

Modulating Effect of *Hypnea musciformis* (Red Seaweed) on Lipid Peroxidation, Antioxidants and Biotransforming Enzymes in 7,12-Dimethylbenz (a) Anthracene Induced Mammary Carcinogenesis in Experimental Animals

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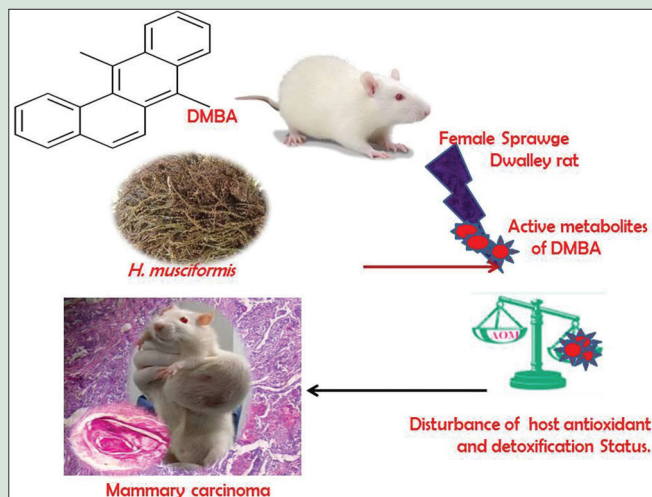
ABSTRACT

Background: Breast cancer is the second most widespread diagnosed cancer and second leading cause of cancer death in women. **Objective:** The present work was carried out to evaluate the chemo preventive potential of *Hypnea musciformis* (ethanol extract) seaweed on oxidative stress markers, bio transforming enzymes, incidence of tumors, and pathological observation in 7,12-dimethylbenzanthracene (DMBA) exposed experimental mammary carcinogenesis. **Materials and Methods:** Female Sprague–Dawley rats were randomly divided into four groups. Rats in the group 1 served as control. Rats in the group 2 and 3 received a single subcutaneous injection of DMBA (25 mg/kg body weight (b.w)) in the mammary gland to develop mammary carcinoma. In addition, group 3 rats were orally administrated with 200 mg/kg between of *H. musciformis* along with DMBA injection and group 4 rats received ethanolic extract of *H. musciformis* every day orally (200 mg/kg b.w) throughout the experimental period of 16 weeks. **Results:** Our results revealed that treatment with *H. musciformis* ethanolic extract to DMBA treated rats significantly reduced the incidence of tumor and tumor volume as compared to DMBA alone treated rats. Moreover, our results showed imbalance in the activities/levels of lipid peroxidation by products, antioxidant enzymes, and bio transforming phase I and II enzymes in the circulation, liver and mammary tissues of DMBA treated rats which were significantly modulated to near normal on treatment with ethanolic extract of *H. musciformis*. All these alterations were supported by histochemical findings. **Conclusion:** The results obtained from this study suggest that chemo preventive potential of *H. musciformis* ethanol extract is probably due to their free radicals quenching effect and modulating potential of bio transforming enzymes during DMBA exposed experimental mammary carcinogenesis.

Key words: Chemo prevention, *Hypnea musciformis*, mammary carcinogenesis, oxidative stress, seaweeds

SUMMARY

- DMBA is a source of well-established site specific carcinogen
- *Hypnea musciformis* act as a free radical quencher
- *Hypnea musciformis* has a definite chemo preventive efficacy in experimental rats
- *H. musciformis* is a resource of prooxidant/antioxidant balance and also its anti-proliferative effects
- *H. musciformis* has a detoxificant in the mammary carcinoma.



Abbreviations Used: BRCA1: Breast Cancer Gene 1; BRCA2: Breast Cancer Gene 2; CYP: Cytochrome P450; DMBA: 7,12-Dimethylbenzanthracene; DMSO: Dimethyl sulfoxide; H₂O₂: Hydrogen peroxides; LPO: Lipid peroxidation; PAH: Polycyclic aromatic hydrocarbon; ROS: Reactive oxygen species; TBARS: Thiobarbituric acid reactive substances; GSSG: Oxidized glutathione.

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INTRODUCTION

Breast cancer is one of the leading causes of morbidity and mortality in women's lives in both developed and developing countries. Worldwide, breast cancer is the second most common type of cancer and fifth most common cause of cancer death.^[1] Several genetic and environmental factors have been identified as causative factors for the development of breast cancer.^[2] Genetic disposition with the presence of genes such as the BRCA1 and BRCA2, increased lifetime exposure to environmental pollutants, mutations in the tumor suppressor genes; hormonal status,

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and sedentary lifestyle are some of the most important risk factors known to influence the incidence of breast cancer.^[3] It has been reported that one in four new cancers diagnosed worldwide each year is a cancer of the female breast. In India, breast cancer has emerged as the leading 70,000 new cases of breast cancer and 35,000 deaths due to this cancer are reported every year.^[4]

Development of mammary tumors involves the aberrant accumulation of cells caused by irreversible genetic alterations, including loss of function of the tumor suppressor, deregulated cell cycle control, excessive proliferation, and diminished apoptosis.^[5] 7,12-dimethylbenzanthracene (DMBA) is a polycyclic aromatic hydrocarbon (PAH) produced during the incomplete combustion of carbon-containing compounds and predominantly found in tobacco smoke and motor vehicle exhaust emissions.^[6] DMBA exposed mammary carcinogenesis was one of the widely studied experimental models in several chemoprevention studies. The conversion of DMBA to its ultimate carcinogenic metabolites is mainly accomplished by the cytochrome P450 (CYP) family enzymes.^[7] In particular, CYP1A isoforms are responsible for bio-activation in the liver, the major organ of DMBA metabolism, while CYP1B enzymes are reported to exert their activity predominantly in extra hepatic tissues such as the mammary gland.^[8]

Many recent studies have been carried out to find several potential chemopreventive agents from edible and natural resources such as fruits, vegetables, and terrestrial plants.^[9] Epidemiological evidence suggests regular seaweed consumption may protect against a range of diseases of modernity. Extracts of seaweeds are believed to contain different chemopreventive or chemotherapeutic compounds, which possess more than one mechanism of action and block tumorigenesis.^[10] Among the diverse pathways, modulation of carcinogen-induced genotoxicity, inhibition of carcinogen activation by altering the activities of phase I and II enzymes, and scavenging of reactive oxygen species (ROS) by antioxidant defense systems have assumed significance.^[11] The active metabolites of most carcinogens are thought to evoke the formation of oxygen-derived free radicals and intermediates of oxygen products such as hydrogen peroxides (H₂O₂).^[12] An imbalance in phase I and phase II xenobiotic metabolizing enzymes has been documented in a wide range of carcinogen-induced malignant tumors models.

Hypnea musciformis is an emerging seaweed and one of the important sources for dietary supplements and potentially active and useful. *H. musciformis* is an effective scavenger of ROS and may also function indirectly as an antioxidant through its effects on enzyme activities, which was recently evaluated in our laboratory.^[13] *H. musciformis* in recent times have gained great importance by virtue of its numerous biological properties such as antibacterial and antioxidant activities.^[14] Based on this background, the present study aimed to evaluate the chemopreventive efficacy of *H. musciformis* major marine seaweeds with special focus on its impact in DMBA exposed biochemical and pathological changes, in an experimental model of mammary carcinogenesis.

MATERIALS AND METHODS

Chemicals

DMBA, thiobarbituric acid (TBA), phenazine methosulfate, nitroblue tetrazolium, 5,5-dithiobis 2-nitro benzoic acid, 1-choloro 2,4-dinitrobenzoic acid, and 2,6-dichlorophenol indophenol were obtained from Sigma Aldrich Chemicals Co., St. Louis, MO, USA. The rest of the chemicals and solvents used were of analytical grade and purchased from HiMedia Laboratories Ltd., Mumbai, India.

Care of experimental animals and diet

The experiment carried out with 24 number of female Sprague–Dawley rats aged 12–14 weeks weighing between 130 and 150 g were obtained

and maintained at the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, Tamil Nadu, India. The rats were maintained as per the principles and guidelines of the Ethical Committee for Animal Care at Annamalai University in accordance with the Indian National Law on Animal Care and use. After making proper approval from the Institutional Animal Ethics by the Committee for the Control and Supervision of Experimental Animal guidelines (Reg. No. 160/1999/CPCSEA: 867). The rats were housed in polypropylene cages at room temperature 27°C ± 2°C with relative humidity 55% ± 5% at experimental room under 12 h light/12 h dark cycle till the end of the experimental period. During the experimental period, animals were fed in commercial pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum* throughout the experimental period of 16 weeks.

Collection and identification of seaweed

H. musciformis wulf. Lamour. (Red seaweed) was collected from the intertidal and subtidal habitat of the Pudumadam coastal area (Lat: 9.27702°N and Long.: 78.9938449°E) located on the Southeast coast of Tamil Nadu, India. The collection was performed during May 2013 when red algal diversity remains dominant. The seaweed was identified by referring the classification of seaweed cited by Krishnamurthy and Joshi.^[15] Tewari.^[16] Live healthy and matured seaweeds were harvested manually and washed thoroughly in running water to remove epiphytes, animal castings, sand and calcareous, and other adhering detritus matters. Cleaned seaweed materials were shade dried for 7 days under an air jet to prevent photolysis and thermal degradation. The completely dried material was weighed and ground coarsely in a mechanical grinder.

Preparation of ethanolic extracts of *Hypnea musciformis*

500 g fine powdered *H. musciformis* sample was extracted in soxhlet apparatus using 40,000 ml as solvents for 8 h at 60°C according to the method of Khan *et al.*^[17] The extracts were filtered using whatman No. 1 filter paper and kept it under hot air oven (40°C) for the solvent evaporation. The residues obtained were stored in a freezer at 5°C. Residues of *H. musciformis* was suspended in 1% DMSO just before treatment and was administered everyday orally at the dose of 200 mg/kg body weight for the 16 weeks study period.

Induction of mammary carcinoma

For inducing experimental mammary cancer, single subcutaneous injection of 25 mg/kg between DMBA, was dissolved in 1 ml emulsion of 0.75 ml of sunflower oil and 0.25 ml of physiological saline, which were accelerated mammary cancer in female Sprague–Dawley rats.^[18]

Experimental design

Rats were randomly divided into four experimental groups of eight rats in each. Rats in group 1 received standard pellet diet along with 1% DMSO and served as normal control. Group 2 rats received 25 mg/kg between of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline by the subcutaneous injection once on 1st week of the experiment, and received no more treatment and served as diseased. Group 3 rats were treated with DMBA injection as in group 2 in addition, oral administration of *H. musciformis* ethanolic extract at the dose of 200 mg/kg between throughout the experimental period of 16 weeks. Group 4 rats received oral administration of *H. musciformis* ethanolic extract alone at the dose of 200 mg/kg between throughout the experimental period of 16 weeks and served as a test control.

Body weight and growth rate changes

During the experimental period, the body weight and growth rate of control and experimental rats were measured. The rats were weighed at the beginning of the experiment, consequently once a week and finally before sacrifice.

Measurement of tumor incidence

To measure the incidence of tumor and tumor burden at the end of the 16th week study, the rat mammary tumor were removed and flushed with saline. The tumors were carefully measured using Vernier Calliper and calculated using the formula $V = 4/3\pi (D1/2) (D2/2) (D3/2)$, where D1, D2, and D3 are the three diameters (mm) of the tumor.

Preparation of sample for oxidative stress markers

The experiment was terminated at the end of the 16th week and animals in different groups were sacrificed under anesthesia (intraperitoneal administration of ketamine hydrochloride, 30 mg/kg body weight) by cervical decapitation after an overnight fasting. Blood was collected in heparinized tubes and plasma was separated by centrifugation at 2000 ×g for 10 min. After the separation of plasma, the buffy coat was removed and packed red blood cells were washed thrice with cold physiological saline (0.9% NaCl). Erythrocyte lysate was prepared by lysing a known volume of red blood cells with hypotonic phosphate buffer, pH 7.4. Centrifuging at 3000 ×g for 10 min at 4°C separated the hemolysate. Immediately after the sacrifice, liver and mammary tissue were dissected out and washed with saline. The tissues were minced and homogenized with 3 volumes (w/v) of the appropriate buffer using a Potter-Elvehjem homogenizer with a Teflon pestle and centrifuged at 1200 ×g for 20 min at 4°C. The supernatant was used for antioxidants and lipid peroxidation (LPO) measurement.

Preparation of cytosolic and microsomal fractions

Liver and mammary tissue were homogenized by using 0.25 M sucrose solution in 10 mM Tris-HCl buffer (pH 7.4), centrifuged at 9000 ×g for 20 min and the supernatant was collected. The collected supernatant was centrifuged at 100,000 ×g for 20 min and the clear cytosolic fractions obtained were used for phase II enzyme analysis such as glutathione S-transferase (GST), reduced glutathione (GR), DT-diaphorase (DTD), and γ -glutamyl transpeptidase (GGT). The pellet after centrifugation at 100,000 ×g was resuspended in ice-cold 0.15 M Tris-HCl buffer (pH 7.4) and centrifuged for 60 min at 100,000 ×g. The microsomal pellet was resuspended in homogenization buffer and used for the assay of phase I enzymes such as CYP and cytochrome b5.

Determination of lipid peroxidation

LPO was estimated by measuring the concentration of TBA reactive substances (TBARS) by the method of Ohkawa *et al.*^[19] in the tissues by the method of Yagi^[20] in the plasma and Donnan^[21] in the erythrocytes.

Determination of antioxidants

Superoxide dismutase (SOD) activity was assayed by the method of Kakkar *et al.*^[22] and catalase (CAT) by the method of Sinha.^[23] Reduced glutathione (GSH) was determined by the method of Ellman^[24] glutathione peroxidase (Gpx) by the method of Rotruck *et al.*^[25] Ascorbic acid (Vitamin C) content was estimated by the method of Roe and Kuether^[26] and α -tocopherol (Vitamin E) content by the method of Baker *et al.*^[27]

Assay of phase I enzymes (microsomal fraction)

CYP (microsomal fraction) and cytochrome b5 were measured by the method of Omura and Sato.^[28]

Assay of phase II enzymes (cytosolic fraction)

GST was assayed by the method of Habig *et al.*^[29] GR by the method of Carlberg and Mannervik,^[30] DTD activity by the method of Ernster *et al.*^[31] and GGT by the method of Fiala *et al.*^[32]

Histopathological study

Slices of the liver and mammary tissue from a different group were fixed in 10% neutral buffered formalin for 1-week at room temperature. Then the specimens were dehydrated by passing graded series of ethanol, cleaned with xylene, and embedded in paraffin wax. Tissue blocks were sectioned and processed by the routine histological methods with hematoxylin and eosin (H and E) staining. Histological changes in the stained sections were viewed under the light microscope.

Statistical analysis

The results were expressed as means \pm standard deviation of six rats per group. Data were analyzed by one-way analysis of variance and any significant difference among the treatment groups was evaluated by Duncan's multiple range test. The results were considered statistically significant when $P < 0.05$. All statistical analyzes were performed using Statistical Package for the Social Sciences (SPSS) version 15.0 software package (SPSS, Tokyo, Japan).

RESULTS

Effect of *Hypnea musciformis* ethanolic extract on body weight and growth rate changes

Table 1 demonstrates the effect of *H. musciformis* ethanolic extract for 16 weeks on mean body weight and growth rate changes in the control and experimental rats. From week 0 to 16, variable changes were observed in the body weight of the rats in the different groups. At the end of the 16th week, DMBA alone (group 2) induced rats showed decreased weight gain as compared to that of control rats (groups 1 and 4). However, this significant ($P < 0.05$) weight loss was prevented by administration with *H. musciformis* ethanolic extract at the dose of 200 mg/kg to DMBA treated rats (groups 3) thereby offering an optimum protection to rats against DMBA exposed mammary carcinogenesis. Besides *H. musciformis* ethanolic extract alone administrated rats did not show any significant complaint and instigated normal growth of rats when compared to control rats (group 1).

Effect of *Hypnea musciformis* ethanolic extract on tumor incidence

Table 2 describes the frequency of tumor volume and burden of control and experimental rats. The incidence of the tumor was higher (100%) in DMBA alone induced rats (group 2). However, administration with

Table 1: Effect of *H. musciformis* extract on initial and final body weight changes of control and experimental rats

Groups	Initial body weight (g)	Final body weight (g)	Weight gain (g)
Control	141.71 \pm 6.81 ^a	215.91 \pm 8.37 ^c	72.80 \pm 3.88 ^a
DMBA	143.38 \pm 7.92 ^a	175.62 \pm 5.83 ^c	31.87 \pm 4.71 ^{ba}
DMBA + <i>H. musciformis</i>	138.84 \pm 10.36 ^a	205.33 \pm 7.06 ^c	66.21 \pm 2.58 ^c
<i>H. musciformis</i> alone	141.25 \pm 9.77 ^a	219.71 \pm 8.56 ^a	78.27 \pm 5.91 ^a

Values are expressed as means \pm SD of eight rats in each group values not sharing common superscript (a-c) differ significantly from each other at $P < 0.05$ (ANOVA followed by DMRT). SD: Standard deviation; *H. musciformis*: *Hypnea musciformis*; DMBA: 7,12-dimethylbenzanthracene; ANOVA: Analysis of variance; DMRT: Duncan's multiple range test

Table 2: Effect of *H. musciformis* extract on the tumor incidence, volume and burden of control and experimental rats

Groups	Total number of rats	Number of tumor bearing rats	Percentage of tumor incidence	Percentage of tumor inhibition	Tumor volume (mm ³)	Tumor burden (mm ³)
Control	8	0	-	-	-	-
DMBA	8	8	100	0	3683.75±254.22 ^a	1514.22±114.63 ^b
DMBA + <i>H. musciformis</i>	8	2	25	66.67	398.58±27.91 ^a	249.86±16.83 ^b
<i>H. musciformis</i> alone	8	0	-	-	-	-

Values are expressed as mean±SD for eight rats in each group. Tumor volume was measured using the formula $V=4/3\pi (D1/2) (D2/2) (D3/2)$, where D1, D2, and D3 are the three diameters (mm) of the tumor. SD: Standard deviation; *H. musciformis*: *Hypnea musciformis*; DMBA: 7,12-dimethylbenzanthracene; ANOVA: Analysis of variance

Table 3: Effect of *H. musciformis* extract on the levels of TBARS in the plasma, erythrocyte and mammary tissue of control and experimental rats

Parameter	Control	DMBA	DMBA + <i>H. musciformis</i>	<i>H. musciformis</i> alone
Plasma TBARS (nmol/mL)	3.12±0.15 ^a	5.67±0.25 ^b	2.80±0.14 ^c	3.19±0.14 ^a
Erythrocyte (pmol/mg Hb)	1.82±0.08 ^a	3.82±0.12 ^b	1.47±0.09 ^c	1.92±0.35 ^a
Mammary tissue (mmol/mg tissue)	1.63±0.12 ^a	0.69±0.03 ^b	1.38±0.11 ^c	1.72±0.28 ^a

Data are presented as the means±SD of eight rats in each group. Values not sharing a common superscript letter (a-e) differ significantly at $P<0.05$ (ANOVA followed by DMRT). SD: Standard deviation; *H. musciformis*: *Hypnea musciformis*; DMBA: 7,12-dimethylbenzanthracene; ANOVA: Analysis of variance; TBARS: Thiobarbituric acid reactive substances; DMRT: Duncan's multiple range test

H. musciformis ethanolic extract at the dose of 200 mg/kg to DMBA treated rats (group 3) the incidence of mammary tumor was significantly lowered (25%) as compared to DMBA alone induced rats (group 2). *H. musciformis* treatment more potentially reduced the tumor volume and burden and also reduced the percentage of tumor incidence in a total number of rats which were more beneficial.

Effect of *Hypnea musciformis* on lipid peroxidation by product (thiobarbituric acid reactive substances)

Table 3, represents the effect of *H. musciformis* ethanolic extract on the level of the LPO by products TBARS of control and experimental rats. DMBA alone induced rats (group 2) the levels of TBARS was significantly ($P < 0.05$) increased in the plasma and erythrocytes whereas in the mammary tissue the levels were significantly decreased as compared to the control and *H. musciformis* ethanolic extract at the dose of 200 mg/kg between alone administrated rats (group 1 and 4). *H. musciformis* at the dose 200 mg/kg body weight (group 4) did not show any changes on the levels of LPO by products. Therefore, it can be inferred that the effect was more pronounced on mammary carcinoma.

Effect of *Hypnea musciformis* ethanolic extract on the activities of antioxidants

Tables 4 and 5 show the activities of the enzymic antioxidants such as SOD, CAT, Gpx and nonenzymic antioxidants GSH, and Vitamin C and Vitamin E of control and experimental rats. Our results indicate that the activities and levels of antioxidants were significantly ($P < 0.05$) diminished in the plasma, erythrocytes, and mammary tissue of DMBA alone induced rats (group 2) as compared to the control rats (group 1). However, the administration of *H. musciformis* ethanolic extract markedly increased the activities of SOD, CAT, and GPx levels when compared to carcinogen alone treated rats (group 3). In addition *H. musciformis* ethanolic extract alone at the dose of 200 mg/kg between administrated rats did not exerts any significant changes on the antioxidants level which showed near normal level as compared to control rats (group 1).

Effect of *Hypnea musciformis* ethanolic extract on phase I (microsomes) xenobiotic metabolizing enzymes

Table 6 illustrate the effect of *H. musciformis* ethanolic extract on the activities of phase I enzymes in the microsomal fraction of control and experimental rats. DMBA treated rats (group 2) showed enhanced activities phase I enzymes such as CYP and cytochrome b5 in the liver and mammary tissues as compared to the control rats (group 1). *H. musciformis* administration to DMBA treated rats (group 3) during the entire experimental period of 16 weeks significantly ($P < 0.05$) reduced the activities of liver and mammary CYP and cytochrome b5 as compared to DMBA alone treated rats. *H. musciformis* alone administration rats did not show any significant changes on the activities of phase I xenobiotic metabolizing enzymes (group 4).

Effect of *Hypnea musciformis* ethanolic extract on phase II (cytosol) xenobiotic metabolizing enzymes

Table 7 exemplifies the specific activities of phase II enzymes in the cytosolic fraction of the liver and mammary tissue of control and experimental rats. Decreased activities of phase II enzymes such as GST, GR, DTD, and GGT were observed in DMBA alone induced rats (group 2) as compared to the control rats (group 1). *H. musciformis* administration at the dose of 200 mg/kg between during the entire experimental period of 16 weeks to DMBA treated rats showed (group 3) significantly ($P < 0.05$) increased activities of liver and mammary phase II enzymes at manifest level as compared to the DMBA alone treated rats (group 2). *H. musciformis* alone administration rats did not show any significant changes in the activities of phase II enzymes (group 4).

Effect of *Hypnea musciformis* ethanolic extract on the histopathology

Figure 1 depicts the photomicrographs of H and E stained liver sections of the control and experimental rats. Liver of control and *H. musciformis* alone administered rats (groups 1 and 4) showed normal liver architecture (NA) (a and d). Rats induced with DMBA alone (group 2) triggered changes in the liver morphology characterized by micro and macro vesicular type of fatty changes, sinusoidal dilation, nuclear pleomorphism, and inflammatory cell infiltration in the portal triad (b). Liver of DMBA treated rats administrated with *H. musciformis* ethanolic extract at the dose of 200 mg/kg between showed very occasionally lymphoid aggregates and fewer neoplastic cells (c).

Effect of *Hypnea musciformis* ethanolic extract on the mammary tissue histopathology

Figure 2 denotes the photomicrographs of H and E stained mammary tissue sections of the control and experimental rats. The mammary tissue of control and *H. musciformis* alone administered rats (groups 1 and 4) showed NA (a and d). DMBA alone induced rat mammary tissue (group 3) showed the sequence of adenocarcinoma

Table 4: Effect of *H. musciformis* extract on the antioxidants status of control and experimental rats

Parameters and sample	Control	DMBA	DMBA + <i>H. musciformis</i>	<i>H. musciformis</i> alone
Plasma				
SOD (50% NBT reduced/min/mg protein)	5.71±0.68 ^a	2.75±0.14 ^b	4.00±0.08 ^c	5.94±0.14 ^a
CAT (μmoles of H ₂ O ₂ utilized/min/mg protein)	53.56±4.30 ^a	27.94±2.04 ^b	29.35±1.77 ^c	40.00±3.63 ^a
Gpx (μmoles of GSH utilized/min/mg protein)	11.73±0.44 ^a	6.17±0.48 ^b	2.92±0.19 ^c	4.83±0.30 ^a
GSH (mmoles/mg tissue)	33.55±2.07 ^a	18.60±2.32 ^b	11.33±0.99 ^c	16.88±1.43 ^a
Vitamin C (mg/dl)	1.22±0.07 ^a	0.64±0.02 ^b	1.03±0.10 ^c	1.27±0.04 ^a
Vitamin E (mg/dl)	1.54±0.03 ^a	0.71±0.02 ^b	1.35±0.09 ^c	1.51±0.07 ^a
Erythrocytes				
SOD (50% NBT reduced/min/mg protein)	6.18±0.36 ^a	2.09±0.16 ^b	5.97±0.48 ^{ac}	6.08±0.59 ^a
CAT (μmoles of H ₂ O ₂ utilized/min/mg protein)	37.64±2.27 ^a	18.33±1.38 ^b	31.55±3.08 ^c	36.77±1.82 ^a
Gpx (μmoles of GSH utilized/min/mg protein)	31.89±1.59 ^a	17.92±1.05 ^b	26.39±2.11 ^c	33.69±2.85 ^a
GSH (mmoles/mg tissue)	47.91±2.84 ^a	23.94±1.53 ^b	43.77±3.65 ^c	45.40±3.93 ^a
Vitamin C (mg/dl)	3.80±0.31 ^a	1.28±0.1 ^b	3.04±0.11 ^c	3.78±0.11 ^a
Vitamin E (mg/dl)	4.06±0.27 ^a	1.99±0.15 ^b	3.58±0.15 ^c	4.15±0.32 ^a

Data are presented as the means±SD of eight rats in each group. Values not sharing a common superscript letter (a-e) differ significantly at $P < 0.05$ (ANOVA followed by DMRT). SD: Standard deviation; *H. musciformis*: *Hypnea musciformis*; DMBA: 7,12-dimethylbenzanthracene; ANOVA: Analysis of variance; SOD: Superoxide dismutase; CAT: Catalase; NBT: Nitroblue tetrazolium; H₂O₂: Hydrogen peroxides; GSH: Glutathione; Gpx: Glutathione peroxidase; DMRT: Duncan's multiple range test

Table 5: Effect of *H. musciformis* extract on the antioxidant status in the mammary tissues of control and experimental rats

Parameters and sample	Control	DMBA	DMBA + <i>H. musciformis</i>	<i>H. musciformis</i> alone
SOD (50% NBT reduced/min/mg protein)	15.74±1.06 ^a	8.91±0.57 ^b	13.63±0.91 ^c	16.08±0.94 ^a
CAT (μmoles of H ₂ O ₂ utilized/min/mg protein)	79.28±4.58 ^a	44.05±2.61 ^b	67.42±4.07 ^c	79.63±6.58 ^a
Gpx (μmoles of GSH utilized/min/mg protein)	16.05±1.11 ^a	7.38±0.38 ^b	13.38±1.14 ^c	15.77±1.03 ^a
GSH (mmoles/mg tissue)	14.83±0.95 ^a	6.88±0.47 ^b	12.55±0.72 ^c	13.59±0.78 ^a
Vitamin C (μg/mg tissue)	5.69±0.31 ^a	2.17±0.13 ^b	4.08±0.26 ^c	5.71±0.52 ^a
Vitamin E (μg/mg tissue)	2.94±0.18 ^a	0.99±0.04 ^b	2.47±0.14 ^{ac}	2.78±0.19 ^a

Data are presented as the means±SD of eight rats in each group. Values not sharing a common superscript letter (a-e) differ significantly at $P < 0.05$ (ANOVA followed by DMRT). SD: Standard deviation; *H. musciformis*: *Hypnea musciformis*; DMBA: 7,12-dimethylbenzanthracene; ANOVA: Analysis of variance; SOD: Superoxide dismutase; CAT: Catalase; NBT: Nitroblue tetrazolium; H₂O₂: Hydrogen peroxides; GSH: Glutathione; Gpx: Glutathione peroxidase; DMRT: Duncan's multiple range test

Table 6: Effect of *H. musciformis* on phase I enzymes in the liver and mammary tissue of control and experimental rats

Parameters samples	Control	DMBA	DMBA + <i>H. musciformis</i>	<i>H. musciformis</i> alone
Liver				
Cytochrome P450 (μmoles/mg protein)	0.83±0.04 ^a	1.98±0.11 ^b	1.13±0.05 ^c	0.85±0.03 ^a
Cytochrome b5 (μmoles/mg protein)	1.05±0.11 ^a	2.12±0.15 ^b	1.39±0.45 ^c	0.98±0.06 ^a
Mammary tissue				
Cytochrome P450 (μmoles/mg protein)	1.28±0.05 ^a	2.35±0.12 ^b	1.57±0.12 ^c	1.19±0.08 ^a
Cytochrome b5 (μmoles/mg protein)	1.31±0.09 ^a	2.17±0.15 ^b	1.48±0.07 ^{ac}	1.27±0.11 ^a

Data are presented as the means±SD of eight rats in each group. Values not sharing a common superscript letter (a-c) differ significantly at $P < 0.05$ (ANOVA followed by DMRT). SD: Standard deviation; *H. musciformis*: *Hypnea musciformis*; DMBA: 7,12-dimethylbenzanthracene; ANOVA: Analysis of variance; DMRT: Duncan's multiple range test

and predominant ductal hyperplasia (DH) (b). The mammary tissue of DMBA treated rats administrated with *H. musciformis* ethanolic extract at the dose of 200 mg/kg between showed DH occasionally (c).

DISCUSSION

Nowadays diet and dietary constituents attributable to their high tolerability and low toxicity square measure receiving additional attention as potential cancer chemo preventive agents.^[33] Edible seaweeds have traditionally been consumed by coastal populations across the world. Today, algae remain a part of the habitual diet in several developed and

developing countries. Evidence regarding the presence of antioxidant properties in marine seaweeds has led many researchers to direct considerable efforts for its use in protection against carcinogenesis. Previous study in our laboratory, we evaluated the major components of *H. musciformis* ethanolic extract, which contain carbohydrate, protein, and mineral and has a moderate concentration of fat and more than 26 biologically important compounds on the ethanolic fraction.^[13] Epidemiological studies strongly suggest that consumption of minerals and antioxidant rich food has attracted a great deal of attention in recent years for their role in the prevention of cancer. Measuring the effect of such agents has now become a major area of experimental cancer research. We selected the DMBA treated rat model of mammary cancer to examine the effect of *H. musciformis* ethanolic extract of red seaweed. Indeed, this was the model used for discovery of antibreast cancer drugs. DMBA-induced mammary tumors in rat are essentially similar in morphology, pathogenesis, and estrogen receptor status to human breast cancer and show estrogen dependent growth.^[34]

During the experimental period of 16 weeks, no adverse effects were observed in *H. musciformis* alone supplemented rats suggesting that prolonged treatment with *H. musciformis* ethanolic extract is well tolerated. Though all the rats in the experimental groups showed an increase in the body weight and growth rate throughout the study period, decreased body weight, and growth rate were observed after 16 weeks in DMBA alone induced rats as compared to the control rats. This may be due to the tumor load, which could elicit host-tumor interactions resulting in multifold increase in the levels of circulating interleukin-6, which can cause severe cachexia as exemplified by loss of muscle weight and fat tissues.^[35] In this context, weight loss is common among people with cancer and is often the first noticeable sign of the disease. There was a significant decrease in the food intake by the rats due to the occurrence

Table 7: Effect of *H. musciformis* on phase II enzymes in the liver and mammary tissue of control and experimental rats

Parameters samples	Control	DMBA	DMBA + <i>H. musciformis</i>	<i>H. musciformis</i> alone
Liver				
Glutathione-S-transferase*	7.82±0.38 ^a	3.59±0.14 ^b	6.31±0.42 ^c	7.73±0.30 ^a
Reduced glutathione*	9.35±0.51 ^a	4.77±0.28 ^b	8.53±0.57 ^c	9.63±0.74 ^a
DTD*	3.41±0.20 ^a	1.09±0.05 ^b	2.89±0.18 ^c	3.52±0.22 ^a
γ-glutamyl transpeptidase*	3.89±0.14 ^a	1.65±0.09 ^b	3.13±0.17 ^c	3.92±0.21 ^a
Mammary tissue				
Glutathione-S-transferase*	4.71±0.31 ^a	2.44±0.13 ^b	4.08±0.27 ^c	4.83±0.19 ^a
Reduced glutathione*	6.82±0.27 ^a	3.08±0.21 ^b	5.92±0.26 ^c	6.94±0.43 ^a
DTD*	0.83±0.02 ^a	0.31±0.01 ^b	0.75±0.04 ^c	0.85±0.06 ^a
γ-glutamyl transpeptidase*	2.16±0.17 ^a	0.76±0.04 ^b	2.04±0.13 ^c	2.18±0.08 ^a

Data are presented as the means±SD of eight rats in each group. Values not sharing a common superscript letter (a-c) differ significantly at $P<0.05$ (ANOVA followed by DMRT). *μmol of CDNB-GSH conjugate formed/min/mg protein, *μmoles of NADPH oxidized/min/mg protein, *μmol of DCPIP reduced/min/mg protein, *nmoles of p-nitroaniline formed/min/mg protein. SD: Standard deviation; *H. musciformis*: *Hypnea musciformis*; DMBA: 7,12-dimethylbenzanthracene; ANOVA: Analysis of variance; DMRT: Duncan's multiple range test; CDNB-GSH: 1-choloro 2,4-dinitrobenzoic acid-glutathione; DCPIP: 2,6-dichlorophenol indophenol; NADPH: Nicotinamide adenine dinucleotide phosphate; DTD: DT-diaphorase

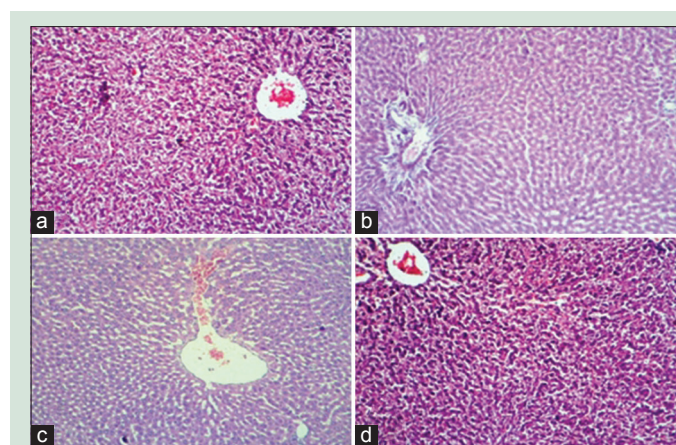


Figure 1: Photomicrographs of hematoxylin and eosin stained liver. Liver of control and *Hypnea musciformis* alone administered rats showed normal liver architecture. (a and d) 7,12-dimethylbenzanthracene alone treated rats showed micro and macro vesicular type of fatty changes, sinusoidal dilation, nuclear pleomorphism, and inflammatory cell infiltration in the portal triad. (b) The liver of 7,12-dimethylbenzanthracene induced rats administered with *Hypnea musciformis* ethanolic extract at the dose of 200 mg/kg between showed very occasionally lymphoid aggregates and fewer neoplastic cells (c)

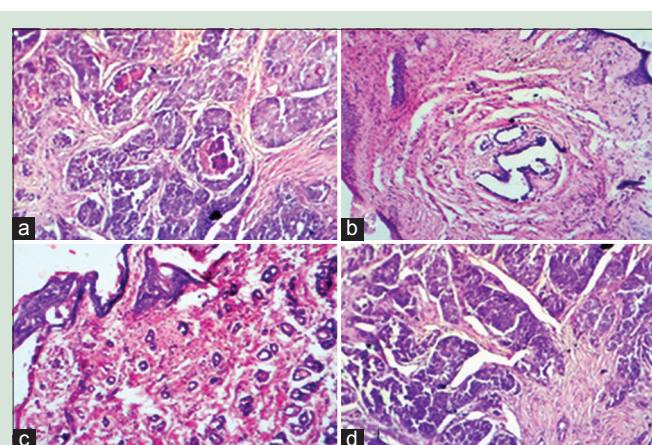


Figure 2: Photomicrographs of hematoxylin and eosin stained mammary tissue. Mammary tissue of control and *Hypnea musciformis* alone administered rats showed normal architecture. (a and d) 7,12-dimethylbenzanthracene alone treated rat mammary tissue showed sequence of adenocarcinoma and predominate ductal hyperplasia. (b) The mammary tissue of 7,12-dimethylbenzanthracene induced rats administered with *Hypnea musciformis* ethanolic extract at the dose of 200 mg/kg between showed ductal hyperplasia occasionally (c)

of a huge number of tumors in the mammary gland, which could again explain the reduced weight gain in DMBA treated rats. Further, the improved body weight gain observed on administration with *H. musciformis* ethanolic extract at the entire period to DMBA exposed rat's correlates with decreased tumor incidence.

ROS are nowadays thought-about as a big category of carcinogens collaborating in cancer initiation, promotion, and progression. It's been clearly established that ROS interfere with the expression of a variety of genes and signal transduction pathways.^[36] The correlation between super molecule peroxidation and look of growth changes is indicative of ROS participation in carcinogenesis. LPO, a well-established mechanism of cellular injury, is used as an indicator of oxidative stress in many chemical carcinogenesis models. The determination of bi-products of LPO in the form of TBARS is one of the most commonly used methods for monitoring LPO levels in the biological samples. Many studies have concluded that LPO was significantly decreased in rat tumor cells and tissues as compared to the corresponding normal tissues.^[37] The site-specific carcinogen DMBA induction to the rats causes deleterious

changes and may induce oxidative stress and production of ROS.^[38] In our study we found that decreased level of tissue TBARS and increased level of TBARS in the circulation (plasma and erythrocyte) which denote that excess production of free radical and released into the circulation, which could be due to the metabolic activation of DMBA. Transformed cancer cells are known to acquire certain characteristics that benefit proliferation,^[39] and they tend to proliferate faster when LPO is low. Therefore, the decreased LPO observed in DMBA treated rats mammary tissue could be due to the increased proliferation, increased resistance and/or decreased susceptibility of the target organs to free radical attack. Moreover, administration with *H. musciformis* significantly elevated the level of mammary TBARS and reduced the levels of TBARS in the circulation which denotes the pro-oxidant potential of *H. musciformis* seaweeds on mammary carcinogenesis.

Phytochemical-rich foods should clearly form part of a healthy balanced diet. However, the human body has a number of physiological, biochemical, and enzymatic processes by which it can combat oxidative stress outside of dietary intake. SOD is a major intracellular catalyst, which protects against the radicals produced by the chemical elements

of catalyzing the dismutation of superoxide free radical and anions to H_2O_2 and oxygen. CAT is an enzyme which is present in most cells and which catalyzes the decomposition of H_2O_2 to water and oxygen. GPx is an equally important antioxidant, which reacts with H_2O_2 thus preventing intracellular damage caused by the same.^[40,41] GSH is an important nonprotein cellular thiol that in conjunction with GPx plays a regulatory role in cell proliferation.^[42] It is essential cofactor for GSH transferases/GSH-peroxidase/Oxidized glutathione (GSSG) reductase system, protects cellular proteins against oxidation through the glutathione redox cycle and also directly detoxifies ROS and neutralizes reactive intermediates generated from exposure to xenobiotics including chemical carcinogen.^[43]

Antioxidant vitamins have a number of biological activities such as immune stimulation, scavenging the free radicals, and alteration in the metabolic activation of carcinogens. They can utilize reactive oxygen metabolites, protect biopolymers and reduce oxidative DNA damage.^[44] In this present study, enzymic and nonenzymic antioxidants such as SOD, CAT, GPx, GSH, and Vitamin C and E levels reduced in the DMBA treated rats when compared to the control rats. However, the administration of *H. musciformis* ethanolic extract the activities of SOD, CAT, and GPx levels are markedly increased when compared to a carcinogen (DMBA) alone induced rats. Whereas the *H. musciformis* ethanolic extract alone administrated rats are well tolerated and there is no difference between the activities of antioxidants when compared to control group. Several studies have reported the decreased activities of antioxidants in various cancer conditions.^[45,46]

In the present study, decreased SOD, CAT, and GPx activities and levels of GSH, Vitamin C and E were observed in DMBA treated mammary carcinoma, which may be due to the altered antioxidant status caused by the chemical carcinogen during their metabolic action. The observed decrease in SOD, CAT, and GPx activities may lead to diminished scavenging of free radicals formed in carcinoma conditions. The increased reactive oxygen radicals themselves reduce the activities of these enzymes. In our study, the administration of *H. musciformis* ethanolic extract to DMBA treated mammary cancer bearing rats restored the anti-per oxidative enzyme (SOD, CAT, and GPx) activities to near normal levels. This antioxidant potency of red seaweeds could be the due presence of pharmacologically active secondary metabolites and the presence of minerals in the ethanolic extract of *H. musciformis*. In addition *H. musciformis* also contain a range of unique phytochemicals which are not present in terrestrial plants, as such, edible *H. musciformis* seaweeds having the capacity is endowed by the presence of polyphenolic compounds and antioxidants components revealing its chemo preventive potential on DMBA mammary carcinogenesis.

Xenobiotic metabolizing enzymes including phase I and phase II enzymes play important roles against toxic chemicals and carcinogens, which are encountered in certain occupational settings and which are not chemically active unless they undergo metabolic activation.^[47] The liver is the most important organ of the body involved in numerous detoxification processes, synthesis of plasma proteins and plays vital role in digestion. DMBA is a potent site-specific procarcinogen and metabolically activated by phase I enzymes (CYP and cytochrome b5) to reactive metabolites (diol epoxides) that can covalently bind to the DNA, thereby stimulating the carcinogenesis process. Reactive metabolites formed during metabolic activation of chemical carcinogen DMBA are detoxified to inactive metabolites and excreted through the excretion system by the action of phase II conjugating enzymes. In the present study, elevated level of CYP and cytochrome b5 were observed in the microsomal fraction of liver and mammary tissue, which could act as metabolic activators of DMBA in the liver, leading to DNA adducts formation.

In addition to the identification of novel anticancer agent, the measurement of phase I and phase II bio transforming enzymes could play an important role in the testing the potential chemo preventive agents.^[48] GST plays a crucial role in the detoxification of electrophilic compounds to protect the tissues against peroxidative damage. GR has an important role in the regeneration of reduced GSH from oxidized GSH in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), thereby preventing the cellular loss of reduced GSH. Measurement of GST and GR in mammary tissues may help to assess the extent of the neoplastic transformation.^[49] GGT, a key enzyme in GSH metabolism provides high intracellular levels of GSH required for conjugation by GST.^[50] DTD is a flavoprotein generally induced along with GST and GGT, which facilitates the metabolism of quinones, thereby protecting cells against the toxins, quinones toxicity, PAHs, and carcinogens.^[51] Therefore stimulation of phase II detoxification system by antioxidants rich marine seaweed.

H. musciformis counteracts the DMBA treated damaging effect. DMBA treated rats showed decreased activities of phase II detoxifying enzymes such as GST, GR, DTD, and GGT whereas this enzyme activity evidently improved on administration with *H. musciformis*. These effects might be due to *H. musciformis* hepatoprotective as well as anticarcinogenic potential exerted by nullifying oxidative injury and restoring antioxidant system thereby protecting the mammary gland and liver from the chemical carcinogen.

CONCLUSION

Chemoprevention directed toward the control of mammary carcinogenesis in its early stages should ultimately provide a higher quality of life for people than waiting to treat end stage disease. Herein, the approach of overall findings of the present biochemical and histological studies provide the evidence that *H. musciformis* ethanolic extract has definite chemo preventive efficacy in experimental rats when supplemented at the dose of 200 mg/kg between as evident by its role in modulating the enzymes activities. *H. musciformis* administration efficiently eliminates the DMBA metabolites and acting as a detoxificant in the mammary carcinoma. This chemo preventive effect could be attributed to its role in maintaining pro-oxidant/antioxidant balance and also its antiproliferative effects. However, further preclinical investigations on the level of molecular cell signaling are warranted to confirm the chemo preventive potential of *H. musciformis* be claimed as a preventive and/or therapeutic agent against mammary carcinogenesis.

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Conflicts of interest

There are no conflicts of interest.

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