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PHARMACOLOGICAL SCREENING OF METHANOLIC EXTRACT OF RED MARINE ALGAE *GRACILARIA CORTICATA* J.Ag.

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

[1]Department of Pharmaceutical Biotechnology, Govt. Polytechnic for Women, Ramanjaneyapuram, Kadapa-516 001 A.P., INDIA.
[2]Department of Biotechnology, P. Rami Reddy Memorial College of Pharmacy, Prakruthi Nagar, Utukur, 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:** tshireeshapharma@gmail.com

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Key words

Gracilaria corticata J.Ag., anti-ulcer, anti-obesity, anti-diabetic and anti-cancer activity.

ABSTRACT

In the present study, methanolic extract of the red seaweed *Gracilaria corticata* J.Ag. (ME of *G. corticata*) at 200 and 400 mg/kg was screened for the activities: Anti-ulcer (Aspirin induced model), anti-obesity (Monosodium glutamate induced model), anti-diabetic (Alloxan induced model) on Wistar albino rats and anti-cancer (MTT assay) against Molt-4 cell lines. ME of *G. corticata* showed significant ($P < 0.05$) dose dependent activities (400 > 200 mg/kg doses) except the anti-diabetic activity. This may prove helpful for developing new drugs from this red marine algae *G. corticata* for managing gastric ulcers, obesity, 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 and anti-cancer drugs.

INTRODUCTION

Among the heterogeneous group of plants, seaweeds or marine algae has a long fossil history and has great medicinal value. They are found in the coastal region and in the sub-tidal regions of the availability of 0.01% of photosynthetic light and also classified into three main categories: brown algae (Phaeophyta), green algae (Chlorophyta), and red algae (Rhodophyta) [1]. More than 600 trace elements are found in high concentration in the seaweeds compared to the terrestrial plants, because of which it has various pharmacological activities [2]. The *Gracilaria corticata* J.Ag. (Fig.1) are generally considered to be important because of their industrial and biotechnological uses due to the presence of constituents like, α-(1,4)-3,6 anhydro-l-galactose and β-(1,3)-d-galactose which is one of the main source of agar with cell wall has slight esterification in it [3]. Discovery of novel moieties using these 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 [4]. 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 [5]. 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 red algae *G. corticata* which is one of the important species and rich in various active constituents.

MATERIALS AND METHODS:

Collection of plant material*:* *G corticata* J.Ag. (Fig.1) is marine red algae / red seaweed belonging to family: Rhodophyceae. They were collected from the Thoothukudi in the south east coast of Tamil Nadu, India. The collected samples were rinsed with marine water to remove debris and epiphytes. The entire epiphytes were removed using soft brush. The plants were brought to the laboratory. In the laboratory, the plants were once again washed in freshwater and stored in refrigerator for further studies[6].

Preparation of methanolic extract of *G. corticata:* The collected red sea weed was washed thoroughly and placed on blotting paper and spread out at room temperature in the 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. 3 g powdered sample was packed in soxhlet apparatus and extracted with methanol for 8 h separately. The excess amount of methanol was evaporated and fine crude powder was obtained and stored in the refrigerator for the further pharmacological screening studies[7].

Statistical analysis of data: All quantitative measurements were expressed as means ± SD for control and experimental animals. The data were analyzed using one-way analysis of variance (ANOVA) on Graph Pad Prism 7.0 free 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 than 0.05.

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. (CPCSEANo.1423/Po/a/11/CPCSEA/04/2013).

Anti-ulcer activity (**Aspirin induced model):** 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 *G. corticata* 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 anesthetic 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.[8] 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 anesthetic 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 were 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 were 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:

$$\%Inhibition\ of\ ulceration = \frac{UI(control)-UI(test)}{UI(control)}\ X\ 100$$

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

Anti-obesity activity (Monosodium glutamate [MSG] induced model): Wistar albino rats of either sex weighing 150-200 g, were taken for the study and they were broadly divided in to five groups of six each (n=6). Groups include normal, control, ME of *G. corticata* 200 mg/kg, ME of *G. corticata* 400 mg/kg, and Orlistat (5 mg/kg) as standard[9]. Experiment was conducted according to Dee man's protocol[10]. All groups, except the normal received MSG 2 mg/kg for the first 5 days through subcutaneous injection along with high fat diet (HFD). They were given HFD for 42 days consecutively. However, the normal was fed by normal diet. All rats had free access to water during the experiment. The composition of both normal and high fat diet was presented in (Table 3). Starting from day 22, either ME of *G. corticata* and Orlistat was given to rats according to their group, meanwhile control group received Na CMC suspension only. 24 h after the last day of experiment, all rats were sacrificed by using carbon-dioxide. Body weight from each group was monitored every week throughout periods. Other parameters which were also determined include food index, urine index, feces index, and serum level of triglycerides. Obtained feces during the study was observed further to examine the presence of oil and/or fat by exposing them to the filter paper. The results of anti- obesity activity as (mean ± SD) are represented in (Table 4). Histology of liver from each group 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 0.1M citrate buffer (pH 4.5) was given. Control group received citrate buffer (pH 4.5) 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). Diabetic rats were kept untreated for four weeks. At the end of 4th week, the rats with plasma glucose ≥ 250 mg/dL was selected for anti-diabetic studies. Alloxan induced diabetes mellitus Wistar albino rats of either sex were divided into five groups of six animals each (n = 6). Negative control group receives normal saline (1 mL/100g/day); positive control group is alloxan induced diabetic rats. Alloxan induced diabetic rats administered with glibenclamide (0.60 mg/kg) orally is the standard group. Alloxan induced diabetic rats administered with ME of *G. corticata* at 200 mg/kg and 400 mg/kg orally are the test group. After 48 h of alloxan induction, and at intervals of: 0, 1, 3, 5 and 7 h after the test drug administration, blood was collected from retro orbital plexus and the mean blood glucose levels were measured[11]. The results of anti- diabetic activity as (mean ± SD) are represented in (Table 5).

Anti-cancer activity (MTT assay): Was conducted at *Biogenix Research Center,* 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.[12].

Methyl Thiazolyl Tetrazolium (MTT) assay: To determine the cytotoxicity of methanolic extract *G. corticata* 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 methanolic extract *G. corticata* for 72 h. The microplate was incubated at 37°C for 4 h and then, the optical density of each well was read by microplate reader (ASYS – EXPERT 96) at 540 nm as per Van de Loosdrecht et al.[13]. The results of MTT assay to determine the anti-cancer activity was represented in (Table 6).

RESULTS & DISCUSSION:

Anti-ulcer activity (**Aspirin induced model):** ME of *G. corticata* showed a dose dependent protection against aspirin (500 mg/kg body weight) induced ulcers in rats. Maximum protection was seen in the Ranitidine treated group. 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 as standard drug. The effect of ME of *G. corticata* on aspirin induced ulceration was shown in (Table 2). Further it was observed that aspirin induction has caused gastric ulcerations and pre-treatment with the ME of *G. corticata* has reduced significantly ($P < 0.05$) in a dose dependent manner. In this model, the percentage inhibition of ulceration was found to be 65.25 and 53.25 at 200 and 400 mg/kg respectively.

Anti-obesity activity (MSG induced model):

The body wt. change: There were significant differences in body weight between control and normal group ($p < 0.05$). Even though the body weight of all groups grew throughout the periods, elevation of that in a group treated with ME of *G. corticata* 200 and 400 mg/kg was lower than that of the control group (Table 4).

Organ Index (in terms of perirenal and perianal fat): Of the group treated with ME of *G. corticata* 200 and 400 mg/kg were lower than that of the control group. At a dose of 400 mg/kg, ME of *G. corticata* possessed the ability to reduce both perirenal and perianal fat compared to the Orlistat treated group (Table 4).

Urine and Feces Index: The group treated with ME of *G. corticata*, 400 mg/kg had significant lower urine index compared to the control group ($p < 0.05$). The similar results were obtained in the measurement of feces index, in which ME of *G. corticata*, 400 mg/kg showed lower value than control ($p < 0.05$). Moreover, there were oil spots when rat feces exposed to the filter paper. Histopathological studies examination on liver histology displayed that, in the normal group, hepatocytes are structurally organized with a few small circles. As shown in (Fig. 2), there were small circles (pointed by an arrow) distributed in the liver tissues that was seemingly used as fat storage. However, the size of each spots was higher in the control group. It is most likely mean that without any treatment, absorbed fat will be stored particularly in the liver with greater size of vesicles. These histopathological findings were relevant to measurement of liver index, where there was a significant increase in the liver index value in all induced group compared to normal group (Table 4).

Table 1. Scoring of ulceration

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

Table 2. Results of anti-ulcer activity

Animal groups	Vol. of gastric juice	pH	Acidity (mEq/L)		Ulcer Index	% Ulcer inhibition
			Free	Total		
Control	4.52±0.2	3.95±0.1	121.7±9.8	132.5±4.5	--	--
Ranitidine (10 mg/kg)	4.70±0.2	4.18±0.2	47.0±1.22	63.50±5.67	74.25±2.9	74.25%
(200 mg/kg) ME	5.41±0.1	5.33±0.1	50.45±1.14	63.58±2.6	65.25±0.8	65.25%
(400 mg/kg) ME	5.73±0.1	5.24±0.16	60.17±1.52	74.9±2.14	53.25±1.3	53.25%

Table 3. Composition of normal and high fat diet

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)			Food Index (% W/W)	Urine Index (% W/W)	Feces Index (% W/W)	Serum TG level (mg/dL)
	1st Wk	2nd Wk	3rd Wk				
Normal control	185.00±7.64	251.00±3.26	290.83±1.62	19.08 ± 1.93	3.32± 1.31	2.51± 0.82	168.33 ± 27.97
HFD+MSG control	184.17±4.12	310.17±3.50	380.50±3.74	30.08 ± 5.16	2.51± 1.38	2.51± 1.25	280.16 ± 30.16
HFD+MSG+(5 mg/kg)Orlistat	183.17±4.71	303.50±4.19	329.33±4.36	22.50 ± 3.66	3.82± 0.82	2.82± 1.31	191.80 ± 25.41
HFD+ MSG+(200 mg/kg) ME	185.33±3.79	305.17±3.84	349.17±3.05	19.67 ± 2.38	3.32± 1.67	2.73± 1.67	203.80 ± 22.45
HFD+ MSG+(400 mg/kg) ME	184.50±3.19	304.00±3.28	310.67±2.75	15.00 ± 1.92	4.64± 1.25	2.92± 1.14	127.50± 28.52

Table 5. Results of anti-diabetic activity

Animal groups	Blood glucose (mg/dL)		Blood glucose after drug administration at (mg/dL)				
	Before	After 48 h	0 h	1 h	3 h	5 h	7 h
Control	70.5±1.1	81.0±5.2	75±5.8	73±3.3	71±0.7	70±1.4	70±1.0
Alloxan 150 mg/kg	72.0±0.7	195±60.8	193±6	222±7	202±6	206±6	207±6
Glibenclamide 0.60 mg/kg	70.2±1.0	258±31.5	79±1.4	76±1.8	73±1.8	71±1.0	70±1.1
Alloxan 150 mg/kg + 200 mg/kg ME	70.7±1.2	196±54.8	192±7	220±8	201±6	207±6	205±9
Alloxan 150 mg/kg + 400 mg/kg ME	70.7±1.0	197±62.8	191±6	221±7	204±5	208±5	207±6

Table 6. Results of anti-cancer activity

OD at 540 nm (molt-4 cells)	Conc. of ME (µg/µL)
0.810	0
0.444	2.723
0.390	4.668
0.370	5.057
0.326	5.835
0.325	6.224
0.315	7.391
0.309	8.167
0.290	8.947
0.290	9.336
0.255	9.726

Fig.1. Photograph of *Gracilaria corticata* J.Ag.

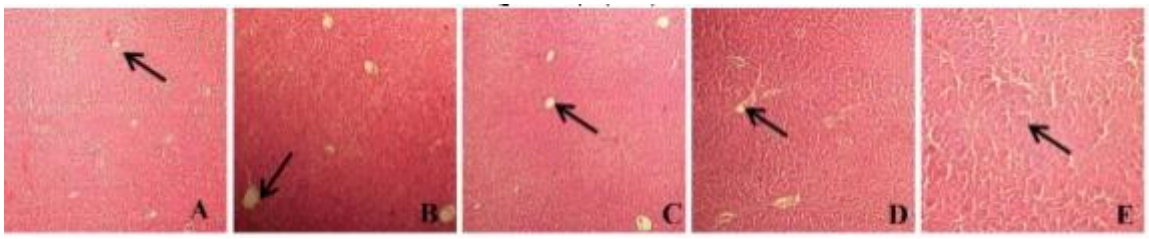

Fig. 2. Histology of liver from each group after 21-day treatment with 40 times magnification in microscope. A: Normal group, B: Control group, C: ME of *G. corticata* 200 mg/kg b.w.; D: ME of *G. corticata* 400 mg/kg b.w.; E: Orlistat 5 mg/kg b.w. The arrow is pointed at small circle where the fat existed.

Serum levels of triglycerides: A significant increase of triglyceride serum levels on the control group comparing to normal group. The results proved that administration of high fat diet caused their elevation. Group treated with ME of *G. corticata* 200 and 400 mg/kg showed lower triglyceride levels remarkably compared to the control group (Table 4).

Anti-diabetic activity (Alloxan induced model): The test group treated with 200 and 400 mg/kg ME of *G. corticata*, does not showed the significant decreased in the blood glucose level when compared to the group treated with alloxan. From the present investigation, it can be concluded that ME of *G. corticata* does not show the anti-diabetic activity (Table 5).

Anti-cancer activity (MTT assay): As shown in (Table 6), the result of MTT assay confirmed that the most effective conc. of ME of *G. corticata* against molt-4 cells was 9.726 µg/µL. Based on previous experience, filtration method is the best way for algal extract sterilization as per Zandi et al.[14]. The heat sensitivity of some biological constituents of algal extract is the most important reason for not using autoclave for sterilizing extract. In this study, the ME of *G. corticata* showed reasonable activity against tumor cells replication. The most effective conc. of ME of *G. corticata* against Molt-4 cells is 9.726 µg/µL. 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 *G. corticata* extract in future studies is recommended.

CONCLUSION:

Anti-ulcer activity (**Aspirin induced model):** From the results obtained, it can be concluded that the ME of *G. corticata*, possess significant anti-ulcer activity. Among the two conc. of ME studied, 400 mg/kg had the highest effect than 200 mg/kg.

Anti-obesity activity (MSG induced model): From the results by HFD-induced and MSG-induced models, it can be concluded that the ME of *G. corticata*, possess significant anti-obesity activity. Among the two conc. of ME studied, 400 mg/kg had the highest effect than 200 mg/kg.

Anti-diabetic activity (Alloxan induced model): From the results obtained, it can be concluded that the ME of *G. corticata* does not show the anti-diabetic activity.

Anti-cancer activity (MTT assay): From the results obtained, it can be concluded that the ME of *G. corticata*, possess significant anti-tumor activity against Molt-4 cell lines. Among the two conc. of ME studied, 400 mg/kg had the highest effect than 200 mg/kg. Also, in regards 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 *G. corticata* 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.

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