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Evaluation of Anti-mitotic and Cytotoxic Potential of Methanolic Extract of *Balanus amphitrite*

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Abstract: ***Balanus amphitrite of phylum Arthropoda, Class Crustacea and family Balanidae are sessile invertebrates found in tropical and sub-tropical regions. They are traditionally looked upon as fouling agents. Our present study examines the cytotoxic and anti-mitotic activity of methanolic extract of Balanus amphitrite (BME) using MTT assay and Allium cepa root tip assay respectively. BME was found to be cytotoxic to Lung cancer cell line (A-549) with IC 50 value of 242*µg/ml *and significantly anti-mitotic on Allium cepa root tip cells.***

Keywords: Barnacle, cytotoxicity, anti-mitotic activity, MTT, Allium cepa assay

1. Introduction

Marine ecosystem is a vast and diverse ecosystem as compared to terrestrial ecosystem. This inspires many researchers to investigate marine ecosystems potential source of raw material for medicines, food, and other purposes [1]. It has been found that marine organisms produce two types of metabolites viz. primary and secondary. Secondary metabolites are biological molecules that have key physiological and behavioural role. Unlike proteins and nucleic acid they are not involved in primary metabolism but they aid in defence and survival of the organism [2]. Secondary metabolites include alkaloids, phenols, steroids, terpenoids etc. [3].The increasing failure of chemotherapeutics and antibiotic resistance shown by many pathogenic microbes have led to screening of several marine organisms [4]. Secondary metabolites are now-a-days being utilized to achieve the aim of manufacturing new and improved drugs[5]. *Balanus Amphitrite belonging* to phylum Arthropoda and class Crustacea and family Balanidae is commonly known as striped barnacle or acorn purple barnacle. They are sessile invertebrates permanently attached to the substrate they live on. Their body is covered by calcareous shell and are prevalent throughout tropical and sub-tropical region[6].They form complex symbiotic associations with larger organisms such as whales, sea snakes, lobsters, medusae, corals and sponges while are considered to be a serious pest because it colonizes speedily on manmade objects immersed in seawater [7,8,9].Till date, efforts have been majorly focussed upon eradication of these organisms owing to their fouling property. Pansch, Christian, et al. reported that diterpene compound derived from a soft coral species has significant anti-fouling property against *Balanus albicostatus*[10]. It has also been observed that certain sponges and tunicates exhibited exemplary anti fouling properties against larval stages of *Balanus amphitrite*[11]. Likewise, many other researches focus on extermination of barnacle sp. using synthetic compounds [12,13]. However, previous findings exemplified high tolerance power of barnacles to certain environmental factors[14,15,16]. This high tolerance of the barnacles may be an indication of the presence of potential bioactive compounds advantageous to mankind in various ways. This urged us to investigate this organism for its bioactive potential. Since past few decades, scientists have reported many marine organisms having significant anti-cancer properties. They are being screened for cytotoxicity as well as genotoxicity to analyse its anti-cancer properties [17,18]. Simmons, T. Luke, et al. in their book have discussed marine products as source of anti-cancer agents in detail[19].Hence the present study is aimed to investigate the anti-mitotic activity and cytotoxic potential of methanolic extract of *Balanus sp*.

2. Material and methods

2.1 Collection and Extraction:

The organism *Balanus Amphitrite* was hand-picked during low tide from Mumbai coast, Maharashtra, India. The soft body of the organisms was, minced and was cold percolated in methanol for 3-4 days. The above procedure was repeated with fresh methanol until colourless methanol was obtained. The filtrate was then reduced under pressure leaving behind a gummy methanolic extract which was named BME.

2.2 Genotoxicity: Allium cepa root tip assay:

The experiment was performed according to the procedure described by Tedesco et.al[20].

2.2.1 Pre- Treatment and Treatment

The *Allium cepa* bulbs were allowed to grow in tap water at room temperature for 2–3 days. Grown onion roots of length approximately 1.5-2cm were treated with different concentrations of the extract (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml) for 4 hours at 24-25°C in a dark place, after which the root tips from each bulb were harvested and fixed in fixative solution (1:3 v/v of Glacial acetic acid: Ethanol) until use. Tap water was taken a negative control while Colchicine (0.04%) served as positive control.

2.2.2 Slide preparation

The root tips were initially washed in distilled water and then hydrolysed in 1N HCl at 60° C for about 5-6 mins. The fragmented meristematic region of the root was stained with 1% acetocarmine, washed with 45% acetic acid, mounted on slides and observed under Optical microscope (KYOWA M.NO.1015) at 40X magnification.

2.2.3 Slide Analysis:
The experiment was performed in triplicate and a total of 2500 cells were analysed for each concentration. The genotoxicity of the extract was determined by calculating Mitotic Index (MI).
The Mitotic Index was calculated by

$$MI = \frac{total\ number\ of\ cells\ in\ mitosis}{total\ number\ of\ cells} \times 100$$

2.3 Cytotoxicity: MTT Assay

Cell culture: Human lung carcinoma (A-549) and Chinese Hamster Ovary (CHO) cell lines were purchased from National Centre for Cell Science (NCCS). A-549 cell line was maintained in HAM's F12medium, CHO cell line was maintained in Roswell Park Memorial Institute (RPMI-1640) medium, each supplied with 10% fetal bovine serum, 1% penicillin-streptomycin and 2% L-Glutamine at 37^0 C in humidified incubator with 5% CO_2.

2.3.1. MTT Assay
The cytotoxic activity of the samples on A-549 and CHO was determined by (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) (MTT) assay[21]with minor modification. Cells of density 2 x 10^3 were seeded in 96-well plate and Incubated for 24 hours. After this, the medium was replaced with fresh serum free medium and the plate was incubated for 24 hours after which the cells were treated with different concentrations of BME (750µg/ml, 500µg/ml, 375µg/ml, 250µg/ml, 187.5µg/ml, 125µg/ml). The medium was then removed carefully and 10µl of MTT was added to each well along with 90µl of complete medium. The plate was then incubated at 37° C in CO_2 incubator for 4 hours, after which, it was replaced by100 µl of DMSO and the plate was read at 595nm on ELISA plate reader (BIO-RAD Microplate reader). The results were calculated using formula of Percentage Viability given by:

$$\frac{Mean\ OD\ Treatment}{Mean\ OD\ Control} \times 100$$

3. Results

The cytotoxic activity of BME against A 549 cells and CHO is depicted in Fig.1. and Fig.2. respectively. BME displayed dose dependent activity on A-549 with IC 50 value being 242.4µg/ml. Less number of viable cells were observed in subsequent concentrations as compared to control. However, in case of CHO cells, the difference of percent viability between control and concentrations is negligible. This implies BME does not affect normal human cells.

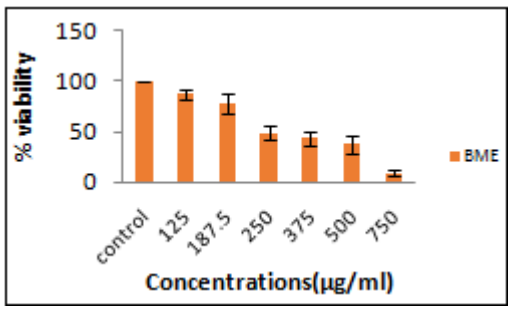

Figure 1: The graph shows in vitro cytotoxic effect of different concentrations (125µg/ml,187.5 µg/ml,250 µg/ml,375 µg/ml,500 µg/ml,750 µg/ml) BME against A-549 cells. Data is expressed as ±SD.

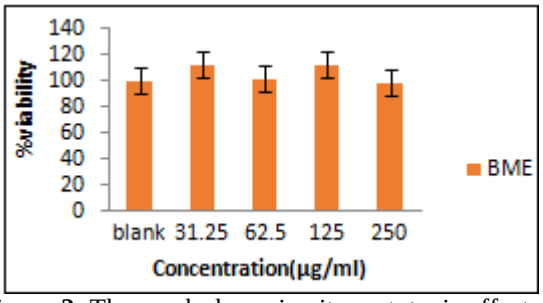

Figure 2: The graph shows in vitro cytotoxic effect of different concentrations (125µg/ml,187.5 µg/ml,250 µg/ml,375 µg/ml,500 µg/ml,750 µg/ml) BME against CHO cells. Data is expressed as ±SD.

Effect of different concentrations (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml) is depicted is Fig 3. Stating that at 500µg/ml, the anti-mitotic effect of the extract is comparable to the control which is Colchicine. The mitotic index gradually decreases from lower concentration to higher concentration

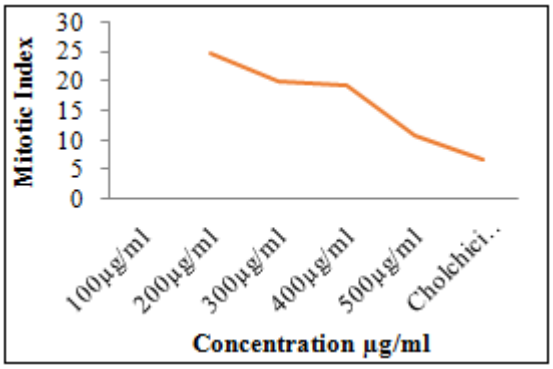

Figure 3: The graph shows the effect of different concentrations (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml) on mitotic index in *Allium cepa* root cells.

From Fig .4 it can be observed that in all the concentrations, cells in prophase are significantly more in number than other phases. It can be concluded that the extract has inhibitory effect on prophase of *Allium cepa* root cells and also produced visible aberrations in certain cells depicted in Fig .5.

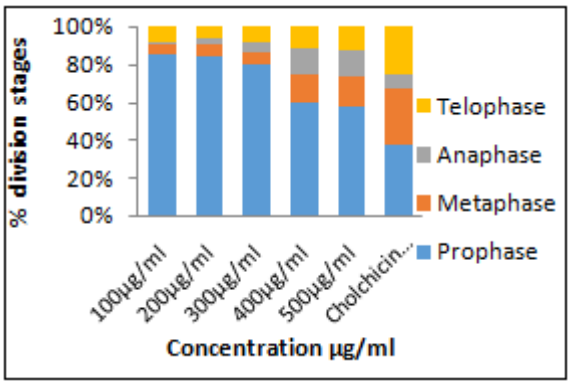

Figure 4: The graph shows effect of BME concentrations (100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml) on

% of cells present in different stages of mitosis in *Allium cepa* root cells.

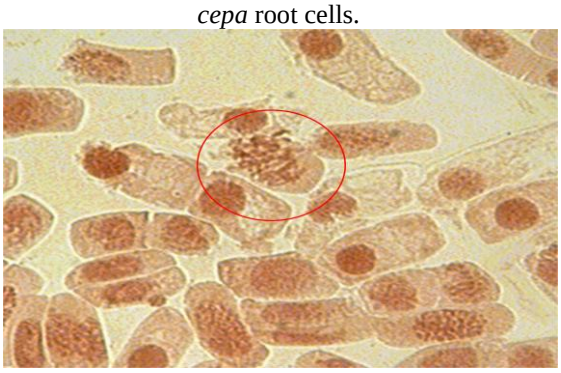

Figure 5: The image represents chromosomal disruption brought about by BME at 400µg/ml.(magnification 40X)

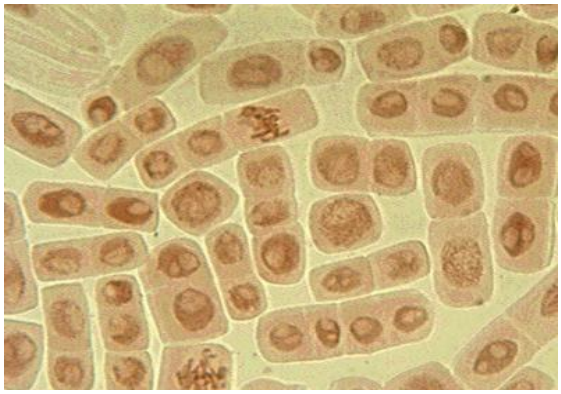

Figure 6: The image indicates normal *Allium cepa* root cells without any aberrations.(magnification 40X)

4. Discussion

Terrestrial plants have proved to be treasure chest for many new and improved drugs over the years[21]. Natural products have always being preferred over their synthetic counterparts owing to their minimal side-effects[22,23]. Since past few decades marine ecosystem has grabbed interest of many researchers. Hence it is under close scrutiny of various researchers which led to discovery of many active compounds. These compounds are playing major role in cancer research [24].Jemma et al. in their studies on crude extract of ink of *Loligo duvauceli* found around 50% cell viability at 250 µg/ml concentration [25]. Findings presented by Moghadasi et al. indicate significant inhibitory effect of Sea anemone nematocyst carpet on Breast cancer cell line MDA MB-231 with IC 50 value being 3.125µg/ml[26] implying significant cytotoxic potential. Substantial cytotoxicity is specifically being recorded in certain organisms and algae [27,28,29]. Although several marine organisms have been investigated previously, rocky shore organisms are comparatively underexplored. They are mostly looked upon as fouling agents. Our present study focuses on bioactive potential of a barnacle species. Our extract, BME, demonstrated prominent cytotoxic effect on Lung carcinoma (A-549) cell line by decreasing the cell viability with increasing concentration. IC 50 value is 242 µg/ml which is comparable to the effect of crude extract of ink of *Loligo duvaceli*. However, in Chinese Hamster Ovary (CHO) cell line, the extract presented negligible effect. This signifies that our extract is non-toxic for normal cells eventually making it a potential anti-cancer agent. In our study we also tested BME for its anti-mitotic potential as it targets cytoskeleton and thus has been of long interest in cancer therapy[30]. According to the work reported by T.Umapoorani et al. of a marine alga on *Allium cepa* root cells, the results conclusively indicated dose-dependent anti-mitotic effect [31]. In a similar study, a sponge species was reported to possess potent anti-proliferative activity against many human cancer cell lines and severe effects on spindle formation in mitotic cells arresting maximum cells in prophase[32]. Our observations state maximum number of cells in prophase indicating that the extract may potentially have arrested the transition of cells from prophase to metaphase and subsequent phases possessing similar effect to that reported by Indap et al[30]. It is also observed that mitotic index decreased in dose dependent manner with higher mitotic index being 24.88% at 100 µg/ml and the lowest being 6.91% at 500 µg/ml which is closely comparable to colchicine suggesting that extract possibly arrests cell division making it probable anti-cancer agent. Related research presented by Kavitha.K.R et al. revealed that there was decrease in mitotic index suggesting change of chromosomal morphology or spindle orientation[33]. Hence we can conclude that BME might also be affecting chromosome morphology by altering the spindle formation or orientation.

5. Conclusion

BME reported cytotoxic activity on A-549 cells with IC 50 value being 242µg/ml while being non-toxic against CHO. BME showed dose dependent anti-mitotic activity with maximum cells arrested at prophase.

6. Declaration

Authors declare that there are no conflicts of interests.

7. Acknowledge

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