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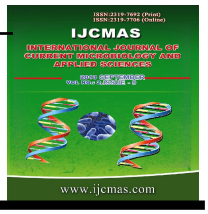


**Original Research Article**

# Evaluation of Anti-Arthritic, Antimicrobial and Amylase activities of *Codium tomentosum* from Andaman and Nicobar islands

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**Keywords**

C57/ Black mice; ELISA; well diffusion method; SDS-PAGE; Dermatophytes

**A B S T R A C T**

*Codium tomentosum*, a marine alga belongs to the family Codiaceae and order Bryopsidales, was collected at a depth of five meters from Pongi Balu area of Andaman Islands. Earlier, blood anticoagulant sulphated polysaccharides and genotoxicity/ antigenotoxicity, anti-oxidative capacities of *C. tomentosum* have been reported. The present investigation was carried out to determine the anti-arthritic, anti-bacterial, anti-fungal and amylase activities of *C. tomentosum*. The anti-arthritic activity was determined in C57/Black mice which were earlier elicited with the disease arthritis, treated using the ethanolic extract of the seaweed and anti-collagen antibody levels were estimated by ELISA. The anti-bacterial and anti-fungal activities were determined using the organic solvent extracts of *C. tomentosum*. The antibacterial activity was tested against both gram-positive and gram-negative bacteria namely *Streptococcus* species, *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Proteus vulgaris.* Moreover the antifungal activity was determined against the clinically important dermatophytes, *Trichophyton mentagrophytes, Trichophyton rubrum and Aspergillus niger, Aspergillus flavus and Candida albicans*. The amylase activity was determined using the aqueous extract. The *C. tomentosum* significantly decreased the antibody response in arthritic mice. In addition, the extracts of *C. tomentosum* were effective against both bacteria and fungi. The amylase activity was found to be stable at a temperature of 60°C and pH 8. This is the first report on anti-arthritic, antimicrobial and amylase activities of *C. tomentosum*. It showed potent inhibition against clinically important dermatophytes.

## Introduction

Marine algae are a source of chemically diversified compounds which have pharmacological importance. Seaweeds have been one of the richest and most promising sources of bioactive primary and secondary metabolites (Faulkner, (2002) and their discovery has significantly expanded in the past three decades (Smit, 2004).

The algae synthesize a variety of compounds such as carotenoids, terpenoids and antioxidants such as polyphenols, alkaloids, halogenated

compounds and polysaccharides such as agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan, galactosyl glycerol and fucoidan (D'Ayala *et al*., 2008; Guven *et al*., 2010; Cabrita *et al*., 2010). It has been realized that the many of these metabolites being biologically active are of biomedical importance and could be used as potential drugs.

Marine macro algae are important ecologically and commercially to many regions of the world[2]. They are a valuable food resource which contains low calories and they are rich in vitamins, minerals, proteins, polysaccharides, steroids and dietary fibers (Darcy, 1993). Seaweeds are harvested or cultivated for the extraction of alginate, agar, carrageenan, chitin and hydrocolloids, which are employed in many biomedical and pharmaceutical applications like cell immobilization, drug delivery, bio adhesives and wound healing (D'Ayala *et al*., 2008). Hydrocolloids have attained commercial significance as food additives because of their gelling, emulsifying properties (Kadam and Prabhasankar, 2010). Seaweeds are also used as a source of biogas (Alberto *et al*., 2008).

These compounds probably have diverse simultaneous functions for the seaweeds and can act as antifouling and herbivore deterrents, or as ultraviolet-screening agents (Praba *et al*., 1997). Since as early as 3000 BC, they were also considered important as traditional remedies (Smit, 2004). The seaweeds are also used by the pharmaceutical industry in drug development to treat diseases as they possess various activities like antitumour (Hong *et al*., 2008), antibiotic (Premila *et al*., 1996), anticoagulant (Shanmugam *et al*., 2001), antiobesity (Hayato *et al*., 2005), anti-inflammatory and antiosteoarthritic (Hyeon *et al*., 2006), antioxidant and antiproliferative (Yvonne and Natalie, 2006), antiprotozoal, antidiabetic and other pharmacological activities (Alejandro and Mark, 2005). Currently, algae represent about 9% of biomedical compounds obtained from the sea (Jha and Zi-rong, 2004).

Seaweed extract is used in some diet pills (Hayato *et al*., 2005). Microbiologists and pharmacologists are having increased attention during the recent years towards marine algae to obtain more bioactive compounds in addition to the existing. The revolutionized therapy of infectious disease by the use of antibiotics has certain limitations due to changing patterns of resistance and side effects together with the demand for improved pharmacokinetic properties which necessitates continued research for the isolation and characterization of antibiotics for the development of drug. There is a need for discovery of new, more effective and less toxic antibiotics for the treatment of fungal and bacterial infections. Similarly, suitable antibiotics are not available in the fields of non-medical areas like plant diseases and as food preservatives. In the present study, the extract of *Codium tomentosum* was evaluated for its anti-arthritic, antimicrobial and amylase activities.

## Materials and Methods

### Collection of Samples

Marine sample collection was done in accordance with Ministry of Environment and Forests, Zoological Survey of India, Andaman and Nicobar Islands. Two field stations, Station-I (Pongi Balu), 11°30′0″–11°34′0″N latitude and 92°38′0″–92°40′0″E longitude and Station-II (Chidiya Tapu), Bay of Bengal, 92° to 94°

East. Longitude 6° to 14° North Latitude, 25km from port Blair within the coastal zone of Andaman and Nicobar were sampled during January 2010. Samples were collected by SCUBA diving, from a depth of 8 – 10 m from the coastal waters or with a gravity corer (66 cm length and 7 cm diameter.) from these two locations. The samples were extruded into alcohol sterilized clean plastic containers.

**Preparation of extract**

The samples were cleaned of epiphytes and necrotic parts were removed. Then the samples were rinsed with sterile water to remove any associated debris. Half of these cleaned fresh materials were air-dried. 3 g of fresh and air-dried sample was extracted in 100 ml of chloroform, diethyl ether, and ethanol individually and 10 g in phosphate buffer and kept for two weeks. The above obtained organic solvent extract samples were concentrated using Rotavapor.

**Anti-arthritic activity**

**Animals**

Male C57/ Black mice (6-8 weeks old) weighing around 30 g were procured from the experimental animal facility from the National Institute of Laboratory Animal Sciences, National Institute of Nutrition , Hyderabad, India. The experimental protocol was approved by the Institutional ethics committee, Andhra University, Visakhapatnam, Andhra Pradesh, India.

**Chemicals and materials**

Chicken type II collagen, Freund's complete adjuvant (CFA), Incomplete Freund's adjuvant (IFA), goat anti-mouse IgG, Bovine serum albumin (BSA), Tetracycline and Streptomycin were obtained from Sigma Chemical Company. O-phenylene diamine (OPD) and a 96-well microtitre flat bottomed ELISA culture plates were procured from local suppliers.

**Preparation of emulsified formulation of type II collagen**

Type II collagen (4 mg) was solublized in 1 ml of 0.05 M acetic acid. Equal volumes of collagen solution were emulsified with CFA and IFA (Trentham et al., 1977).

**Development and evaluation of arthritis in mice**

On day 0, the mice (five mice per group) were immunized with 0.1 ml of collagen (emulsified in CFA) by intradermal injection at the base of the tail. On day 21, mice were given a booster dose of collagen (emulsified in IFA) through the same route. The ethanolic extract of *Codium tomentosum* at a dose of 33mg/kg body weight) dissolved in olive oil was given intraperitonially two times a week for 4 weeks, starting from day 1 to day 42. Control mice received olive oil alone.

**Determination of anti- type II collagen antibodies by ELISA**

Sera were collected from tail vein by tail cut method from arthritic mice on the 3$^{rd}$, 5$^{th}$ and 7$^{th}$ weeks after primary immunization. Sera samples were diluted with saline. The microtitre plate was coated with 100 µl type II collagen and incubated overnight at 4 °C and then blocked with 100 µl of 2% BSA in PBS for 90 mins at room temperature. The plate was washed twice with wash buffer (PBS, pH 7.5 and 0.05 Tween 20). 100 µl of dilute sera were added to each well and incubated for 45mins at room temperature. The plate was washed again twice with

wash buffer. 100 µl of peroxidise labeled rabbit anti-mouse IgG was added to each well and incubated for 90mins at room temperature. The reaction was stopped by adding $H_2SO_4$ and the plate was read at 493nm(Peng *et al*., 1995). *Codium tomentosum* significantly suppressed the anti-collagen antibody response in collagen induced arthritic mice (C57/Black).

**Anti-microbial activity**

The antibacterial and antifungal activity was determined by the well diffusion method. Nutrient agar plates in case of bacteria and sabouraud dextrose agar media in case of fungi were inoculated by spreading the respective cultures over the entire sterile agar surface. This procedure was repeated by spreading two more times, rotating the plate approximately 60° each time to ensure even distribution of the inoculums and a sterile borer is used to prepare wells in the media .

Each of the 3 wells of a plate is filled with 25 µl crude supernatant, 25 µl of standard antibiotics like Tetracycline and Erythromycin (100 µg/ml) for antibacterial activity and Fluconazole and Clotrimazole (250 µg/ml) for antifungal activity. The plates were inoculated and wells were filled with crude sample as well as standard antibiotics and were placed in refrigerator for 1hr so that the antibiotics diffuse into the media. Later, they were placed in the incubator at 28±2 °C for 3-5 days. After the incubation period plates were observed for the inhibition zone diameter (IZD) measured to the nearest millimeter.

**Amylase Activity**

Amylase was assayed according to the procedure of Bernfeld (1995). Gelatinized soluble starch, (1%) in phosphate buffer saline, pH 6.8 incubated with aliquots of enzyme appropriately diluted .The reaction was stopped by the addition of DNS reagent. One unit of enzyme activity was defined as micro (µ) mol maltose equivalent released/ minute under the assay conditions. The specific activity was expressed as activity units/mg protein.

**Estimation of protein**

Protein content was determined according to Lowry method using bovine serum albumin (BSA) as standard.

**Purification of α-Amylase from *Codium tomentosum***

**Preparation of crude extract**

The amylase crude extract was prepared by homogenization of 10 g *Codium tomentosum* with 0.1 M phosphate buffer saline (pH 6.8, 100ml) using mortar and pestle. The homogenate was centrifuged using cold centrifuge at 6500 rpm for 20 minutes to remove coarse particles. The supernatant was designated as the crude extract.

**Ammonium sulphate fractionation**

To the crude extract (80 ml) solid ammonium sulphate (31.2 g) was added gradually with constant stirring at 4 °C to obtain 60% saturation. The mixture was allowed to stand overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 15 minutes at 4 °C. Then dissolved in 20 ml of 0.1 M PBS buffer pH 6.8 and dialyzed against the same diluted buffer. A small amount of precipitate formed during dialysis was removed by centrifugation.

**Removal of contaminants by dialysis**

Dialysis is commonly used for removing salt from the protein. The presence of salt in the protein interferes in many ways. The protein solution to be desalted was taken inside a dialysis bag and the two ends are secured tightly to prevent leakage. The bag was then suspended in a large vessel containing about 100 fold of water of preferable diluted buffer and the contents were stirred. Salt molecules pass freely and get eluted by large volumes of fluid in the external medium. Repeated changes of the dialysis fluid help in reducing the salt concentration inside the bag to negligible level. Water being freely permeable would have entered the bag and diluted the proteins also. There are several methods of concentrating.

**Ion exchange chromatography on DEAE cellulose**

The dialyzed sample (20 ml) was loaded on to a DEAE cellulose column (2×30 cm) previously equilibrated with 0.1 M sodium phosphate buffer pH 7.6. The unbound proteins were eluted with 0.1-0.3M NaCl in 0.1M phosphate buffer pH 7.6. Fractions of 5 ml were collected at a flow rate of 60ml per hour. These fractions were assayed for protein by measuring their absorbance at 280 nm using a spectrophotometer as well as enzyme activity using starch as substrate. The fraction containing enzyme activity was pooled dialyzed against distilled water and lyophilized.

**Gel filtration on Sephadex G 100**

The lyophilized sample was dissolved in 0.1 M phosphate buffer pH 7.6 and loaded on Sephadex G -100 column (1.8×60 cm) the column was previously equilibrated with 0.1M Phosphate buffer pH 7.6 and eluted with the same buffer. 2 ml fractions were collected at a flow rate of 12 ml per hour and protein was monitored by measuring the absorbance at 280 nm. The enzyme activity was assayed.

**Molecular weight determination: SDS PAGE**

SDS polyacrylamide gel electrophoresis was carried out in 12% slab gels using markers in the molecular mass range from 97.4 to 14.3 kDa. After staining and destaining a calibration curve was constructed by plotting the distance migrated by the standard protein against their log molecular weights. The molecular weight of amylase was read from the calibration curve.

**Determination of Kinetic Constants**

To determine the effect of substrate concentration on enzyme activity, substrate concentrations ranging from 0.2 to 2.0% were used. Kinetic constants, $K_m$ were calculated from Lineweaver-Burk plot.

**Effect of temperature on amylase activity**

To study the effect of heat on amylase activity 1ml aliquots of enzyme extract in Phosphate buffer saline pH 6.8 were exposed to different temp (25-100 °C) in a water bath for 15 min. After cooling on ice, the enzyme activity was determined by the standard assay using the above heat treated extracts.

**Effect of pH on amylase activity**

1 ml of samples of enzyme extract in phosphate buffer saline pH 6.8 was

separately incubated with 2 ml of appropriate buffer for 24 hrs at 4 °C. Aliquots were diluted with phosphate buffer and used for enzyme activity. Each experiment was done at least three times and the results of typical experiments were presented.

## Results and Discussion

*Codium tomentosum* at 33 mg/kg body weight significantly decreased the anti-collagen antibody response in collagen induced arthritic model. As shown in Figure 1, ethanolic extract of *Codium tomentosum* suppressed the antibody response in sera samples collected at 3, 5, 7 week intervals when compared to Indomethacin, a standard anti-inflammatory drug.

Antibacterial activity was determined against gram positive and gram negative bacteria using ethanolic, chloroform and diethyl ether extracts of *Codium tomentosum*. Chloroform extract was effective against *Streptococcus* sps, whereas the diethyl ether extract was active against *Bacillus subtilis* and *Proteus vulgaris.* (Figure 2) Antifungal activity of the diethyl ether extract was prominent against *Trichophyton mentagrophytes, Candida albicans, Aspergillus niger* whereas the chloroform extract was effective against *Aspergillus flavus i*n comparison with the standard drugs. (Figure 3). α-amylase is present in trace amounts in *Codium tomentosum*.

α-amylase crude enzyme extract was subjected to ammonium sulphate 30-60% saturation, dialyzed and fractionated on to a DEAE-cellulose column. Bound proteins have been eluted with 0.2 M sodium chloride with a recovery of 39.4% and a purification of 9.87-fold (Table-1). The overall yield of amylase activity was 34.4%. The homogeneity of the preparation has been checked by SDS-PAGE (Figure 7).

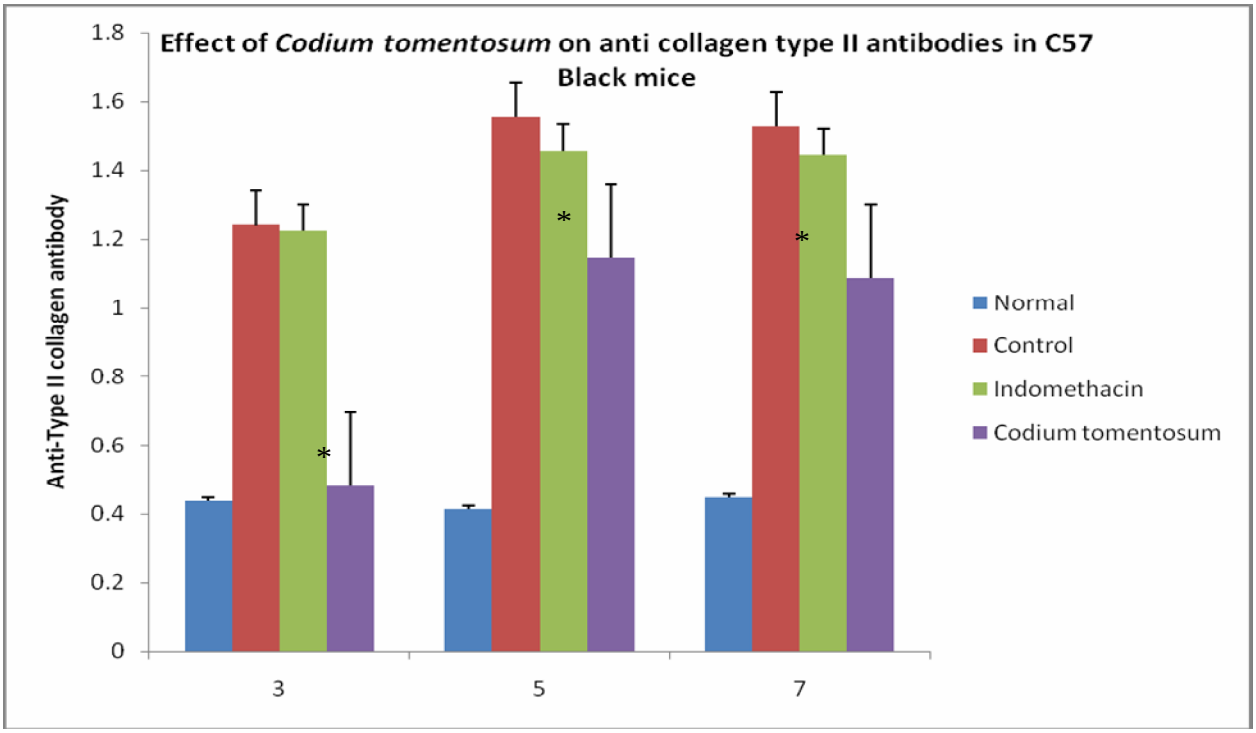
The enzyme appears to move as a single band, indicating the absence of hetero-subunits. The molecular mass of the protein was determined to be 48 kDa by SDS-PAGE. The apparent Michaelis constant ($K_m$) has found to be 0.0016g/ml.The enzyme was active over a wide pH range with a pH optimum of 8.0 (Figure 5). Purified amylase was found to have a temperature optimum of $60^0$C (Figure 6).

Trentham *et al.,* (1977) induced arthritis in animal model by injecting type II collagen as an experimental model that is similar to human rheumatoid arthritis in several of its clinical and immunological manifestations. In the present study, ethanolic extract of *Codium tomentosum* (33.3 mg/kg body weight) could suppress the total anti collagen IgG responses significantly as determined by ELISA (Poosarla *et al*., 2007). Interactions between cytokines and sulphated polysaccharides from *Codium fragile* were also reported. The degree of antibiotic property depends upon the suitable solvents used for extraction, condition of the sample and season in which the algae was collected .There are several factors such as age of the plant, duration of

**Table.**1 Summary of purification of α-amylase from *Codium tomentosum*

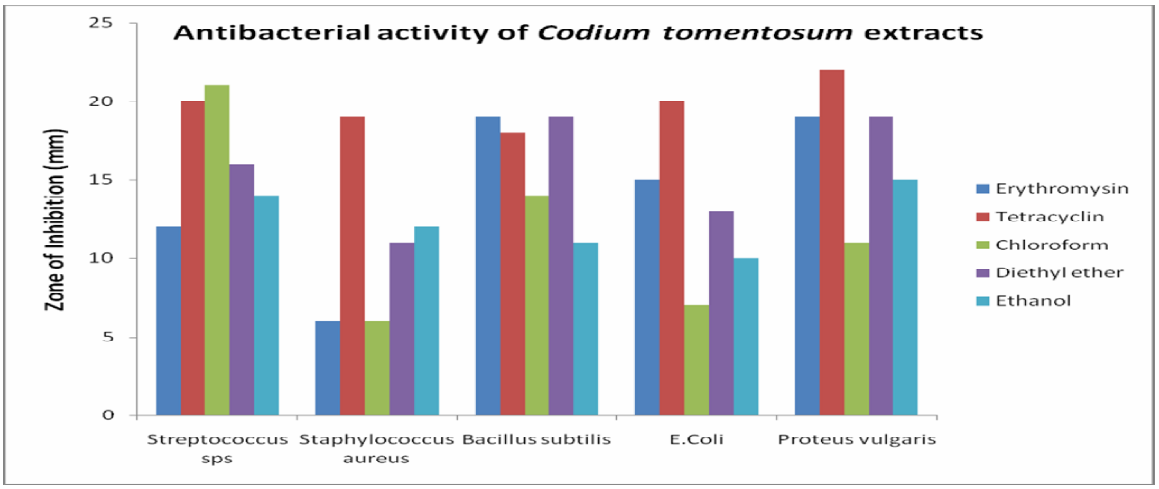
| Step | Volume (ml) | Total activity (U)×$10^3$ | Total Protein (mg) | Specific activity (U/mg)×$10^3$ | Fold purification | Percentage recovery |
|---|---|---|---|---|---|---|
| Crude | 80 | 638.8 | 4018 | 0.159 | 1 | 100 |
| Ammonium Sulphate (65%) | 30 | 355.5 | 1150 | 0.309 | 1.94 | 55.6 |
| DEAE-Cellulose | 20 | 252.1 | 160.5 | 1.57 | 9.87 | 39.4 |
| SephadexG-150 | 15 | 220.5 | 115.5 | 1.91 | 12.0 | 34.4 |

**Figure.1** Anti-arthritic activity of *Codium tomentosum*



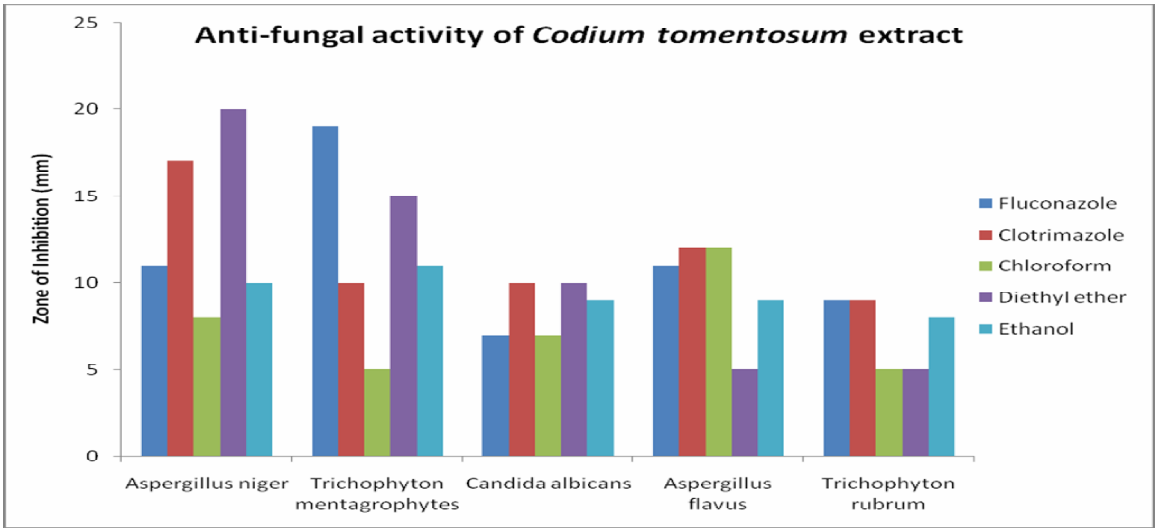

Three groups of C57/Black mice (five mice per each group) were immunized with 0.1 ml of bovine type II collagen at the base of the tail. Booster dose was given 3 weeks after primary immunization. One group was treated with 3 mg/kg body weight of Indomethacin, another group was treated with *Codium tomentosum* 33 mg/kg body weight and control group was treated with vehicle alone. Sera were collected at 3, 5, 7 week intervals after primary immunization for the IgG sub classes by ELISA. *$p<0.05$ was considered as significant.

**Figure.2** Anti-bacterial activity of *Codium tomentosum*




The different extracts of *Codium tomentosum* namely the ethanolic, chloroform and diethyl ether extracts were subjected to both gram positive bacteria *Streptococcus sps*, *Staphylococcus aureus* and *Bacillus subtilis* and gram negative bacteria like *Escherichia coli* and *Proteus vulgaris* and its anti-bacterial activity was observed after 48 hrs of incubation. The activity of the chloroform extract was effective more against *Proteus vulgaris* and *Bacillus subtilis*.

**Figure.3** Anti-fungal activity of *Codium tomentosum*




The different extracts of *Codium tomentosum* namely the ethanolic, chloroform and diethyl ether extracts were subjected to the fungi, namely *Aspergillus niger*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Aspergillus flavus* and *Candida albicans*. The diethyl ether extract activity was highest against *Aspergillus niger* followed by *Trichophyton mentagrophytes* having a zonal inhibition diameter of 20 mm and 15 mm.

**Figure.4** Effect of temperature on Amylase activity

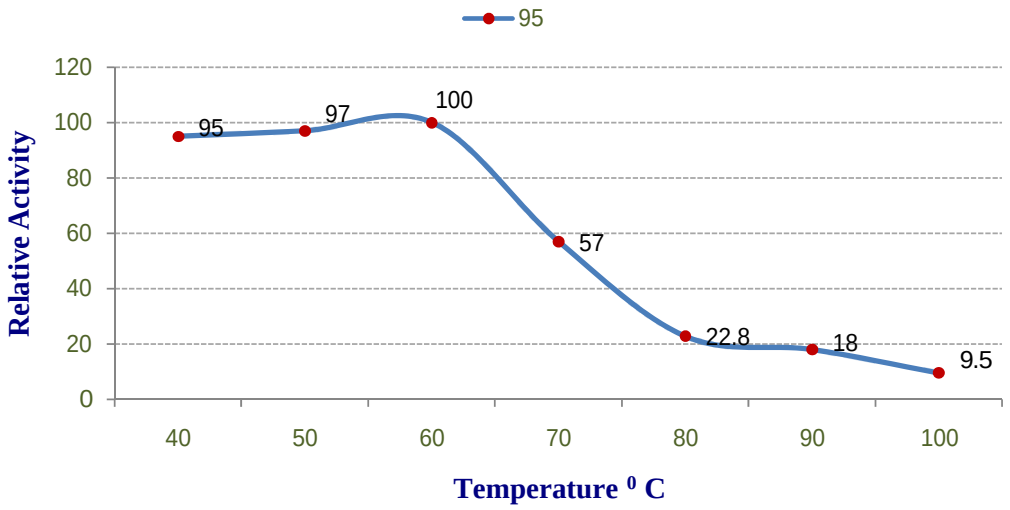



**Figure.5** Effect of pH on Amylase activity

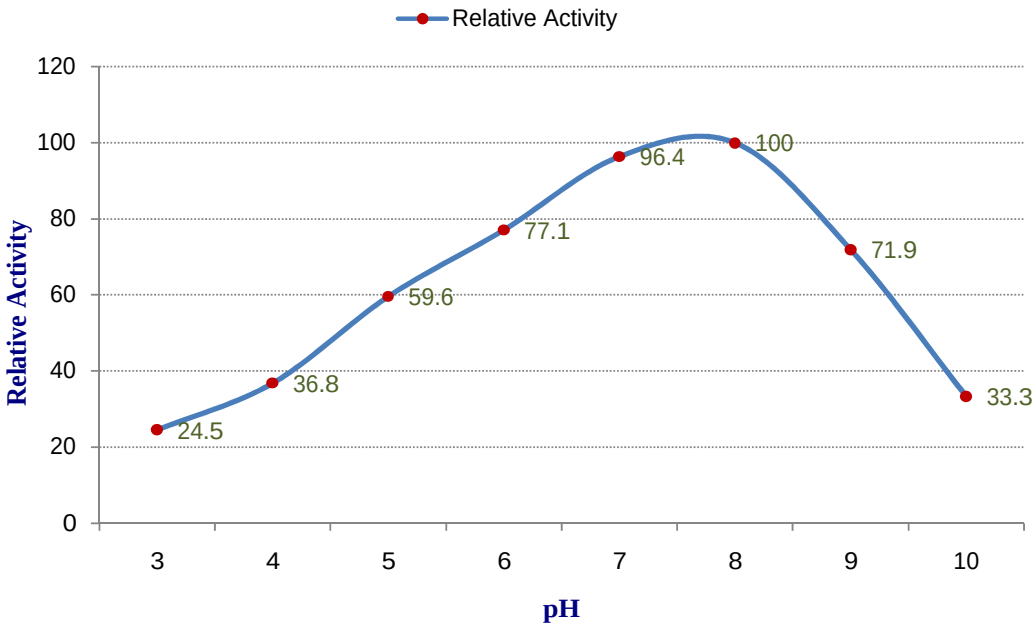
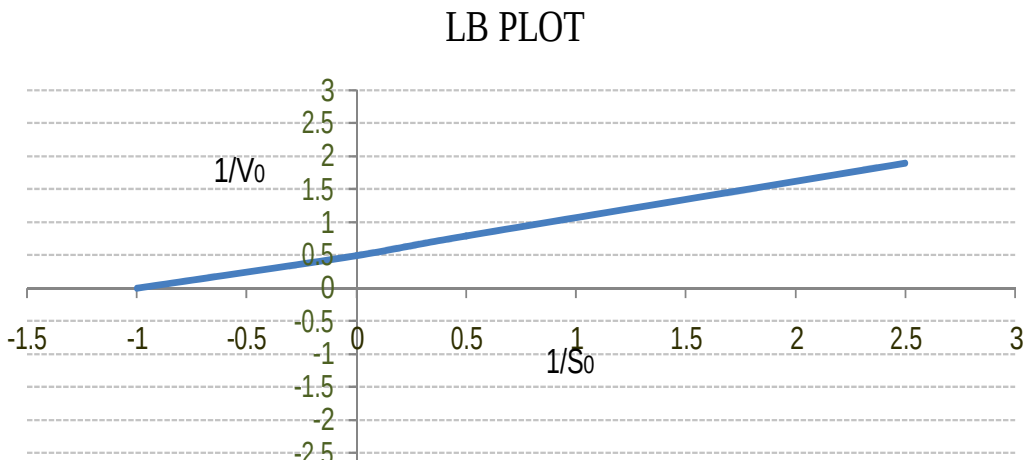
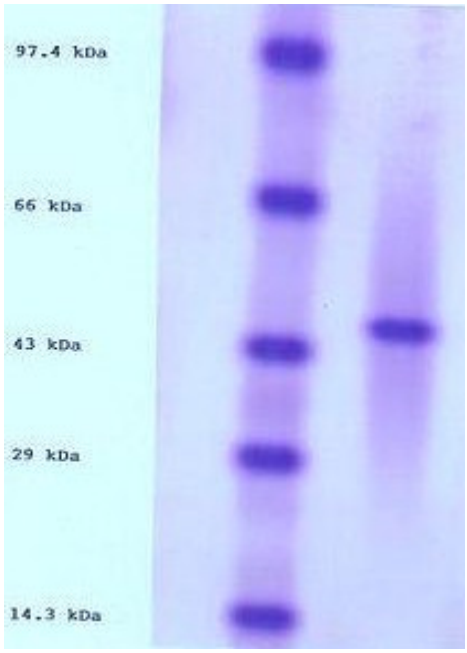


**Figure.6** Effect of substrate concentration on amylase activity of *Codium tomentosum*




SDS PAGE was carried out in 12% slab gel using markers, Phosphorylase b (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Lysozyme (14.3 kDa), Purified amylase (48 kDa). A calibration curve was constructed by plotting the distance migrated by the standard protein against log molecular weights.

**Figure.7** SDS PAGE on 12% slab gel




The molecular weight of amylase was storage, temperature, preparation of the media and pH which sometimes indirectly effect the activity (Nika *et al*., 2003). Martinez-Nadal *et al*., (1966) mentioned that benzene and diethyl ether were suitable solvents for extracting on antibiotic principle. Our present investigation different from the earlier investigation, here chloroform, diethyl ether and ethanol were used simultaneously to check the activity.

The diethyl ether extract of the naturally available seaweed were maximally effective against both gram positive and gram negative bacteria like *Bacillus subtilis* and *Proteus vulgaris* known to be the pronounced human pathogens. The extract of *Codium tomentosum* was found very effective almost competing with the standard drugs taken, which were fluconazole and clotrimazole, the ethanolic extract of the above mentioned seaweed was maximally effective against *Trichophyton mentagrophytes* and the diethyl ether extract was maximally effective against *Trichophyton rubrum*. *Candida albicans*, a diploid fungus and a causal agent of opportunistic oral and

genital infections in humans. The diethyl ether extract of the *Codium tomentosum* was found to be effective against *Candida albicans* showing a competitive potency than the standard drugs. Chiheb *et al*., (2009) reported the antibacterial activity of *Codium dichotomum*.

In the present study, α-amylase have been isolated and purified to apparent homogeneity by ammonium sulphate fractionation, DEAE-Cellulose Ion exchange chromatography and Gel filtration from the *Codium tomentosum*. The molecular weight has been found to be 48 kda by SDS-PAGE. The Michaelis constant ($K_m$) using starch as substrate was $1\times10^{-3}$g/ml.

Analysis on the influence of various physico-chemical parameters on the purified enzyme revealed the optimum temperature and pH to be 60 °C and 8.0 respectively which is relatively stable than the salivary amylase hence can be useful industrially and thereby is economically beneficial. Fibrinolytic and nitrogenase enzyme activities were reported earlier from *Codium* species (Kiminori *et al.*, 2002). In the present study, the antiarthritic, antimicrobial and amylase activities of *Codium tomentosum* were evaluated for the first time and significant results were observed. However, further studies are required to identify the specific compounds responsible for activity.

## Statistical Analysis

The values are expressed as Mean±SEM. The results were calculated statistically using Graph Pad Prism 5.0 software. Two-way ANOVA was performed between groups. $P<0.05$ was considered as significant.

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