant species. The extract may exert its effect by reducing the oxidative damage due to free radicals. The EtOAc fraction exhibited the highest radical scavenging activity.

Table 1. Rf [x 100] values of the constituents of the leaves of *S. littoreus* (Whatman No : 1, Ascending, 30 ± 2˚ C)

Compound	DEVELOPING SOLVENTS							
	a	b	c	d	e	f	g	h
Glycoside from EtOAC fraction	13	40	42	68	77	70	71	53
Astragalin (authentic)	13	40	42	68	77	70	71	53

Table 2. Rf [x 100] values of the constituents of the leaves of *S. littoreus* (Whatman No : 1, Ascending, 30 ± 2˚ C)

Compound	DEVELOPING SOLVENTS								
	a	b	c	d	e	f	g	h	i
Aglycone from Et_2O fraction	-	-	5	15	49	93	67	62	87
Kaempferol (authentic)	-	-	5	15	48	93	67	62	87

Table 3. ^{13}C NMR spectral data and their assignment for the glycoside from the flowers of *S. littoreus*

Compound	C2	C3	C4	C5	C6	C7	C8	C9	C10	C'_1	C'_2	C'_3	C'_4	C'_5	C'_6
Glycoside from EtOAc fraction (δ ppm)	156.75	133.06	177.41	161.62	98.71	164.14	93.60	156.35	104.10	121.04	130.70	115.06	159.81	115.06	130.70
Astragalin (from Literature)	156.75	133.06	177.41	161.62	98.71	164.14	93.60	156.35	104.10	121.04	130.70	115.06	159.81	115.06	130.70

Compound	C''_1	C''_2	C''_3	C''_4	C''_5	C''_6
Glycoside from EtOAc fraction (δ ppm)	100.71	74.22	77.38	69.89	76.45	60.81
Astragalin (from Literature)	100.71	74.22	77.38	69.89	76.45	60.81

Table 4. Antioxidant activity of the isolated flavonoid glycosides

S.No	Grouping	Absorption λ at 517 nm	% of inhibition of oxidation
1	**Ascorbic acid**	**0.04**	**98%**
2	CB	2	--
8	Astragalin	0.82	89%

CONCLUSION

Phytochemical studies of *S. littoreus* leaves led to the identification of bioactive flavonoids (Kaempferol, astragalin). So they have the valuable source of flavonoids with antioxidant activity.

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