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Oriental Pharmacy and Experimental Medicine 2006 **6(4)**, 355-361

Antioxidant, antinociceptive activity and general toxicity study of *Dendrophthoe falcata* and isolation of quercitrin as the major component

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SUMMARY

Plants have been used in traditional medicinal system for centuries. Bangladeshi medicinal plants have received considerable attention from the researchers for evaluation of their bioactivity. As a part of our ongoing research of screening the Bangladeshi medicinal plants, the ethanolic extract of *Dendrophthoe falcata* have been chosen for the present study. The ethanolic extract of the leaves of the plant have been assessed for their antioxidant, antinociceptive, and general toxicity. The extract showed potent antioxidant activity (IC_{50} 5.1 μg/ml) using DPPH radical scavenging assay, which is comparable to the standard ascorbic acid (IC_{50} 4.6 μg/ml). The extract significantly and dose dependently inhibited the acetic acid induced writhing in mice (71.2%, $P < 0.001$ and 28.0%, $P < 0.05$ for 500 and 250 mg/kg body weight, respectively). A general toxicity was assessed by a simple and low cost assay using brine shrimp lethality as an indicator. The extract showed low level of toxicity (LC_{50} 100 μg/ml). Using different chromatographic techniques, quercitrin (quercetin 3-*O*-α-rhamnoside) was separated as the major component from the extract. The structure was elucidated by detailed 1D and 2D NMR and mass spectral analysis.

Key words: *Dendrophthoe falcata*; Loranthaceae; Antioxidant; DPPH; Antinociceptive; Toxicity; Brine shrimp; Quercetrin

INTRODUCTION

Dendrophthoe falcata Linn. (Loranthaceae), commonly known as 'Porgassa', is a woody parasite in tree crowns, leaves are dull green, stem are narrow but woody found in southern region in Bangladesh and also widely distributed in Australia, Indiam China, Malaysia and Myanmar (Nair and Krishnakumary, 1990; Agrawal, 2001). This is an angiospermic parasitic plant found in different host trees. The leaves of *D. falcata* used in spermatorrhoea (Agrawal, 2001). A number of enzymes are separated from the leaves of *D. falcata* such as L-Threonine dehydratase, hexokinase, Glucan phosphatase (Khanna *et al.*, 1971; Baijal and Sanwal, 1976; Malhotra *et al.*, 1984). The plant is rich in flavonoids and quercitrin was the major constituent of *D. falcata* on different host plants.

As part of our ongoing pharmacological screening of randomly selected Bangladeshi medicinal plants (Shilpi *et al.*, 2004; Uddin *et al.*, 2004, 2005), we now first time report on the investigation of a number of

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pharmacological activity (antioxidant, antinociceptive, and general toxicity) and separation of quercitrin from the leaves of *D. falcata* on *Heritiera fomes* host tree.

MATERIALS AND METHODS

Plant material

Leaves of *D. falcata* on *H. fomes* host tree were collected from the tidal forest in the coastal Sundarbans (a swamp region in the Ganges delta) at the November, 2004 when the plant was fully flowered, and identified by experts of the Bangladesh National Herbarium, Dhaka, Bangladesh. Voucher specimens (DACB. 31155) representing the collections have been deposited in the Bangladesh National Herbarium, Dhaka, Bangladesh.

Extraction and isolation

Shade-dried and ground leaves (200 g) were extracted by maceration over 24 - 72 h using 90% ethanol (EtOH) at room temperature for 7 days. The extract was filtered and dried using a rotary evaporator at a temperature not exceeding 55°C and the yield was approximately 5.5% w/w on dry weight basis. The extract (2.14 g) was subjected to silica gel column chromatography using $CHCl_3$-MeOH with increasing polarity. The major fraction, 3 : 1 eluate (179 mg), yielded precipitate using $CHCl_3$-MeOH 1 : 1 solvent mixture, which was separated as 1 (64.8 mg). Compound 1 ($C_{21}H_{22}O_{11}$, 448) was isolated as yellow powder.

Animals

Swiss albino mice of either sex (20 - 25 g) were obtained from the Animal house, Pharmacy Discipline, Khulna University, Khulna. The animals were housed under standard laboratory conditions (relative humidity 55 - 65%, room temperature 23.0 ± 2.0°C and 12 h light-dark cycle). The animals were fed with standard diet and water *ad libitum*.

Antioxidant assay (DPPH assay)

1, 1-Diphenyl-2-picrylhydrazyl (DPPH, $C_{18}H_{12}N_5O_6$) (95%, Aldrich, USA) and Ascorbic acid (Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs) were used.

Qualitative

Qualitative assay was performed by the method of Sadhu *et al*. (2003). Test samples were developed with a suitable solvent system on a TLC plate and sprayed with 0.004% w/v DPPH solution in MeOH using an atomizer. The positive activity was detected by the discolored (pale yellow) spots on a reddish purple background. Ascorbic acid was used as the positive control.

Quantitative

The method used by Gupta *et al.* (2003) was adopted with suitable modifications to our particular circumstance. Stock solutions (10 mg/ml) of the plant extracts were prepared in ethanol. Serial dilutions were carried out to obtain concentrations of 1, 5, 10, 50, 100 and 500 μg/ml. Solution of each concentration (1 ml) were mixed with DPPH (3 ml 0.004% w/v solution in MeOH) and allowed to stand for 30 min for reaction to occur. The absorbance was recorded at 517 nm. Sample of each concentration was tested in triplicate and the average absorption was taken. Ascorbic acid was used as the positive control and percent inhibition was calculated as follows:

$$\% \text{ inhibition of DPPH} = A(b) - A(s)/A(b) \times 100$$

where, A(b) = absorbance of blank, and A(s) = absorbance of sample.

Antinociceptive activity study using acetic acid induced writhing assay

The method of Uddin *et al.* (2005) was adopted with minor modification. The animals were orally fed with the extracts, vehicles (for control groups) at the specified doses (500 μg/kg body weight). Thirty minutes after administration of the extract and the vehicle, each animal was given 0.7% (v/v)

solution of acetic acid (0.1 ml/10 g body weight) intraperitoneally (i.p.) to induce abdominal contractions or writhing. Five minutes after the administration of acetic acid, the number of writhing for each animal was counted for 15 min. The number of writhings in the control was taken as 100% and percent inhibition was calculated as follows:

% inhibition of writhing = 100 - (treated mean/ control mean) × 100

For comparison, the same experiment was carried out with a positive control group treated orally with Paracetamol (Square Pharmaceuticals Ltd., Bangladesh) at the dose of 50 mg/kg body weight.

Brine shrimp lethality assay for general toxicity

The method of Sarker *et al.* (2003) was adopted to study the general toxicity of the compound. Waterlife® brand brine shrimp (*Artemia salina*) eggs were purchased from The Waterlife Research Industries, Bath Road, Longford, Middlesex, UK. The eggs were hatched in a conical flask containing brine shrimp medium (300 ml). The flasks were well aerated with the aid of an air pump, and kept in a water bath at 29 - 30°C. A bright light was left on. The nauplii hatched within 48 h. The extracts were dissolved in DMSO to obtain a concentration of 5 mg/ml. Serial dilution technique was performed to make solutions of 10 concentrations. Solution of each concentration (1 ml) was transferred into clean sterile universal vials with a pipette and aerated seawater (20 ml) was added. About 10 - 15 nauplii were transferred into each vial with a pipette. A check count was performed. The number alive after 24 h was noted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 s of observation. The experiment was carried out in triplicate and the average values were noted. The controls used were DMSO in normal saline. Abbots formula was used to correct the values, i.e., $P = Pi - C/1 - C$, where P denotes the observed non-zero mortality rate and C represents the mortality rate of the control group.

Statistical analysis

All data were expressed as mean ± S.E.M. The Student's *t*-test was used to analyze data obtained from *in vivo* experiments.

RESULTS AND DISCUSSION

The DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. In the TLC-based qualitative antioxidant assay using DPPH spray, the ethanolic extracts *of D. falcata*, showed very potent free radical scavenging properties indicated by the presence of a yellowish spot on the reddish purple background of the TLC plate. The potent antioxidant activity observed with the extracts (IC_{50} 5.1 μg/ml) might be due to the major compound 1, a standard antioxidant. Comparison of the antioxidant activity of the extract and ascorbic acid was shown in Fig. 2.

In acetic acid induced writhing test, the extract significantly and dose dependently suppressed the frequency of acetic acid induced writhing in mice. At the dose 250 mg/kg body weight the extract of *D. falcata* showed 28.0% writhing inhibition ($P < 0.05$) where as at 500 mg/kg body weight produced 71.2% writhing inhibition ($P < 0.001$), which is comparable to a standard drug and all the result are statistically significant (Table 3). Paracetamol, used as the positive control exhibited a writhing inhibition of 75.2% as compared to control and the result was statistically significant ($P < 0.001$).

Acetic acid is a pain stimulus and intraperitoneal (i.p.) administration of acetic acid (0.7% v/v) causes localized inflammation, which causes contraction of the body in mice and referred to as 'writhing'. Such pain stimulus causes the release of free

Table 1. ^{1}H-NMR and ^{13}C-NMR spectral data of compound 1

Position	^{13}C NMR (125 MHz, CD_3OD)	^{1}H NMR (500 MHz, CD_3OD)
2	159.3	
3	136.2	
4	179.6	
5	163.2	
6	99.8	6.19 (1H, d, *J* = 2.1 Hz)
7	165.8	
8	94.7	6.35 (1H, d, *J* = 2.1 Hz)
9	158.5	
10	105.9	
1'	123.0	
2'	116.9	7.33 (1H, d, *J* = 2.1 Hz)
3'	146.4	
4'	149.8	
5'	116.4	6.90 (1H, d, *J* = 8.2 Hz)
6'	122.9	7.29 (1H, dd, *J* = 8.2, 2.1 Hz)
Rhm		
1	103.5	5.34 (1H, d, *J* = 1.5 Hz)
2	71.9	4.22 (1H, dd, *J* = 3.4, 1.8 Hz)
3	72.0	3.75 (1H, dd, *J* = 9.5, 3.4 Hz)
4	73.2	3.35 (1H, br. d, *J* = 9.5 Hz)
5	72.1	3.38 - 3.44 (1H, m)
6	17.6	0.94 (1H, d, *J* = 6.4 Hz)

arachidonic acid from tissue phospholipid by the action of phospholipase A_2 and other acyl hydrolases. There are three major pathways in the synthesis of the eicosanoids from arachidonic acid. All the eicosanoids with ring structures that is the prostaglandins, thromboxanes and prostacyclines are synthesized via the cyclooxygenase pathway. The leucotrienes, HETEs (hydroxyeicosatetraenoic acids), and HPETEs (hydroperoxyeicosatetraenoic acids) are hydroxylated derivatives of straight-chain fatty acid and are synthesized via the lipooxygenase pathway (Mary *et al.*, 1997).

The prostaglandins, mainly prostacyclin (PGI_2) and prostaglandin E have been reported to be responsible for pain sensation by exciting the A∂-fibres. Activity of the A∂-fibres causes a sensation of sharp well localized pain (Rang and Dale 1993). Any agent that lowers the number of writhing will demonstrate analgesia preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition.

These extract possess a very potent free radical scavenging activity which is comparable to the standard ascorbic acid (Table 2), and it is now well established that free radicals (e.g. superoxide, hydroxyl radical, nitric oxide) and other reactive species (e.g. hydrogen peroxide, single oxygen, perxynitrite, hypochlorous acid) contribute to the pathology of many disorders including atherogenesis, neurodegeneration, chronic inflammation, and function of the immune system (Hemani and Panihar, 1998). Recent studies suggest that the inflammatory tissue damage is due to the liberation of reactive oxygen species form phagocytes invading the inflammation sites (Winrow *et al.*, 1993; Conner and Grisham, 1996; Parke and Sapota, 1996). The

Table 2. Antioxidant activity and general toxicity of *D. falcata* extract

Assay	EtOH extract of *D. falcata* (μg/ml)
Antioxidant activity (IC_{50})	5.1
Ascorbic acid (IC_{50})	4.6
General toxicity (LC_{50})	100

reactive oxygen species may act as toxins, mediators and modulators of inflammatory gene activation, efforts have been directed to investigate antioxidant molecules as potential therapeutic agent (Conner and Grisham, 1996). In that context, polyphenols are becoming increasingly important (Gonclaves *et al.*, 2005) for their antioxidant properties. Many natural and synthetic antioxidants are in use to prevent the lipid peroxidation. Search for new antioxidant remains a highly research area because the antioxidants play a very important role in reducing the risk of various chronic disorder. So it can be assumed that their antioxidant activity may reduce the production of free arachidonic acid from phospholipid or may inhibit the enzyme system, which is responsible for the synthesis of prostaglandins, and ultimately relive pain sensation.

The general toxicity of the extracts was assessed by a simple and low-cost assay using brine shrimp lethality as an indicator of toxicity. The brine shrimp lethality assay is not specific to any pharmacological activity but can certainly provide an indication of toxicity. Anticancer compounds show high level of toxicity to brine shrimps, however, it is important to note that not all compounds/extracts that show toxicity in this assay can be considered as a potential source of anticancer drugs. The extracts of *D. falcata* showed low level of general toxicity in the brine shrimp lethality assay (LC_{50} 100 mg/ml) (Table 2) and could possibly assure its safety in relation to its use in traditional medicine preparations.

From the extract of *D. falcata* a major compound 1 was separated as yellow powder (Fig. 1). Its 1D

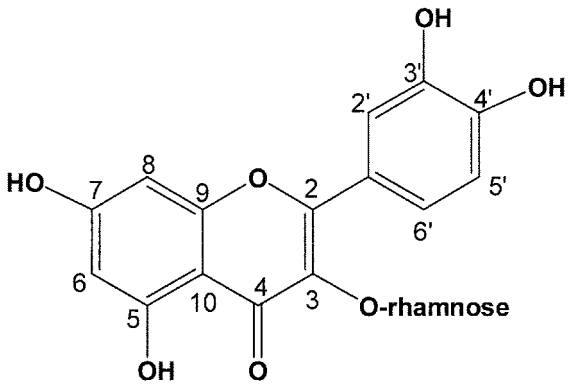

Fig. 1. Structure of compound 1.

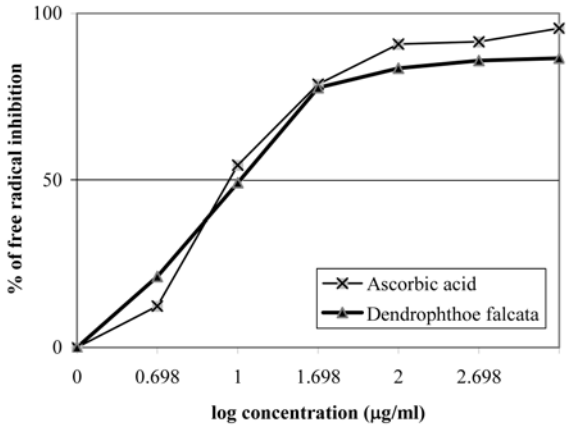

Fig. 2. Antioxidant assay of *D. falcata* extract and ascorbic acid.

NMR spectra (Table 1) indicated the presence of a flavonoid and a rhamnose sugar in its structure. From 2D NMR (HMQC, HMBC and COSY) analysis, its structure was established, which was also supported by mass spectral data {FABMS (NBA) *m/z*: 449 $[M+H]^+$}. In HMBC experiment, the rhamnose 1-H at 5.34 ppm (1H, d, J = 1.5 Hz) showed a cross peak

Table 3. Effect of the *D. falcata* extract on acetic acid induced writhing in mice

Treatment	Dose[a] (mg/kg, p.o.)	Writhings[b]	Inhibition (%)
Control (1% Tween 80, 10 ml/kg, p.o.)	-	25.0 ± 2.06	-
Diclofenac- Na	25	6.2 ± 0.74***	75.2
D. falcata extract	250	18.0 ± 0.50*	28.0
	500	7.2 ± 0.74***	71.2

[a]Administered 45 min before 0.7% v/v acetic acid administration (ml/kg, i.p.); [b]Counted for 15 min, starting 5 min after acetic acid administration; values are mean ± S.E.; *P < 0.02; **P < 0.01; ***P < 0.001 vs. control, Student's t-test; n = 5.

with C-3 at 136.2 ppm, which indicated its connection position with the aglycone. Compound 1 was identified as quercitrin or quercetin 3-*O*-α-rhamnoside comparing the spectral data with published reference (Harborne, 1994) (Fig. 1).

CONCLUSION

From the well known antioxidative potency of quercitrin and the results obtained in the present study, it can be postulated that the antinociceptive activity of the extract may be linked to their free radical scavenging activity. However, further study could be carried out to find out the actual mechanism of the antinociceptive activity of the extract and that of the major compound quercitrin.

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