1. INTRODUCTION

India being one of the richest repositories of biodiversity includes nearly 7000 species of medicinal herbs with explicit and wide usage in traditional medicine for over centuries. These plants form the origin and source of drugs to more than 80% of the population to sustain their essential primary health care. However records on ancient healing does not account for standardized studies of the medicinal plants in terms of chemo-pharmacology and toxicology.
Hence the utility of such medicinal herbs becomes limited in modern medicine unless specific protocols are validated for isolation, characterization and elucidation of bioactivity in them. It is therefore imperative to conduct a thorough research to understand the underlying bioactive principles in these herbs which could serve as valuable aid in drug designing and pharmacological kinetics.

Keeping in view of the above aspects the present investigation has been directed to elucidate the bioactivities in *Eryngium foetidum* L. The plant is an aromatic and medicinal plant belonging to the family Apiaceae. It is commonly known by the name culantro or wild coriander and is a popular condiment widely used in continental cuisine like soups, salads, sauces, salsa, sofrito, noodles and curries among the ethnic populations. The plant contains aldehyde rich essential oils valuable to perfumery industry. The essential oil of the plant is also an essential ingredient of drug formulation to treat arthritis and skin diseases (NEIST, Imphal and CSIR). The scientific validations for the medicinal attributes of the plant have been obtained for its analgesic and anti-inflammatory, anti-diabetic, antioxidant and anticlastogenic activities. The anti-helminthic property of the plant (Eryngial) against parasitic trypanosome *Strongyloides stercoralis* has been patented. The plant is being used in formulation for skin whitening under Japanese patent.

It has been recorded that the triterpenoid saponins from the plants exhibit antioxidant and anticarcinogenic activity due to their complex structure and chemical heterogeneity. The same is true of essential oils which are known to possess high levels of free radical scavenging activity preventing oxidative damage and inflammation owing to their mosaic combination of terpene constituents. Since reactive oxygen species activate signal pathways contributing to tumor development, the essential oils are considered to be active against cancer cells. Therefore, during the present investigation, a pioneer study has been carried out to extract the essential oil and triterpenoid saponin from the leaves of *E. foetidum* and to subsequently test their potential for the *in vitro* antioxidant and anti-proliferative activities. The above tests were also carried out using the methanolic extract of the leaf in order to obtain a comparative profile (bioactivity of the individual bioactive components and the total plant extract) and to determine the synergistic bioactive effects of the plant.

2. MATERIAL AND METHODS

**Collection of plants:** The plants of *E.foetidum* were collected from Hassan, Karnataka in the months June and July, identified and preserved at Regional Research Institute, Bangalore.

**Methanolic extract:** The leaves of *E. foetidum* were excised, shade dried and coarsely powdered. 20g of dried plant material was extracted with 250 ml of methanol with continuous agitation at room temperature for 48 h, filtered and centrifuged at 5000g for 15 min. The supernatant collected was evaporated to obtain the methanolic extract which was stored in air tight bottles at 4°C till further use.

**Leaf essential oil:** The leaves were shade dried and hydrodistilled in Clavenger-type apparatus for 4 h. The essential oil was collected and dried over anhydrous Na₂SO₄ and stored in refrigerator until further use.

**Saponin:** About 20g of coarsely powdered leaves of *E. foetidum* were used for the extraction of saponin according to the procedure elaborated by Obadani and Ochuko, 2001. The saponin thus obtained was loaded on a silica gel column (mesh size 60-120) and eluted with chloroform followed by chloroform: methanol with increasing polarity. The fractions were collected at regular intervals and subjected to TLC on 0.25 mm Silica gel.
F254 (Merck) precoated aluminium plates. The chloroform:methanol:water (7:2:1) was used as developing solvent system and detection was carried out using Vanillin reagent (15g vanillin in 250 ml ethanol+2.5 ml con. Sulfuric acid) or p-anisaldehyde reagent (0.5ml p-anisaldehyde in 50 ml glacial acetic acid+1 ml con. Sulfuric acid). Fractions with similar Rf values were pooled, concentrated and used for bioactivity.

**DPPH radical scavenging assay:** The ability of the samples (the leaf essential oil, methanolic extract and saponin) to scavenge synthetic free radical 1, 1-diphenyl 2-picrylhydrazyl (DPPH) was determined by the method described by Bondet et al, 1997 and Hsu et al, 2007. Different concentrations of the leaf essential oil, methanolic extract and saponin (10-50µg/ml) in DMSO were added to 5 ml of methanolic DPPH (0.1mM) solution. The mixture was shaken vigorously and incubated in the dark at room temperature for 20 minutes and the absorbance read at 517nm against a blank (methanol). Ascorbic acid (1mg/ml) was used as the reference standard (positive control). The radical scavenging activity of the samples was determined from a calibration curve. The tests were run in triplicates and averaged. The DPPH radical scavenging activity was calculated as follows:

\[
\text{DPPH Radical-scavenging activity} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100
\]

Where, \(A_{\text{control}}\) and \(A_{\text{test}}\) are the absorbance of the control (methanolic DPPH) and the sample (methanolic DPPH + samples) respectively.

The concentration of the samples at which 50% inhibition (IC\(_{50}\)) occurred was calculated from the graph.

All the results obtained were expressed as means ± SD. Analysis of variance (ANOVA) was used to determine the significant differences for multiple comparisons which was completed using the Tukey Honestly Significant Different (HSD) test at \(\alpha = 0.05\). All of these were carried out using MATLAB 7.5.0 (R2007b).

**MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay**

**Cell lines and Culture medium:** HeLa (Cervix carcinoma), A-549 (Lung carcinoma) and PC-3 (prostate carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. The stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% \(\text{CO}_2\) at 37°C until 75-80% confluence was achieved. TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS) was used to dissociate the cells. The stock cultures were grown in 25 cm\(^2\) culture flasks and all experiments were carried out in 96 microtitre plates.

**Preparation of samples:** The samples of *E. foetidum* viz. methanolic extract, leaf essential oil and crude saponin were dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

**MTT assay:** After trypsinization of the monolayer cell culture, the cell count was adjusted to 1.0 x 10\(^5\) cells/ml using Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS. 0.1 ml of the diluted cell suspension was seeded to each well of the 96 well microtitre plate. After 24 h incubation, the monolayer was washed with DMEM medium and 100 µl of various concentrations of methanolic extract, leaf essential oil and saponin extract were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37\(^\circ\) C for 3 days in 5% \(\text{CO}_2\)
atmosphere and tracked through microscopic examination carried out at 24 h interval. After 72 h, the extracts and the essential oil in the wells were discarded and 50 µl of MTT (5mg/ml) in Phosphate Buffer Solution (PBS) was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO₂ atmosphere. The supernatant was removed; followed by the addition of 100 µl of propanol and subsequent gentle agitation to solubilize the formazan formed. The absorbance was read using a microplate reader at a wavelength of 540 nm (Francis and Rita, 1986). The percentage growth inhibition was determined using the formula:

\[
\% \text{ growth inhibition} = 100 - \frac{\text{mean OD of sample}}{\text{mean OD of control}} \times 100
\]

All the assays were done in triplicates and expressed as means ± SD. Analysis of variance (ANOVA) was used to determine the significant differences for multiple comparisons which was completed using the Tukey’s Honestly Significant Different (HSD) test at \( \alpha = 0.05 \) in SPSS version 20.

3. RESULTS AND DISCUSSION

The radical scavenging abilities of the essential oil, methanolic extract and the saponin samples were determined using the DPPH radical scavenging assay. High DPPH radical scavenging potential confers superior antioxidant activity to the samples. The highest percentage of inhibition of DPPH radicals was observed in the methanolic extract and essential oil closely followed by the saponin (Table 1). The essential oil, saponin and methanolic extract recorded an IC\(_{50}\) of 22.14µg/ml, 23.79µg/ml and 22.00 µg/ml respectively as against an IC\(_{50}\) of 19.07µg/ml for the standard ascorbic acid indicating high antioxidant property in all the samples tested (Fig.1). All samples showed significantly different (p<0.05) antioxidant activity for the assay.

The essential oil, methanolic extract and saponin demonstrated varying levels of dose dependent cytotoxicties against HeLa, A549 and PC3 cell lines in MTT antiproliferative assay. The comparative levels of cytotoxicties of the extracts against the various cell lines have been represented in Fig.2. The cytotoxic effects of the essential oil against the cell lines were significantly different at p<0.05 levels, the highest CTC\(_{50}\) values recorded against PC3 cell lines (42.5 µg/ml) (Table 2). The saponin evoked least response for Hela and PC3 cell lines, but a moderate response was observed against the A549 cell lines at higher dosages. The methanolic extract generated a low cytotoxic response for Hela and PC3 cells but a significant inhibitory effect against A549 cell lines. Going by the American National Cancer Institute, the LC\(_{50}\) limit to consider for a crude extract promising for further purification to isolate biologically active (toxic) compounds should be lower than 30 g/ml 19. It has also been stated that oils and extracts from plants presenting LC\(_{50}\) values below 1000 g/ml are known to contain physiologically active principles 20. A number of prior reports have indicated a possible significant role of reactive oxygen species being responsible for stimulating growth related responses triggering tumorigenesis and subsequent malignancy 21, 22, 23. It has been also established that the bioactive principles in plants like phenolics, flavonoids, terpenoids, saponins, ascorbic acid and essential oils are potent inhibitors of reactive oxygen species thereby acting as anti inflammatory, anticarcinogenic and anti proliferative agents 24, 25, 26. A high phenolic, flavonoid and ascorbic acid content has already been reported in E.foetidum 27, 28 and could be the stemming factor for the high antioxidant properties being presently observed in the extracts and leaf essential oil of E.foetidum. Earlier investigations have also recorded the antioxidant potential of E. foetidum 29, 30, 31 and
species of Eryngium viz. E.creticum and E.maritimum. However the antioxidant properties have been recorded for the first time in the saponin extract and essential oil of E.foetidum.

In traditional medicine, the decoction of E.foetidum leaves has been documented as an effective remedy against malignant prostate adenoma. This goes in accordance with our study as the essential oil from leaves was found to be highly cytotoxic against the PC-3 cells (CTC$_{50}$ - 42.5µg/ml). However further systematic investigations are obligatory to understand the pharmacology and toxicity of the essential oil of E.foetidum. The saponin extract failed to generate adequate response against any of the cell lines thereby instigating intensive standardization of protocols for isolation and its purification. The in vitro cytotoxicity studies of methanolic extract, saponin and essential oil of E.foetidum against various carcinogenic cell lines have also been a maiden endeavor.

### Table 1: DPPH radical scavenging activity of leaf essential oil, saponin and methanolic extract with ascorbic acid (standard).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration (µg/ml)</th>
<th>Inhibition (%)</th>
<th>IC$_{50}$ (µg/ml)*</th>
<th>DPPH IC$_{50}$</th>
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</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>10</td>
<td>14.945</td>
<td>9.07±0.02</td>
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<tr>
<td></td>
<td>20</td>
<td>57.126</td>
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<td></td>
<td>30</td>
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</tr>
<tr>
<td></td>
<td>40</td>
<td>96.572</td>
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<tr>
<td></td>
<td>50</td>
<td>97.653</td>
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<tr>
<td>Leaf essential oil</td>
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<td>22.14±0.04</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>50</td>
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<td>Saponin</td>
<td>10</td>
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<td>Methanolic extract</td>
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<td>94.343</td>
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</table>

Data are expressed as mean ± SD. *Values statistically differ at P > 0.05 as measured by Tukey HSD test.

### Table 2: CTC$_{50}$ values of leaf essential oil, methanolic extract and saponin against various cell lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>CTC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>A-549</td>
</tr>
<tr>
<td>Leaf essential oil</td>
<td>203.33±1.9*</td>
</tr>
<tr>
<td>Methanolic extract</td>
<td>340.00±1.4*</td>
</tr>
<tr>
<td>Saponin</td>
<td>810.00±1.2*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. *Values statistically differ at P > 0.05 within the row as measured by Tukey HSD test.

### 4. CONCLUSION

This work is an attempt to provide strong evidence for the bioassay guided pharmacological activities in E.foetidum. The antioxidant potential of extracts and essential oil of E.foetidum proves its possible usage in therapeutics as a natural antioxidant. The above study also provides preliminary data for the use of essential oil and the methanolic extract in further confirmatory studies as anti-cancer drugs against respective cell lines.
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17. Bondet V, Brand-Williams W and Berset C. Kinetics and mechanisms of antioxidant activity
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