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ISOLATION AND STANDARDISATION OF ANTIOXIDANT ACTIVITY OF *BHALLATAK (SEMECARPUS ANACARDIUM LINN.)*

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ABSTRACT

DPPH, nitric oxide and Superoxide scavenging assay to investigate the antioxidant activity of crude ethyl acetate extract from the stem bark of bhallatak, it was found that the ethyl acetate extract exhibited a stronger antioxidant activity compared to the other (hexane, chloroform and methanol) extracts. On the basis of DPPH, nitric oxide and inhibition of superoxide anion radical scavenging assay, bioassay guided isolation of the ethyl acetate extract of bhallatak stem bark was carried out by silica gel column chromatography. It afforded a bright-yellow solid crystal, which was identified as butein. This compound exhibited antioxidant activity which was compared to rutin, taken as a standard.

KEYWORDS: Bhallatak, nitric oxide, DPPH, Scavenging Activity, Antioxidant activity.

INTRODUCTION

Reactive oxygenic species in the form of superoxide anion (O2 -), hydrogen peroxide (H2O2) and hydroxyl radical (HO.) 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 antiinflammatory 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: N-Hexane, Ninhydrin, Petroleum Ether, Pot. Dichromate, Pot. Iodide, Pyridine, Silica gel(60-120), Silica gel Gel, Sod. Chloride, Sod. Hydroxide, Sulphuric Acid, Zinc Chloride, Ethanol, Ethyl Acetate, Acetone, Benzene, Calcium Chloride fused, Chloroform, Copper Sulphate, Cyclohexane, from C.D.H Ltd. Delhi.Glacial Acetic acid, Ammonia Solution, dimethyl sulfoxide, Hydrochloric Acid. from Rankem Ltd, New Delhi. Dichloromethane, Iodine, Methanol. from Qualigens Ltd, Mumbai. All the chemicals used were of pure analytical grade.

Plant material

The drugs were collected from Sharma Ayurved Mandir Kashipur (Uttarranchal) & identification of the drugs was done with the help of qualified professor Mr. S.R. Gupta, Vipin Bihari Degree College (Botany Department) Bundelkhand University, Jhansi / asst.

professor Mr. A.K. Gupta, Baidhanath Pvt. Ltd., Jhansi. Also authentication of drugs was carried out at Shri Baidyanath Ayurved Ltd. Jhansi. And a voucher specimen has been deposited in herbarium Dept. of Pharmacognosy, I.T.S Pharmacy college murad nagar Ghaziabad. (sp.no 502/10)

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 (4% w/w); chloroform (3% w/w); ethyl acetate (4% w/w) and methanol (7% w/w). The extract, a powdered mass of red color was obtained and kept in a desiccator.

Analysis of total phenolic compound

Total soluble phenolics in the Hexane, Chloroform, Ethyl acetate and Methanol 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 Na2CO3 (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

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 Hexane, Chloform, Ethyl acetate and Methanol. After 10 minutes the absorbance was read at 515 nm using a spectrophotometer (Perkin-Elmer). The inhibition curve was plotted and IC50 values obtained (14, 15); where rutin was considered as a standard. The % of inhibition was calculated by using the equation;

% Inhibition= [(Absorbancecontrol – Absorbancesample)/Absorbancecontrol] 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% H3PO4 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= [(Absorbancecontrol – Absorbancesample)/Absorbancecontrol] x 100.

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= [(Absorbancecontrol – Absorbancesample)/Absorbancecontrol] x 100.

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.

STANDARDIZATION OF EASA OF BHALLATAK

Ethyl acetate was dissolved in CH3OH (1 mg/ml) and 10 µl of it was applied on the silica gel 60 F254 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 Rf = 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 CDCl3) 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 Hexane, Chloroform, Ethyl acetate and Methanol of bhallatak stem bark were determined as shown in Table 1. Hexane, Chloroform, Ethyl acetate and Methanol extracts exhibited antioxidant activity on DPPH and nitric oxide and Superoxide (Table 2) which has been widely used to measure the radical scavenging

ability of various plant extracts and constituents. Ethyl acetate was shown the potent antioxidant activity than the rest of those extracts as Hexane, Chloroform and Methanol. Hence, Ethyl acetate 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; where rutin was taken as a standard. The structural elucidation of the compound-1 was based on the spectroscopic evidences and comparison with literature data. 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 Ethyl acetate 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 IC50 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 IC50 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 Hexane, Chloroform, Ethyl acetate and Methanol of Bhallatak stem bark.

Different extract	Polyphenol content (equivalent of Pyrocatechol/ mg)	% of Polyphenols
Hexane	28.76 µg/ mg	2.87
Chloroform	113.3 µg/ mg	11.33
Ethyl acetate	686.7 µg/ mg	68.67
Methanol	387.8 µg/ mg	38.78

Table 2. Inhibitory effect (IC50) of Hexane, Chloroform, Ethyl acetate and methanol of S. anacardium stem bark on DPPH, nitric oxide, superoxide scavenging in vitro antioxidant assay.

Parameters	Hexane (µg/ml)	Chloroform (µg/ml)	Ethyl acetate (µg/ml)	Methanol (µg/ml)	Standard (µg/ml)
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]

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

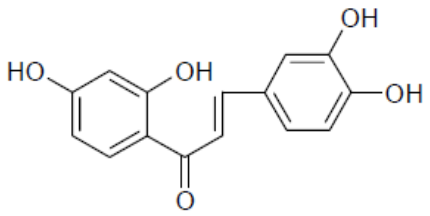

Fig. 1. Chemical structures of compound-1 (Butein) isolated from *S. anacardium* stem bark

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