In Vitro Antioxidant Potential of *Semecarpus Anacardium* L.

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Summary

Using Lipid peroxidation, DPPH and nitric oxide, Superoxide and Hydroxyl radical scavenging assay to investigate the antioxidant activity of crude ethyl acetate extract from the stem bark of *Semecarpus anacardium* L., it was found that the ethyl acetate extract exhibited a stronger antioxidant activity compared to the other (hexane, chloroform and methanol) extracts, where total phenolic content of ethyl acetate extract was highest with 68.67 % measured as pyrocatechol equivalent. On the basis of Lipid peroxidation, DPPH, nitric oxide and inhibition of superoxide anion radical scavenging assay, bioassay guided isolation of the ethyl acetate extract of *S. anacardium* stem bark was carried out by silica gel column chromatography. It afforded a bright-yellow solid crystal, which was identified as butein (1). This compound exhibited antioxidant activity (IC_{50} values of 43.28 ± 4.34 μg/ml) which was comparable to rutin, taken as a standard.

Key words: *Semecarpus anacardium* L., Butein, DPPH, Rutin, Antioxidant activity.

Introduction

Reactive oxygenic species in the form of superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are natural by-products of human metabolism. The free radicals and reactive oxygen species have been proposed to induce cellular damage and to be involved in several human diseases, such as cancer, arteriosclerosis inflammatory disorders, as well as in aging processes (1, 2). Of various kinds of natural antioxidants, phenolic compounds have received much attention (3, 4). Antioxidants, such as phenolic compounds including flavonoids, chalcones, lignoids, stylbenoids, tannins, and diarylheptanoids, are distributed in the plant kingdom and may prevent oxidative damage by scavenging ROS. Therefore, the phenolic constituents of plants are of interest as potential chemo preventive agents, and plants may be an attractive alternative to currently available commercial antioxidants, because they are biodegradable to non-toxic products (5, 6). In view of these we aimed to evaluate the antioxidant activity of *S. anacardium* through different *in vitro* test models so as to prove the antioxidant activity of another well known traditional plant to be effective therapeutically, considering the presence of polyphenols in the stem bark of *S. anacardium*.

Semecarpus anacardium (SA) L. F. (Anacardiaceae) is a deciduous tree distributed in the sub-Himalayan tract and in hotter parts of India (7). Commonly known as "Bhelwa", is a deciduous tree, up to ten meters tall. Different parts of this plant have been traditionally used to treat rheumatism, asthma, neuralgia, anthelmintic infection, cancer and psoriasis (8). Most of the work was performed on the nut and fruit parts of the *S. anacardium* L. (Bhallatak, nut shell) as fruit extract exhibited hypocholesterolemic action and prevented cholesterol induced atheroma in hypercholesterolaemic rabbit (9). The *in vitro* acetyl cholinesterase activity (AChE) of methanolic extracts of stem bark of *S. anacardium* was investigated (10). The ethyl acetate extract showed *in vivo* anti-inflammatory activity in carrageenin-induced rat paw edema (11). Till date, however little work has been carried out on the stem bark of this plant. Phytochemical screening of the extracts showed the presence of flavonoids, tannins, steroids in the ethyl acetate extract of this plant. Literature review indicated that the *in vitro* antioxidant activity of this species has not been clinically evaluated so far. Considering the presence of % of polyphenol contents and phytochemical screening of ethyl acetate extract of *S. anacardium* plant, the present study were undertaken to evaluate the antioxidant potential of *S. anacardium* L.

Materials and Methods

Chemicals

All chemicals were of analytical and highest purity and were purchased from Sigma Chemical Co. (St., Louis, USA), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA). Hi-Media, India Ltd. and, Merck Co. (Germany).

Plant material

The stem bark of *S. anacardium* were collected from field areas of Narendrapur, Kolkata and the authenticity of the stem specimen was confirmed through Government Arts College, Ooty, Tamil Nadu. A voucher specimen was deposited in the School of Natural

Product Studies for the future references (Access No. SNPS-1022/2006-2007); Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal.

Extraction

Extraction procedure was based on the method of Selvam et al., 2004 (11). The air-dried stem barks 2 kg were roughly ground and subjected to extraction in a Soxhlet apparatus successively with hexane (HESA) (4% w/w); chloroform (CHSA) (3% w/w); ethyl acetate (EASA) (4% w/w) and methanol (MESA) (7% w/w). The EASA extract, a powdered mass of red color was obtained and kept in a desiccator.

Analysis of total phenolic compound

Total soluble phenolics in the HESA, CHSA, EASA and MESA extracts of *S. anacardium* extract were expressed as microgram of pyrocatechol equivalents, determined with Folin-Ciocalteu reagent (FCR) according to the method of Slinkard and Singleton, 1977 (12). 1ml of solution (containing 1mg) of the extracts in methanol was transferred into 100 ml Erlenmeyer flask containing 46 ml of distilled water. Afterward, 1 ml of FCR was added into this mixture and after 3 min, 3 ml of Na_2CO_3 (2%) was added. Subsequently, mixture was shaken intermittently for 2 h at room temperature and then absorbance was measured at 760 nm. The concentration of the total phenolic compounds was calculated by using an equation that was obtained from standard pyrocatechol graph: Absorbance= 0.001 X Pyrocatechol (µg) + 0.0033. Total phenolic content were measured as pyrocatechol equivalent of all the above extracts of *S. anacardium*.

In vitro assay

Lipid peroxidation:

The peroxide formation was measured by the method of Ohkawa, (1979) (13) by measuring the color of thiobarbituric acid reactive substances (TBARS) formed at the end of the reaction. Malonaldehyde (MDA) which is formed as end product in lipid peroxidation react with thiobarbituric acid (TBA) to give TBARS which is pink in color and measured at 530 nm. The reaction mixture contained rat liver homogenate (0.1 ml, 25%, w/v) in Tris-HCl buffer (20 mM, pH 7.0), KCl (150 mM), ferrous ammonium sulphate (0.8 mM), ascorbic acid (0.3 mM) and concentrations of the HESA, CHSA, EASA and MESA extracts at (10, 25, 50, 75 and100 µg/ml) in a final volume of 0.5 ml, was incubated for 1 hr at 37 ^{0}C (14; 15). The incubated reaction mixture (0.4 ml) was treated with 0.2 ml of 8% sodium dodecyl sulphate (SDS), thiobarbituric acid (1.15 ml, 8%) and acetic acid (1.5 ml, 20%, pH 3.5). The total volume was then made upto 4 ml by adding distilled water and kept in a water bath at 100 ^{0}C for 1 hr. After cooling, 1ml of distilled water and 5ml of a mixture of n-butanol: pyridine (15:1 v/v) was added and shaken vigorously. The absorbance of the organic layer was measured at 560 nm using (Perkin-Elmer Lambda UV-Visible spectrophotometer) after centrifugation. The % inhibition of lipid peroxide formation was determined by comparing the results of the extract and control samples. Curcumin (1-100 µg) was used as reference. The % of inhibition was calculated by using following equation;

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Free radical scavenging activity (DPPH)
The free radical scavenging capacity of the extracts was determined using DPPH. A methanolic DPPH solution (0.15%) was mixed with serial dilutions (10-100 μg/ml) of the extracts of HESA, CHSA, EASA and MESA. After 10 minutes the absorbance was read at 515 nm using a spectrophotometer (Perkin-Elmer). The inhibition curve was plotted and IC_{50} values obtained (14, 15); where rutin was considered as a standard. The % of inhibition was calculated by using the equation;
% Inhibition= [(Absorbance$_{control}$ – Absorbance$_{sample}$)/Absorbance$_{control}$] x 100.

Inhibition of Nitric oxide radical
Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (16, 17). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the drug in different concentrations (10-100 μg/ml) was incubated at 25 ^{0}C for 150 minutes. At intervals samples (0.5 ml) of incubation solution were removed and 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was measured at 546 nm. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test compounds, whereas rutin was taken as standard. The % of inhibition was calculated by using following equation;
% Inhibition= [(Absorbance$_{control}$ – Absorbance$_{sample}$)/Absorbance$_{control}$] x 100.
Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by HESA, CHSA, EASA and MESA extract of stem bark of *S. anacardium*.

Inhibition of Superoxide anion radical
Measurement of superoxide anion scavenging activity of different extracts was done based on the method described by Nishimiki (18) and slightly modified. About 1 ml of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of sample solution of *S. anacardium* in water were mixed. The reaction started by adding 100 μl of phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 ^{0}C for 5 minutes and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The % of inhibition was calculated by using following equation;
% Inhibition= [(Absorbance$_{control}$ – Absorbance$_{sample}$)/Absorbance$_{control}$] x 100.

Hydroxyl radical scavenging activity: Deoxyribose assay
The assay was performed as described by Halliwell (1987) (19) with minor changes. All solutions were prepared freshly. 1.0 ml of the reaction mixture contained 100 μl of 28 mM 2-deoxy-2-ribose (Fluka, dissolved in KH_2PO_4-K_2HPO_4 buffer, pH 7.4), 500 μl solution of various concentration of the extracts *S. anacardium* (50-250 μg/ml in buffer and Tween 80), 200 μl of 200 μM $FeCl_3$ and 1.04 mM EDTA (1:1, v/v), 100 μl H_2O_2 (1.0 mM) and 100 μl ascorbic acid. After an incubation period of 1 hour at 37 ^{0}C the extent of

deoxyribose degradation was measured by the TBA reaction. 1.0 ml of TBA (1% in 50 mM NaOH) and 1.0 ml of TCA were added to the reaction mixture and the tubes were heated at 100 ^{0}C for 20 minutes. After cooling the absorbance was read at 532 nm against a blank (containing only buffer and deoxyribose). The absorbance read at the end of the experiment was used for the calculation of the % inhibition of deoxyribose degradation by the test compound. Catechin was used as a positive control. Inhibition of deoxyribose degradation in percent was calculated in following way:

$$I = A_0 - A_1/A_0 \times 100$$

Where A_0 is the absorbance of the control reaction (containing all reagents except the test compound), and A_1 is the absorbance of the test compound. The IC_{50} value represented the concentration of the compounds that caused 50 % inhibition.

Isolation

A glass column, 75 cm in length, 5.5 cm inside diameter and small column 25 cm in length, 3.5 cm inside diameter fitted with a stopcock was used. Silica gel 230-400 mesh size, 0.040-0.063 mm (E. Merck and Co. Ltd) was activated by heating at 120 ºC for 1 h and was used as adsorbing material. The solvent system of chloroform: ethyl acetate (10:0 to 0:10) was used. The combined ethyl acetate extracts (15 g) was subjected to chromatography on a silica gel column using gradient elution of chloroform: ethyl acetate (10:0 to 0:10) (chloroform: 10; chloroform-ethyl acetate: 9:1; 8:2; 7:3, 6:4; 5:5; 4:6; 3:7; 2:8; 1:9 v/v; ethyl acetate:10). Several samples were collected and monitored by TLC (dichloromethane: methanol: acetic acid (4.3: 0.2: 0.2, v/v); UV 254 nm). Similar samples were combined into three fractions (Fr A: 21-32; Fr B: 38-53; Fr C: 59-77). Each fraction was tested for *in vitro* antioxidant activity as in Table 3. Among these fractions, Fraction C which was eluted with chloroform: ethyl acetate (6:4, 300 ml); showed the most significant *in vitro* antioxidant activity by DPPH of 43.28 ± 4.34 at µg/ml concentration; rutin is taken as a standard 21.22 µg/ml. Fraction C (1.4 g) was again subjected to silica gel column chromatography eluted with methanol; resulted in the isolation of phenolic compound-1 (0.000665% w/w, with the dry plant material), which was purified by re-crystallization ethanol. The structure of the phytoconstituent is given in Fig. 1.

Statistical analysis

The data were expressed as mean ± SD., which for the biochemical and physiological parameters were analyzed statistically using one way ANOVA procedures followed by by Dunnett's tests. Values of P-values <0.05 were regarded as significant, P-values <0.01 regarded as very significant and P-values <0.001 regarded as most significant.

Standardization of EASA of S. anacardium

EASA was dissolved in CH_3OH (1 mg/ml) and 10 µl of it was applied on the silica gel 60 F_{254} HPTLC plates. After development the plates were scanned at 254 nm. In dichloromethane: methanol: acetic acid solvent system (4.3: 0.2: 0.2, v/v). The standardization of extract was done by the isolated compound-1 with R_f = 0.40; with the solvent system dichloromethane: methanol: acetic acid with the ratio (4.3: 0.2: 0.2, v/v); shown yellow spot at UV-254 nm.

Identification of Compound-1

Bright-yellow solid crystal, odorless, practically insoluble in water, freely soluble in ethanol, ethyl acetate and methanol, m.p. 211-215° C.

UV (Ethanol): Absorbance peaks 234, 280, 310 μm.

IR (KBr) cm^{-1} : 3450 (OH), 1650 (-C=O), 1645 (C=C), 1600, 1530, 1280, 1120, 1100, 1015, 995 cm^{-1}.

^{1}H NMR (500 MHz, in $CDCl_3$) δ ppm: indicated the presence of a trans-α,β-unsaturated ketone peak with δ 7.09 (1H, d, J = 16.1 Hz) and δ 7.51 (1H, d, J = 16.1 Hz) and an m,p-3 substitution benzene structure with δ 7.01 (1H, d, J = 2.2 Hz, H-3), δ 7.11 (1H, dd, J = 2.2, 8.6 Hz, H-5′), δ 7.71 (1H, d, J = 8.6 Hz, H-6′), δ 7.42 (1H, d, J = 2.1 Hz, H-2), δ 7.23 (1H,d, J = 8.4 Hz, H-5), and δ 7.45(1H, dd, J = 2.1, 8.4 Hz, H-6).

EIMS: m/z (rel int.) = m/z 272 $(M+H)^+$, pattern 272 (99), 163(22), 150 (16), 135 (17), 111 (10), 94 (52), 77 (70).

Results and Discussion

Total phenolic content of the three extracts as HESA, CHSA, EASA and MESA of *S. anacardium* stem bark were determined as shown in Table 1. EAMT was found to have higher phenolic content as compared to the other extracts. HESA, CHSA, EASA and MESA extracts exhibited antioxidant activity on lipid peroxidation, DPPH and nitric oxide, Superoxide and Hydroxyl radical scavenging assay (Table 2) which has been widely used to measure the radical scavenging ability of various plant extracts and constituents (20,21). EASA was shown the potent antioxidant activity than the rest of those extracts as HESA, CHSA and MESA. Hence, EASA was considered for the Column chromatography through bioassay guided isolation technique. Different fractions were collected and pulled into 3-fractions on the basis of TLC separation method; using the solvent system dichloromethane: methanol: acetic acid with the ratio (4.3: 0.2: 0.2, v/v). Individual fractions were assayed by DPPH antioxidant procedure (14, 15); where rutin was taken as a standard. It was found that; fraction-C was shown potent antioxidant activity with IC_{50}-value 43.28 ± 4.34 at μg/ml than other fractions. Then fraction-C was further purified to get the compound-1. The structural elucidation of the compound-1 was based on the spectroscopic evidences and comparison with literature data (22). Compound-1 was obtained as a bright-yellow solid crystal. As far as we know, compound-1 is a new natural phenol (chalcone) found for the first time by bioassay guided isolation way in the EASA extract of the plant material. The name of the compound is ‘Butein’. The DPPH radical-scavenging activity of compound-1 was carried out and it exhibited an IC_{50} value of 43.28 ± 4.34 (Table 3). It was noticed that most of the isolated fractions showed obvious scavenging activity on DPPH radicals. Comparing with rutin, the flavan-3-ol derivative displayed stronger activities where it was considered as standard; the IC_{50} value was shown in Table 3**.** However, both rutin and butein showed distinguished scavenging activity on DPPH radicals in our work. Butein is the main constituent isolated from *S. anacardium* and may play an important role for the antioxidant activity of this plant. Finally, the above results will provide the evidences to evaluate the biological functions of *S. anacardium* and promote the reasonable usage of this plant.

Table1. Total phenolic content of HESA, CHSA, EASA and MESA of *S. anacardium* stem bark.

Different extract	**Polyphenol content (equivalent of Pyrocatechol/ mg)**	**% of Polyphenols**
HESA	28.76 µg/ mg	2.87
CHSA	113.3 µg/ mg	11.33
EASA	686.7 µg/ mg	68.67
MESA	387.8 µg/ mg	38.78

Hexane (HESA), chloroform (CHSA), ethyl acetate (EASA) and methanol (MESA) of *S. anacardium.*

Table 2. Inhibitory effect (IC_{50}) of HESA, CHSA, EASA and MESA of *S. anacardium* stem bark on lipid peroxidation, DPPH, nitric oxide, superoxide and hydroxyl radical scavenging *in vitro* antioxidant assay.

Parameters	**HESA (µg/ml)**	**CHSA (µg/ml)**	**EASA (µg/ml)**	**MESA (µg/ml)**	**Standard (µg/ml)**
Lipid Peroxidation	205.08**± 14.45	187.43**± 11.43	102.34**± 9.67	165.21**± 9.82	10±1.13 [curcumin]
DPPH radical scavenger	103.69*± 9.98	82.45*± 7.77	44.03*± 4.12	60.23*± 5.68	20±1.87 [rutin]
Nitric oxide radical scavenger	176.33**± 15.32	132.43**± 13.21	80.75**± 8.11	119.23**± 10.41	20±1.15 [rutin]
Superoxide radical scavenger	89.91*± 7.48	73.23**± 6.65	68.55**± 6.62	78.21**± 7.71	5±0.45 [curcumin]
Hydroxyl radical scavenging	187.94*± 17.78	143.24*± 14.32	92.43**± 8.97	129.51**± 11.75	5±0.55 [catechin]

Hexane (HESA), chloroform (CHSA), ethyl acetate (EASA) and methanol (MESA) on lipid peroxidation, DPPH, nitric oxide, superoxide and hydroxyl radical scavenging *in vitro* antioxidant assay. *Represents $P<0.05$ and **Represents $P<0.01$. The data on all antioxidant activity tests are the average of triplicate analyses. Statistical analysis of variance was performed by one way ANOVA procedures followed by Dunnett's test. IC_{50} values were calculated from the concentration-effect linear regression curve. The rest groups HESA, CHSA, EASA and MESA were compared with the respective standard drugs.

Table 3. *In vitro* antioxidant assay of isolated fractions of *S. anacardium* stem bark by DPPH method.

***In Vitro* assay**	**Fraction-A (µg/ml)**	**Fraction-B (µg/ml)**	**Fraction-C (µg/ml)**	**Standard (µg/ml)**
DPPH	107.45 ± 7.32*	85.47 ± 4.45*	43.28 ± 4.34*	Rutin 21.22 ±0.75

*Represents $P<0.05$; the data on all antioxidant activity tests are the average of triplicate analyses. Statistical analysis of variance was performed by one way ANOVA procedures followed by Dunnett's test. Standard drug rutin was compared with the rest of the groups.

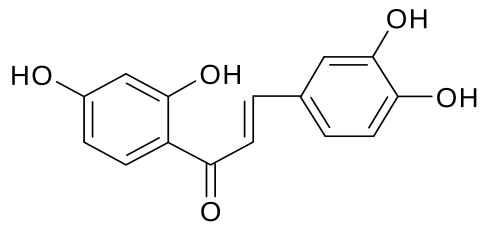

Fig. 1. Chemical structures of compound-1 (Butein) isolated from *S. anacardium* stem bark.

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