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INHIBITION OF PROTEASES AND ANTI-CANCER ACTIVITIES OF ETHANOLIC TUBER EXTRACT OF *GLOBBA BULBIFERA*

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ABSTRACT: Studies on anticancer and protein inhibition process play a vital role in regulating the cellular process of life and diseases. The present studies have been conducted on ethanolic tuber extract from *Globba bulbifera* for protease inhibition and breast cancer inhibition. The extract has shown good inhibition for Trypsin, Chymotrypsin and protease K. The extract has also shown MCF-7 (Breast Cancer) inhibition, IC_{50} value at 235 µg/ml. The inhibition is less compared to the standard compound, tamoxifen (IC_{50}: 37 µg/ml). Hence the present studies have shown good protease inhibition and anticancer (breast cancer) activities.

INTRODUCTION: There are several proteins accessible in the living systems present in various parts of the cells that perform numerous functions within the cells of living organisms [1]. Biological scientists proposed that enzymatic activity is associated with proteins [2]. Proteolytic enzymes show many physiological functions varies from protein digestion to specific regulated processes like blood coagulation, activation of zymogens, the release of bio pharmacologically active peptides and hormones, transport of secretory proteins through membranes, *etc*. [3] The evolution of proteolytic enzymes changes in structure and function and their inhibitors show diverse and complex functions in higher organisms [4].

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Many proteolytic enzymes are synthesized as inactive precursors (Zymogens) with subsequently converted active enzymes by the selective cleavage by peptide bonds and prevent unwanted protein degradation, to enable spatial and temporal regulation of proteolytic activity [5]. These enzymes include proteinases like trypsin, proteinase K, Chymotrypsin, *etc*. [6] The trypsin catalyzes the hydrolysis of dietary protein of the internal peptide bonds that are formed by the basic amino acids due to lysine, arginine and their corresponding amino acid derivatives [7]. Human trypsin is strongly inhibited by inhibitory activity of compounds like mercuric chloride, calcium salts, *etc*. [8]

The chymotrypsin catalyzes the hydrolysis of specific ester substrates [9] that are readily soluble and stable to Tris buffer at pH 8 [10] The biocatalytic activity of chymotrypsin (CT) ethanol/water solution containing small amounts of metal salts. Calcium acetate accelerates transesterification of amino acid to six fold at 100 µM.

Metal salts can change the secondary and tertiary structures of CT [11]. The main proteolytic enzyme was proteinase consists of a single peptide chain containing 277 amino acids concerning its keratin hydrolyzing activity. The specificity of the enzyme for peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids was observed [12].

Cancer is a disease occurring in a series of steps, arising as a consequence of activating mutations (oncogenes) or deactivating mutations (tumor suppressor genes) in proliferating cells [13]. Cyclooxygenase-2 (COX-2) recognized as a molecular target of many chemopreventive as anti-inflammatory agents. Previous studies on COX-2 had shown the regulated mechanism by the transcription factor NF-κB. Curcumin, derived from the rhizome of *Curcuma longa* L. having anti-inflammatory properties, and inhibits chemically induced carcinogenesis in the skin and colon [14]. The Tumour-associated macrophages (TAM) are a major inflammatory component of the stroma of tumors and can affect different aspects of the neoplastic tissue [15]. The Plant-derived compounds an important source of several clinically useful anti-cancer agents such as vincristine, camptothecin, the vinblastine derivatives, irinotecan, topotecan, and etoposide, derived from paclitaxel an epipodophyllotoxin [16]. Some promising new agents are in clinical development and based on selective activity against cancer-related molecular targets, including flavopiridol while some agents who failed in earlier clinical studies are stimulating interest [17].

MATERIALS AND METHODS:

Plant Materials: The fully matured *Globba bulbifera* plant tubers were collected from Kerala. Plant materials for the study were washed thoroughly in distilled water and air-dried. A tuber from *Globba bulbifera* is taken and thoroughly washed with double distilled water and cut into pieces and air dried.

Preparation of Ethanolic Extract: The powder from *Globba bulbifera* tuber is obtained by using a grinder. The powder is placed in a refrigerator. Nearly 30 gm of air-dried powder is taken in a 100ml of ethanol in a 500 ml conical flask by thoroughly mixing by incubating the contents at room temperature in a rotary shaker for 48hrs at 120 rpm. The slurry was then filtered through cheesecloth and Whatman no. 1 filter paper with three times, and then the filtrate was centrifuged at 10,000 rpm for 15 min at 4 °C to remove any cell debris that remains in the preparation. Then the solvent is evaporated through rotavapor and make the final volume one-fourth of the original volume and stored at the 4 ºC in airtight containers. Protease inhibitor activity and anticancer activity is tested for *Globba bulbifera* plant tubers.

Protein Inhibition Activity: Different metals have been tested for protein inhibition activity. Various metals (5mM) like $CaCl_2$, $MgSO_4$, $ZnSo_4$, $CuSo_4$, $FeSO_4$, $MnSO_4$, $HgCl_2$, $BaCl_2$, $CdCl_2$, Na_2MO, Al_2O_3, Na_2EDTA, $(CH_3COO)_2Pb$, Na_2CrO_4, H_3BO_3 and KI has been used in the present experimentation. By using these metals, inhibition of protease has been observed with tuber extract of *Globba bulbifera* Linn. Approximately 10 µl of protease inhibitor (plant extract) was mixed with 10µl of protease (0.5 mg/ml) and spotted on to a strip of X-ray film. 10µl of the protease was mixed with 10µl phosphate buffer 0.l M (pH 7.0) as the control and spotted on to the X-ray film. Incubation of X-ray film at 37 °C for 10 min has to be done. Wash the film under tap water for the zone of gelatin hydrolysis to protease activity visualization.

Anti-cancer Activity: Human cancer cell lines used in this study were produced from National Centre for Cell Science, Pune. All cells were grown in Minimal Essential Medium (MEM, GIBCO) and supplemented with 4.5 g/L glucose, 2 mM L-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37 ºC in 5% CO_2 incubator.

The trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5×10^3 cells/well in growth medium and cultured at 37 ºC in 5% CO_2 to adhere. After 48 h incubation, the supernatant was discarded, and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (12.5, 25, 50, 100, 200 and 250 µg/ml) in triplicates to achieve a final volume of 100 µl and cultured for 48 hours. The compound separately is prepared as 1.0 mg/ml concentrations of stock solutions using DMSO.

The culture medium and solvent are used as controls. Each well then received 5 microliters of fresh MTT (0.5 mg/ml in PBS) followed by incubation for 2hr at 37^0c. The supernatant growth medium was removed from the wells and replaced with 100 micro liters of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance (OD) of the culture plate is measured at a wavelength of 492 nm on ELISA reader, Anthos 2020 spectrophotometer.

RESULTS: Protease inhibition activity of *Globba bulbifera* Linn. has been presented. Comparative studies on different proteases and different metals upon plant extract shown small variation. The control (10µl of Chymotrypsin+10µl of phosphate buffer) has been used with enzyme Chymotrypsin and phosphate buffer. There is no inhibition observed in control. Different metals have been tested with enzyme Chymotrypsin. Strong inhibition is not observed in metals. The test sample has been experimented (10µl of Chymotrypsin+10µl of each metal + 10µl of tuber extract) for chymotrypsin inhibition. $FeSO_4$ is acting as a strong activator to *Globba bulbifera* tuber extract. Remaining tested metals was not shown any inhibition with *Globba bulbifera* tuber extract.

The control (10µl of protease K+10µl of phosphate buffer) has been used with enzyme protease K and phosphate buffer. There is no inhibition observed in control. Different metals have been tested with enzyme protease K. Strong inhibition is shown by $FeSO_4$ and $(CH_3COO)_2Pb$ only but remaining metals not shown inhibition. The test sample has experimented (10µl of protease K + 10µl of each metal + 10µl of tuber extract) for protease K inhibition. The $CaCl_2$ shown slight inhibition, $FeSO_4$ and $HgCl_2$ shown strong inhibition means they are acting as strong activators to *Globba bulbifera* tuber extract, but $(CH_3COO)_2Pb$ not shown any inhibition with *Globba bulbifera* tuber extract and remaining metals not shown any inhibition with enzyme protease k.

The control (10µl of trypsin+10µl of phosphate buffer) has been used with enzyme trypsin and phosphate buffer. There is no inhibition observed in control. Different metals have been tested with enzyme trypsin. Strong inhibition is shown by $FeSO_4$ and $(CH_3COO)_2Pb$ only, but remaining metals has not shown inhibition. The test sample has been experimented (10µl of trypsin+10µl of each metal + 10µl of tuber extract) for trypsin inhibition. Except for $FeSO_4$ and $(CH_3COO)_2Pb$ remaining all metals acting as activators to the *Globba bulbifera* tuber extract and shown strong inhibition with trypsin **Fig. 1**.

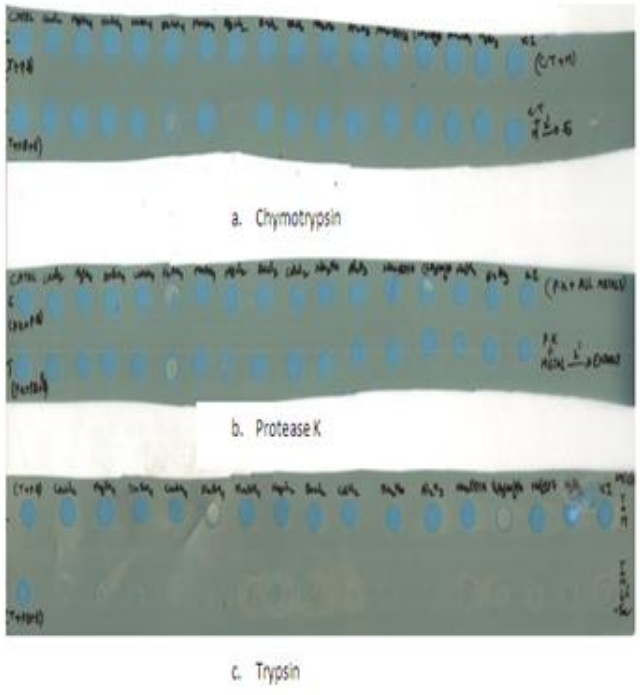

FIG. 1: PROTEIN INHIBITION ASSAY

TABLE 1: DOSE-RESPONSE OF ETHANOLIC EXTRACT OF *GLOBBA BULBIFERA* ON MCF-7 (BREAST CANCER) CELL LINE

Conc Microgm/ml)	OD of Tamoxifen at 450 nm	% of Cell survival for Tamoxifen	% of Cell inhibition for Tamoxifen	OD of extract at 450nm	% of cell survival for extract	% of cell inhibition for extract
12.5	0.468	89.3	10.7	0.452	94.3	5.7
50	0.201	33.6	66.4	0.380	77.8	22.2
100	0.126	17.9	82.1	0.323	64.8	35.2
200	0.110	14.6	85.4	0.280	54.9	45.1
250	0.105	12.7	87.3	0.245	47.8	52.2

The OD of the blank is 0.040 and control is 0.447. **Table 1** shows the %cell survival and %cell inhibition for the standard (tamoxifen) and the plant extract. There is a gradual decrease in the OD values that shown good inhibition results. **Fig. 2** shows the IC_{50} Standard at 37 µg/ml and the IC_{50} Plant extract at 235 µg/ml.

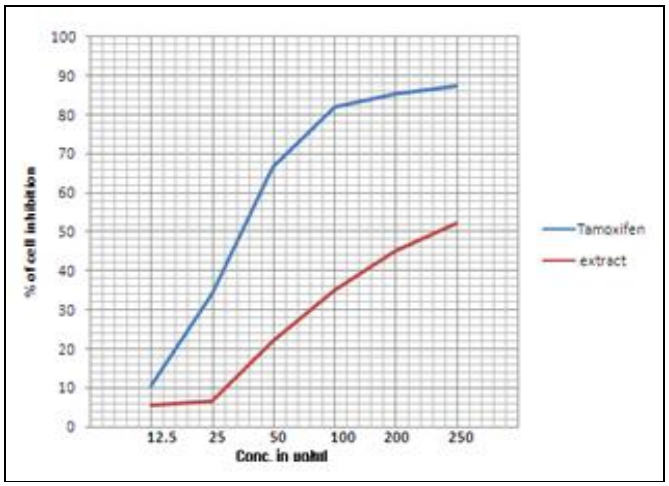

FIG. 2: ANTICANCER ACTIVITY OF ETHANOLIC EXTRACT OF *GLOBBA BULBIFERA*

DISCUSSION: *Globba bulbifera* belongs to the Zingiberaceae family. Most of the medicinal plants are widely used for the treatment of several diseases in India and China [18]. The usage of plants like turmeric may reduce the risk of several kinds of cancers and provide other protective biological effects in humans. These biological effects are due to constituent curcumin that widely studied for its wound healing, anti-oxidant, anti-inflammatory and anti-cancer effects [19].

The no steroidal anti-inflammatory drugs (NSAIDs), including aspirin, have clinically significant in anti-carcinogenic effects in the gastrointestinal tract. The epidemiological data indicate that use of these drugs is associated with the risk of sporadic colorectal cancer, and clinical trials of patients with familial polyposis coli show that NSAIDs can lead to the regression of large bowel adenomas [20].

The response of the body to cancer is not a unique process but has many parallels with inflammation and wound healing. The inflammatory cells and cytokines found in tumors are more contribute to tumor growth, progression, and immune suppression than they are to mount an effective host antitumor response. The cancer susceptibility to severity may be associated with functional polymorphisms of inflammatory cytokine genes, deletion or inhibition of inflammatory cytokines inhibits the development of cancer [21].

Pigeon-pea seed extracts have already been analyzed for the protease inhibitors using x-ray film protease inhibitor method [22]. Seed extracts of *Cajanus cajan* were analyzed by Pichare & Kachole, 1996, for protease inhibitor activities using caseinolytic assay and are determined by PAGE. The relative amounts of different trypsin inhibitors and the total trypsin inhibitor activity varied with different mining media. The trypsin inhibitors were not detectable in pigeon pea leaves. The profiles of trypsin and chymotrypsin inhibitors in almost all the cultivars of pigeon pea analyzed were similar those in wild relatives were quite variable [23]. The evaluate has been conducted in various plants and animals like *Helicoverpa armigera* [24] *Periplaneta americana* [25] *Moringa oleifera* [26].

The *Globba bulbifera* is a one of the medicinal plants which is used for the various disorders. The various inhibitors inhibit the proteolytic enzymes, but the present studies indicate that proteolytic enzymes are inhibited by the ethanolic extract of *Globba bulbifera* along with various metals. The enzyme trypsin showed strong inhibition with various metals along with *Globba bulbifera* ethanolic extract. The protease K and chymotrypsin showed slight inhibition with ethanolic extract of *Globba bulbifera*. This study also explains anti-cancer activity by ethanolic extract of *Globba bulbifera* but shown less activity with standard tamoxifen.

CONCLUSION: *Globba bulbifera* ethanolic tuber extract has shown good activity for protease inhibition and anti-cancer activity. Hence the tuber extract shows protease inhibition and anti-cancer components.

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CONFLICT OF INTEREST: Nil

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