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PHARMACOLOGICAL SCREENING OF METHONOLIC EXTRACT OF RED MARINE ALGAE *HYPNEA MUSCIFORMIS*

Shireesha .T[*1], Ravindra Reddy.K[2], Jayaveera.K.N[3]

1. Department of Pharm. Biotechnology, Govt. Polytechnic for Women, Ramanjaneyapuram, Kadapa-516 001 A.P., INDIA.
2. Department of Biotechnology, P. Rami Reddy Memorial College of Pharmacy, Kadapa-516 003, A.P., INDIA.
3. Department of Chemistry, Vemu Institute of Technology, P. Kothakota-517 112, Near Pakala, Chittoor Dist, A.P., INDIA.

[*]Corresponding author E-mail***: siirishabujji06@gmail.com***

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ABSTRACT

In the present study, methonolic extractof *Hypnea musciformis* (ME of *H. musciformis*), a red seaweed at 200 and 400 mg/kg was screened for the activities: Anti-ulcer (Pylorus ligation method), anti-obesity (high fat diet [HFD] induced model), anti-diabetic (Alloxan induced model) on Wistar albino rats and anti-cancer by (MTT assay). ME of *H. musciformis* showed a significant dose dependent effect on studied pharmacological activities. This may prove helpful for developing new drugs from this red seaweed *H. musciformis* for managing gastric ulcers, obesity, diabetes, cancers and their associated complications. However further studies required to elucidate the exact mechanism of action and the structure of the secondary metabolites which are responsible for these activities for the development as potent anti-ulcer, anti-obesity, anti-diabetic and anti-cancer drugs.

INTRODUCTION:

The written record on the study of uses of marine plants was available from the third century B.C. by the Greek naturalist Theophrastus, who gave descriptive account of certain useful seaweeds. Seaweeds have been reported to be important sources of certain lifesaving drugs. India has vast costal area and about 740 species of marine algae were recorded, 60 species of them are of economic value. They are majorly classified into three main classes: Brown algae (Phaeophyceae), green algae (Cladophoraceae), and red algae (Rhodophyceae)[1].Seaweeds have been one of the richest and most promising sources of bioactive primary and secondary metabolites. These compounds have diverse simultaneous functions for the seaweeds and can act as antimicrobial, antifouling, and herbivore deterrents, or as ultraviolet-screening agents. They are also used by the pharmaceutical industry in drug development to treat diseases like cancer, acquired immune-deficiency syndrome

(AIDS), infection from virus, bacteria and fungus, inflammation, pain, arthritis etc. Currently, algae represent about 9% of biomedical compounds obtained from the sea[2].*Hypnea musciformis* (Fig.1) belongs to Class: Rhodophyceae, Family: Hypneaceae, whose plants are bushy, spreading, cylindrical, 10-30 cm high, purplish green in colour, cartilaginous, much branched, branches irregular, giving a bushy look to the plant[3]. The hooked and swollen tendrils are the characteristic feature of this species. These are also collected as drift materials. It has been reported to possess K-carrageenan. Carrageenan is extensively used as a food additive in a wide range of products including cheese, cream, chocolate and ice creams. Its chief use is as a suspending and stabilizing agent, and has a number of pharmacological properties[4]. Discovery of novel moieties using the natural sources is an immense assignment and was successful to a great extent, thus serve as a source of many useful drugs with fewer side effects has reached about 30% of pharmaceutical market[5]. According to the previous literature review, around one thousand molecules entered into the market, in which approximately 49% of substances were isolated, characterized and identified from natural origin including seaweeds and the skeleton of these structures can be used as a template for the synthetic and semi-synthetic derivatives[6]. Ever since scientists faces a great challenge in identifying new effective medicines for many life threatening diseases. Therefore, all over the place in the world, many scientists have an eye on the natural sources for new molecules identification. Hence, this research article aims in the pharmacological screening of methonolic extract of red algae *H. musciformis* which is one of the important species and rich in various active constituents.

MATERIALS AND METHODS:

Collection of plant material*:* *H. musciformis* (Fig.1) the red seaweed was collected from the Rameshwaram, in the south east coast of, Tamil Nadu, India. The collected plants were rinsed with fresh water to remove debris and epiphytes. The entire epiphytes were removed using soft brush. In the laboratory, the plants were once again washed in freshwater and stored in refrigerator for further studies[7].

Preparation of methonolic extract of *H. musciformis:* The collected red sea weed was washed thoroughly and spread on blotting paper at room temperature, under shade for drying. The shade dried samples were grounded to fine powder using a tissue blender. The powdered samples were then stored in the refrigerator for further use. Powdered sample of wt. 3 g was packed in soxhlet apparatus and extracted with methanol for 8 h. The excess amount of methanol was evaporated and fine crude powder was obtained and stored in the refrigerator for the further pharmacological screening studies[8].

Statistical analysis of data: All quantitative measurements were expressed as means ± SD for control and experimental animals. The data were analysed using one-way analysis of variance (ANOVA) on Graph Pad Prism 7.0 trail version software and the group means were compared by Duncan's multiple range test (DMRT). The results were considered statistically significant if the *p* value is less than0.05.

Animal handling and experimental protocols: Were approved by the Institutional Animal Ethics committee, P. Rami Reddy Memorial College of Pharmacy, Kadapa-516 003, A.P., INDIA. (CPCSEA No.1423/Po/a/11/CPCSEA/04/2013).

Anti-ulcer activity (**Pylorus ligation method):** Wistar albino rats of either sex weighing 150-200 g, were divided into four groups of six animals each (n=6). Animals

were fasted for 24 h before the study, but had free access to water. Animals in the control group received only distilled water. ME of *H. musciformis* at 200 and 400 mg/kg were given to the animals in the treatment group. Ranitidine (10 mg/kg) was used as a standard. After 1 h of drugs treatment, they were anaesthetized with anaesthetic ether and the abdomen was opened by a small midline incision. Pyloric portion of the stomach was slightly lifted out and ligated according to method of (Shay et al.)[9] avoiding traction to the pylorus or damage to its blood supply. The stomach was replaced carefully and the abdominal wall was closed by interrupted sutures. Rats were sacrificed by an over dose of anaesthetic ether after 4 h of pyloric ligation. The abdomen was opened, cardiac end of the stomach was dissected out and the contents were drained into a glass tube. The volume of the gastric juice was measured and centrifuged at 2000 rpm for 10 min. From the supernatant, 1 mL of aliquots was taken for the determination of pH, total and free acidity. The stomachs were opened along the greater curvature, rinsed with saline to remove gastric contents and blood clots and examined by a 10X magnifier lens to assess the formation of ulcers. The number of ulcers was counted. Scoring of ulcers will be given as per (Table 1). Mean ulcer score for each animal will be expressed as ulcer index which is expressed as follows:

UI = [(UN + US + UP) X 10]$^{-1}$ Eq. No. 1

Where: UI= Ulcer Index; UN = Avg. No. of ulcers per animal; US = Avg. No. of severity score; UP = % of animals with ulcers

% inhibition of ulceration is expressed as follows:

% Inhibition of ulceration= $\frac{UI(control)-UI(test)}{UI(control)}$ *X 100* ***Eq. No. 2***

The results of anti-ulcer activity as (mean±SD) are represented in (Table2).

Anti-obesity activity (High fat diet [HFD] induced model): Wistar albino rats of either sex weighing 150-200 g, were taken for the study. The test animals were broadly divided into five groups of six each (n=6). Groups include: Normal or negative control group which receives only sodium carboxymethyl cellulose orally, HFD induced obese rats as positive control group, HFD induced obese rats administered with Orlistat (5 mg/kg) orally is the standard group and HFD induced obese rats administered with ME of *H. musciformis* at 200 mg/kg, 400 mg/kg orally are the test groups[10]. Experiment was conducted according to (Dee man's protocol)[11]. All groups, except the normal or negative control group received high fat diet (HFD) for 42 days consecutively. However, the normal or negative control group was fed by normal diet pellets. All rats had free access to water during the experiment. The composition of both normal and high fat diet pellets was presented in(Table 3). Starting from day 22 after HFD induced obesity, either ME of *H. musciformis* or Orlistat was given to rats orally as a suspension with sodium carboxymethylcellulose (SCMC) according to their group up to 21 days, meanwhile normal or negative control group received SCMC suspension only. Body weight from each group was monitored every week throughout various periods. Other parameters which were also determined include fatpad index, urine index, faeces index, and serum level of triglycerides (from the blood samples collected from retro orbital plexus). Obtained faeces during the study were observed further to examine the presence of oil and/or fat by exposing them to the filter paper. The consolidated results of anti-obesity activity as (mean ± SD) are represented in (Table 4). 24 h after the last day of experiment, all rats were sacrificed by using CO_2gas, lever was dissected and preserved for histology studies. Histology of liver(s) from each group of the study after 21-day treatment was shown in (Fig. 2).

Anti-diabetic activity (Alloxan induced model): Wistar albino rats of either sex were fasted for 18 h before the study, but had free access to water. To the test group alloxan (150 mg/kg), in freshly prepared normal saline was given intra peritonially. Normal or negative control group received normal saline only. 48 h after alloxan induction, blood samples were collected from retro orbital plexus and plasma glucose was determined. The induction of diabetes mellitus was confirmed by determination of plasma glucose level (≥250 mg/dL). The rats with plasma glucose (≥ 250 mg/dL) were selected for anti-diabetic studies. The test animals were broadly divided into five groups of six each (n=6). Groups include: Normal or negative control group which receives only normal saline (1 mL/100 g/day) orally, alloxan induced diabetic rats as positive control group, alloxan induced diabetic rats administered with glibenclamide (2mg/kg) orally is the standard group and alloxan induced diabetic rats administered with ME of *H. musciformis* 200 mg/kg, 400 mg/kg orally are the test groups. After 48 h of alloxan induction, the blood glucose levels were measured on 0^{th},7^{th}, 14^{th} and 21^{st}day after the test drug administration, blood was collected from retro orbital plexus and the mean blood glucose levels were measured[12]. The results of anti-diabetic activity as (mean ± SD) are represented in (Table 5).

Anti-cancer activity (MTT assay): Was conducted at Biogenix Research Centre, Thiruvananthapuram-695 012, Kerala, India.

Cell lines: Human Molt-4 (lymphoblast-like) cell lines were chosen as proper representatives of human leukemic cell lines.

Cell culture: The cells were cultured in 50 mL cell culture flasks (Orange Scientific) or 96 wells cell culture microplates (Orange Scientific) by using RPMI 1640 (Gibco) containing 10% fetal bovine serum (Gibco) and were incubated at 37°C in the presence of 5% CO_2 as per (Morgan et al.)[13].

Methyl Thiazolyl Tetrazolium (MTT) assay: To determine the cytotoxicity of ME of *H. musciformis* against studied cancer cell lines, MTT assay test was used as a quantitative and approved method. In this method, 10 µL of MTT stock solution (5 mg/mL in PBS) was added to 90 µL medium of wells which were treated by different conc. of ME of *H. musciformis* for 72 h. The micro plate was incubated at 37°C for 4 h and then, the optical density of each well was read by micro plate reader (ASYS – EXPERT 96) at 540 nm as per (Van de Loosdrecht et al.)[14]. The results of MTT assay to determine the anti-cancer activity was represented in (Table 6).

RESULTS & DISCUSSION:

Anti-ulcer activity (Pylorus ligation method):ME of *H. musciformis* exhibits an effective protection against pylorus induced ulcer in rats. Maximum protection was seen in the ranitidine treated group (standard). The volume of gastric secretion and total acidity was significantly reduced in all drug treated groups as compared to control. Gastric pH was also found to be increased in all drug treated groups as compared to control, with maximum increase being produced by ranitidine treated group (standard). The effect of ME of *H. Musciformis* against pylorus induced ulceration was shown in (Table 2). The ME of *H. musciformis* has reduced the ulceration significantly ($P<0.05$) in a dose dependent manner. In this model, the percentage inhibition of ulceration was found to be 52.32 % and 64.13 % with 200 and 400 mg/kg of ME of *H. musciformis* respectively.

Anti-obesity activity (HFD induced model):

The body wt. change: Even though the body weight of all groups grew throughout the period of 3 weeks. Elevation of that in a

groups treated with ME of *H. musciformis (*200 and 400 mg/kg) was significant lower ($p < 0.05$) than that of the control group, in a dose dependent manner (Table 4).

Fat pad Index (in terms of peri renal and peri anal fat): Of the groups treated with ME of *H. musciformis* (200 and 400 mg/kg) was significantly lower ($p < 0.05$) with values 28.32 ± 1.42 and 24.32 ± 2.45 respectively, when compared with the positive control and standard groups, in a dose dependent manner (Table 4).

Urine and faeces Index: The group treated with 400 mg/kg of ME of *H. musciformis*, had significant lower ($p < 0.05$) urine index and faeces index with values 2.97±1.54 and 2.68±1.23 respectively, when compared with the positive control and standard groups, in a dose dependent manner (Table 4). Moreover, there were oil spots when rat faeces were exposed to the filter paper indicating the consumption of HFD. Results of liver histopathological studies from each group after 21-day treatment reveals that, in the normal group the hepatocytes are structurally organized with a few small circles. There were small circles (pointed by an arrow) distributed in the liver tissue, indicating the fat storage. However, the size of each spots was higher in the positive control group than treatment groups, as without any treatment, absorbed fat will be stored in the liver with as greater size vesicles. (Fig. 2)

Serum levels of triglycerides: A significant increase of serum triglyceride levels in the control group was observed, compared to normal group, due to the administration of HFD. The groups treated with ME of *H. musciformis* (200 and 400 mg/kg) had significantly lower ($p < 0.05$) serum triglyceride levels with values 178.15±1.23 and 174.32±1.16 respectively, when compared with the positive control and standard groups, in a dose dependent manner (Table 4).

Anti-diabetic activity (Alloxan induced model): Administration of alloxan produced increased blood glucose levels of diabetic control rats compared to the normal control rats. Administration of ME of *H.* musciformis to alloxan induced diabetic rats over a period of three weeks produced a significant blood glucose reduction(Table 5). At the end of the 21st day the ME of *H. musciformis* 400mg/kg reduced the glucose levels to 155±0.28. Maximum decreased in the blood glucose level was seen in the glibenclamide treated group (standard).

Anti-cancer activity (MTT assay): As shown in (Table 6), the result of MTT assay confirmed that the most effective conc. of ME of *H. musciformis* against molt-4 cells was 8.947µg/µL. Based on previous experience, filtration method is the best way for algal extract sterilization as per (Zandi et al.)[15]. The heat sensitivity of some biological constituents of algal extract is the most important reason for not using autoclave for sterilizing the extract. In this study, the ME of *H. musciformis* showed significant ($p < 0.05$) activity against tumor cells replication. In this study, the effective conc. is higher than other similar studies in which the purified biological active compound(s) were used instead of crude extract. Therefore, fractionation and purification for *H. musciformis* extract is recommended in further studies.

CONCLUSION:

Anti-ulcer activity (pylorus ligation method): From the results, it can be concluded that the ME of *H. musciformis*, possess significant anti-ulcer activity. Among the two conc. of ME studied, 400 mg/kg had the highest anti-ulcer effect.

Table 1: Scoring of ulceration

Observation	Score
Normal colored stomach	0.0
Red coloration	0.5
Spot ulcer	1.0
Haemorrhagic streak	1.5
Deep ulcers	2.0
Perforation	3.0

Table 2: Results of anti-ulcer activity

Animal groups	Vol. of gastric juice	pH of gastric juice	Acidity (mEq/L)		Ulcer Index	% Ulcer inhibition
			Free	Total		
Control	4.52±0.21	3.95±0.10	121.7±1.34	132.5±1.56	8.2±2.3	--
(10 mg/kg) Ranitidine (standard)	4.70±0.23	5.42±0.13	47.10±1.22	63.50±1.67	0.2±0.91	74.25%
200 mg/kg ME (test)	5.36±0.14	4.18±0.20	51.23±0.22	61.35±1.32	6.32±0.68	52.32%
400 mg/kg ME (test)	5.55±0.12	5.23±0.11	58.23±0.46	71.24±1.22	4.13±0.76	64.13%

Table 3: Composition of normal and high fat diet pellets

Ingredient	Normal diet (g/ kg)	High fat diet (g/ kg)
Casein	80.0	80.0
Corn starch	60.0	60.0
Sucrose	200.0	122.6
Corn oil	45.0	0.0
Lard	0.0	219.2
AIN-76 vitamin mix	4.0	4.0
DL-methionine	1.2	1.2
Energy (kcal/100 g)	390.2	487.0
Calories from fat (%)	11.5	45.0

Table 4: Results of anti-obesity activity

Animal groups	Body weight (g)			Fat pad Index (% W/W)	Urine Index (% W/W)	Feces Index (% W/W)	Serum TG level (mg/dL)
	1st Wk	2nd Wk	3rd Wk				
Normal (-ve control)	185.23±1.47	251.24±1.61	290.83±1.62	19.12±1.83	3.32±1.31	2.51±1.52	148.33±1.75
HFD (+ve control)	188.34±1.23	310.22±1.37	380.50±1.67	30.11±1.62	2.82±1.38	2.78±1.25	280.16±1.52
HFD+(5 mg/kg) Orlistat (standard)	186.78±1.65	303.42±1.52	357.33±1.72	19.52±1.54	3.26±1.62	2.65±1.24	151.32±1.35
HFD+ (200 mg/kg) ME(test)	186.13±1.45	285.17±1.13	342.17±1.25	28.32±1.42	2.91±1.54	2.71±1.35	178.15±1.23
HFD+ (400 mg/kg) ME(test)	185.52±1.22	267.32±1.52	310.67±1.77	24.32±2.45	2.97±1.54	2.68±1.23	174.32±1.16

Table 5: Results of anti-diabetic activity

Animal groups	Blood glucose (mg/dL)		Blood glucose after drug administration (mg/dL)			
	Before	After 48 h	0^{th}day	7^{th}day	14^{th} day	21^{st}day
Normal (-ve control)	115±1.12	113±1.21	113±1.24	112±1.31	111±0.73	110±1.42
Alloxan 150 mg/kg (+vecontrol)	110±0.74	273±1.52	273±1.51	297±1.14	312±1.61	325±1.31
Alloxan 150 mg/kg + 2mg/kg Glibenclamide (standard)	115±1.11	273±1.54	273.±1.43	216±1.23	134±1.82	121±1.06
Alloxan 150 mg/kg +200 mg/kg ME (test)	110±0.72	285±1.81	285±1.81	253±1.42	212±1.33	162±0.69
Alloxan 150 mg/kg +400 mg/kg ME (test)	112±1.15	276±1.32	276±1.28	221±1.35	198±1.63	155±0.28

Table 6: Results of anti-cancer activity

OD of Molt-4 cells at 540 nm	Conc. of ME(µg/µL)
0.832	0
0.452	2.723
0.387	4.668
0.368	5.057
0.318	5.835
0.304	6.224
0.214	7.391
0.287	8.167
0.231	8.947

Fig.1. Photograph of *Hypnea musciformis*

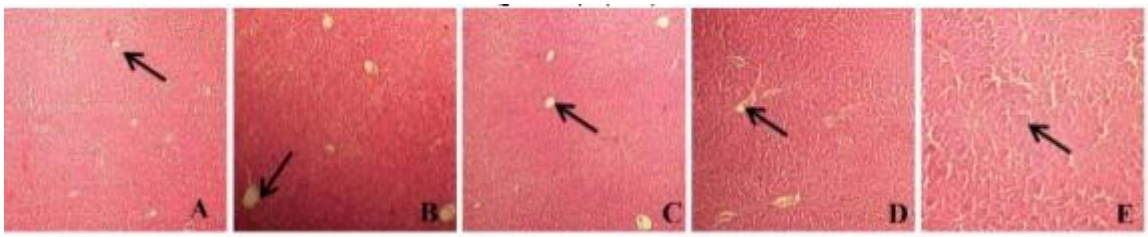

Fig.2. Histology of liver from each group after 21-day treatment with 40 times magnification in microscope. A: Normal group (-ve control), B: Control group (+ve control), C: Orlistat 5 mg/kg body wt. (standard), D: ME of *H. musciformis* 200 mg/kg body wt.,E: ME of *H. musciformis* 400 mg/kg body wt. (The arrow is pointed at small circle where the fat existed).

Anti-obesity activity(HFD induced model): From the results, it can be concluded that the ME of *H. musciformis*, possess significant anti-obesity activity in a dose dependent manner.

Anti-diabetic activity (Alloxan induced model): From the results, it can be concluded that the ME of *H. musciformis* showed significant anti-diabetic activity when compared with the standard group treated with glibenclamide.

Anti-cancer activity (MTT assay): From the results, it can be concluded that the ME of *H. musciformis*, possess significant anti-tumor activity against Molt-4 cell lines. The most effective conc. of ME of *H. musciformis* against molt-4 cells was found to be 8.947 µg/µL, which is very potent when compared with the similar studies. Also, with regard to the significant results of these studies, the isolated and purified biological active compound(s) must be used instead of crude extract. This may prove helpful for developing new drugs from this marine algae *H. musciformis* for managing gastric ulcers, obesity, diabetic cancers and their associated complications. However further studies are required to elucidate the exact mechanism of action and the structure of the secondary metabolites which are responsible for these activities for the development as potent anti-ulcer, anti-obesity anti diabetic and anti-cancer drugs.

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