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ORIGINAL ARTICLE

Rasam (South Indian Spice Soup) - Attenuates the Mammary Tumor Induction Magnitude of 7,12-Dimethylbenz[a] anthracene in Sprague–Dawley Rats

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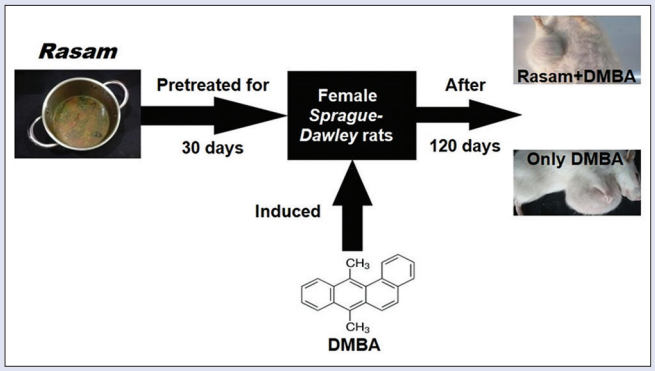
ABSTRACT

Background: Recently, we have reported *rasam* (South Indian spice soup) for antiproliferative activity against Michigan Cancer Foundation-7 cell lines. **Objectives:** Breast cancer seems to be more common in the younger age group. Considering the deviation of the younger group from the traditional food habits as one of the many reasons of breast cancer incidence, *rasam* was investigated for its chemopreventive effect on mammary carcinoma. **Materials and Methods:** *Rasam* at 3 and 4 mL/kg dose was administered to female Spraque–Dawley rats for 30 days before 7,12-dimethylbenz[a] anthracene (DMBA) induction of mammary carcinoma. Tamoxifen was used as the standard drug. Body weight, thiobarbituric acid reactive substances (TBARSs), phase I enzymes, hexokinase (HK), aldolase (AD), glucose 6-phosphatase (G6P), fructose 1,6-bisphosphatase (F16P), citric acid cycle (CAC) enzymes, histopathology, tumor weight, tumor latency, and tumor incidence were studied. **Results:** In all the studied parameters such as body weight, TBARS, phase I enzymes, HK, AD, G6P, F16P, and CAC enzymes, *rasam*-pretreated groups showed better prevention than tamoxifen-treated groups. Histochemical findings also clearly confirm the chemopreventive effect of *rasam*. Particularly at the dose of 4 mL/kg, *rasam* was efficient in reducing the percentage of incidence of tumor, number of tumor, and tumor weight. **Conclusion:** The present study reveals that *rasam* attenuates the mammary tumor induction magnitude of DMBA in female rats.

Key words: *Chaaru*, chemoprevention, *saaru*, spices, tamoxifen, tumor incidence, tumor latency

SUMMARY

- Preventive effect of *rasam* on 7,12-dimethylbenz[a] anthracene (DMBA)-induced mammary carcinoma in female Spraque–Dawley rats was evaluated
- After 30 days of pretreatment with *rasam* (3 and 4 mL/kg), mammary carcinoma was induced by DMBA (25 mg/kg)
- Tamoxifen (10 mg/kg) was used as a standard drug in this experiment
- On the 120th day, the rats were sacrificed; the mammary tumors were excised out for the analysis of biochemical and histological parameters
- Enzymatic and non-enzymatic antioxidants levels were restored to normal levels by the pretreatment of *rasam*
- Modulation of cytochrome (cyt) P450 and cytb_5 by *rasam* prevents the reactive metabolites of DMBA
- *Rasam* pretreatment showed 67.33% of tumor incidence and 65 days of tumor latency
- *Rasam* at a dose of 4 mL/kg showed better chemoprevention than tamoxifen.



Abbreviations used: WHO: World Health Organization; ER: Estrogen receptor; DMBA: 7,12-Dimethylbenz[a] anthracene; SS 316: Stainless steel 316 grade; CPCSEA: Committee for the Purpose Control and Supervision in Experimental Animals; TBARS: Thiobarbituric acid reactive substances; SOD: Superoxide dismutase; CAT: Catalase; GP_X: Glutathione peroxidase; GSH: Reduced glutathione; HK: Hexokinase; AD: Aldolase; G6P: Glucose 6-phosphatase; F16P: Fructose 1,6-bisphosphatase; SDH: Succinate dehydrogenase; IDH: Isocitrate dehydrogenase; MDH: Malate dehydrogenase; cyt: Cytochromes; CAC: Citric acid cycle; ROS: Reactive oxygen species; DNA: Deoxyribonucleic acid, SD: Standard deviation; ANOVA: Analysis of variance; USA: United States of America.

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INTRODUCTION

Breast cancer is the most commonly occurring cancer in women. Moreover, the increase in the incidence of breast cancer worldwide is alarming. Although various therapies are available, affordability of high healthcare cost is beyond the reach of common people. Hence, the better approach would be to prevent and/or to reduce the increasing incidence of breast cancer. There is a growing interest in the chemopreventive approach coupled with multiple health benefits among researchers for breast cancer. The current drugs approved to prevent breast cancer

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are raloxifene hydrochloride and tamoxifen citrate, which result in several major side effects.[1] Chemopreventive therapy of tamoxifen does not reduce the estrogen receptor (ER)-negative cancer incidence. Moreover, ER-positive precancerous lesions can also resist tamoxifen chemopreventive therapy.[2] The World Health Organization suggests a long-term measure to have an influence in the reduction of breast cancer incidence, which is to control specific modifiable risk factors such as healthy diet, overweight, and obesity.[3]

Epidemiological, preclinical, and clinical studies indicate that spices have potential possibilities with multiple anticancer characteristics.[4] Spices play an important role in digestive function, and traditionally, in India, spices have been used in food as medicines to prevent and treat diseases.

Rasam is a spice soup indigenous to South India, also called as *chaaru* or *saaru* in different South Indian languages. It is traditionally consumed on daily basis in every South Indian home along with rice. *Rasam* is traditionally prepared using tamarind juice and a number of spices such as coriander, garlic, curry leaves, tamarind, cumin, black pepper, mustard, turmeric, red chili, and asafoetida, which are considered to be good for health. *Rasam* has been reported for health maintenance, as a cure for mineral deficiency and treatment for cold, cough, and diabetes.[5] *Rasam* is a functional food because the ingredients used are traditionally claimed for various therapeutic indications. Recently, *sambar*, a South Indian traditional dish, has been reported for preventive effect against colon cancer.[6] Hence, there is a need to understand traditional systems and to visualize the future of preventive medicine. Methodical chronic consumption of traditional foods with functional ingredients may prevent and/or delay the incidence of numerous diseases.[7] Linking the medicinal knowledge from the past to the future can provide new directions towards health, disease prevention, and possible medicines.[8]

Food has always played an expanded role apart from providing nutrition. Most of the time-tested traditional foods consumed in different cultures always contain functional ingredient/s. It would not be incorrect to label traditional foods as functional foods. There has been a cultural deviation from traditional food habits (rice with *sambar*, *rasam*, and butter milk) among South Indians, particularly in the younger people. Studies have shown that there is a sharp rise in the incidence of cancer in the younger group, specifically breast cancer in younger women.[9] A study on *rasam*, which is being consumed from time immemorial, is only an approach of "drug rediscovery." Recently, we have reported *rasam* for antiproliferative activity against Michigan Cancer Foundation-7 (MCF-7) cell lines beyond its culinary and nutritional effect.[10] In continuation of our ongoing research in exploring the Indian traditional functional food, *rasam* as a preventive medicine hitherto unreported, we herein aim to investigate the preventive effect of *rasam* on 7,12-dimethylbenz[a] anthracene (DMBA)-induced mammary carcinoma in female Spraque–Dawley rats.

MATERIALS AND METHODS

Ingredients and utensils

All ingredients used for the preparation of *rasam* were purchased from Arokya Organic Shop, Vellore, Tamil Nadu, India. The ingredients were authenticated and voucher specimens were deposited. Botanical source of all ingredients used is presented in Supplementary Table 1. All utensils used for the preparation of *rasam* were of stainless steel 316 grade.

Preparation of *rasam*

Rasam was prepared as per the standardized procedure reported by Devarajan and Raja.[11] Traditionally, *rasam* is received in palm and filtered through the gaps in fingers to the eating plate. Hence, the supernatant of *rasam* was collected and used for further studies. The volume of *rasam* studied is based on an average consumption of a human adult-to-animal dose conversion.[5]

Chemicals

DMBA was acquired from Sigma Chemicals (Mumbai, India). Other chemicals of analytical grade were obtained from SD Fine-Chem Limited (Mumbai, India).

Animals

Female Sprague–Dawley rats (6–8 weeks) weighing 160 ± 20 g were used for the present study. Throughout the study, all rats had access to food and water *ad libitum*. They were maintained at 12 h alternative light/dark cycles, with a temperature and humidity of 22°C ± 3°C and 50% ± 10%, respectively. Experiments were performed strictly in accordance with the Committee for the Purpose Control and Supervision in Experimental Animals (approval number: KMCRET/PhD/15/2017-18).

Study plan

Animals were segregated into five different groups (n = 6, for each group) and designated as group numbers 1, 2, 3, 4, and 5. The detailed experimental protocol for all groups is shown in Table 1. The animals of all groups were treated with saline or tamoxifen or *rasam* from the 1st day till the 30th day. *Rasam* was freshly prepared every day and administered to respective groups. At the end of the 30th day, tumor was induced by administrating DMBA (25 mg/kg) by single gastric intubation in 1 mL olive oil. On the 120th day, the rats were sacrificed through cervical decapitation, and the mammary tumors were excised out for the analysis of biochemical and histological parameters.

Estimation of body and tumor parameters

Weights of all rats were recorded at the beginning of the experiment and before their sacrifice. The changes in the body weight were recorded and analyzed.[12] Cancer masses and their progression in the rat mammary tissue were monitored by palpation twice a week. During this time, the latency period was recorded. The number of days between DMBA induction and the appearance of the first tumor was recorded as tumor latency. After 120 days, the tumor number, tumor weight, and tumor incidence were recorded.[13]

Estimation of oxidative stress parameters

The mammary tissues were weighed, and a homogenate was prepared with 0.1 M Tris-HCl buffer (pH 7.5, 10% w/v). After centrifugation at 15,000 *g* for 20 min at 5°C, the obtained clear supernatant tissue homogenate was used for the estimation of enzymatic antioxidants, non-enzymatic antioxidants, and lipid peroxidation (evaluated by measuring thiobarbituric acid reactive substances [TBARS]). Superoxide dismutase (SOD),[14] catalase (CAT),[15] and glutathione peroxidase (GPx)[16] were estimated as per previous reports. The non-enzymatic antioxidant, reduced glutathione (GSH)[17] and lipid peroxidation were measured by estimating TBARS as reported earlier.[18]

Estimation of phase I and mitochondrial enzymes

Cytochrome (cyt) P450 and cytb_5 (phase I enzymes) were estimated from the microsomal fraction. Mitochondrial fraction was isolated from the mammary tissues,[19] and hexokinase (HK), aldolase (AD), glucose 6-phosphatase (G6P), fructose 1,6-bisphosphatase (F16P), succinate dehydrogenase (SDH), isocitrate dehydrogenase (IDH), and malate dehydrogenase (MDH) were estimated as per previous reports.[20,21]

Histopathological studies

The 10% neutral buffered formalin was used to fix the mammary tissues for 24 h at room temperature. Graded series of ethanol was used to dehydrate the tissues followed by paraffin wax embedding. Sectioned tissue blocks were deparaffinized in xylene and rehydrated. The sections were histologically assessed after staining with hematoxylin and eosin.[22,23]

Statistical analysis

Values are expressed as mean ± standard deviation. One-way ANOVA followed by Tukey–Kramer multiple comparisons test was carried out to assess the statistical significance using version 5 of GraphPad Software Inc., San Diego, California, USA. A $P < 0.05$ was considered statistically significant. All groups were compared with group number 1 (negative control) and also with group number 2 (positive control) to identify the correlations among normal, induced-untreated, induced standard-pretreated, and induced *rasam*-pretreated rats.

RESULTS

Effect of *rasam* on body weight

The study showed an increase in the body weight of all the rats throughout. A reduced weight gain was observed after 120 days in DMBA alone-induced rats [Figure 1]. In the final body weight assessment, group number 2 (DMBA alone induced) showed significant ($P < 0.001$) decrease in weight gain as compared to group number 1. In addition, *rasam* at both dose levels of 3 and 4 mL/kg (group numbers 4 and 5) showed a significant ($P < 0.001$) restoration of body weight as compared to negative control (group number 1). The numerical values are presented in Supplementary Table 2.

Effect of *rasam* on antioxidants levels (enzymatic and non-enzymatic)

The effect of *rasam* on antioxidants SOD, CAT, and GPx (enzymatic) and GSH (non-enzymatic) indicates that the activities and levels of these antioxidants were significantly ($P < 0.001$) diminished in group number 2 (DMBA only) as compared to group number 1 (negative control). However, the pretreatment of *rasam* showed significant ($P < 0.001$) increase in the levels of SOD, CAT, GPx, and GSH as compared to group number 2 (DMBA only) [Table 2]. In addition, *rasam* at the dose of 4 mL/kg (group number 5) showed almost normal levels of antioxidants as compared to negative control (group number 1).

Effect of *rasam* on lipid peroxidation

The effect of *rasam* on the level of TBARS as a parameter of lipid peroxidation in controls and experimental groups is represented in Table 2. In DMBA alone-induced rats (group number 2), the TBARS levels were significantly ($P < 0.001$) decreased as compared to the negative control (group number 1). *Rasam* at the dose of 3 and 4 mL/kg (group numbers 4 and 5) showed significant restoration of TBARS level

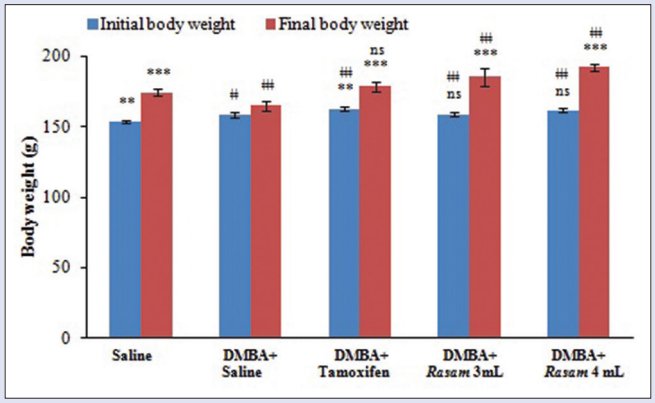

Figure 1: Effect of pretreated *rasam* on initial and final body weight of rats after 7,12-dimethylbenz[a]anthracene induction in controls and experimental groups. [ns]not significant; *calculated by comparing all groups with group number 2; [‡]calculated by comparing all groups with group number 1; [ns]not significant; [###]or***significantly different, $P < 0.001$; [##] or **significantly different, $P < 0.01$

Table 1: Detailed experimental protocol of all groups

Group number	Name of the group	Treated with	Dose per day	Treatment period	After 30 days of treatment
1	Negative control	Saline (p.o.)	4 mL/kg	30 days	Nil
2	Positive control	Saline (p.o.)	4 mL/kg	30 days	DMBA (25 mg/kg) in 1 mL olive oil
3	Standard control	Tamoxifen (p.o.)	10 mg/kg	30 days	DMBA (25 mg/kg) in 1 mL olive oil
4	Treated 3	Rasam (p.o.)	3 mL/kg	30 days	DMBA (25 mg/kg) in 1 mL olive oil
5	Treated 4	Rasam (p.o.)	4 mL/kg	30 days	DMBA (25 mg/kg) in 1 mL olive oil

p.o.: Orally; DMBA: 7,12-dimethylbenz[a] anthracene

Table 2: Effect of pretreated *rasam* on antioxidants (enzymatic and non-enzymatic), thiobarbituric acid reactive substances and phase I enzymes after 7,12-dimethylbenz[a] anthracene induction

Parameters	Groups				
	1	2	3	4	5
SOD[a]	0.32±0.01***	0.24±0.01‡‡‡	0.31±0.01ns,***	0.29±0.01‡‡‡,***	0.33±0.01ns,***
Catalase[b]	0.15±0.01***	0.11±0.01‡‡‡	0.15±0.01ns,***	0.11±0.02‡‡‡,ns	0.16±0.01ns,***
GPX[c]	0.17±0.01***	0.12±0.01‡‡‡	0.16±0.01ns,***	0.12±0.01‡‡‡,ns	0.18±0.01ns,***
GSH[d]	0.17±0.03*	0.14±0.01‡	0.15±0.01ns,ns	0.14±0.01‡,ns	0.19±0.01ns,***
TBARS[e]	0.46±0.03***	0.28±0.03‡‡‡	0.52±0.01‡,***	0.51±0.01ns,***	0.48±0.04ns,***
cytP450[f]	0.58±0.01***	0.78±0.01‡‡‡	0.70±0.04‡‡‡,***	0.68±0.03‡‡‡,***	0.61±0.04ns,***
cytb_5[f]	0.47±0.01***	0.68±0.01‡‡‡	0.48±0.02ns,***	0.51±0.01‡‡,***	0.48±0.03ns,***

[a]unit/min/mg protein; [b]µmol of H_2O_2 consumed/min/mg protein; [c]µmol of glutathione oxidized/min/mg protein; [d]µg/mg protein; [e]nmoles of MDA formed/mg protein; [f]nmol/min/mg protein; *Calculated by comparing all groups with Group 2; ‡Calculated by comparing all groups with Group 1; nsNot significant; ‡‡‡,***Significantly different, $P<0.001$; ‡‡,**Significantly different, $P<0.01$; ‡,*Significantly different, $P<0.05$. SOD: Superoxide dismutase; GPx: Glutathione peroxidase; GSH: Reduced glutathione; TBARS: Thiobarbituric acid reactive substances; MDA: Malondialdehyde

as compared to positive control (group number 2). Moreover, *rasam* at both dose levels of 3 and 4 mL/kg (group numbers 4 and 5) did not show any significant change on the levels of TBARS as compared to the negative control (group number 1) [Table 2].

Effect of *rasam* on cytochrome P450 and cytochrome b_5

A significant increase of cytP450 and cytb_5 in DMBA alone-induced group was observed [Table 2]. Adversely, the levels of cytP450 and cytb_5 were significantly decreased in both *rasam*-treated groups as compared to group number 2. However, *rasam* at dose of 4 mL/kg was able to restore the levels of cytP450 and cytb_5, near to normal when compared to group number 1. The *rasam*-treated groups showed marked decline in the levels of cytP450 and cytb_5 [Table 2].

Effects of *rasam* on glycolytic, gluconeogenic, and citric acid cycle enzymes

The effects of *rasam* on glycolytic (HK and AD), gluconeogenic (G6P and F16P), and mitochondrial CAC enzymes (SDH, IDH, and MDH) indicate that the levels of glycolytic enzymes were significantly ($P < 0.001$) increased and gluconeogenic enzymes were decreased significantly ($P < 0.001$) in DMBA only rats (group number 2) as compared to group number 1 (the negative control) [Table 3]. DMBA alone-induced group showed elevated levels of HK and AD. However, the *rasam*-treated groups showed significant ($P < 0.001$) restoration of both glycolytic and gluconeogenic enzymes when compared to DMBA alone-treated group [Table 3]. In addition, *rasam* at the dose of 4 mL/kg (group number 5) showed almost normal levels when compared to negative control (group number 1). *Rasam*-treated groups nearly normalized the elevated levels of glycolytic (HK and AD) and diminished the levels of F16P and G6P [Table 3]. The CAC enzyme (SDH, IDH, and MDH) levels were significantly decreased in DMBA alone-induced group. In DMBA alone-induced rats (group number 2), the levels of CAC enzymes were significantly ($P < 0.001$) decreased as compared to the negative control (group number 1). *Rasam*-treated groups showed significant increase in CAC enzymes than group number 2 (positive control). There are no significant changes on CAC enzymes observed in *rasam*-pretreated at dose of 4 mL/kg (groups number 5) as compared to group number 1 (negative control) [Table 3].

Effect of *rasam* on tumor parameters

The DMBA alone-induced group showed higher tumor incidence, number of tumor, and tumor weight while lowest tumor latency. All the other experimental groups showed significant change ($P < 0.001$) in tumor incidence, tumor latency, number of tumors, and tumor weight as compared to DMBA alone-induced group [Figure 2]. Tamoxifen treated (group number 3) showed a tumor incidence of 67.33% and a tumor latency of 64.86 days. However, *rasam*-treated group number 5 (4 mL/kg) showed lower tumor incidence (66.66%) and higher tumor latency (85.43 days) than tamoxifen [Figure 2a and b]. Tamoxifen treated (group number 3) and *rasam* treated (group numbers 4 and 5) showed lesser and similar number of tumors among experimental groups [Figure 2c]. Tumor weight was found to be least (13.62 mg/kg) in tamoxifen-treated group. However, *rasam*-treated group numbers 4 and 5 showed 18.5 and 18.43 mg/kg, respectively [Figure 2d]. The numerical values are presented in Supplementary Table 3.

Effect of *rasam* in the histopathological studies of mammary tissues

Histological studies on group number 2 (DMBA alone induced) showed a sequence of adenocarcinoma. Predominant ductal hyperplasia [Figure 3a] and an infiltrating neoplasm were also observed. Round-to-oval individual cells with moderate eosinophilic cytoplasm and vesicular nuclei were observed with some showing nucleoli. Sections of group number 3 (tamoxifen treated) showed a circumscribed lesion showing closely packed uniform tubules, myoepithelial cells, separated by dense fibrous stroma and mast cells infiltration, consistent with tubular adenoma [Figure 3b]. The sections of *rasam* (3 mL/kg)-treated group number 4 showed areas of necrosis, inflammatory infiltrates, and fibrous stroma with infiltration [Figure 3c]. *Rasam* (4 mL/kg)-treated group number 5 showed circumscribed lesions, single layer of epithelial cells packed tubules, attached myoepithelial cells, separated by dense fibrous stroma and mast cells infiltration with occasional ductal hyperplasia [Figure 3d].

DISCUSSION

Prevention of body weight loss by *rasam*

The weight loss is generally associated with cancer. The changes of difference in the effect of *rasam* in body weight between zero day and 120th day in controls and experimental groups were studied. The increase in the circulating interleukin-6 can cause loss of muscle weight and fat tissues.[21] The reduction in weight gain in DMBA only group may be due to increase in the levels of circulating interleukin-6. This significant weight loss was prevented by the prophylactic administration of *rasam* in the treated groups, 3 and 4 mL/kg (group numbers 4 and 5). These results indicate that pretreatment with *rasam* provided an optimal protection to rats against DMBA-induced mammary carcinogenesis by preventing weight loss.

Restoration of enzymatic and non-enzymatic antioxidants levels by *rasam*

In the present study, both the enzymatic and non-enzymatic antioxidants (SOD, CAT, GPx, and GSH) levels were reduced in

Table 3: Effect of pretreated *rasam* on glycolytic, gluconeogenic, and citric acid cycle enzymes after 7,12-dimethylbenz[a] anthracene induction

Enzymes	Groups				
	1	2	3	4	5
HK[a]	11.16±0.36***	29.33±1.21‡‡‡	15.3±0.21‡‡‡,***	16.3±0.54‡‡‡,***	12.23±0.43ns,***
AD[b]	18.13±0.45***	35.13±0.53‡‡‡	21.76±0.46‡‡‡,***	23.05±1.02‡‡‡,***	16.96±0.57‡,***
G6P[c]	6.31±0.33***	1.87±0.53‡‡‡	4.43±0.53‡,***	3.36±0.39‡‡‡,ns	6.58±1.89ns,***
F16P[c]	71.86±2.05***	30.73±0.69‡‡‡	65.26±0.53‡‡‡,***	57.06±1.06‡‡‡,***	69.66±2.84ns,***
SDH[d]	10.66±0.29***	4.13±0.41‡‡‡	11.4±0.20‡‡,***	9.33±0.14‡‡‡,***	10.43±0.52ns,***
IDH[e]	1.11±0.05**	0.76±0.07‡‡	0.95±0.22ns,ns	1.04±0.12ns,*	1.14±0.19ns,***
MDH[f]	252.63±5.92***	194.67±9.32‡‡‡	251.97±2.09ns,***	229.33±5.27‡‡‡,***	254.4±14.28ns,***

[a]min/mg protein; [b]min/mg protein; [c]nmol of Pi liberated/min/mg protein; [d]μmol of succinate oxidized/min/mg protein; [e]nmol of α-ketoglutarate liberated/min/mg protein; [f]nmol of NADH oxidized/min/mg protein; [ns]Not significant; *Calculated by comparing all groups with Group 2; ‡Calculated by comparing all groups with Group 1; ‡‡‡,***Significantly different, $P<0.001$; ‡‡,**Significantly different, $P<0.01$; ‡,*Significantly different, $P<0.05$. HK: Hexokinase; AD: Aldolase; G6P: Glucose 6-phosphatase; F16P: Fructose 1,6-bisphosphatase; SDH: Succinate dehydrogenase; IDH: Isocitrate dehydrogenase; MDH: Malate dehydrogenase

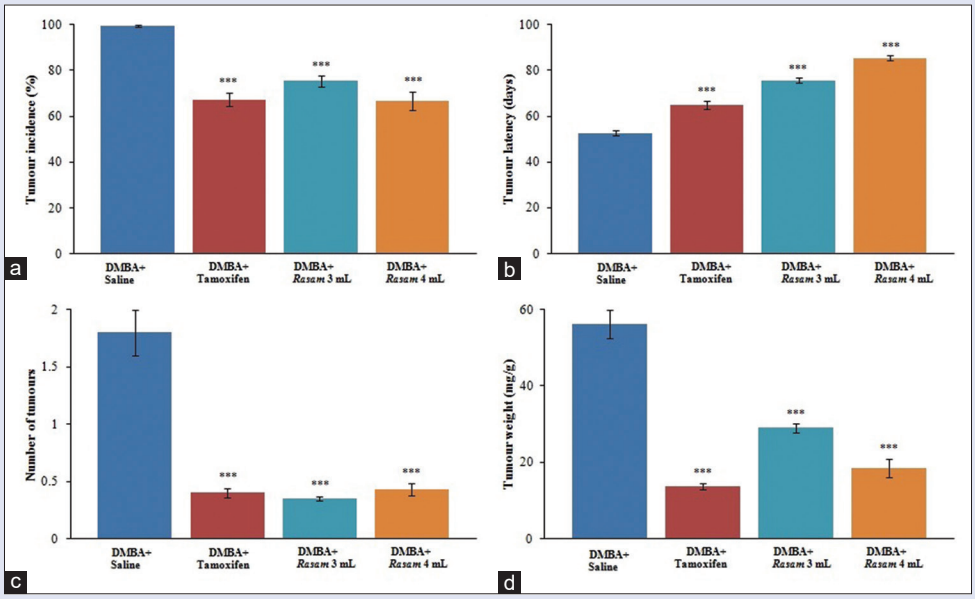

Figure 2: Preventive effect of *rasam* on tumor incidence (a), tumor latency (b), number of tumors (c), and tumor weight (d) in 7,12-dimethylbenz[a] anthracene-induced mammary carcinoma of rats. ***significantly different from positive control (group number 2), $P < 0.001$

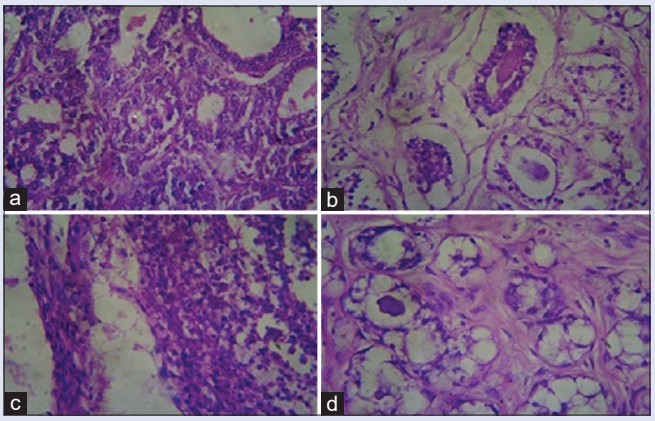

Figure 3: Photomicrographs of hematoxylin and eosin-stained mammary tissue of group number 2, no treatment + 7,12-dimethylbenz[a] anthracene (a); group number 3, tamoxifen treated + 7,12-dimethylbenz[a] anthracene (b); group number 4, *rasam* treated (3 mL/kg) + 7,12-dimethylbenz[a] anthracene (c); and group number 5, *rasam* treated (4 mL/kg) (d) + 7,12-dimethylbenz[a] anthracene

the DMBA alone-induced group as previously reported.[24] DMBA induces the production of reactive oxygen species (ROS) and oxidative stress.[25,26] The experimental group pretreated with *rasam* particularly, at the dose of 4 mL/kg (group number 5), showed almost normal levels of antioxidants. There was no significant change observed in group number 5 as compared with group number 1, which is an evidence for the protective effect of *rasam* at 4 mL/kg on oxidative stress.

Prevention of pro-oxidant potential of DMBA by *rasam*

DMBA alone-induced group showed decreased level of TBARS which denotes the excess production of free radical due to the metabolic activation of DMBA. Cancer cells are known to proliferate faster when lipid peroxidation is low.[27] Decreased TBARS levels observed in the mammary tissue of DMBA only group may be due to either an increase in proliferation rate or an increase in resistance and/or a decrease in the susceptibility to attacks by the free radicals. The pretreatment with *rasam* significantly elevated the level of TBARS, which denotes the prevention of pro-oxidant potential of DMBA.

Modulation of cytochrome P450 and cytochrome b_5 by *rasam*

Phase I enzymes (cytP450 and cytb_5) cause metabolic degradation of DMBA into reactive metabolites (diol epoxides)[28] to bind with DNA covalently, forming DNA adducts[29] and thereby initiating the carcinogenesis process. The restoration of cytP450 and cytb_5, near to normal levels at the dose of 4 mL/kg confirms that *rasam* was effective in modulating phase I enzymes levels in a dose-dependent manner and thereby preventing reactive metabolites (diol epoxides) of DMBA. The prevention of formation of DNA adducts may be indicated as possible mechanism of *rasam* for exerting potent anti-mammary carcinoma activity.

Antioxidative stress effect of *rasam* on glycolytic, gluconeogenic, and citric acid cycle enzymes

Carbohydrate metabolism can be an indicator of the excessive energy demand required by the rapidly proliferating tumor cells. The downregulation of F16P and G6P activity increases the concentration of its substrates, fructose 1,6-bisphosphate and glucose 6-phosphate. Tumor cells utilize a high rate of glucose and show an increased lactic acid production. The decreased levels of gluconeogenic enzymes in DMBA-induced alone group may be due to high production of lactic acid in tumor cells.[30] *Rasam* inhibited accelerates glycolytic enzymes and ameliorates gluconeogenic enzyme activities in tumor cells. Weight loss can be attributed to the gluconeogenic enzymes as they are part of excessive energy utilization. The improved body weight gain observed with *rasam* may be due to the amelioration of gluconeogenic enzymes. The source of cellular ROS that inhibit CAC enzymes is mitochondria.[31] DMBA alone-induced group showed significant decrease in the CAC enzyme levels. Peroxidation

of macromolecules, leading to the alteration in membranes of mitochondria, its morphology, and organelles, can be the reason behind decreased CAC enzymes.[32] The significant increase of depleted levels of CAC enzymes in *rasam*-treated groups may be due to restored mitochondrial function. The damages to the membrane due to free radicals generated by oxidative stress can be prevented by the restored mitochondrial function.

Preventive effect of *rasam* on tumor parameters

Rasam was efficient in reducing the percentage of incidence of tumor, number of tumor, and tumor weight. Moreover, it showed highest tumor latency than tamoxifen. The decreased tumor incidence and increased tumor latency observed with *rasam* pretreatment correlate with the improved body weight gain. These results clearly suggest a broad chemopreventive effect of *rasam* against the DMBA-induced mammary carcinoma.

Histopathological evidence for preventive effect of *rasam*

Overall, the histological examination has revealed that DMBA alone-induced rats showed signs of malignancy such as high infiltrating cellular morphology, abnormal mitotic cells, and loss of extracellular matrix, whereas the *rasam*-treated rats particularly at dose of 4 mL/kg showed near-to-normal cellular architecture. These results clearly reveal the chemopreventive potential of *rasam* against mammary carcinoma.

CONCLUSION

The imbalance in the levels of SOD, CAT, GPx, GSH, TBARS, phase I, glycolytic, gluconeogenic, and CAC enzymes as observed in the DMBA alone-induced rats was significantly modulated in *rasam*-pretreated rats to near-to-normal levels, particularly at a dose of 4 mL/kg. In most of the studied parameters such as body weight, TBARS, phase I enzymes, HK, AD, G6P, F16P, CAC enzymes, tumor weight, tumor latency, and tumor incidence, *rasam*-treated groups showed better chemoprevention than tamoxifen-treated group. Tumor incidence and latency observed in pretreated groups supported by the histochemical findings clearly confirm the chemopreventive effect of *rasam* against DMBA-induced mammary carcinogenesis. It is very clear that *rasam* attenuates the mammary tumors induction magnitude of DMBA in female rats.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: Botanical source of the ingredients used in the preparation of *rasam*

Common names	Morphological part used	Nature of the material	Botanical name	Family
Tamarind	Ripped fruit pulp	Dried	*Tamarindus indica* L.	*Fabaceae*
Turmeric	Rhizome powder	Dried	*Curcuma longa* L.	*Zingiberaceae*
Sea salt	NA	Solid	NA	NA
Tomato	Ripped fruit	Fresh	*Solanum lycopersicum* L.	*Solanaceae*
Chili pepper	Crushed fruit of long chili pepper	Dried	*Capsicum annuum* L.	*Solanaceae*
Cumin	Ripped fruit	Dried	*Cuminum cyminum* L.	*Apiaceae*
Garlic	Bulb	Dried	*Allium sativum* L.	*Amaryllidaceae*
Black pepper	Unripe drupe	Dried	*Piper nigrum* L.	*Piperaceae*
Indian sesame oil	Seed	Oil	*Sesamum indicum* L.	*Pedaliaceae*
Black mustard	Seed	Dried	*Brassica nigra* L.	*Brassicaceae*
Chili pepper	Whole fruit of long chili pepper	Dried	*Capsicum annuum* L.	*Solanaceae*
Curry leaves	Leaves	Fresh	*Murraya koenigii* (L.) Sprengel	*Rutaceae*
Portable water	NA	Liquid	NA	NA
Coriander	Leaves	Fresh	*Coriandrum sativum* L.	*Apiaceae*
Asafoetida	Dried latex (oleo gum resin) exuded from the rhizome or tap root	Powder	*Ferula assa-foetida* L.	*Apiaceae*

NA: Not applicable

Supplementary Table 2: Effect of pretreated *rasam* on initial and final body weight of rats after 7,12-dimethylbenz[a] anthracene induction

Groups	1	2	3	4	5
Initial body weight (g)	153.83±1.16**	158.5±1.99‡‡	162.66±1.76‡‡‡,**	158.8±1.57‡‡‡,ns	161.61V1.63‡‡‡,ns
Final body weight (g)	174.33±2.53***	164.83±3.33‡‡‡	178.5±3.36ns,***	185.21±6.23‡‡‡,***	192.03±2.79‡‡‡,***

nsNot significant; *Calculated by comparing all groups with Group 2; ‡Calculated by comparing all groups with Group 1; ‡‡‡,***Significantly different, $P<0.001$; ‡‡,**Significantly different, $P<0.01$

Supplementary Table 3: Preventive effect of *rasam* on tumor incidence, tumor latency, number of tumors, and tumor weight in 7,12-dimethylbenz[a] anthracene-induced mammary carcinoma of rats

Groups	2	3	4	5
Tumor incidence (%)	99.33±0.42	67.33±2.95***	75.33±2.56***	66.66±4.02***
Tumor latency (days)	52.7±1.01	64.86±1.65***	75.66±1.11***	85.43±1.25***
Number of tumors	1.8±0.20	0.40±0.04***	0.35±0.02***	0.43±0.05***
Tumor weight (mg/g)	56.23±3.78	13.62±0.75***	29.06±1.20***	18.43±2.40***

***Significantly different from positive control (Group 2), $P<0.001$