ORIGINAL ARTICLE

ANTITUMOR AND ANTIBACTERIAL ACTIVITY OF ETHYLACETATE EXTRACT OF *LUDWIGIA HYSSOPIFOLIA* LINN AND ITS ACTIVE PRINCIPLE PIPERINE

BANIBRATA DAS, JUTHIKA KUNDU*, SITESH CHANDRA BACHAR, MOHAMMAD AFTAB UDDIN AND JOYDEB KUMAR KUNDU**

Department of Pharmaceutical Technology, Faculty of Pharmacy, Dhaka University, Dhaka-1000, Bangladesh
**Department of Pharmacy, East West University, Dhaka, Bangladesh*
***Department of Biochemistry and Molecular Biology, Dhaka University, Dhaka-1000*

ABSTRACT

An alkaloid constituent 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine, trivial name piperine was isolated from *Ludwigia hyssopifolia* Linn. (Family-Onagraceae). The ethylacetate extract of the plant and the isolated compound piperine were studied for antitumor and *in vitro* antibacterial activity. Ethylacetate extract showed 73.05 and 84.14% inhibition of *Agrobacterium tumefaciens*-induced crown gall tumor formation in potato disc. Piperine exhibited antitumor activity with IC_{50} value of 13.50 μg/disc. Both ethylacetate extract and piperine showed mild to moderate antibacterial activity against selected Gram-positive and Gram-negative bacteria.

Keywords: *Ludwigia hyssopifolia* Linn., antitumor, *in vitro* antibacterial activity.

INTRODUCTION

Ludwigia hyssopifolia Linn. (synonym *Jussiaea linifolia* Vahl. or *Jussiaea hyssopifolia* Linn., Family-Onagraceae; Bengali Name- Lalbunlonga) is extensively grown in Bangladesh, in all parts of India and Ceylon (Hooker, 1973; Huq, 1986). The plant is considered as astringent, anthelmintic, carminative and diuretic. A decoction is used in diarrhoea and dysentery, flatulence, leucorrhoea, and spitting of blood. The leaves are used for poulticing in orchitis and glands in the neck. A decoction is also used as a vermifuge and purgative (Ambasta, 1986; Ghani, 1990). Previous phytochemical investigation of the plant revealed the presence of chemical constituents namely vitexin, isovitexin, orientin and isoorientin (Huang, 1985).

In continuation of our work on chemical and biological characterization of different medicinal plants of Bangladesh, attempt was made to investigate the biological activity of piperine isolated from the plant *Ludwigia hyssopifolia* Linn. The results of antitumor and antibacterial activity of piperine, isolated for the first time from the plant, have been reported in the present communication.

MATERIALS AND METHODS

Plant material

The whole plant *Ludwigia hyssopifolia* Linn. was collected at flowering stage from Dhaka and was identified (voucher specimen No. DUH-163) by the Department of Botany, University of Dhaka, Bangladesh. After collection, whole plant was sun-dried for one week and made into a coarse powder by grinding. The dried coarse powder (800g) of the plant was successively cold-extracted with *n*-hexane and ethylacetate.

Isolation of piperine

On the basis of TLC analysis, the crude ethylacetate extract was subjected to vacuum liquid chromatography (VLC) for fractionation. By subsequent TLC analysis of all VLC eluates, a fraction designated as EF-3 was obtained by using the solvent system *n*-hexane and ethylacetate in ratio of 40:60. The fraction EF-3 was then investigated by preparative thin layer chromatography (P.T.L.C.) using the solvent system tolune:ethylacetate (90:10). This yielded a clear band at R_f 0.25, which was isolated as white crystals and termed as LE.

The structure of LE was confirmed as 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]-piperidine or piperine by spectral analysis and comparing the values with the reference data (De Araujo-Junior *et al*., 1997). The method of isolation of respective alkaloid piperine is depicted in scheme 1. The IR spectrum of the isolated compound was taken on U-270-30 infrared spectrophotometer. ^{1}H-NMR spectrum was measured on Varian VXR-500 spectrometer and JEOL FX (500 MHz) spectrophotometer in $CDCl_3$. ^{13}C-NMR spectrum was

Corresponding author: E-mail address: kundujk@yahoo.com - Tel.: +880-2-9665144

recorded on JEOL FX (125 MHz) spectrophotometer using $CDCl_3$. Chemical shifts were reported in δ (ppm) units downfield from tetramethylsilane, and the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. The FAB-MS was taken on a Hitachi M80B mass spectrophotometer. Column chromatography was performed on silica gel (Merck).

Antitumor activity

The antitumor activity of piperine was evaluated by potato disc bioassay method as described by McLaughlin (1991). Broth culture of Cambia SR009 EHA105 strain of *Agrobacterium tumefaciens* was collected from Department of Biochemistry, University of Dhaka, Bangladesh. Potato (*Solanum tuberosum*) discs (1.5 cm x 1.5 cm), previously surface sterilized by 50% sodium hypochloride (colorax), were made aseptically. The discs were inoculated with *Agrobacterium tumefaciens* by immersing the discs in 48 h old broth culture of the bacterium in a growth medium contained 0.5 g sucrose, 0.8 g nutrient broth (DIFCO, USA) and 0.1 g yeast extract (DIFCO, USA) in water q.s. to 100 ml. *A. tumefaciens*-containing potato discs were placed into a petridish containing 1.5% agar. Freeze-dried ethylacetate extract (2 mg) and piperine (1 mg) were dissolved separately in 0.1 ml dimethylsulfoxide (DMSO) and final volume was adjusted to 0.5 ml with distilled water. Ethylacetate extract (500 and 1000 µg) and piperine (5, 10, 15 and 20 µg) were added to each potato disc. Vincristine (6.25 µg/disc) was used as the standard drug. After application of test compounds, the petridishes were sealed with paraffin film and kept at room temperature for 15 days at room temperature for the growth of *A. tumefaciens* and the development of crown-gall tumors on potato discs. Each set of experiment was carried out in triplicate. The crown-gall tumors developed on potato discs were stained with Lugol's solution (5% iodine plus 10% potassium iodide in water). The tumor cells lack starch, so these cells had no stains against the stained background of normal potato cells rich in starch. The crown-gall tumors on the potato discs were observed with the aid of Olympus SZPT Microscope with Olympus PM20 camera and the average number of tumors for the sample and negative control were counted on the individual discs. Antitumor activity was quantified as percentage inhibition (Ti) from the expression: $Ti = 100 \times (1-n_e/n_c)$, where n_e is the number of tumors for an experimental compounds, and n_c is the number of tumors on the appropriate DMSO/water control discs. A Ti value of more than 20% in two or more independent assays considered as beneficial for further investigation (Ferrigni *et al.*, 1982).

Antibacterial activity

The antibacterial activity of ethylacetate extract of *L. hyssopifolia* Linn. and piperine was studied against Gram-positive (*Staphyllococcus aureus, Bacillus subtilis, Bacillus cereus, Bacillus megatorium*) and Gram-negative (*Shigella flexneri, Shigella dysenteriae, Shigella boydii, Salmonella typhi, Vibrio cholerae, Escherichia coli*) pathogenic bacteria by disc diffusion technique (Bauer *et al.*, 1966). Streptomycin (100 µg/disc) was used as the reference standard. Nutrient agar media (DIFCO) was used for preparing fresh cultures and sensitivity tests. The test organisms (stored at -20° C in presence of 3-4 drops of glycerol) were collected from the Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh and subculturing in nutrient broth medium was performed in our laboratory. One loop of broth culture was added per nutrient agar plate and sensitivity assay with test materials was performed by disc diffusion method.

RESULTS AND DISCUSSION

According to a report by World Health Organization (WHO), the use of herbal medicines is in increasing trend in both developing and industrialized countries. Considering the fact that over one-third of the population in developing countries lack access to essential medicines and the provision of safe and effective traditional therapies could become a critical tool to increase access to health care, WHO launched its first ever comprehensive traditional medicine strategy in 2002 (http://www.who.int/mediacentre/factsheets/fs134/en/).

Table 1: Antitumor activity of ethylacetate extract of *L. hyssopifolia* and piperine[b]

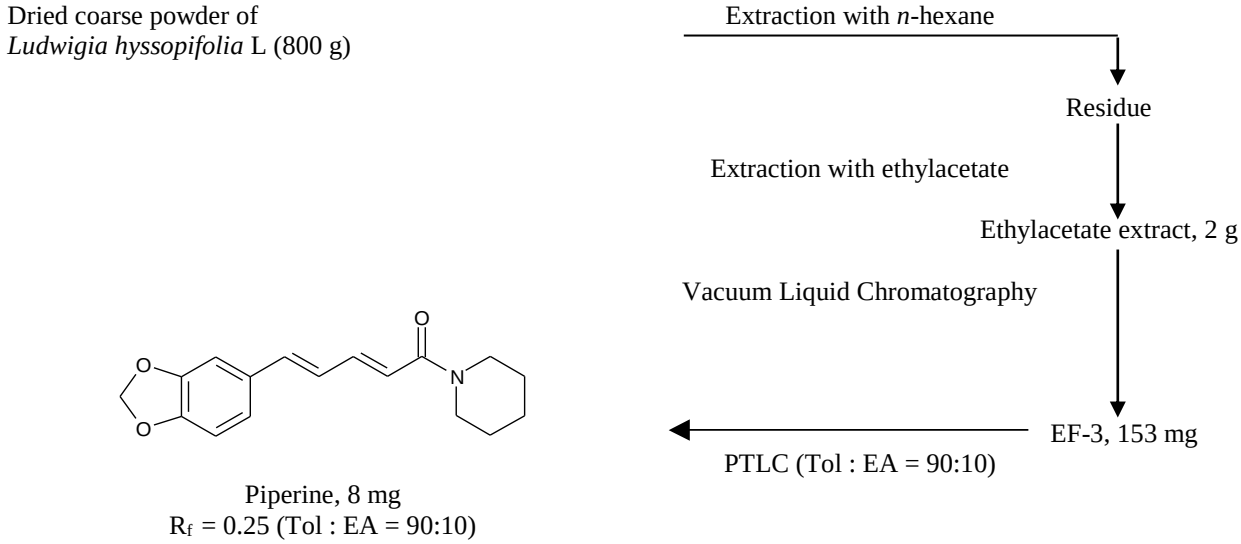
Test material	Dose (µg/disc)	Average number of tumor per disc	% Inhibition of tumor growth	IC_{50} (µg/disc)
Ethylacetate extract	500	05.66± 0.30	71.05	----
	1000	03.33 ± 0.36	84.14	
Piperine	20	07.33 ± 1.01	66.68	
	15	09.00 ± 0.33	59.09	
	10	13.66 ± 0.54	37.90	13.50
	05	16.66 ± 0.89	24.27	
Vincristine	6.25	00.00 ± 0.00	100	
Control	Saline	21.00 ± 1.53	------	-----

[b] Values are average of three replicates and vincristine (6.25 µg/disc) was used as standard drug which showed 100% inhibition of tumor growth.

Table 2: *In vitro* antibacterial activity of ethylacetate extract of *L. hyssopifolia* Linn. and piperine[c]

Test Organism	A	B	C
Vibrio cholerae	12.33 ± 0.58	11.67 ± 0.58	18.00 ± 1.00
Shigella flexneri	13.00 ± 1.00	14.33 ± 1.00	19.33 ± 1.53
Shigella boydii	13.67 ± 0.58	10.67 ± 0.58	18.67 ± 1.15
Shigella dysenteriae	20.00 ± 2.00	15.00 ± 1.00	19.67 ± 1.15
Salmonella typhi	13.67 ± 1.53	N	18.67 ± 1.15
Escherichia coli	12.67 ± 1.53	N	20.33 ± 1.52
Staphylococcus aureus	18.33 ± 0.58	14.00 ± 0.58	24.33 ± 0.58
Bacillus subtilis	18.33 ± 1.53	12.67 ± 1.53	20.33 ± 0.58
Bacillus cereus	15.33 ± 0.58	11.33 ± 0.58	19.33 ± 1.53
Bacillus megaterium	13.67 ± 0.58	10.00 ± 0.58	19.33 ± 0.58

[c]Values are zone of inhibition (mm) presented as Mean ± SD from triplicate set of experiments. A = Ethylacetate extract (400 µg/disc), B = piperine (200 µg/disc) and C = streptomycin (100 µg/disc), N = not performed.



Scheme 1

The plant *Ludwigia hyssopifolia* Linn. is one of the traditionally used medicinal plants of Bangladesh. In a previous study, we have reported the antidiarrheal activity of the methanol extract of this plant in experimental animals (Shaphiullah *et al.*, 2003). In an effort to make biological and chemical characterization of indigenous medicinal plants of Bangladesh, we have isolated an alkaloidal constituent, piperine, from the ethylacetate extract of *L. hyssopifolia* Linn. In the present study, the antitumor and antibacterial activity of the crude ethylacetate extract and that of piperine was studied. The ethylacetate extract (yield, 0.25%) was obtained by successive cold extraction of whole plant of *Ludwigia hyssopifolia* Linn. with *n*-hexane and ethylacetate. Piperine was isolated (yield, 0.001%) from ethylacetate extract as described in materials and methods. The structure of piperine was confirmed by various spectral analyses as mentioned in experimental section. The IR spectrum of the compound gave characteristic absorption band at ν_{max} 1630 cm^{-1}, which indicated the presence of an amide carbonyl group. The positive ion FAB MS spectroscopy revealed the presence of protonated molecular ion $[M+1]^+$ peak at 286.2. The FAB^+ MS, ^1H spectral analysis of the compound led to establish the molecular formula of the isolated compound as 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine ($C_{17}H_{19}NO_3$), which was further confirmed by comparing with published spectral data of the alkaloid pipreine. *Piperine* (**1**). ^1H-NMR (500 MHz, $CDCl_3$): δ 3.55 (2H,br *s*, H-2), 1.57 (2H, *m*, H-3), 1.63 (2H, *m*, H-4), 1.57 (2H, *m*, H-5), 3.55 (2H, br *s*, H-6), 6.60 (1H, *d*, *J* 14.5 Hz, H-2'), 7.31 (1H, *dd*, *J* 14.6,11.4 Hz, H-3'), 6.89 (1H, *dd*, *J* 14.6, 11.4 Hz, H-4'), 6.81 (1H, *d*, *J* 11.0 Hz, H-5'), 5.95 (2H, *s*, H-2"), 6.78 (1H, *d*, *J* 1.6 Hz, H-4"), 6.94 (1H, *dd*, *J* 1.6,8.0 Hz, H-5"), 7.08 (1H, *d*, *J* 8.0 Hz, H-7"); ^{13}C-NMR (125 MHz, $CDCl_3$): 44.58 (C-2), 26.87 (C-3), 25.53 (C-4), 27.86 (C-5), 48.16 (C-6), 167.76 (C-1'), 120.58 (C-2'), 144.62 (C-3'), 126.41 (C-4'), 140.21 (C-5'), 102.71

(C-2"), 149.81 (C-3a"), 106.71 (C-4"), 132.42 (C-5"), 123.87 (C-6"), 109.36 (C-7"), 149.76 (C-7a"); FABMS *m/z*: 286.2 $[M+H]^+$. $C_{17}H_{19}NO_3$ (285.34).

In vitro antitumor activity of ethylacetate extract and of isolated compound, piperine was performed by potato disc bioassay method (McLaughlin, 1991). Ethylacetate extract exhibited 73.05 and 84.14% inhibition of *Agrobacterium tumefaciens*-induced crown gall tumor formation on potato discs at concentrations of 500 and 1000 µg/disc, respectively. In the same study, piperine inhibited the formation of crown-gall tumor by 66.68%, 59.09%, 37.90% and 24.27% at concentrations of 20, 15, 10 and 5 µg/disc, respectively. The concentration at which 50% inhibition (IC_{50}) of crown-gall formation observed with piperine was found to be 13.50 µg/disc (table 1). Vincristine, used as the standard drug, showed 100% inhibition at a dose of 6.25 µg/disc. In a recent study, piperine was also found to inhibit growth of Dalton's lymphoma ascites (DLA)-induced solid tumors in Swiss-albino mice (Sunila and Kuttan, 2004). Moreover, cytotoxic activity of piperine was reported in several recent studies (Padmaja *et al*., 2002; Sunila and Kuttan, 2004).

Crown-gall is a neoplastic disease of plants, in which autonomous plant tumor cells are produced from normal, wounded plant cells by the action of bacteria-borne tumor-inducing plasmids. The method is independent of antibiosis (Fadli *et al.*, 1991). It is caused by a specific strain of Gram-negative bacterium *Agrobacterium tumefaciens* ((Pelczar and Reid, 1965). As certain mechanisms of tumorigenesis, such as the intracellular incorporation of extraneous nucleic acids, are common in both plants and animals (McLaughlin, 1991), the fundamental concept of developing this method was that the antitumor drugs might inhibit the initiation and growth of tumors in both animal and plant systems. Thus the inhibitory effect of the ethylacetate extract of *L. hyssopifolia* Linn. and its active constituent piperine on crown-gall tumor formation indicated the rationale of exploiting this plant species for the prevention and/or treatment of cancer.

In vitro antibacterial activity of ethylacetate extract and piperine was studied by disc diffusion method (Bauer *et al.*, 1966) at a concentration of 400 and 200 µg/disc, respectively, taking streptomycin (100 µg/disc) as the reference standard. Among the bacterial strains tested, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus subtilis* exhibited good sensitivity (17-20mm) to ethylacetate extract, while piperine showed only weak antibacterial activity (13-15 mm) against *Shigella flexneri*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus subtilis* (table 2).

ACKNOWLEDGEMENTS

The authors wish to thank Dr. M. A. Rashid, Senior Scientist, Laboratory of Drug Discovery Research and Development, National Cancer Institute, Frederick Cancer Research (currently, the Dean, Faculty of Pharmacy, Dhaka University, Bangladesh) for spectral analysis.

REFERENCES

Ambasta SP (1986). The useful plants of India: Publications and Directorate, CSIR, New Delhi.

Bauer AW, Kirby WMM, Sherries JC and Turck M (1966). Antibiotic susceptibility testing by standardized single disc method. *Am. J. Clin. Pathol.* 45, 493-496.

De Araujo-Junior JX, Da-Cunha EVL, De O Chaves MC and Gray AI (1997). Piperdardine, a piperidine alkaloid from Piper tuberculatum. *Phytochemistry*, **44**: 559-561.

Fadli M, Aracil JM, Jeanty G, Banaigs B and Francisco C (1991). Novel meroterpenoids from Cystoseira mediterranea: use of the crown-gall bioassay as a primary screen for lipophilic antineoplastic agents. *J. Nat. Prod.*, **54**: 261-4.

Ferrigni NR, Putnam JE, Anderson B, Jacobsen LB, Nichols DE, Moore DS, McLaughlin JL, Powell RG and Smith CR Jr (1982). Modification and evaluation of the potato disc assay and antitumor screening of euphorbiaceae seeds. *J. Nat. Prod.* **45**: 679-86.

Ghani A (1990). Traditional Medicine: Asiatic Society of Bangladesh, Dhaka.

Hooker JD (1973). Flora of British India. New Delhi: Periodical Book Agency.

Huang S (1985). Flavonoid constituents in the systematics of *Ludwigia* (Onagraceae) in Taiwan. *Shih. Ta. Hsueh Pao. (Taipei)* **30**: 547-569.

Huq MM (1986). Name changes in Bangladesh angiosperms. Dhaka: Bangladesh National Herbarium, BARC.

McLaughlin JL (1991). Methods in plant biochemistry. London: Academic Press.

Padmaja R, Arun PC, Prashanth D, Deepak M, Amit A and Anjana M (2002). Brine shrimp lethality bioassay of selected Indian medicinal plants. *Fitoterapia*, **73**: 508-10.

Pelczar JMJ and Reid RD (1965). Microbiology. New York: McGraw Hill Book Company Inc.

Shaphiullah M, Bachar SC, Kundu JK, Begum F, Uddin MA, Roy SC and Khan MTH (2003). Antidiarrheal activity of methanol extract of L. hyssopifolia Linn. *Pak. J. Pharm. Sci.*, **16**: 7-13.

Sunila ES and Kuttan G (2004). Immunomodulatory and antitumor activity of Piper longum Linn. and piperine. *J. Ethnopharmacol.*, **90**: 339-46.

Received: 23-01-2006 – Accepted: 24-03-2007