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ISOLATION, CHARACTERISATION AND EVALUATION OF ANTIOXIDANT AND *IN-VITRO* ANTICANCER ACTIVITY OF CHITIN AND CHITOSAN FROM CARAPACE OF CULTURED *PENAEUS MONODON SPECIES*

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ABSTRACT

Exploration of the sea and its organisms is still at a relatively early stage. Although the oceans contain much greater biodiversity, identifying of new biological activities have hardly begun. Chitin and Chitosan are proteinaceous substances that can be isolated from the marine sources like crustaceans. In the present study Chitin & Chitosan have been isolated from the carapace of the cultured *Penaeus monodon species*. Chitin is a non-toxic, biodegradable polymer of high molecular weight and a modified polysaccharide that contains nitrogen. Chitosan is deacetylated chitin, a hydrophillic biopolymer, naturally occurring, non-toxic, biodegradable polysaccharide. The goal of the present study is to isolate Chitin and Chitosan from the eco-waste of the cultured species of *Penaeus monodon* and to further study its antioxidant and *in-vitro* anticancer activity. Chitin and Chitosan are used as nutritional supplements in the market. Hence this study identifies opportunities to develop value added products from crustacean processing by-products with different biological activity.

OBJECTIVE

To isolate chitin and chitosan from the carapace of cultured *Penaeus monodon species* and to further evaluate it for the antioxidant activity by DPPH scavenging activity, Nitric Oxide Scavenging activity & Reducing Power activity and the *in-vitro* anticancer activity on MDA cancer cell lines (human breast cancer cell lines) by MTT assay and DAPI.

Experimental work

Isolation

The carapace of cultured *Penaeus monodon species* was taken, dried at 50°C and powdered. 150gm of powder was washed with water, filtered and to the residue 4%HCl was added and kept for 6 hrs for decalcification, filtered and 3%NaOH was added and kept over night and filtered. To the above residue 0.5% $KMnO_4$ and 0.5% oxalic acid were added separately for decolourisation and filtered. To the decolourised residue 40% NaOH was added and kept in hot air oven for 4hrs, filtered and neutralised by washing with distilled water. The obtained sample is called Chitin. The chitin obtained was mixed with 5%NaOH, kept in hot air oven at 90-95°C for 2hrs, alkali is drained washed with distilled water and dried at 30±2°C.[1]

Characterisation: Chitin and Chitosan were characterised by IR spectroscopy. The peaks for chitin and chitosan were found at 1578.45cm^{-1} and 1603.52cm^{-1}. When chitin deacetylation occurs, the band observed at 1603.23cm^{-1} decreases, while a growth at 1578.45cm^{-1}occurs, indicating the prevalence of NH_2 groups.[6]

DPPH Scavenging activity: A stock solution of 3.9mg DPPH(1,1-diphenyl-2-picryl hydrazine) was prepared in 100ml of ethanol. To 1ml of chitin and chitosan of different concentrations (50,100,200,400,600,800,1000µg/ml) in DMF (dimethyl formamide) 1ml of DPPH in ethanol was added. Control without test compound was prepared and ethanol was used as blank. The reaction was allowed to complete by keeping in dark for 20mins and absorbance was read at 517nm. Ascorbic acid was used as standard.[2]

%DPPH inhibition =

$$\frac{\text{O.D of control} - \text{O.D of test}}{\text{O.D of control}}$$

Nitric Oxide Scavenging activity: The nitric oxide scavenging activity of Chitin and Chitosan was determined according to the method (Green et al., 1982). Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrate ions which was measured colorimetrically. 3ml of reaction mixture containing 2ml of sodium nitroprusside, (10mM) in phosphate buffered saline (PBS) and 1ml of various concentrations of the chitin and chitosan were incubated at 37^0C for 4 hours. Control without test compound was kept in an identical manner. After incubation 0.5mL of Griess reagent was added. The absorbance of the chromophore formed was read at 546nm. The percentage inhibition of nitric oxide generation was measured by

comparing the absorbance values of control and those of test compounds. Ascorbic was used as standard. The results were tabulated.

% Inhibition was calculated using the following formula.

$$\% \text{ Inhibition} = \frac{\text{Control-Test}}{\text{Control}} \times 100.$$

Determination of Reducing Power Activity: The measurement of reductive ability was determined by the Fe^{+3} -Fe^{+2} transformation in the presence of the samples such as chitin , chitosan and ascorbic acid at different doses (50, 100, 200, 400, 600, 800, 1000µg/ml). The different concentrations of chitin, chitosan and ascorbic acid were taken in a test tube and to it, 2.5ml of phosphate buffer pH 6.6 and 2.5ml of 1% potassium ferricyanide were added and incubated at 50°C for 20 minutes. To the incubated sample 2.5ml of 10% trichloroaceticacid (TCA) was added and centrifuged for 10 minutes at 3000r.p.m. After centrifugation 2.5ml of the supernatant was diluted with 2.5ml of water and shaken with 0.5ml freshly prepared 0.1% ferric chloride. The absorbance was measured at 614nm.The control solution was prepared without the test sample. The increase in absorbance indicated higher reductive ability.[3]

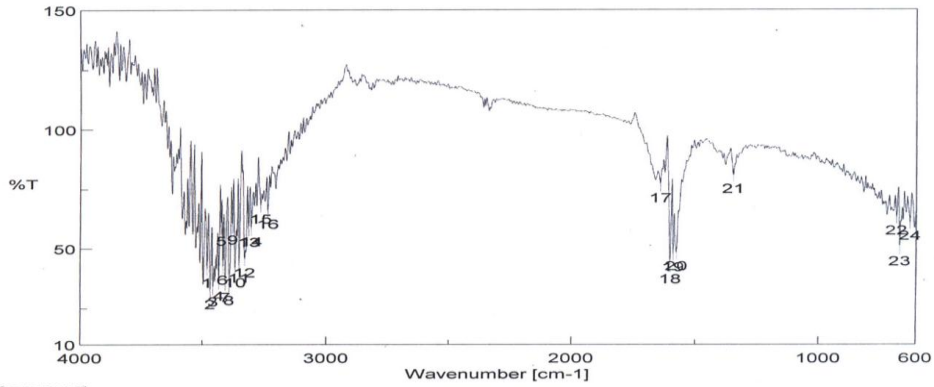
***IN-VITRO*ANTICANCER ACTIVITY AGAINST MDA CANCER CELL LINE**

MTT Assay: MDA cells (human breast cancer cell lines) were seeded in 96 well plates at a density of 8000 cells/200µL/well and treated with different concentrations (1ng-1×10^{6}ng/mL) of chitin and chitosan in 0.5% DMSO after 24hrs following plating and incubated at 37°C for 1 day. After 20hrs of chitin and chitosan exposure, the cells were incubated at 37°c with 5mg/ml MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) for 4hrs, and at the end medium was removed and insoluble formazan product was dissolved in DMSO(200µL) and kept in dark for 15mins. MTT reduction was quantified at 570nm & 630nm in Multiskan spectrophotometer, USA.[5]

DAPI: Apoptotic morphological changes were evaluated by DAPI (4',6'-diamidino-2-phenylindole) staining. Preconfluent MDA cells were seeded in 12-well plates at a density of 25,000 cells/ml/well. Cells were treated with different concentrations of test drug after 24h following plating and incubated at 37^{0}C for 1h. After incubation, cells were gently washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15minutes. The plates were washed twice with PBS and incubated for 30min with 1µg DAPI at 37°C. Finally, the cells were washed three times with PBS and examined by fluorescence microscopy.

RESULTS

IR spectrum of chitin



[Comment]
Sample Name
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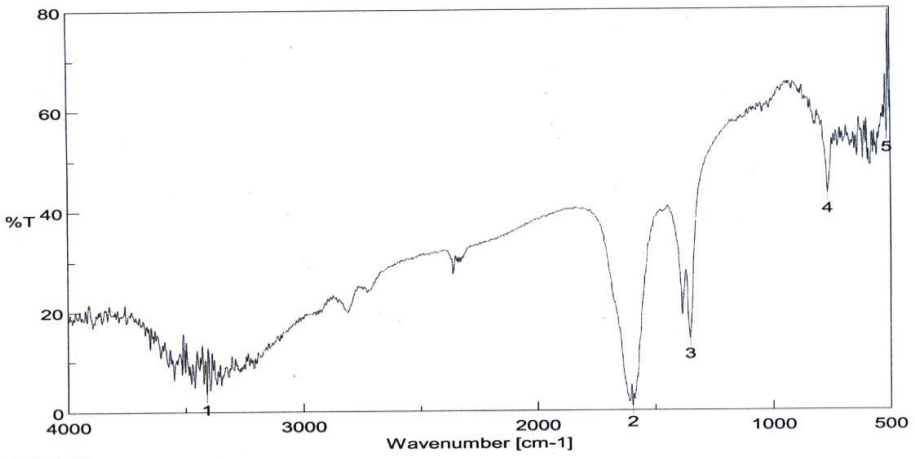
User
Division
Company SRI RAMACHANDRA MEDICAL UNIVERSITY

[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity
1	3483.78	41.1064	2	3469.31	32.1855
3	3458.71	33.2416	4	3435.56	35.6601
5	3426.89	58.7368	6	3420.14	42.3302
7	3409.53	35.197	8	3394.1	33.8532
9	3380.6	59.2441	10	3370	41.2531
11	3353.6	43.1485	12	3332.39	45.3705
13	3315.03	58.2211	14	3305.39	58.5113
15	3266.82	68.1134	16	3237.9	65.9231
17	1644.02	76.8585	18	1603.52	42.6976
19	1590.99	47.7881	20	1578.45	48.1586
21	1349.93	80.7259	22	681.713	63.2008
23	668.214	50.1714	24	625.788	61.0004

IR spectrum of chitosan



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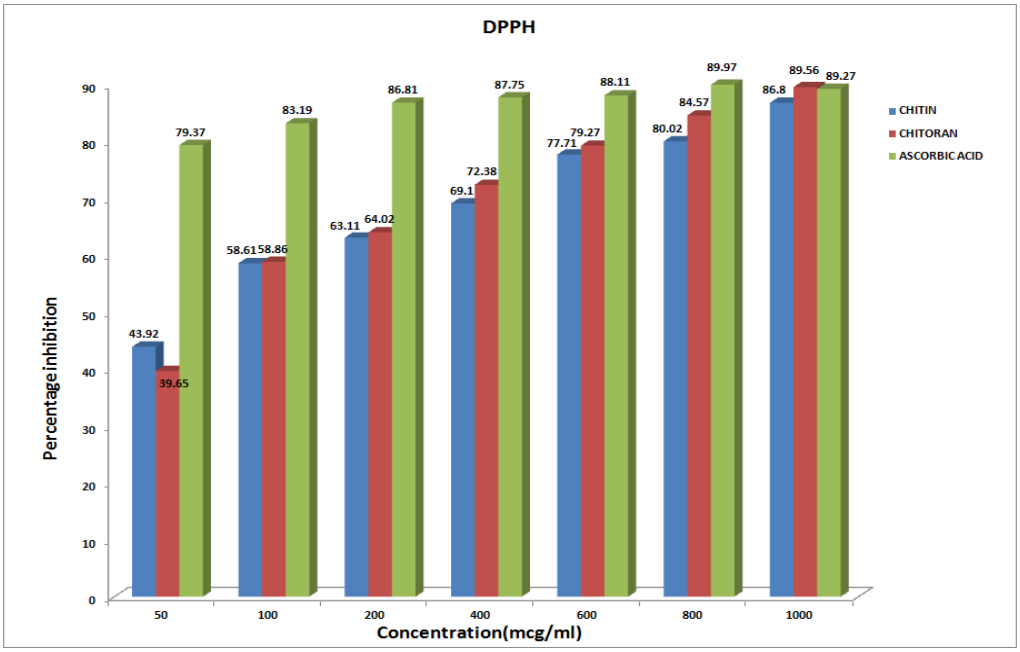
Sample Name chitosan 2
Comment
User
Division
Company SRI RAMACHANDRA MEDICAL UNIVERSITY

[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity
1	3410.49	3.53881	2	1595.81	0.762155
3	1351.86	14.2108	4	769.458	43.2535
5	515.865	55.2211	6	490.795	36.4127
7	479.224	52.9852	8	461.868	50.2284

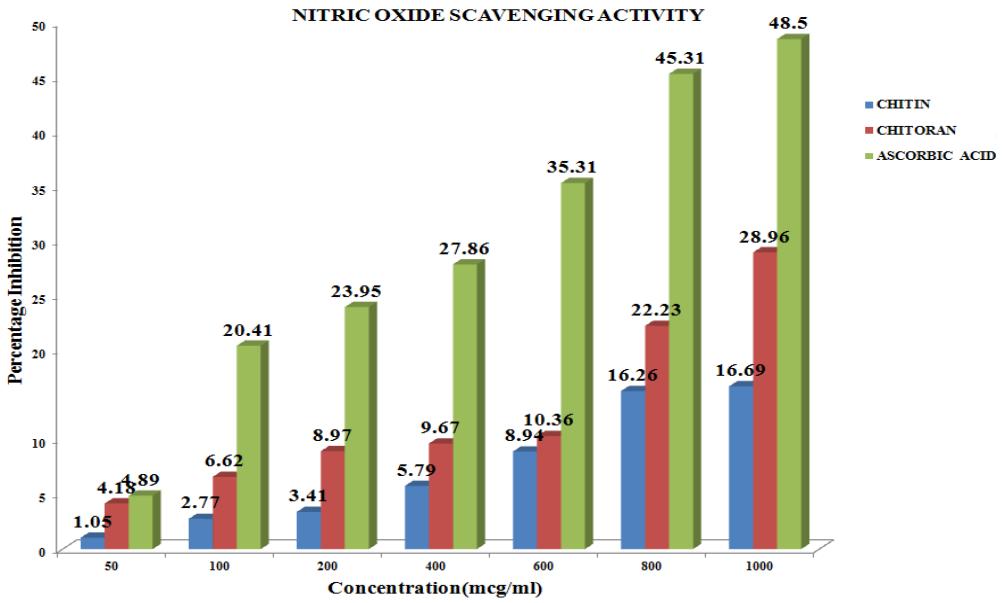
DPPH SCAVENGING ACTIVITY

Concentration (µg/ml)	%Inhibition		
	Chitin	Chitosan	Ascorbic acid
50	43.92	39.65	79.37
100	58.61	58.86	83.195
200	63.11	64.025	86.815
400	69.1	72.385	87.75
600	77.71	79.275	88.115
800	80.02	84.575	88.975
1000	86.805	89.565	89.27



NITRIC OXIDE SCAVENGING ACTIVITY

Concentration µg/ml	% Inhibition		
	Chitin	Chitosan	Ascorbic acid
50	1.05	4.18	4.98
100	2.77	6.62	20.41
200	3.41	8.97	23.95
400	5.79	9.67	27.86
600	8.94	10.36	35.31
800	16.26	22.23	45.31
1000	16.69	28.96	48.5

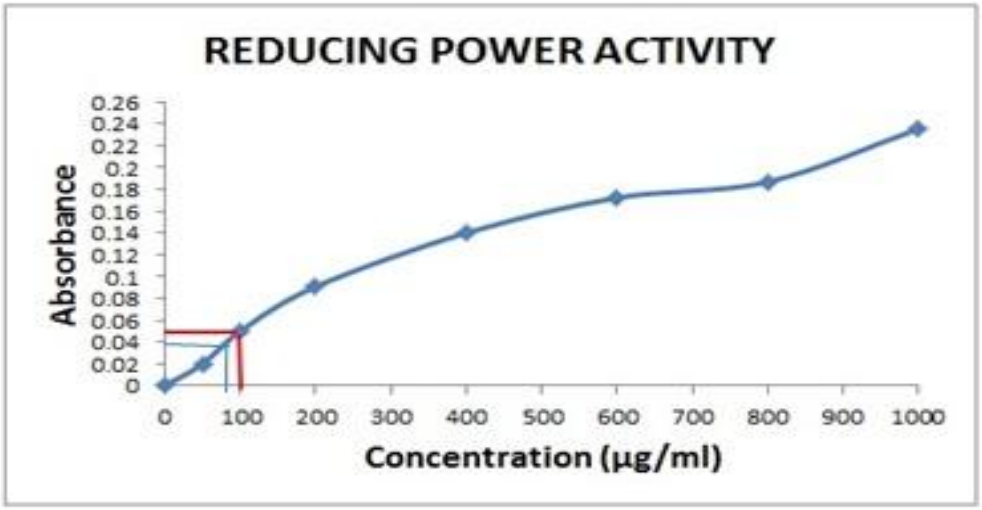


REDUCING POWER ACTIVITY

ASCORBIC ACID

S.NO	CONCENTRATION µg/ml	ABSORBANCE
1	50	0.0213
2	100	0.0543
3	200	0.0912
4	400	0.1321
5	600	0.1651
6	800	0.1842
7	1000	0.2231

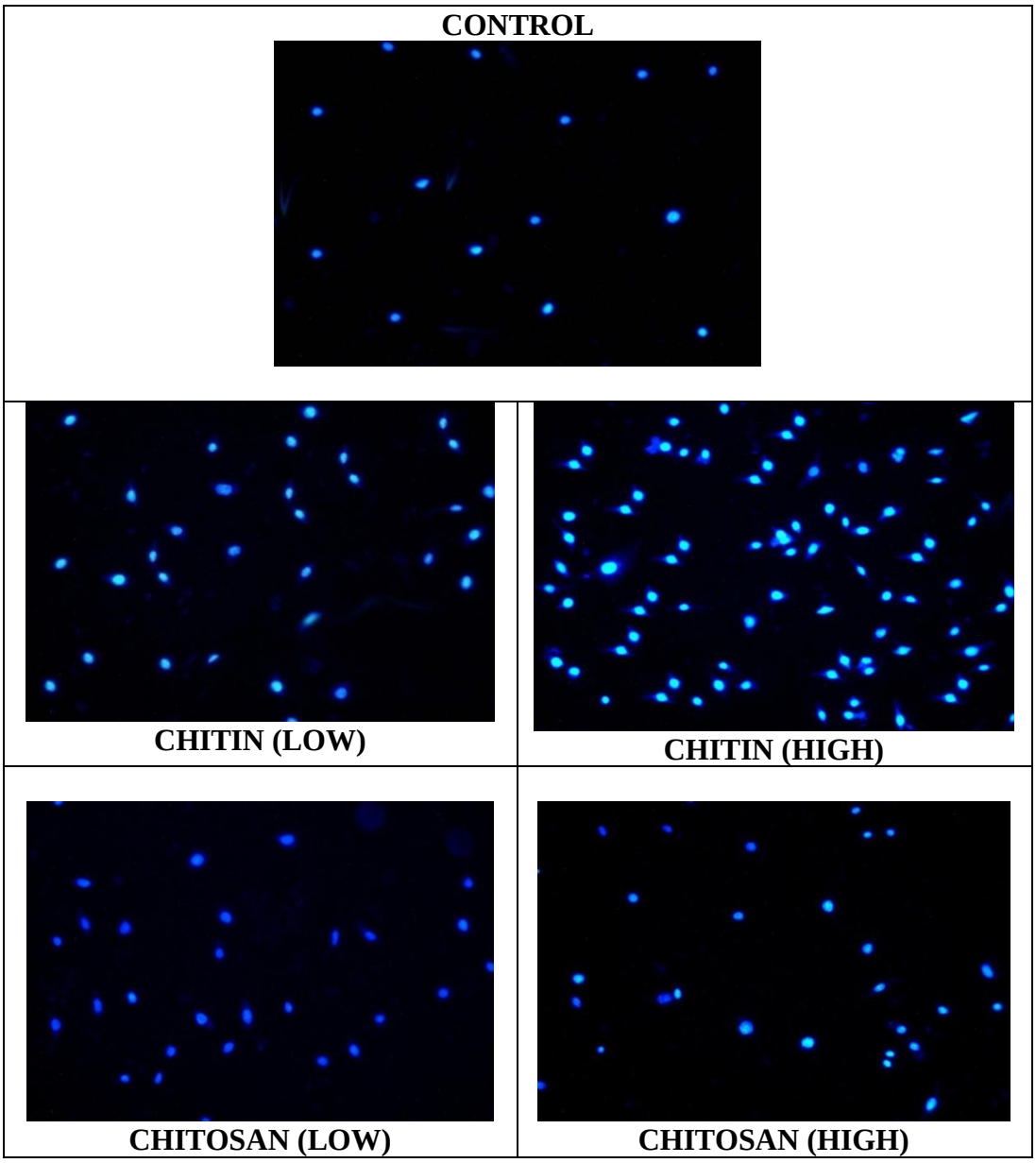
S.No	DRUG	CONCENTRATION µg/ml	ABSORBANCE
1	Chitin	1000	0.0508
2	Chitosan	1000	0.0454



MTT ASSAY

Test drug	Concentration	% cytotoxic (mean±sem)
Chitin	1ng/ml	14.02±1.17
	10ng/ml	24.58±1.78
	100ng/ml	25.21±0.15
	1µg/ml	27.88±2.74
	10µg/ml	33.74±1.50
	100µg/ml	34.01±0.29
	1mg/ml	36.65±1.70
IC_{50}		**105.4**

Test drug	Concentration	% cytotoxic (mean±sem)
Chitosan	1ng/ml	14.83±3.64
	10ng/ml	12.74±2.21
	100ng/ml	21.40±2.07
	1µg/ml	23.84±0.18
	10µg/ml	24.52±4.44
	100µg/ml	35.61±1.50
	1mg/ml	36.05±1.47
IC_{50}		**1499**



DISCUSSION

Chitin and Chitosan were isolated from the carapace of cultured *Penaeus monodon species* by using reagents like 4% HCl for decalcification, 3% NaOH, 0.5% $KMnO_4$ & 0.5% oxalic acid for decolourisation and 40%NaOH. The antioxidant activity was carried out using DPPH sacvenging activity, Nitric Oxide Scavenging activity & Reducing Power activity and Chitosan shows greater DPPH inhibitory activity, Chitosan showed a better Nitrogen Scavenging activity than Chitin & Chitin and Chitosan showed a similar Reducing activity. The *in-vitro* anticancer activity was carried out against MDA cancer cell lines (human breast cancer cell lines) using MTT Assay and DAPI. Chitin is highly cytotoxic to MDA cancer cell lines than Chitosan. DAPI assay suggested that Chitin shows a better apoptotic activity.

CONCLUSION

Many of the marine derived natural products which are being isolated exhibit exceptional levels of biological activity combined with unique modes of action. This study identifies opportunities to develop value added products from crustacean processing by-products with different biological activity such as anti oxidant and *in-vitro* anticancer activity.

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