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Effect of Bio Assay Guided Isolation of 1-Phenanthrene Carboxylic Acid from *Eulophia herbacea* Lindl. Tubers on Human Cancer Cell Lines

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ABSTRACT

The tuber of *Eulophia herbacea* L. (Orchidaceae) is utilized as food particle with health benefit used by the tribes of Toranmal region, Maharashtra, India and also valued in management of cancer, in folklore medicine. The present study was undertaken to explore the cytotoxic potential of *Eulophia herbacea* L. tubers. The methanol extract and its fractions were tested using Brine Shrimp Lethality Test (BSLT) and human cancer cell line study. Furthermore the phytoconstituents was isolated from EA fraction and identified as 1-phenanthrenecarboxylic acid 1, 2, 3, 4, 4a, 9, 10, 10a-octahydro-1, 4a-dimethyl-, methyl ester (PCA). Mixtures of Phytosterols (MPS) and stigmasterol were obtained from n-hexane fraction. ME, EAF HEX, MPS and stigmasterol showed cytotoxic potential in brine shrimp lethality assay. PCA demonstrated most potent anticancer activity with GI₅₀ concentration between 26.1 and 53.7 µg mL⁻¹ for human cell line. HEX was active only against human Leukemia (K562) cell line with concentration <10 µg mL⁻¹ of GI₅₀. Overall, ethno pharmacological evaluations proved potency of activity in successive sequence of EAF>HEX>ME. The ethyl acetate fraction of methanol extract was found to be active in BSLT with LC₅₀ 168.3 µg mL⁻¹, while the phenanthrene type of isolated compound from ethyl acetate fraction was showed as more active against a panel of three human cancer cells. TGI (Total Growth Inhibition) and GI₅₀ of all samples were found to be less than 80 µg mL⁻¹. Hence, the present study was confirmed the traditional anticancer effect of *Eulophia herbacea* tuber and could be a potential source for anticancer drugs.

Key words: *Eulophia herbacea* tubers, cell lines, brine shrimp, SRB assay

INTRODUCTION

Several plants consist of ethno medicinal claims for their cytotoxic and immunomodulatory potential (Pezzuto, 1997). Scientific evidence divulges that natural product isolated from plant source posses anticancer activity with immunomodulatory effect (Bhandari *et al.*, 1985; Prozesky *et al.*, 2001; Govindarajan *et al.*, 2005). In the discovery of anticancer drugs, plants have been played significant role, as over 60% of anticancer agent are derived from natural sources (Newman *et al.*, 2003). For prevention of neoplastic diseases, immunomodulation may

considered as an alternative therapy (Mitchell, 2003). India is also one of rich source for anticancer and immunomodulatory plants (Balekar and Jain, 2006). Ayurveda also perk up the concept of defence mechanism against various diseases (Thatte and Dahanukar, 1986). *Eulophia herbacea* Lindl. (Family: Orchidaceae) is terrestrial herbs with fleshy subglobose tubers, growing in hilly slopes of Himalaya forest, Bengal, Toranmal, Eastern part of India and other different parts of world. In India it is known as Kukad-kand (Anonymous, 2003). The genus *Eulophia* includes 200 species of orchids, out of this, 26 species native to India (Bhattacharjee, 2001). Traditionally *Eulophia herbacea* used as substitute for salep (Khare, 2007), used for ailment of pimples (Patil and Patil, 2007). Decoction of tubers is used on spermatorrhoea, urinary complaints and menses. Tubers are also used as tonic. Tubers are beneficial to treat the aforesaid disease (Patil and Patil, 2006). Other species of genus *Eulophia* used as anticancer, immunomodulatory, aphrodisiac, tonic appetizer, blood purifier, emetics, bronchitis, cardiac problem, infertility and anthelmintic (Gerstner, 1941; Tuchinda *et al.*, 1988; Bhattacharjee, 2001; Nadkarni, 1991; Steenkamp, 2003; Jain *et al.*, 2005; Datla *et al.*, 2010). Scientific data revealed the presence of different chemical constituents from other species of *Eulophia* as; phenanthrenes, phytosterols, mucilage, phenolics, zinc, iron (Blitzke *et al.*, 2000; Nadkarni, 1991).

Although, various ethnomedicinal claims for *Eulophia herbacea* Lindl. have been described by folklore and extensively used by local healers but till now no scientific validation has been established for cytotoxic potential. Hence this study was to investigate cytotoxic activity of extract and fractions by brine shrimp nauplii lethal test and SRB assay on human cancer cell lines.

MATERIALS AND METHODS

Collection of plant material: *Eulophia herbacea* Lindl. (Orchidaceae) tubers were collected in August 2012 from subtropical hilly area of Toranmal region, Nandurbar District, Maharashtra, India. The tubers were authenticated by authority from Botanical Survey of India (BSI), Pune, India. A voucher specimen was kept at R.C. Patel Institute of Pharmaceutical Education and Research, Shirpur with the number BANDEUH1 for reference purpose.

Preparation of plant extracts and fractions for bioassay: The air-dried tubers powder (4×100 g) of *Eulophia herbacea* (Lindl) was extracted with methanol (4×1000 mL) by using soxhlet extractor till the active constituents were extracted. All the extract was filtered and concentrated under reduced pressure in rotary vacuum evaporator. The dried crude methanol extract was then subjected to fractionation by solubilisation in methanol:water (2:3) and sequential extraction with n-hexane (HEX) and Ethyl Acetate (EA). Each fraction thus obtained was evaporated to dryness and subjected to bioassay.

Phytochemical screening: This was carried out as described by Harbone (2005) and Trease and Evans (1983) for detection of class of phytochemicals.

Bioassay guided isolation and identification of compounds: Bioassay guided separation and isolation of active compounds was done by column chromatography. Hexane and ethyl acetate fraction was subjected to isolation of chemical compound. The 2.1 g of ethyl acetate fraction and 4 g of n-hexane fraction was chromatographed on 60-120 mesh size silica gel with glass column (30 cm/3 cm) and both the fractions were gradiently eluted with n-hexane:ethyl

acetate separately. Fractions with similar pattern in TLC were pooled together and concentrated. Further purification of compound was done by preparative TLC. Identification of isolated compound was carried out by spectroscopic and chromatographic analysis. HPLC and HPTLC chromatogram recorded on Shimadzu HPLC system and HPTLC-CAMAG, respectively. GC-MS analysis performed with autosystem XL GC plus Perkin Elmer with NIST mass spectra library. Molecular mass of compound was confirmed by liquid chromatography/mass spectrophotometer, LC-MS (Varian Inc, USA), IR (Shimadzu FT-IR 8400S), UV (Shimadzu UV 2401).

Cancer cell lines: Human nasopharynx (KB), human lung (HOP62), human cervix (ME180) and human leukemia (K562) cell lines were purchased from National Cancer Institute, USA and National Centre for Cell Science (NCCS), Pune. They were maintained in the logarithmic phase of growth in RPMI 1640 medium supplemented with 2 mM l-glutamine (sigma) 10% fetal bovine serum at 37°C under humidified air with 5% CO₂.

Brine shrimp lethality test: The brine shrimp nauplii lethality test (BST) was used to predict the cytotoxicity of *Eulophia herbacea*. EA, HEX, ME, MPS and isolated stigmaterol were evaluated for lethality to Brine Shrimp Larvae (BST) as per the procedure described by Anderson *et al.* (1991). In the assay, brine shrimp eggs were bred on saline medium (artificial sea salt water). Some shrimps were hatched after 48 h which were ready for testing. The 24 h old larvae (10 per vial) were transferred into 5 mL vials containing the test sample and saline solution. The dose range of test materials were optimized for initial concentration at 10, 100 and 1000 µg mL⁻¹. The concentration of DMSO was used to dissolve the test sample and adjusted to 0.5%. In each test sample, assay was repeated in three replicate for each concentration. After 24 h, the numbers of survivors were counted and percentage of death calculated. LC₅₀ in µg mL⁻¹ was determined using probit analysis method reported by Ghosh (2005).

Anticancer activity by SRB assay: Anticancer activity of test samples by sulforhodamine B assay was evaluated against 4 human cell lines at Cancer Research Institute of Tata Memorial Centre, Mumbai, India according to the reported method (Skehan *et al.*, 1990). In SRB assay, isolated 1 Phenanthrene Carboxylic Acid (PCA) compound from ethyl acetate fraction, hexane fraction and methanol extract were tested. Cancer cells were plated (5×10⁵ cells well⁻¹) for 24 h before treatment with test samples to allow the attachment of cells to the plate. Samples were dissolved in DMSO and different concentrations of test samples were added to the cells monolayer. Triplicate wells were repeated for each dose and the cells incubated for 48 h at 37°C with 5% CO₂, then the cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with 10 mM trizma base and the absorbance was read on a micro plate reader at a wavelength of 540 nm with 690 nm as reference wavelength. LC₅₀, TGI and GI₅₀ was calculated in comparison to Doxorubicin as reference standard (Pharmacia Ltd-India).

Statistical analysis: Results were presented as Mean±Standard Error of Mean (SEM) of 6 observations. Dunnett test was to calculate the level of significance for comparison made within a control vs. test groups using statistical software program, INSTAT.

RESULTS

Isolation, purification and identification of compound: The yield of methanol extract, n-hexane fraction and ethyl acetate fraction was 13, 1.73 and 2.11% w/w, respectively. The crude

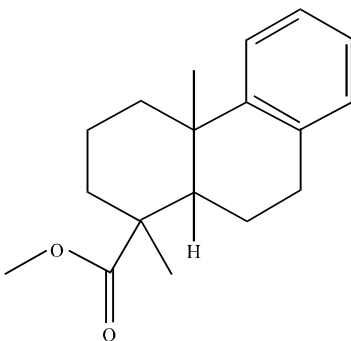


Fig. 1: PCA(1-phenanthrenecarboxylic acid 1, 2, 3, 4, 4a, 9, 10, 10a-octahydro-1, 4a-dimethyl-, methyl ester)

Table 1: Effect of *Eulophia herbacea* tubers on brine shrimp lethality test

Extracts	r ²	LC ₅₀ (24 h) (µg mL ⁻¹)
ME	0.9798	205.0
EAF	0.9863	168.3
HEX	0.9798	205.0
MPS	0.9671	865.3
Stigmasterol	0.9460	200.0

ME: Methanol extract, EAF: Ethyl acetate fraction, HEX: Hexane fraction, MPS: Mixture of phytosterols fractions

methanol extract of fresh *Eulophia herbacea* tubers was fractionated in ethyl acetate fraction which obtained a single compound and it was characterized as 1-phenanthrenecarboxylic acid 1, 2, 3, 4, 4a, 9, 10, 10a-octahydro-1, 4a-dimethyl-, methyl ester (Fig. 1) by extensive data of LC-MS and GC-MS. UV spectra shown λ_{max} at 308 nm. In IR study, absorption peak at 1081.45 and 1215.19 cm^{-1} suggested C-O stretch of carboxylic acid and ester. LC-MS spectrum supported a molecular formula for molecular weight 272. HEX fraction was passed through C-C to obtained mixture of phytosterols (MPS) which gave positive test of salkowaskii and Liebermann burchard test. Similarly single compound isolated from n-hexane fraction was characterized as stigmasterol with m/e 413 by GC-MS analysis and other chromatographic and spectroscopic techniques. UV spectra of stigmasterol shown λ_{max} at 288 nm with characteristic peaks of IR as 3431.48 cm^{-1} for O-H stretching, 1700.31 cm^{-1} for carbonyl group.

Phytochemical screening: The results of preliminary phytochemical test showed that methanolic extract contains flavonoids, steroids, triterpenoids, tannins, mucilage, carbohydrates and starch whereas, n-hexane fraction contains triterpenoids, steroids, vitamin-E and ethyl acetate fraction contains flavonoids, tannins, carbohydrates and vitamin E, B₆ and B₁.

Brine shrimp lethality test: The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which, in most cases, correlates reasonably well with cytotoxic and anti-tumor properties. Preliminary cytotoxic study was performed by using artemia nauplii brine shrimp lethality test which demonstrated that the various fractions of *Eulophia herbacea* exhibited cytotoxic potential in varying concentration of LC₅₀ value. LC₅₀ for all test samples were between 168.3-865.3 $\mu\text{g mL}^{-1}$. Ethyl acetate fraction showed most active with LC₅₀ 168.3 $\mu\text{g mL}^{-1}$ for cytotoxic testing (Table 1).

Table 2: Anticancer activity of *Eulophia herbacea* tubers on human cancer cell lines

Parameters ($\mu\text{g mL}^{-1}$)												
Drugs	Human nasopharyngeal cancer cell line (KB)			Human lung cancer cell line (Hop62)			Human cervix cancer cell line (ME180)			Human leukemia cell line (K562)		
	LC ₅₀	TGI	GI ₅₀	LC ₅₀	TGI	GI ₅₀	LC ₅₀	TGI	GI ₅₀	LC ₅₀	TGI	GI ₅₀
PCA	>80	57.5	26.1	>80	>80	53.7	>80	79.3	43.8	>80	>80	36.6
EAF	>80	68.7	47.3	>80	>80	76.2	>80	>80	53.4	>80	>80	45.9
HEX	>80	>80	>80	>80	>80	>80	>80	>80	>80	71.2	41.4	10
ME	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80	>80
Std	40.4	6.7	<10	78.8	34	<10	54.0	18.8	<10	44.5	<10	<10

PCA: 1-phenanthrenecarboxylic acid 1, 2, 3, 4, 4a, 9, 10, 10a-octahydro-1, 4a-dimethyl-, methyl ester, EAF: Ethyl acetate fraction, HEX: Hexane fraction, ME: Methanol extract, Std: Standard doxorubicin, LC₅₀: Lethal concentration, TGI: Total growth inhibition, GI₅₀: 50% growth inhibition

Anticancer activity by SRB assay: To investigate the effect of anticancer activity on human cell lines, the methanol extract, n-hexane fraction and isolated phenanthrene type compound was assayed and all test sample showed cytotoxic effect. The isolated phenanthrene type (PCA) compound was most active against all 4 cell lines and showed 50% inhibition of growth (GI₅₀) with concentration 26.1, 36.6, 43.8 and 53.7 $\mu\text{g mL}^{-1}$ for human nasopharynx (KB), leukemia (K562), cervix (ME180) and lung (HOP62) cell line, respectively. PCA also showed concentration-dependent growth inhibition of human cell-lines. n-hexane fraction was active only against human leukemia (K562) cell line with GI₅₀ concentration <10 $\mu\text{g mL}^{-1}$ whereas, methanol extract was >80 $\mu\text{g mL}^{-1}$ for all cell lines. Present anticancer study on human cell lines demonstrated that isolated PCA compound was most active as compared to ME and HEX (Table 2).

DISCUSSION AND CONCLUSION

This is the first report describing the scientific evidence and support to the claims laid by the folklore use of *E. herbacea* tubers against cancer in Toranmal region of Nandurbar district, Maharashtra, India. As immune system plays vital role in antitumor potential, some reports suggested that *Eulophia* species exerts antitumor property with complimentary augmentation of immunomodulating effect (Datla *et al.*, 2010). Natural or semisynthetic compounds may be used to block, reverse or prevent the development of invasive cancers. Cellular carcinogenesis forms the biological basis for the identification of preventive products, the assessment of their activity and ultimately the success or failure of a therapy (Reddy *et al.*, 2003). *E. herbacea* consist diterpenes, triterpenoids, polysaccharides, flavonoids and phenolics which have been closed to immunomodulating effect which, worthwhile, also act as antitumor agents (Tan and Vanitha, 2004; Moradali *et al.*, 2007).

Phenanthrene type of compound 1-phenanthrenecarboxylic acid 1, 2, 3, 4, 4a, 9, 10, 10a-octahydro-1, 4a-dimethyl-, methyl ester was isolated from *Eulophia herbacea*, might be laid to anticancer potential as other species of *Eulophia* showed the presence of phenanthrene compound with potent anticancer activity (Tuchinda *et al.*, 1988; Blitzke *et al.*, 2000; Shriram *et al.*, 2010). A fairly large number of phenanthrene has been reported from mainly Orchidaceae family and their numerous derivatives are promising and expanding group of biologically active natural compound with anticancer, spasmolytic, anti allergic, anti-inflammatory

and antibacterial potential. But their screening has not yet been investigated sufficiently and which have not been exploited by the pharmaceutical industry (Kovacs *et al.*, 2008). Finally, we can conclude by carrying further study with thorough characterization of isolated Phenanthrene for evaluating pharmacodynamic at multiple levels of biological study and target for lead compound with safety, efficacy and acceptability based on relevant science and can emerge new drug candidate from *Eulophia herbacea* Lindl.

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