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Anticancer activity of unexplored species of *Atuna*

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ABSTRACT

The present study is the first report of the anticancer activity of the wild plants *Atuna indica* (Bedd.) Kosterm and *Atuna travancorica*(Bedd.) Kosterm from Western Ghats, India. Different plant parts of *Atuna indica* (leaves and flowers) and *Atuna travancorica* (leaves) were collected, chopped, shade dried and extracted in ethanol by maceration process and evaporated to dryness with the use of rotary evaporator. The ethanol extracts of the test plant parts of the two species were subjected to the anticancer therapeutic nature with the help of *In vitro* cytotoxicity studies using Dalton's Lymphoma ascites (DLA) and K562 Cell Line through Trypan blue dye exclusion and MTT assay methods. Similarly, antioxidant activity evaluation was done by DPPH Assay of the two species to strengthen the anticancer therapeutic findings obtained. The results suggested that the plant parts such as the leaves of the two species possess anticancer activity; in particular leaves of *A. travancorica* shows prominent anticancer activity when compared with the other parts of the two species examined.

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INTRODUCTION

The two species of *Atuna* distributed in the Southern Western Ghats are *Atuna indica (*Bedd.) Kosterm and *Atuna travancorica (*Bedd.) Kosterm. The specimens of *Atuna indica* were collected from Nadukani (Nilambur North Forest Division, Kerala State, India) and it was first collected by Beddomei from Carcoorghats of Wayanad, and it was the rediscovery of this species, about 150 years later. Similarly, *Atuna travancorica* was described based on Hooker's collection, collected from Near the valara waterfalls, under Munnar Forest Division, Kerala State, India. The taxonomical as well as morphological characterization of the species was clearly done (Sasidharan and Sujanapal, 2011).

Atuna indica, a 20m tall tree with smooth, thin, brown bark having elliptic-oblong or elliptic ovate leaves with 17-21cm long and 5.5-7.5cm wide with white flowers. Similarly, in the case of *Atuna travancorica, a* 25m tall tree with smooth thin grayish brown bark having alternate, lanceolate leaves with 7-16.5cm long and 1.7-4.5cm wide with pale lavender or white flowers. A unique feature of the family (Chrysobalanaceae) is seen such as they are prominently keeled (Ghilleane*t al.*, 2003; Flowering Plants, 1989). In the case of *Atuna indica*(Bedd.) Kosterm; Flowering and Fruiting: November to February; Habitat: West Coast tropical evergreen forests (Western Ghats in India) whereas in the case of *Atuna travancorica* (Bedd.) Kosterm; Flowering and Fruiting: January to May; Habitat: West Coast tropical evergreen forests, usually riparian (Southern India, Travancore region) Both the species are belonging to the endangered category (Sudhakare*t al.*, 2007; SasidharanN, 2002; Malin *et al.*, 2015; Jean *et al.*, 2003)

Therapeutically potent drugs such as Digitalis, Atropine, Morphine, Ergot and Quinine were of Plant origin and still Pharmaceutical Scientist derives therapeutically useful leads from plant sources. It has been found that still there is a sorrow from the affected human population due to the severe diseases such as Advanced Cancer (Metastatic Cancer). (Satoskar*et al*., 2015; Ahmed *et al*., 2010; Remington).In the species of *Atuna indica*, Presence of Umbelliferone, an active coumarin having a number of reported pharmacological activities found to possess therapeutically better antioxidant activity as reported (Asish *et al*., 2013).The search to find the pharmacological as well as the therapeutical outcome of this powerful antioxidant property synergistic with the free radical scavenging activity present in this wild species, *Atuna indica* and *Atuna travancorica* is the root of this study.

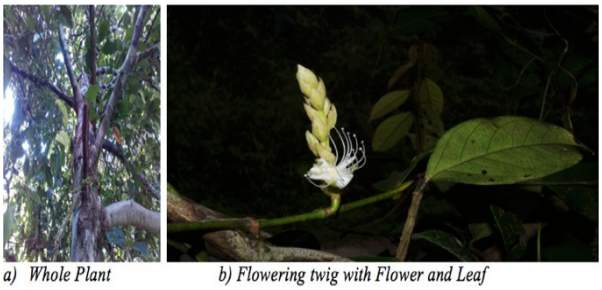
MATERIALS AND METHODS

Plant Materials

leaves of *A. travancorica* and leaves and flowers of *A. indica* were collected from Western Ghats of Kerala; *A. travancorica* collected from Near the valara waterfalls, under Munnar Forest Division, Kerala State, India. and *A.indica* collected from Nadukani (Nilambur North Forest Division, Kerala State, India).

a) Whole Plant *b) Leaf* *c) Flowering twig with Flower and Fruit*

Figure 1: Habit of *Atuna travancorica* (Bedd.) Kosterm



a) Whole Plant *b) Flowering twig with Flower and Leaf*

Figure 2: Habit of *Atuna indica* (Bedd.) Kosterm

Specimen Voucher-Reference number

Voucher specimens of *Atuna indica* (Bedd.) Kosterm, Collected from Malabar Wild Life Sanctuary, Near Kakkayam Dam, Kerala and *Atuna travancorica* (Bedd.) Kosterm, collected from Near the Valara Water Falls, Munnar, Kerala were deposited at Calicut University Herbaria, Dated 23.10.2016 with Herbarium reference numbers CU143967 and CU143986 Voucher specimens of *Atuna indica* (Bedd.) Kosterm, Collected from Malabar Wild Life Sanctuary, Near Kakkayam Dam, Kerala and *Atuna travancorica* (Bedd.) Kosterm, collected from Near the Valara Water Falls, Munnar, Kerala, were deposited at Centre for Medicinal Plants Research (CMPR) Herbaria, Kottakkal-676503, Kerala Dated 26.10.2017 with the Herbarium reference numbers CMPR08694 and CMPR08695 respectively.

Extraction of Plant Parts

The collected parts (leaves and flowers) of the two species were chopped into suitable smaller size and shade dried, powdered in a mixer and stored in suitable air tight containers. Weighed a quantity of 10 gram in three separate stoppered conical flasks with proper labeling on it. To that added 100 ml of 50% aqueous ethanol solution and kept aside with occasional shaking for a period of 24 Hours. After a period of 24 Hours the supernatant solvent replaced with fresh 100 ml 50% aqueous ethanol solution and the mixture again kept for a period of 24 Hours. (Molnar *et al.,* 2017). After the completion of the prescribed time, both the supernatant liquids mixed together and concentrated for the complete removal of the solvent using rotary evaporator. The extracts obtained from each species properly labeled and transferred to air tight vials.

Anticancer activity

In-vitro cytotoxicity study on DLA Cells by trypan blue exclusion method

Dalton's lymphoma ascites cell (DLA) line procured from ACI, Chennai and maintained in the peritoneal cavity of tumour bearing mice from which it is aspirated during the study. Cell viability was determined by trypan blue exclusion method. (Talwar GP, 1971). Viable cell suspension (1x 10^6cells in 0.1 ml) was added to tubes containing various concentrations of the test compounds of Leaf extracts of *Atuna indica* and *Atuna travancorica* and the volume was made up to 1 ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixtures were incubated for 3 hour at 37^0C. Further cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 minutes and loaded on to the haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The numbers of stained and unstained cells were counted separately.

The percentage cytotoxicity calculated as follows:

$$\frac{\text{No. of dead cells x 100}}{\text{No. of live cell + No. of dead cell}}$$

Cell viability assay on K562 Cells by MTT assay

K562 cell line procured from NCCS Pune. The viability of the cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazoliumbromide (MTT) assay (Mosmann,1983) which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. In this method K562 (chronicmyelogenous leukemia) cells were seeded in a 96 well microtitre plate with a density of 15000 cells/well (Raghavan*et al.,* 2014) and incubated overnight. After incubation, fresh medium containing varying concentration of the sample (100 - 400µg/ml) was added to respective wells and incubated for 48 hours in a CO_2 incubator. At the end of incubation period medium was aspirated and replaced with fresh medium containing MTT (0.5mg/ml) and incubated again for 3-4 hours. The formazan crystals thus formed were dissolved in DMSO and the absorbance measured at 570 nm (MULTISKAN EX Thermo Scientific).

The IC_{50} values were calculated using the ED50 plus v1.0 software programme.

Antioxidant Evaluation by DPPH free radical scavenging method:

To different volume of extract, 0.5 ml of methanolic solution of DPPH was added and made up to 2ml using methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the extracts, served as the positive control. After 30 minutes of incubation, the discoloration of the purple colour was measured at 518nm in a spectrophotometer (GENESYS™ 10S UV-Vis Spectrophotometer). The radical scavenging activity was calculated as follows:

% of inhibition= (Control- Test)/Control X 100

RESULTS AND DISCUSSION

In the present study, an amount of 10gram of powdered leaves of *Atuna indica* and *Atuna travancorica* in 100ml of 50% aqueous ethanol solution as solvent were used for the extraction. From each sample, ~2gram extracts were collected.

Anticancer evaluation on DLA Cell line

Table 1: Concentration Vs Percent Cell Death (DLA Cell Line) of leaf extract of *A.indica*

S.No.	Concentration (µg/ml)	Percent Cell Death (DLA)
1.	200	70
2.	100	52
3.	50	40
4.	20	32
5.	10	25

***Atuna indica* leaves:** Action of the sample on DLA Cell line indicated that a percent cell death of 70% was obtained at a concentration of 200µg/ml and IC_{50} value of the sample is above 200µg/ml as shown below in Table 1.

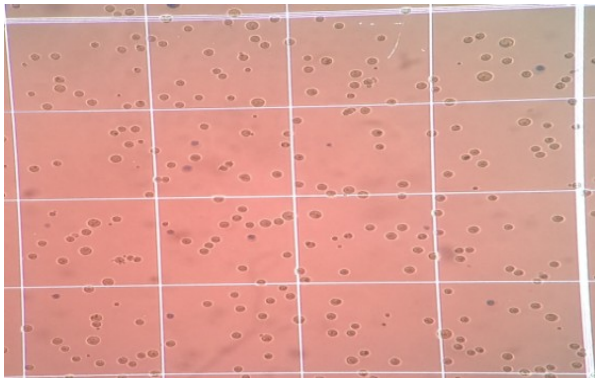


Figure 3: Control with DLA cell suspension

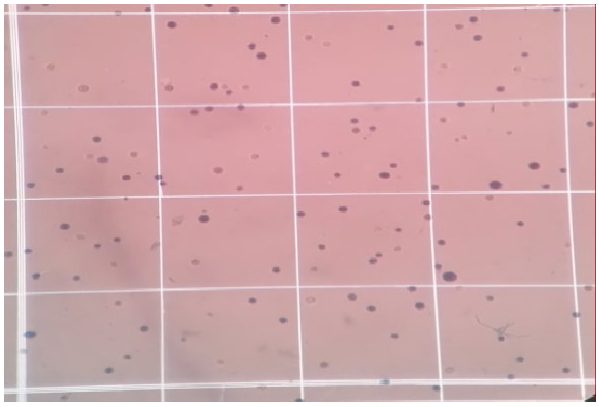


Figure 4: Percent cell death with *A.travancorica* leaf extract

***Atuna travancorica* leaves:** Action of the sample on DLA Cell line indicated that a percent cell death of 88% was obtained at a concentration of 200µg/ml and IC_{50} value of the sample is found to be 50µg/ml as shown below in Table 2 and Figures 3 and 4.

Table 2: Concentration Vs Percent Cell Death (DLA Cell Line) of leaf extract of *A.travancorica*

S.No.	Concentration (µg/ml)	Percent Cell Death (DLA)
1.	200	88
2.	100	62
3.	50	50
4.	20	38
5.	10	28

Anticancer Evaluation - MTT assay on K562 Cell Line with Ethanol Extract: In this *In vitro* Cytotoxicity evaluation, it uses 96 well microtitreplates with a density of 15000 cells per well.

***Atuna travancorica* leaves:** Action of the sample on K562 Cell line indicated that the IC_{50} value of the sample is found to be 321.667µg/ml as shown above in Figure 5.

Antioxidant activity by DPPH Assay: As indicated in the above Table No.3 *A. travancorica* leaf extract has a better percentage scavenging activity at a concentration of 5mg as seen in the tabular representation.

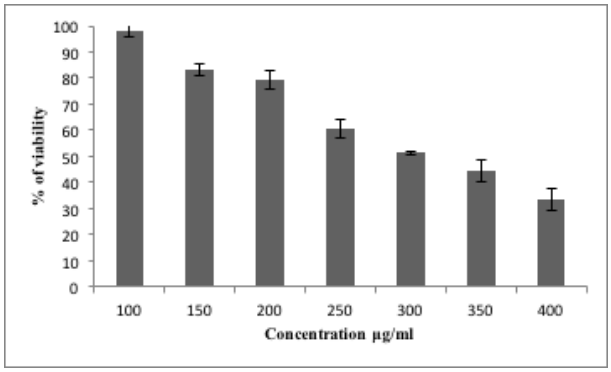


Figure 5: Concentration versus percent viability of of *A.travancorica* leaf extract -K562 cell line- IC50 321.667µg/ml

Table 3: Percentage Scavenging Activity of *A.travancorica* leaf extract By DPPH Assay

Conc. mg	OD at 518nm	% of scavenging activity
0.5	0.067	29.47
1	0.044	53.68
5	0.018	81.05

CONCLUSION

The results suggested that the plant parts such as the leaves of the two species possess anticancer activity; in particular leaves of A. travancorica shows prominent anticancer activity when compared with the other parts of the two species examined and the current study concluded that there is a remarkable anticancer activity in the leaves of the endangered tree species *Atuna travancorica* in a therapeutical threshold manner, which can be associated with the percentage scavenging antioxidant activity as seen from the results of DPPH Assay. In this context, there is an immense requirement of a detailed and authoritative Phytochemical screening about the Phytoconstituents present in the leaves of *Atuna travancorica* by using highly sophisticated analytical methods to drive the mechanism based activity.

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