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Phytochemical and pharmacological evaluation of the flowers of *Sarcostemma brevistigma Wight*

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SUMMARY

Shade-dried flowers of *Sarcostemma brevistigma Wight Syn* (SBF) belonging to Asclepiadaceae yielded a rare flavonol glycoside viz., Quercetin 5a prenyl, 3-O-glucosyl, 7-O-(4c-p-coumaroyl) neohesperidoside which were characterized based on chemical and spectral (including 2D NMR) studies. The ethyl acetate fraction of alcoholic concentrate (test sample) of flowers of this plant (SBF) was evaluated for its hepatoprotective and inhibition of lipid peroxidation activities to investigate the scientific basis of the traditional uses. The oral administration in varying doses viz., 125 and 250 mg/kg of aqueous suspension of SBF to rats for 7 days produced significant ($P < 0.01$) hepatoprotective effect comparable to that of standard drug silymarin. The SBF afforded good hepatoprotection against CCl_4 induced elevation levels of serum marker enzymes, serum bilirubin and liver weight. The free radical scavenging effects of SBF and flavonol glycoside (SA) were assigned by Fe^{2+}/ascorbate induction method (in vitro), which revealed the inhibition of lipid peroxidation. The SBF and SA showed prominent anti-lipid peroxidation activity (IC_{50} about ~ 180 mg/ml and 11.0 mg/ml respectively), which was comparable to standard drug curcumin (IC_{50} about ~ 8.25 mg/ml). Thus the hepatoprotective activity of SB could be correlated to the free radical scavenging property of the flavonol glycoside.

Key words: *Sarcostemma brevistigma Wight Syn*; Flavonol glycoside; Hepatoprotective activity; Free radical scavenging effect

INTRODUCTION

Sarcostemma brevistigma Wight Syn (SB); *S.acidum Roxb Voight*, *Asclepias acida Roxb*; (Family: Asclepiadaceae) is a leafless trailing jointed shrub which grows throughout India and other tropical regions of the world (Kirtikar and Basu, 1987). Flowers and leaves of S. brevistigma are used as an antivenom (Selvanayahgam *et al.*, 1994). Preliminary ethnobotanical survey carried out by us revealed the use of aerial parts of this plant for liver disorders in Kolli Hills, Tamil Nadu. Hence the study on phytochemical constituents and hepatoprotective activity of flowers of this plant was undertaken.

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MATERIALS AND METHODS

Plant material

The Shade-dried flowers of *Sarcostemma brevistigma*

Wight Syn (SBF) were collected in January 2002 from Kolli Hills of Tamil Nadu and authenticated by P. Daniel, Botanical Survey of India, Coimbatore, Tamil Nadu. A voucher specimen GUHS-02-12 of the plant has been deposited in the Department of Chemistry at Gandhigram Rural University, Gandhigram.

Extraction, isolation and characterization

The fresh flowers (2 kg) were shade dried and extracted with petroleum ether (60 - 80°C) [3.5 l] and 80% (v/v) ethyl alcohol [3.5 l] successively in hot condition for 6 × 6 h each. The extracts were dried using a rotary evaporator at a temperature not exceeding 55°C and the yield were approximately 2.7% and 5.2% w/w respectively on wet weight basis. The aqueous concentrate of the alcoholic extract was successively extracted with benzene (4 × 300 ml), diethyl ether (3 × 300 ml) and ethyl acetate (4 × 300 ml) and the respective fractions were collected and concentrated in vacuo and the yield were approximately (10 g) 0.5%, (15 g) 0.75%, (20 g) 1.0% w/w respectively.

The ethyl acetate fraction (20 g) gave yellow solid (SBF) which was non homogenous in TLC and was chromatographed in silica gel (60 - 120 mesh, 400 g, 100 × 5 cm) column, using gradient elution with the solvents of increasing polarity. The 100% ethyl acetate eluent fractions afforded the pale yellow solid (SA), which responded positively for ferric chloride test, gave yellow colouration when fumed with ammonia (characteristic of polyphenolics) and gave violet colouration with Molisch's reagent (characteristic of glycoside) (Kokate, 2003). SA was subjected to acid hydrolysis and the aglycone and sugars were separated and the mobility of these in various solvents was studied (Markham, 1982). UV, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and EI-MS were also recorded for SA.

Hepatoprotective activity

The ethics committee of Vinayaka Mission's College of Pharmacy, Salem has approved the experimental protocol VM-02-42 used in this study. The LD_{50} value of SBF was determined in mice by Miller and Tainter method. (Miller and Tainter, 1944). The SBF and silymarin were suspended in 0.5% (w/v) gum acacia and used for studying antihepatotoxic activity. Male Wistar rats weighing between 150 - 170 g were fed on commercial diet (Hindustan lever, Bangalore) and water ad libitum during experiments and were divided into five groups, each group consisting of six animals. Hepatoprotective activity of SB was evaluated using CCl_4-induced model (Saraf and Dixit, 1991). In view of the fact that the SBF did not produce 50% mortality even at a dose of 5000 mg/kg and the content of flavonoids, which were suspected to be the active principle (Handa, 1986) is only 0.0025%, the two doses viz., 125 and 250 mg/kg were chosen for SBF. Group I, kept on normal diet, received 0.5% (w/v) gum acacia at a dose of 2 ml/kg and served as control, the group II received CCl_4 (1.25 ml/kg p.o.), the group III received silymarin, the standard drug (100 mg/kg p.o.), while the groups IV and V received the test drug at a dose of 125 and 250 mg/kg p.o. respectively daily for seven days.

On the seventh day, the animals were given CCl_4 by oral route 30 min after the administration of silymarin and test samples. After 36 h of CCl_4 administration, blood samples were collected and serum was separated for various biochemical analysis. Biochemical parameters like serum glutamic oxaloacetate transaminase (SGOT) (Reitman and Frankel, 1957), serum glutamic pyruvate transaminase (SGPT) (Reitman and Frankel, 1957), alkaline phophatase (ALP) (Kind and King, 1954), total bilirubin (Mallay and Evelyn, 1937), and gamma glutamate transpeptidase (GGTP) (Szasz, 1969) were analyzed. The liver was examined grossly, weighed and stored in formalin (10% v/v) and was processed for paraffin embedding using the standard microtechnique (Galigher and Kozloff,

1971). A section of the liver (5 m) stained with alum haemotoxylin and eosin was observed microscopically for histopathological studies. All values were expressed as mean ± S.E.M. The data were statistically analyzed by one-way ANOVA followed by Dunnett multiple comparison's test. *P* values < 0.05 were considered significant.

Determination of lipid peroxidation inhibiting activity (Induction by $FeCl_2$/ ascorbate)

The lipid peroxidation effects of SBF and SA were studied in vitro, (Kimuya *et al*., 1981) using curcumin as the reference compound. The solution of SBF, SA and curcumin were prepared by dissolving in dimethyl sulfoxide. Protein content was determined (Lowry *et al*., 1951), using bovine serum albumin as standard. The experiment was repeated for five times. The results were expressed in terms of malonodialdehyde (MDA) formed and the IC_{50} values were determined by graphical extrapolation as reported earlier (Ghosh, 1971).

RESULTS

Structure elucidation

The 100% ethyl acetate eluent fractions of column chromatography of SBF afforded the pale yellow solid (SA), which responded positively for flavonoid glycoside (m.p 1951 °C, yield 50 mg, 0.0025% w/w). It showed intense absorption maxima at 258 nm (band II) and 357 nm (band I) indicating a flavonol type skeleton with substitution at 3-OH. The ^1H NMR, HSQC and HMBC data of SA are tabulated in Table 1.

Hepatoprotective activity

Table 2 shows that the animals of group II, which received only CCl_4 were found to develop significant hepatic damage which was observed from elevated levels of SGPT, SGOT, ALP, total bilirubin, GGTP and liver weight as compared to group I normal animals. SBF treatment at a dose of 250 mg/kg, p.o could prevent the increase in the levels of various marker enzymes after CCl_4 administration.

Histological section of control animals (Group I)

Table 1. ^1H NMR, HSQC and HMBC data of SA

Proton signal δ H (ppm)	Correlated Carbon signal in HSQC dppm	Correlated carbon signal in HMBC δ (ppm)
7.59 H-2a/6a	115.1 and 121.6	115.1 (C-2a), 121.6 (C-6a), 120.9 (C-5a), 122.0 (C-1a), 156.4 (C-2), 144.8 (C-3a)
7.45 H-2f/6f	131.0	131.0 (C-2f/6f), 127.6 (C-1f), 116.2 (C-3f/5f)
7.36 H-β	144.7	144.7 (C-β), 116.0(C-α), 127.6 (C-1f)
6.80 H-3 f/5f	116.2	116.2 (C-3f/5f), 131.0 (C-2f/6f), 159.9 (C-4f)
6.43 H-8	93.7	93.7 (C-8), 156.4 (C-9), 162.5(C-7)
6.32 H-α	116.0	116.0(C-α), 144.7 (C-β), 166.1 (C=O) of coumaroyl
6.22 H-6	98.7	98.7 (C-6), 162.5(C-7), 161.2(C-5)
5.80 H-1b	101.7	101.7 (C-1b), 133.2 (C-3), 73.4 (C-2b)
5.40 H-1c	101.8	101.8 (C-1b), 162.5 (C-7), 77.8 (C-2c)
5.39 H-2e	121.1	121.1(C-2e), 29.0 (C-1e), 129.4 (C-3e)
4.90 H-1d	102.1	102.1(C-1d), 77.8 (C-2c) 69.5 (C-2d)
4.60 H-4c	71.2	166.1 (C=O) of coumaroyl, 71.2(C-4c)
4.0-3.3	60-80	60-80 (sugar carbon)
3.20 H-1e	29.0	29.0 (C-1e), 121.1 (C-2e), 120.9 (C-5a)
1.50 H-4e, H-5e	15.1 and 25.0	15.1 (C-4e), 25.0 (C-5e), 129.4 (C-3e)
1.20 H-6d	20.1	20.1 (C-6d), 67.9 (C-5d)

Table 2. Effects of SBF on levels of biochemical parameters and liver weight variation in CCl_4 treated rats

Design of treatment	Dose (mg/kg)	SGPT U/l	SGOT U/l	ALP U/l	Total Bilirubin (mg %)	GGTP U/l	Liver wt /100 g body wt (g)
Group I control	2 ml/kg	131.50 + 0.80	45.30 + 0.80	195.60 + 10.60	0.70 + 0.03	123.00 + 4.10	4.10 ± 0.01
Group II CCl_4	1.25 ml/kg	217.30 + 4.50[a]	341.00 + 3.80[a]	388.60 + 19.20[a]	1.20 + 0.07[a]	257.30 + 5.30[a]	6.50 ± 0.28[a]
Group III silymarin	100	138.0 + 2.10[a]	81.30 + 9.10[a]	218.60 + 5.40[a]	0.80 + 0.07[a]	124.60 + 5.20[a]	4.10 ± 0.26
Group IV SBF	125	142.10 + 1.80[a]	90.10 + 3.40[a]	324.00 + 4.90[a]	0.94 + 0.03[a]	130.30 + 2.40[a]	4.60 + 0.18[a]
Group V SBF	250	132.30 + 1.90	76.00 + 1.10[a]	222.60 + 5.30[a]	0.90 + 0.01[a]	126.00 + 2.40[a]	4.10 ± 0.10

n = 6 animals in each group; [a]$P < 0.01$; when compared to CCl_4. Data were analyzed by one-way ANOVA followed Dunnett multiple comparison's test. Values are expressed as mean ± S.E.M.

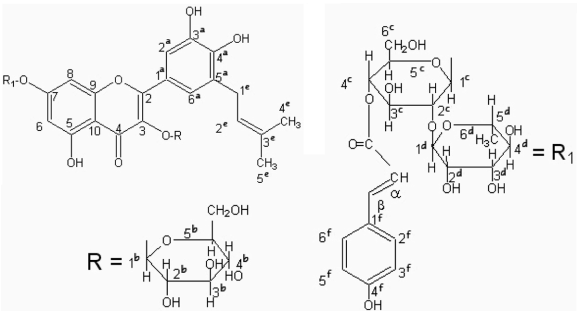

Fig. 1. Structure of SA.

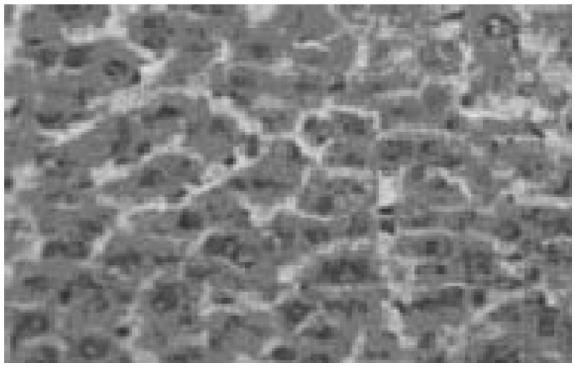

Fig. 2. Biopsy study of the liver of normal group.

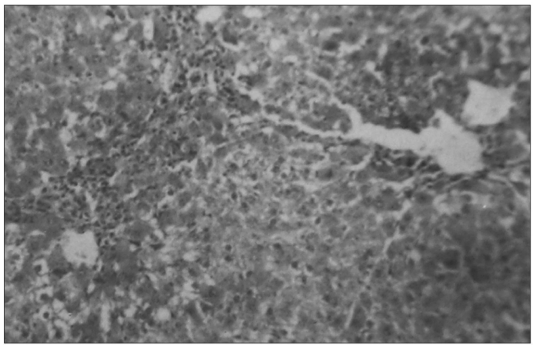

Fig. 3. Biopsy study of the CCl_4 intoxicated liver.

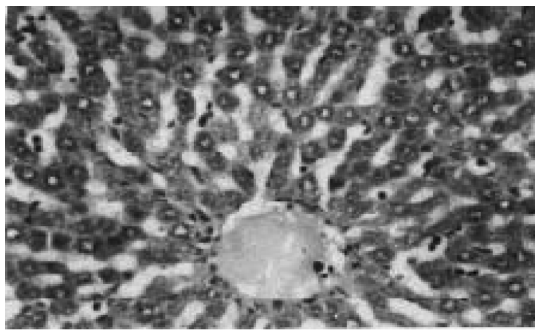

Fig. 4. Biopsy study of the CCl_4 intoxicated liver treated with the standard drug silymarin.

showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus and conspicuous central vein (Fig. 2). Histological section of Group II animals showed high degree of damage characterized by cell vacuolation, pyknotic and degenerated nuclei and damage to wall of capillaries (Fig. 3). Histopathological profile of the Group III animals showed the recovery against the CCl_4 induced damage as compared to control (Fig. 4). The histological section of the Group IV animals showed that nuclei were not clear as in normal hepatocytes but when compared to the CCl_4 damage ones, the hepatocytes with normal nucleus were more. Endothelium was disrupted at places but in lesser numbers than CCl_4 intoxicated rats. Hepatic cells adjoining the interlobular vein showed atrophy. Pyknotic nucleus and vacuolation in cytoplasm were observed to be low. There seemed to be a satisfactory recovery (Fig. 5).

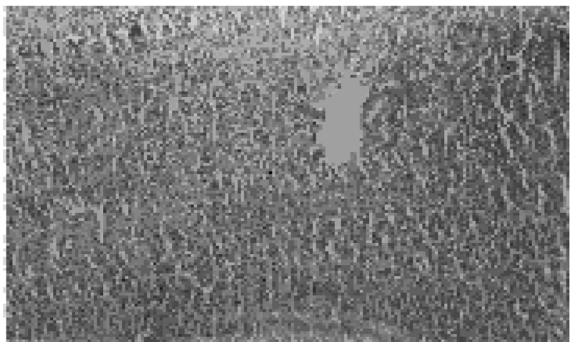

Fig. 5. Biopsy study of the CCl_4 intoxicated liver treated with ethyl acetate concentrate of *SB*.

Lipid peroxidation activity

The results of antioxidant studies carried out using Fe^{2+}/ascorbate induction method clearly reveals that SBF at 200 mg/ml and SA at 20 mg/ml act as a radical scavenger and inhibits lipid perioxidation (53.42% and 67.69% respectively) comparable to curcumin at 20 mg/ml (68.53%), the standard drug used. The SBF and SA showed prominent anti-lipid peroxidation activity (IC_{50} about 180 µg/ml and 11.0 µg/ml respectively), which were comparable to standard drug curcumin (IC_{50} about 8.25 µg/ml).

DISCUSSION

SA (m.p 1951°C, yield 50 mg, 0.0025%) showed intense absorption maxima at 258 nm (band II) and 357 nm (band I) indicating a flavonol type skeleton with substitution at 3-OH. A bathochromic shift of +51 nm of band I on addition of NaOMe showed the presence of hydroxyl group at C-4a. The characteristic bathochromic shift was not observed on band II on the addition of NaOAc, which suggested the non-existence of free hydroxyl group at C-7. Further no correlation of H-6 proton (6.22 ppm) or H-8proton (6.43 ppm) with C-7 was observed in ^{1}H-^{1}H COSY spectrum of SA and it was taken as the further evidence for the presence of substitution at C-7. A bathochromic shift of band I on addition of NaOAc/H_3BO_3 (+22 nm) confirmed the presence of ortho-dihydroxyl group in ring B. Band I underwent a bathochromic shift of +39 nm on the addition of $AlCl_3$/HCl which indicated the presence of 5-OH in SA.

In ^{1}H NMR a downfield signal at 12.65 ppm showed the presence of a chelatogenic OH at C-5. Glycosidic nature of SA was ascertained by colour reactions (Kokate, 2003) and hydrolytic studies. The Rf values in various solvent systems were supportive of a trioside. He estimation of sugars revealed the presence of glucose and rhamnose in the ratio of 2:1(Markham, 1982).

The ^{1}H NMR and ^{13}C NMR also suggested the presence of two glucopyranosyl moieties with β configuration and one rhamnopyranosyl residue with α configuration. The signals displayed at 4.9 ppm (^{1}H, d, J = 2 Hz, H-1d) and ppm (^{1}H, d, J = 6 Hz, H-6d) in ^{1}H NMR and signal at 61.1 ppm in ^{13}C NMR revealed the neohesperidoside nature of the glycoside (Mabry *et al*., 1970).

After acid hydrolysis, a mixture of aglycone and coumaric acid was obtained. From the studies of ^{1}H NMR, ^{13}C NMR (Matsuda, *et al*., 2002), ^{1}H-^{1}H COSY, HSQC and HMBC (Table 1), the presence of coumaroyl group was evident. A large coupling constant of (J = 15.8 Hz) observed between the olefinic protons (H- and H-) in acyl moiety of SA confirmed the trans configuration of H- and H- (Chaudhuri and Thakur, 1986). The fragment lines of m/z 164 and 147 implied the presence of p-coumaroyl moiety (Mizuno *et al*., 1987).

The anomeric proton at d 5.4 ppm (glucose H-1c) was correlated with C-7 (d 162.5 ppm) and the anomeric proton at d 4.9 ppm (rhamnose H-1d) was correlated with C-2c (d 77.8 ppm) in HMBC spectrum, which revealed the attachment positions of neohesperidoside at C-7 (Table 1). Further correlation observed between the anomeric proton signal at d 5.8 ppm (glucose H-1b) and d 133.2 ppm (C-3 signal) in HMBC spectrum revealed the attachment of glucose at C-3 (Table 1).

The attachment of coumaroyl group with the glucose moiety of neohesperidoside was confirmed by the correlation of H-4c signal (4.6 ppm) of glucose with carbonyl carbon signal (166.0 ppm)

of coumaroyl group, in the HMBC spectrum (Table 1). The presence of prenyl group was established by ^{1}H and ^{13}C NMR spectroscopic analysis whose assignments were facilitated by HSQC and HMBC experiments and were in agreement with values reported in the literature for the presence of prenyl group (Tahara *et al.*, 1994). This was further evidenced by the appearance of mass spectral line of m/z 69 in EI-MS spectrum of SA.

Since ^{1}H NMR spectrum revealed the presence of only H-2a, 6a protons and further UV studies had already confirmed the presence of o-diOH groups at C-3a, 4a, the position of attachment of prenyl group could be only at C-5a. The EI-MS of SA exhibited molecular ion peak at m/z 369 ($M-1+^{2}$), with diagnostic retro-Diels-Alder cleavage of ring C at m/z 153 and 205 supporting the presence of two hydroxyl in ring A and two hydroxyl and one prenyl in ring B.

Thus on the basis of UV, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC and EI-MS, SA has been characterized as quercetin 5a prenyl, 3-O- glucosyl, 7-O-(4c-p-coumaroyl) neohesperidoside.

The hepatotoxicity induced by CCl_4 is due to its metabolites viz., trichoromethyl (CCl_3^{2}) and trichloromethyl peroxy ($CCl_3O_2^{2}$), radicals that bind to lipoprotein and lead to peroxidation of lipids of endoplasmic reticulum (Recknagael, 1967). The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Protection of hepatic damage caused by CCl_4 administration observed by changes in the marker enzymes such as SGPT, SGOT, ALP, total bilirubin and GGTP in normal, toxin treated, standard drug (silymarin) treated and SBF treated (both 125 mg/kg and 250 mg/kg) groups. The disturbance in the transport function of the hepatocytes as a result of hepatic damage causes the leakage of enzymes from cells due to altered permeability of membrane (Zimmerman and Seeff, 1970). This results in decreased levels of SGOT and SGPT in the hepatic cells and a raise in their serum level. From the Table 2, it was clear that SBF showed greater protection at a dose of 250 mg/kg and the values are comparable to those of silymarin, the standard drug used. The weight of the liver remained normal in the cases of silymarin and SBF treated groups. A significant protection ($P < 0.01$) in liver weights supports this finding.

The histopathological studies offer direct evidences of efficacy of SBF as hepatoprotective agent. The SBF treated group showed good recovery of the hepatocytes. The liver sections showed almost disappearance of fatty deposit and necrosis, comparable to the standard drug silymarin. The lesions developed on treatment with CCl_4 (Fig. 3), were found to be normalized with near normal histoarchitecture of liver cells, in standard drug (Fig. 4) and SBF treated animals (Fig. 5).

The SBF and SA showed prominent anti lipid per oxidation activity (IC_{50} about in 180.0 μg/ml and 11.0 μg/ml respectively), which were comparable to standard drug curcumin (IC_{50} about 8.25 μg/ml). Many plants exhibit efficient antioxidant properties owing to their phenolic constituents. Most of the tannins and flavonoids are phenolic compounds and are responsible for antioxidant properties of many plants (Larson, 1998). The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial bio-molecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions. (Halliwell and Gutteridge, 1985). Drugs that act against free radical or scavenge it may be very useful in various free radical induced debilitating diseases such cancer or carcinoma, diabetes, hypertention, liver disorders and tumour. Thus the hepatoprotective activity of SB could be correlated to the free radical scavenging property of the flavonol glycoside.

CONCLUSION

The study clearly proves that the radical scavenging

property of SA (Quercetin 5a prenyl, 3-O-glucosyl, 7-O-(4c-p-coumaroyl) neohesperidoside) is responsible for the observed hepatoprotective activity of SBF. It is a known fact that flavonoids have a high safety margin and are free from side effects such as ulcerogenicity (Alvaraz *et al*, 1999). The results of the present study advocate the full exploitation of S. brevistigma as a hepatoprotective agent in the management of liver disorders after proper clinical trials.

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