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Phytochemical Screening and Cytotoxicity of Root Extracts of *Eupatorium odoratum* Against the Shrimp Nauplii of *Artemia salina*

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Abstract. Extracts of roots of *Eupatorium odoratum* were fractionated using various organic solvents. The fractions so obtained were petroleum ether extract (F1), solid residue left after ethanol extraction (F2), liquid residue left after ethanol extraction (F3), hexane soluble sub-fraction of F2 (F2-1), dichloromethane-soluble sub-fraction of F2 (F2-2), ethyl actate-soluble sub-fraction of F2 (F2-3), and 80% aqueous methanol soluble sub-fraction of F2 (F2-4). The root extract fractions were screened for such phytochemicals as sterols, triterpenoids, polyphenols, carbohydrates and reducing sugurs, alkaloids, flavonoids, quinones, and tannins. These root extract fractions were further tested for their cytotoxicity in the brine shrimp lethality test. The LC_{50} values indicated that F1, F2-1 and F2-2 were significantly cytotoxic against the brine shrimp nauplii. The fractions F3, F2-3 and F2-4 showed no cytotoxicity, whereas F2 was mildly cytotoxic in the brine shrimp lethality test.

Keywords: *Eupatorium odoratum,* brine shrimp lethality test, phytochemical cytotoxicity, *Artemia salina*, shrimp nauplii

Introduction

Eupatorium odoratum is a perennial shrub. In its new habitats it spreads quickly, forms dense thikets and seriously interferes not only with natural vegetation, but also with forestry, pasture and plantation crops. The plant is accordingly known as the forest killer, "banamara", in the Nepali language. The plant is distributed in the central and eastern regions of Nepal from 400-1500 m altitude (Press *et al.,* 2000). Juice of aerial parts of the plant is used on cuts and wounds to arrest bleeding and promote healing (Taylor *et al.,* 1996). The plant is used as fish poison (Talpatra *et al.,* 1977). It is also used as an ingredient in the preparation of antimalarial mixtures and cough suppressants; chloroform extract of leaves has shown antimicrobial activity (Iwu and Chiori, 1984). Okunji *et al.* (2000) have reported that the plant was suitable for treating fungal and protozoan diseases. Essential oil of leaves form Ivory Coast showed notable antimicrobial activity against the gram-negative bacteria (*Klebsiella pneum-oniae, E. coli, Pseudomonas aeruginosa*), but no activity was noted towards gram-positive species (Bamba *et al.,* 1993). Ethanol extract of the leaves showed antioxidant activity to protect cultured skin cells (Phan *et al.,* 2001). Triratana *et al.* (1991) reported that a compound 4,5,6,7-tetramethoxyflavone, isolated from this plant, was found as a blood clotting enhancer factor when studied *in vitro*. Roots of *Eupatorium odoratum* contain the N-oxides of pyrrolizidine alkaloids (Biller *et al.,* 1994). Most plants are toxic to domestic animals and humans (Cheeke, 1989).

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Researchers have isolated different classes of compunds from its aerial parts. Some of these are sesquiterpenoids (Chowdhry, 2002; Bedi *et al.,* 2001; Dung *et al.,* 1992), triterpenoids (Ding *et al.,* 2001; Talpatra *et al.,* 1977), pyrrolizidine alkaloids (Biller *et al.,* 1994), flavonoids (Ding *et al.,* 2001; Wollenweber and Roitman 1996; Hai *et al.,* 1995; 1991; Metwally and Ekejiuba, 1981; Barua *et al.,* 1978), fatty acids (Barua *et al.,* 1993), and tannins (Ahmad, 1969).

Very few phytochemical studies have been done on its root portion. The available literature indicates that no previous cytotoxic study has been done on roots of *E. odoratum.* The present study investigated the cytotoxic properties of its root extracts against *Artemia salina* (brine shrimp), along with its phytochemical screening. Brine shrimps have been previously used in bioassay studies for a variety of toxic substances, while this method has been applied to plant extracts in order to facilitate the isolation of biologically active compounds for therapeutics by De Giulio *et al.* (1992). It has been reported that over 300 novel antitumour and natural pesticidal products were isolated using this bioassay technique for prescreening (McLaughlin *et al.,* 1998).

Materials and Methods

Plant material. *E. odoratum* was collected from Tinpipalay, Kabhrepalanchok District, Nepal, in November 2001 and identified by Prof. K.K. Shrestha, a taxonomist in the Central Department of Botany, Tribhuvan University,

Kathmandu, Nepal and a voucher specimen was deposited under number 19-TUCH for record and reference.

Brine shrimp lethality test. This test was performed as described by Meyer *et al.* (1982). Each extract of the fraction solution was tested at a concentration level of 1000, 100 and 10 µg/ml. The eggs of brine shrimp (15 mg, Red Jungle Brand, USA) were hatched in a small beaker, filled with artificial seawater which was prepared from 40 g/l sea salt (Instant Ocean Aquarium System, Inc., USA). The eggs were incubated for about 48 h, at 27-30 °C in a waterbath. After 48 h, nauplii were collected with a pasture pipette by attracting the organisms to one side of the glass beaker, opposite to the light source, and transferred to a small beaker containing artificial seawater. Samples were prepared by dissolving 50 mg of each fraction of the root extract in 5 ml of a suitable solvent (chloroform or mehtanol). Chloroform was used as the solvent for petroleum ether extract (F1), solid residue of ethanol fraction (F2), and sub-fractions: hexane solubles (F2-1) and dichloromethane solubles (F2-2). Methanol was used as the solvent for ethyl acetate soluble fraction (F2-3), and 80% aqueous methanol soluble fraction of F2 (F2-4), as well as the liquid residue of ethanol fraction (F3). From these sample solutions, 500, 50 and 5 µl were transferred to test tubes (10 cm x 1 cm), corresponding to 1000, 100 and 10 µg/ml, respectively. Five replicates were made for each dose level. The contents of each test tube were evaporated completely at a temperature of 50 °C in Rotavapor at reduced pressure. A volume of 4 ml of artificial seawater was added to each test tube as noted above. Ten brine shrimp nauplii were tansferred to each test tube and the volume adjusted to 5 ml by adding more seawater. Solvent controls were also prepared for all the three-dose levels. Test tubes were kept in a room illuminated by using 15 W bulb, maintaining temperature at 20-22 °C for 24 h. The number of survivours were counted after 24 h and deaths of nauplii, in each dose level, were determined. The LC_{50} values were calculated by Probit analysis (Finney, 1971). The LC_{50} values were expressed as mean of three independently performed experiments in µg/ml, with 95% confidence interval.

Extraction process. Root powder (50 g) was first defatted with 300 ml petroleum ether (40-60 °C) for 7 h. The procedure was repeated four times. The extracts were combined and evaporated to obtain yellow oil (F1). The dried defatted marc (100 g) was then extracted with 500 ml ethanol for 7 h. This process was repeated twice. Combined extracts were concentrated in Rotavapor at reduced pressure at 40 °C. The so concentrated ethanol extract was treated with 100 ml water and the supernatant liquid was filtered off through cotton plugs leaving the solid residue in the flask (F2). It was repeated twice. The filtrate was evaporated to dryness to yield reddish brown liquid (F3). The solid residue (F2) was subsequently fractionated by dissolving it in succession with hexane, dichloromethane, ethyl acetate and 70% aqueous methanol. The successive fractionation yielded hexane soluble sub-fraction (F2-1), dichloromethane soluble sub-fraction (F2-2), ethyl acetate soluble sub-fraction (F2-3), and 80% aqueous methanol soluble sub-fraction (F2-4).

Results and Discussion

Phytochemical screening of root extracts. Preliminary chemical tests for the indication of classes of organic compounds were performed on roots of E. *odoratum* extracted in various solvents (F1, F2, F3, F2-1, F2-2, F2-3 and F2-4), as outlined by Harborne (1973) and Dhoubhadel and Shrestha (1978).

Petroleum ether extract (F1) showed the presence of sterols/triterpenoids and quinones (detected by spraying the TLC with 10% alcoholic KOH). Solid ethanolic extract (F2) revealed the presence of sterols/triterpenes, reducing sugars, alkaloids, carbohydrates and quinones. Polyphenols and tannins were not detected in both F1 and F2 fractions. Liquid residue (F3) showed the presence of polyphenols, reducing sugars, quinones and tannins (Table 1). Both hexane-soluble sub-fraction of F2 (F2-1) and dichloromethane-soluble sub-fraction of F2 (F2-2) showed the presence of sterols/triterpenes and quinones, whereas alkaloids were detected in sub-fraction F2-2. Similarly, ethyl acetate-soluble sub-fraction (F2-3) showed the presence of reducing sugars, alkaloids, and the 80% aqueous methanol-soluble sub-fraction (F2-4) showed reducing sugars and carbohydrates, but quinones were not detected in both these cases. Flavonoids were not detected in any of the extracts or fractions (Table 1).

Table 1. Phytochemical screening of root extracts of *Eupatorium odoratum*

Classes of chemicals indicated	Root extact fraction*						
	F1	F2	F3	F2-1	F2-2	F2-3	F2-4
Sterols/triterpenoids	+	+	-	+	+	-	-
Polyphenols	-	-	+	n	n	n	n
Reducing sugars	-	+	+	-	-	+	+
Alkaloids	-	+	-	-	+	+	-
Carbohydrates	-	+	-	-	-	-	+
Flavonoids	-	-	-	n	n	n	n
Quinones	+	+	+	+	+	-	-
Tannins	-	-	+	-	n	n	n

+ present; - absent; n = not tested; * see Brine shrimp lethality test, Extraction process sections, and footnote of Table 2 for details about fractions

The cytotoxicity of root extract was evaluated by the brine shrimps lethality test. Significant activity (LC_{50} < 1000 µg/ml) was exhibited by F1, while F3 (liquid residue) was found to be non-cytotoxic (LC_{50}>1000 µg/ml) (Table 2). The LC_{50} value of petroleum ether extracts (F1) was 237.2 µg/ml and of the solid residue of ethanol fraction (F2) was 81.1 µg/ml, while hexane-soluble sub-fraction of F2 (F2-1) displayed LC_{50} value of 263.1 µg/ml and dichloromethane-soluble sub-fraction of F2 (F2-2) displayed LC_{50} value of 207.7 µg/ml. However, F2-3 and F2-4 sub-fractions of F2 were found to be non-cytotoxic against brine shrimps. It may also be seen from Table 2 that cytotoxicity of F2 was greater than that of its own sub-fractions F2-1 and F2-2. This difference is likely to be due to the synergistic effect of hexane-soluble and dichloromethane-soluble compounds. Phytochemical analysis (Table 1) showed that cytotoxicity of F1, F2, F2-1 and F2-2 may be due to the presence of sterols, triterpenes, alkaloids and quinones. Therefore, the present observations have indicated that root extracts of *E. odoratum* may be used for cytotoxic applications.

Table 2. Brine shrimp *(Artimia salina)* lethality of root extracts of *Eupatorium odoratum*

Extract fraction	Extract yield (%)	LC_{50} (µg/ml)	Confidence interval (95%)
F1	0.31	237.2	304.4-169.8
F2	0.72	81.1	131.7-31.4
F3	10.23	>1000	-
F2-1	0.07	263.1	289.5-236.8
F2-2	0.17	207.7	249.2-166.3
F2-3	0.01	>1000	-
F2-4	0.12	>1000	-

F1: petroleum ether extract; F2: solid residue of ethanol fraction; F3: liquid residue of ethanol fraction; F2-1: hexane-soluble sub-fraction of F2; F2-2: dichloromethane-soluble sub-fraction of F2; F2-3: ethyl aceate-soluble sub-fraction of F2; F2-4: 80% aqueous methanol soluble sub-fraction of F2

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