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# Phytochemical analysis and anticancer capacity of Shemamruthaa, a herbal formulation against DMBA– induced mammary carcinoma in rats

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## ABSTRACT

**Objective:** To investigate the bioactive constituents of Shemamruthaa (SM), a herbal combination and its therapeutic effects on the mitochondrial functions with reference to lipid peroxidation (LPO), antioxidant status, citric acid cycle enzymes and electron transport chain enzymes in mammary tissues of 7,12–dimethylbenz(a)–anthracene (DMBA) induced mammary carcinoma in rat model. **Methods:** Adult female Sprague–Dawley rats were used for the study and were divided into four groups. Group I served as control and Group II rats were induced mammary carcinoma by administration of DMBA (25 mg/kg b.w.) orally. The normal and cancer–induced rats (Group III) were treated with SM (400 mg/kg b.w./day) orally by gastric incubation for 14 days. Group IV rats served as SM–treated control animals. **Results:** Cancer–induced rats showed a considerably increased level of LPO with concomitant decreased levels of antioxidants, citric acid cycle enzymes, electron transport chain enzymes and cytochrome contents in the mammary tissue. Treatment with SM brought back the aforementioned biochemical parameters to near normal. **Conclusions:** From the results, it can be inferred that Shemamruthaa possesses significant anticancer effect through its role in attenuation of LPO, prevention of membrane damage and restoring membrane integrity.

## 1. Introduction

In women, breast cancer is the most common cancer and second most common cause of cancer–related death[1]. It is frequently treated with radiotherapy and chemotherapy, which are known risk factors for the development of a second malignancy[2]. More recently, resistance to anticancer drugs has been observed. Therefore, the research and development of more effective and less toxic drugs by the pharmaceutical industry has become necessary. Many substances derived from dietary or medicinal plants are known to be effective chemopreventive and

antitumour agents in a number of experimental models of carcinogenesis[3].

*Hibiscus rosa–sinensis* Linn (Family: Malvaceae) (*H. rosa–sinensis*) is a conspicuous, ornamental, evergreen, glabrous shrub, native to Tropical Asia and has several forms with varying colours of flowers. In medicine, however, the red flowered variety is preferred[4]. In South Asian traditional medicine, various parts of the plant are used in the preparation of a variety of foods. The flowers are considered to be aphrodisiac, emollient and emmenagogic[5] and are reported to possess various pharmacological actions such as anti–analgesic, anti–inflammatory[6], wound healing[7], anti–oxidant and anti–diabetic[8], cardioprotective[9], anti–genotoxic[10] and anticancer[11,12] activities. *H. rosa–sinensis* contains numerous compounds like anthocyanins, flavonoids, cyclopeptide alkaloids and vitamins[13], which are known to possess therapeutic value in various disorders.

*Phyllanthus emblica* Linn. (Syn: *Embllica officinalis* Gaertn., family: Euphorbiaceae) (*P. emblica*) is one of the

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notable plants used in many local traditional medicine systems including Ayurvedic medicine of India, Chinese herbal medicine and Thai traditional medicine<sup>[14]</sup>. The fruits of *Embllica officinalis* (*E. officinalis*) have beneficial role in cancer, ulcer, diabetes, anaemia, liver, heart and various other diseases. Similarly, it has application as antioxidant, immunomodulatory, antipyretic, analgesic, cytoprotective, antitussive and gastroprotective, memory enhancing, ophthalmic disorders and lowering cholesterol level<sup>[15]</sup>. The fruits of *E. officinalis* (EO) contain tannins, alkaloids, phenolic compounds, amino acids, carbohydrates and highest vitamin C content. Compounds isolated from EO were gallic acid, ellagic acid, 1-O-galloyl- $\beta$ -D-glucose, 3,6-di-O-galloyl-D-glucose, chebulinic acid, quercetin, chebulagic acid, corilagin, 1,6-di-O-galloyl- $\beta$ -D-glucose, 3, ethylgallic acid (3, ethoxy 4,5-dihydroxy benzoic acid) and isostrictiniin. It also contains flavonoids such as kaempferol-3-O- $\alpha$ -L (6'' methyl) rhamnopyranoside and kaempferol-3-O- $\alpha$ -L (6'' ethyl) rhamnopyranoside<sup>[16,17]</sup>.

The mixture of several crude extracts, when used in formulation enhances the beneficial effects through synergistic amplification and diminishes any possible adverse effects and offers advantage over a single isolated ingredient<sup>[18,19]</sup>.

Hence, in the present study Shemamruthaa (SM), a phytochemical combination constituting flowers *H. rosa-sinensis* and fruits of (*P. emblica*) was formulated and evaluated for the first time with a view to potentiate more intense anticancer property. In order to promote intellect and prevent senility and for longevity, honey was also added in SM. Both *H. rosa-sinensis* and *E. officinalis* were independently proved to exhibit a vital role in traditional and modern medicines because of their wider pharmacological activities. In addition to that, they also exhibit anticancer effect against various types of cancer<sup>[11, 20-22]</sup>.

Therefore, this study was undertaken to investigate the synergic effects of SM on the mitochondrial functions with reference to lipid peroxidation (LPO), mitochondrial antioxidants status, critical citric acid cycle enzymes, respiratory chain enzymes and cytochrome contents in mammary tissues of 7,12-dimethyl benz(a)anthracene (DMBA)- induced mammary carcinoma bearing Sprague-Dawley rats.

## 2. Materials and methods

### 2.1. Chemicals and reagents

DMBA (7,12-Dimethylbenz[a]anthracene) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade and highest purity.

### 2.2. Preparation of SM Drug

The flowers of *H. rosa-sinensis* were collected from a local garden in southern part of India (Kancheipuram District, Tamil Nadu) and the pharmacognostic authentication was done by Department of Plant Sciences, University of Madras, Chennai-600 025. The fruits of *Embllica* (*P. emblica* L.), at the commercially mature stage, were purchased from the market and the rinds were carefully removed from the seeds. The flowers of *H. rosa-sinensis* and the rinds were air dried under shade, pulverized to fine powder using a cutting mill and mixed with pure honey in definite ratio.

### 2.3. Animals

Adult female albino rats of Sprague-Dawley strain weighing (180 $\pm$ 10) g were provided from Central Animal House facility, University of Madras, Taramani Campus, Chennai-600 113, Tamil Nadu, India. The animals were maintained under standard conditions of humidity, temperature [(25 $\pm$ 2) °C] and light (12 h light/dark). They were fed with standard rat pellet diet and water *ad libitum*. The experimental design was performed in accordance with the current ethical norms approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment & Forests (Animal Welfare Division), Government of India and Institutional Animal Ethics Committee Guidelines (IAEC. No. 01/030/2010).

### 2.4. Experimental design

The rats were randomized and divided into 4 groups of 6 animals each. Group I was kept as normal control and received 1 mL olive oil, a vehicle. Group II rats were induced with a single dose of DMBA (25 mg in 1 mL of olive oil) by oral gavage at the first day of experimental period. Group III rats received SM (400 mg/kg b.w. /day) orally for 14 days after 90 days of DMBA induction. Group IV received SM as drug control. At the end of the experimental period, all animals were killed by cervical decapitation. The mammary tissue samples were cut and placed in isotonic saline to remove the blood and then rinsed in ice-cold 0.25 M sucrose, blotted, weighed and minced. The homogenate was prepared in ice-cold 0.25 M sucrose at 4 °C.

### 2.5. Assay of mitochondrial enzymatic and nonenzymatic antioxidants

Mitochondrial fraction of mammary tissue was isolated according to the method of Johnson and Lardy<sup>[23]</sup> and the following parameters were analysed. Protein was estimated by the method of Lowry *et al*<sup>[24]</sup>. LPO was assayed by the method of Ohkawa *et al*<sup>[25]</sup> in which the malondialdehyde (MDA) formed served as the index of LPO. Superoxide dismutase (SOD) was assayed according to the method of

Marklund and Marklund<sup>[26]</sup>. Catalase (CAT) activity was assayed by the method of Sinha<sup>[27]</sup>, glutathione peroxidase (GPx) was determined by the method of Rotruck *et al*<sup>[28]</sup>. Glutathione reductase (GR) was assayed by the method of Beutler<sup>[29]</sup>. Vitamin C was measured by the method of Omaye *et al*<sup>[30]</sup>, vitamin E was estimated by the method of Desai<sup>[31]</sup> and reduced glutathione (GSH) was assayed by the method of Moron *et al*<sup>[32]</sup>.

## 2.6. Assay of citric acid cycle, respiratory chain enzymes and cytochromes

Citric acid cycle enzymes namely iso citrate dehydrogenase (ICDH) was assayed by the method of King<sup>[33]</sup>, alpha-keto dehydrogenase (alpha-KDH) by the method of Reed and Mukherjee<sup>[34]</sup>, succinate dehydrogenase (SDH) by the method of Slater and Bonner<sup>[35]</sup> and malate dehydrogenase (MDH) by the method of Mehler *et al*<sup>[36]</sup>. The activities of NADH dehydrogenase was determined by the method of Minakami *et al*<sup>[37]</sup> and Cytochrome c oxidase by the method of Pearl *et al*<sup>[38]</sup>. Mitochondrial cytochrome contents were estimated by the method of Williams<sup>[39]</sup> and ATP contents were measured by the method of Williamson<sup>[40]</sup>.

## 2.7. Gas chromatography–mass spectrometry (GC–MS) analysis of SM extract

Powdered herbal constituents of SM (5 g) were soaked in absolute alcohol overnight and then filtered through a Whatmann® No. 41 filter paper along with 2 g of sodium sulphate to remove the sediments and traces of water and the filtrate is then concentrated by bubbling nitrogen gas into the solution.

GC–MS analysis of the extract was performed using a Perkin Elmer GC Claurus 500 system and gas chromatograph interfaced to a mass spectrometer equipped with a Elite–5MS fused silica capillary column (30 m × 0.25 mm ID, ×1 μm df, composed of 5% Diphenyl/ 95% Dimethyl polysiloxane). For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min and an injection volume of 3 μL was employed (split ratio of 10:1). Injector temperature 250 °C; Ion–source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min) with an increase of 10 °C/min to 200 °C, then 5 °C/min to 280 °C ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV, a scan interval of 0.5 seconds and fragments from 45 to 450 Da. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a TurboMass Ver 5.2.0.

Interpretation on mass–spectrum GC–MS was conducted using the database of National Institute Standard and Technology (NIST) having more 62 000 patterns. The spectrum

of the unknown components was compared with the spectrum of known components stored in the NIST library and the molecular weight and structure of the components of the test materials were ascertained.

## 2.8. Determination of polyphenolics by HPLC

Chromatographic analysis was carried out by HPLC Shimadzu CLASS–VP V6.14 SP2. The operating parameters were as follows: Column: C18; Mobile phase: Solvent A–Water–Acetic acid (25:1), Solvent B–Methanol; Pumps (Binary Gradient); T.Flow: 1.000 mL/min; P.Max: 400.0 kgf/cm<sup>2</sup>; P.Min: 0.0 kgf/cm<sup>2</sup>; CTO–10ASvp, Temperature: 40 °C; SPD–10 Avp (Det.A); Lamp: D2; Polarity: +Ve.

The extraction was carried out using 2 g of powdered plant material with 50 mL of 95% ethanol under 80 KHz, 45 °C in ultrasonic extraction device for 30 min, repeated twice. The extract was collected and filtered; the filtrate was dried at 50 °C under reduced pressure in a rotary evaporator. The dried crude extract was dissolved in the 100 mL mobile phase. After filtering through a filter paper and a 0.45 mm membrane filter (Millipore), the extract was injected into HPLC by auto sampler. The gradient elution of solvent A [water–acetic acid (25:1 v/v)] and solvent B (methanol) had a significant effect on the resolution of compounds. As a result, solvent gradients were formed, using dual pumping system, by varying the proportion of solvent A [water–acetic acid (25:1, v/v)] to solvent B (methanol). Solvent B was increased to 50% in 4 min and subsequently increased to 80% in 10 min at a flow rate of 1.0 mL/min. Detection wavelength was 280 nm.

Flavonoid components were identified by comparing their retention times to those of authentic standards under identical analysis conditions and UV spectra, using in house PDA library.

## 2.9. Histopathological analysis

Formalin–fixed mammary tumour samples were paraffin embedded, sectioned (3 mm thickness) and placed on glass slides. Paraffin–embedded sections of tissue were deparaffinised, rehydrated with graded alcohol and stained with Harris' haematoxylin and eosin (Dako, Glostrup, Denmark) in a Leica Autostainer (Wetzlar, Germany) and examined under a microscope.

## 2.10. Statistical analysis

The data is expressed as mean±standard deviation (SD). Statistical comparisons for biochemical parameters were performed by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. The results were considered statistically significant if the  $P < 0.05$ .

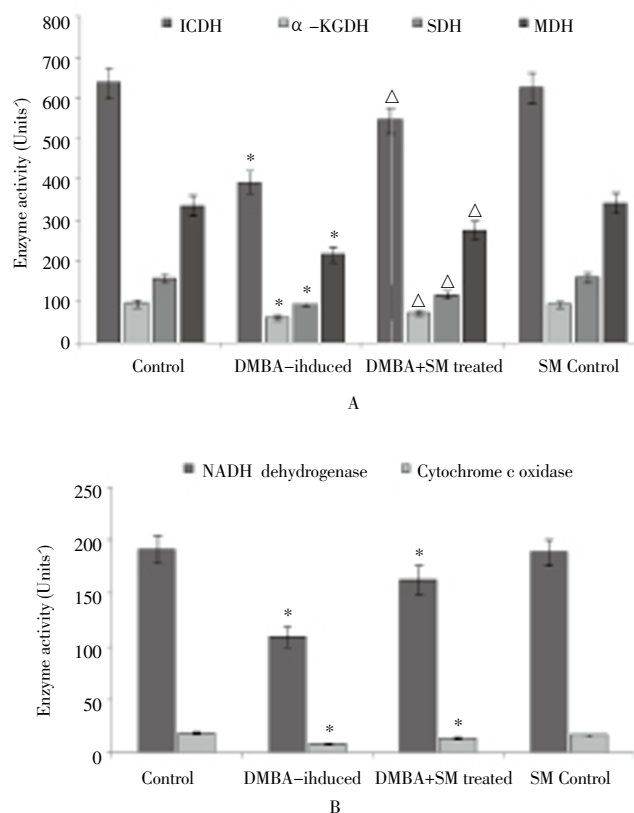
### 3. Results

The tumour volume in control and experimental animals were measured using standard formula. We have observed 100% tumour formation with mean tumour volume of 4283.76 mm<sup>3</sup> in DMBA alone treated animals (Group II). Oral administration of SM at a dose of 400 mg/kg b.w. /day for 14 days significantly reduced (1.44 fold) the mean tumour volume in Group III rats, compared to DMBA induced rats. No tumour was observed in control animals (Group I) as well as SM alone administered animals (Group IV). The effect of SM treatment on mammary mitochondrial LPO in which the MDA released served as the index of LPO was significantly increased in DMBA-induced (Group II) animals. SM treatment resulted in free radical scavenging and there by significantly ( $P < 0.05$ ) decreased MDA content (1.69 fold) compared to DMBA-induced rats.

Table 1 displays the activities of enzymatic antioxidants (SOD, CAT, GPx and GR) and the levels of non-enzymatic antioxidants (Vit C, Vit E and GSH) in the mammary mitochondria of normal and experimental rats. DMBA-treated (Group II) animals showed significantly ( $P < 0.05$ ) decreased activities of antioxidant enzymes and reduced levels of Vit C, Vit E and GSH, compared to control rats (Group I). Upon SM treatment the levels of these antioxidants were restored to near normal. Moreover, there were found to be no significant difference in the levels of LPO and antioxidants status between control (Group I) and SM control (Group IV) rats.

The effect of SM treatment on the activities of critical citric acid cycle enzymes and respiratory chain enzymes in the mammary mitochondria of normal and experimental rats is summarized graphically in Figure 1A and 1B, respectively. DMBA-induced animals (Group II) showed significantly decreased activities of ICDH,  $\alpha$ -KDH, SDH, MDH (38.42%, 36.48%, 38.52%, 35.38% respectively) and respiratory chain enzymes NADH dehydrogenase (45.49%), cytochrome c oxidase (53.91%), compared to the normal control rats.

Treatment with SM significantly restored the levels of enzyme activities to near normal levels, compared to those of the DMBA-induced rats.



**Figure 1.** Effect of SM treatment on the activities of citric acid cycle key enzymes and respiratory chain enzymes in the mammary mitochondria of control and experimental rats.

Each value is expressed as mean±SD for six rats in each group.

\*Units: ICDH– nmoles of NADH oxidised/h/mg of protein;  $\alpha$ -KDH– nmoles of potassium ferrocyanide liberated/h/mg of protein; SDH– nmoles of succinate oxidized/min/mg of protein; MDH– nmoles of NADH oxidized/ min/mg of protein; NADH dehydrogenase– nmoles of NADH oxidized/min/mg of protein; Cytochrome c oxidase– nmoles/min/mg of protein. \* $P < 0.05$  DMBA group vs. control,  $\Delta P < 0.05$  DMBA+SM group vs. MDBA group.

**Table 1**

Effect of SM on enzymatic and non-enzymatic antioxidant levels in mammary mitochondrial fractions of control and experimental group of rats.

Parameter	Group I (Control)	Group II (DMBA Induced)	Group III (DMBA + SM)	Group IV (SM Control)
Enzymatic antioxidants				
SOD	11.85±0.89	7.44±0.07*	9.36±0.08*	11.47±0.75
CAT	36.77±1.66	20.59±1.24*	27.20±1.43*	37.29±1.58
GPx	4.86±0.65	3.14±0.28*	3.79±0.35*	5.05±0.47
GR	5.87±0.55	3.01±0.26*	4.05±0.36*	5.79±0.48
Non-enzymatic antioxidants				
Vitamin C	1.98±0.15	1.40±0.22*	1.76±0.16*	2.04±0.24
Vitamin E	2.73±0.22	2.07±0.23*	2.26±0.24*	2.78±0.26
GSH	1.56±0.12	1.20±0.13*	1.36±0.13*	1.58±0.13

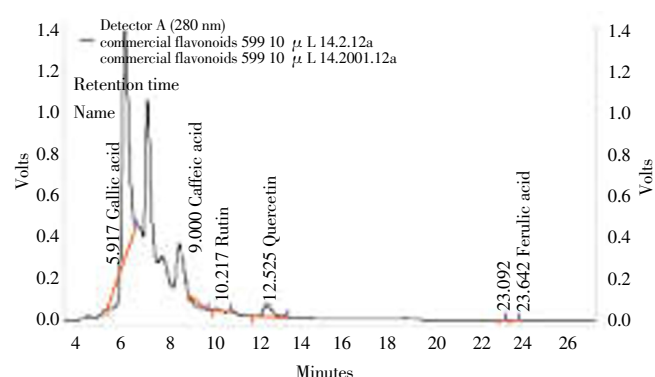
Each value is expressed as mean±SD for six rats in each group. \* $P < 0.05$  group II vs. group I, \* $P < 0.05$  group II vs. group III.

Units: SOD– units/min/mg protein (one unit is equal to the amount of enzyme required to inhibit autoxidation of pyrogallol by 50%); CAT– nmoles of H<sub>2</sub>O<sub>2</sub> consumed/min/mg protein; GPx–  $\mu$  g of reduced GSH utilised/min/mg protein; GR– nmoles of NADPH oxidized/min/mg protein; Vitamin C, Vitamin E and GSH were expressed as  $\mu$  g/mg protein.

Table 2 depicts the levels of cytochrome aa<sub>3</sub>, b, c and c<sub>1</sub> in control and experimental rats. A significant ( $P < 0.01$ ) decrease was observed in cytochrome content in mammary mitochondria of DMBA-induced group II rats when compared with control group I rats. However DMBA-induced rats treated with SM retained their mitochondrial cytochrome content.

The GC-MS study of herbal constituents of SM has shown many phytochemicals which contributes to the wider pharmacological activities. The prevailing bioactive components with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in Table 3.

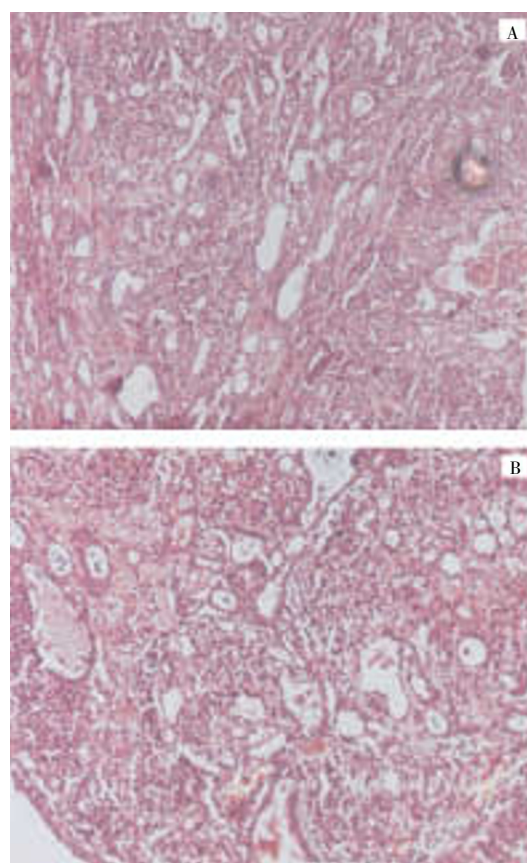
Figure 2 shows the polyphenolics constituents (Gallic acid, caffeic acid, rutin, quercetin and ferulic acid) present in the extract of herbal constituents of Shemamruthaa.



**Figure 2.** HPLC analysis of polyphenolic constituents (Gallic acid, caffeic acid, rutin, quercetin and ferulic acid) present in the extract of herbal constituents of Shemamruthaa.

Figure 3 reveals the effect of SM on histological alterations in mammary gland of control and experimental rats. Normal rats showed mammary gland skin with underlying fibro fatty tissue containing scattered mammary gland ducts and bundles of skeletal muscles are seen in one area, whereas DMBA-induced rats show parts of a tumour composed of hyperchromatic, pleomorphic, vesicular nuclei and moderate cytoplasm arranged in nests, sheets and acinar

structures with numerous mitotic figures (Figure 3A). Mammary carcinoma bearing rats treated with SM show regression in tumour cells and fibro fatty tissue with few ducts are seen (Figure 3B). The drug control rats' mammary tissue showed normal architecture.



**Figure 3.** Histological alterations in mammary gland of control and experimental rats.

3A: DMBA-induced rats (group II) show parts of a tumour composed of hyperchromatic, pleomorphic, vesicular nuclei and moderate cytoplasm arranged in nests, sheets and acinar structures with numerous mitotic figures.

3B: Tumour induced + SM treated rats (group III) show fatty tissue with small lobules and tumour regression.

**Table 2**

Effect of SM on the activities of cytochrome c<sub>1</sub>, c, b and aa<sub>3</sub> in mammary mitochondrial fractions of control and experimental group of rats.

Parameter	Group I (Control)	Group II (DMBA induced)	Group III (DMBA + SM)	Group IV (SM control)
Cytochrome aa <sub>3</sub>	0.17±0.04	0.082±0.004*	0.11±0.02*	0.18±0.03
Cytochrome b	0.53±0.05	0.32±0.03*	0.44±0.04*	0.52±0.04
Cytochrome c	0.38±0.03	0.21±0.02*	0.29±0.03*	0.36±0.03
Cytochrome c <sub>1</sub>	0.29±0.02	0.13±0.02*	0.21±0.02*	0.27±0.03

Each value is expressed as mean±SD for six rats in each group. Cytochromes are expressed as nmoles/mg protein. \*  $P < 0.05$  group II vs. group I, \*  $P < 0.05$  group II vs. group III.

**Table 3**

Major phyto-components identified through the GC/MS study of herbal constituents of SM.

Sl. No.	Retention time (min)	Name of the compound	Molecular formula	Molecular weight	Peak Area %
1.	2.46	2-cyclohexen-1-one, 4-hydroxy	C <sub>6</sub> H <sub>8</sub> O <sub>2</sub>	112	8.04
2.	4.65	2-furancarboxaldehyde, 5-(hydroxymethyl)- (5-Hydroxymethylfurfural)	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	126	16.42
3.	6.72	1,2,3-benzenetriol (Pyrogallol)	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	126	57.62
4.	8.39	1,6-anhydro- $\alpha$ -D-glucopyranose (Levoglucozan)	C <sub>6</sub> H <sub>10</sub> O <sub>5</sub>	162	13.32
5.	9.68	7,8-epoxylanostan-11-ol, 3-acetoxy-	C <sub>32</sub> H <sub>54</sub> O <sub>4</sub>	502	1.26
6.	13.61	dipalmitin	C <sub>35</sub> H <sub>68</sub> O <sub>5</sub>	568	2.34
7.	16.44	Trilinolein	C <sub>57</sub> H <sub>98</sub> O <sub>6</sub>	878	1.00

#### 4. Discussion

It is vital to enhance our understanding of the role of naturally occurring antioxidants in cancer prevention and their possible use in intervention trials for the prevention of cancer in humans. Frequent production of reactive oxygen species (ROS) in animals treated with carcinogens cause free radicals and electrophiles mediated oxidative stress favouring the progression of carcinogenesis. These highly reactive species causes oxidative modification of DNA, proteins, lipids and small intracellular molecules[41]. Mitochondria play a central role as a regulator of energy balance and appear to be the primary intracellular target for oxidative stress-induced damage during cancer[42]. Thus, the study of mitochondrial status and functions during carcinoma is of particular interest.

The present study demonstrates that DMBA causes marked increase in mitochondrial LPO in DMBA-induced animals (Group III) is in line with similar findings from our laboratory and other workers[43,44]. This study also demonstrates that the herbal formulation Shemamruthaa induce a significant ( $P < 0.01$ ) reduction in LPO, tumour multiplicity and tumour volume in a defined experimental rat mammary carcinogenesis model. *H. rosa-sinensis* and *P. emblica* (the herbal constituents of SM) are reported to contain a variety of phenolic compounds such as chlorogenic acid, hydrolysable tannins, flavonols and their glycosides, anthocyanins, quercetin 3- $\beta$ -D-glucopyranoside, kaempferol 3- $\beta$ -D-glucopyranoside, isocorilagin, quercetin and kaempferol[45,46]. These polyphenolic compounds elicit oxidative defence against ROS-induced oxidative stress by their potent antioxidant role in protecting cells against a variety of endogenous and exogenous toxic compounds.

The current work substantiates ROS generated during DMBA metabolism may be responsible for the increased LPO and compromised antioxidant status. SOD is the first antioxidant enzyme to deal with oxyradicals by accelerating the dismutation of superoxide ( $O_2^{\bullet-}$ ) to hydrogen peroxide ( $H_2O_2$ ). CAT is a peroxisomal haem containing protein that catalyses the removal of  $H_2O_2$  to undistruptive water. Thus, SOD and CAT act mutually and protect the cellular constituents against oxidative damage[47,48]. GPx,

a selenium-containing enzyme, works together with glutathione (GSH) in the decomposition of hydrogen peroxide or other organic hydro peroxides to non-toxic products at the expense of GSH[49]. The decrease in activities of SOD, CAT, GPx and GR in mammary carcinoma rats obtained from the present investigation indicates the weak free radical defence system against oxidative stress which was consistent with published studies[50,51]. The activities of these enzymes were markedly elevated to near normal upon SM administration. This might be due to the action of SM in decreasing the free radical generation due to the presence of phenolic compounds.

Mitochondrial GSH plays a critical role in maintaining cell viability through regulation of mitochondrial inner membrane permeability by maintaining sulfhydryl groups in the reduced state. The observed lower level of glutathione in mammary carcinoma rats may result in enhanced lipid peroxidation and excess utilization of this antioxidant for tumour cell proliferation, which is in line with earlier reports[52].

There is evidence that a higher intake of vitamin C is associated with a reduced risk of cancer and cardiovascular disease, probably due to its antioxidant effects[53]. Thus, the concentration of vitamin C in tissue is an indicator of oxidative stress[54]. The concentration of vitamin E has been inversely correlated to LPO[55]. In the present study, vitamin C and vitamin E levels were found to be reduced in mammary carcinoma bearing rats signifying an increase in LPO. The levels of these vitamins in rats induced with DMBA were reverted to near normal levels upon SM treatment.

Decreased activities of mitochondrial citric acid cycle enzymes ICDH,  $\alpha$ -KDH, SDH and MDH were observed in cancer-induced rats. Decreased levels of these enzymes in DMBA-induced cancer rats are due to defect in aerobic oxidation of pyruvate, which might cause the low production of ATP molecules[56]. Decreased activity of these enzymes might also be due to the alteration in the morphology and ultra structure of cancer cells[57] and decrease in mitochondrial membrane fluidity, increase in the negative surface charge distribution and alterations in membrane ionic permeability including proton permeability, which uncouples oxidative phosphorylation from electron

transport chain<sup>[58]</sup>. SM administration has influential effect and protects the mitochondria from oxidative stress and subsequent inactivation of enzymes caused by mammary carcinoma.

Enhanced oxidative stress and lipid peroxidation are implicated in carcinogenic processes. Lipid peroxidation is a chain reaction that occurs in membranes mediated by ROS and is an indicator of oxidative cell damage<sup>[59]</sup>. Lipid peroxides that are usually formed by the auto-oxidation of polyunsaturated fatty acids in the cell membrane reflect an increase in membrane damage<sup>[60]</sup>. Lipid peroxides are capable of reducing membrane fluidity, and loss of this feature of the mitochondrial membrane leads to decreased activities of NADH dehydrogenase and Cytochrome C oxidase<sup>[61]</sup> in DMBA-induced group. Also a significant decline in the activities of cytochrome c<sub>1</sub>, c, b and aa<sub>3</sub> were seen in DMBA-induced (Group II) animals due to increased LPO activity of DMBA. Treatment with SM attenuates LPO and restored membrane integrity by restoring the above-mentioned enzymes levels.

The ethanol extract of herbal constituents of SM contains rich phytochemical constituents which in turn resulted in the identification of seven different compounds by GC/MS and HPLC analysis. Trilinolein has been identified as one of the active constituents isolated from SM. Trilinolein is a triacylglycerol, which carries two unsaturated bonds (C 18:2, MW = 890), at all three esterified positions of glycerol. It has been reported to provide a number of beneficial effects including reducing thrombogenicity<sup>[62]</sup>, anti-ischemic<sup>[63]</sup>, anti-arrhythmic, and displaying antioxidant effects in various experimental models<sup>[62]</sup>. It is reported that trilinolein induced apoptosis in human lung carcinoma cells. The pro-apoptotic response was correlated with the increase of Bax, decrease of Bcl-2, cytochrome c release, caspase-3 activation and poly (ADP ribose) polymerase (PARP) degradation. Furthermore, the inactivation of PI3K/Akt, activation of p53/p21 protein expression and NF- $\kappa$ B inhibitory activity may play an important role in trilinolein-induced apoptosis<sup>[64,65]</sup>.

The increased levels of antioxidants status, attenuation of LPO, restoration of mitochondrial membrane integrity and modulation of TCA cycle/ respiratory chain enzymes in the drug treated rats (Group III) could be due to trilinolein and phenolic compounds (flavonoids) present in SM. It has been reported earlier that the flowers of *Hibiscus rosa-sinensis* contains the total phenolic content of 3.15 g/100 g dry weight, which contribute to the potential antioxidant and free radical scavenging activity<sup>[45]</sup>. Compelling data from laboratory studies, epidemiological investigations, and human clinical trials indicate that flavonoids have important effects on cancer chemoprevention and chemotherapy. Many mechanisms of action have been identified, including carcinogen inactivation, antiproliferation through inhibition of signal transduction enzymes such as protein tyrosine

kinase, protein kinase C and phosphoinositide-3-kinases, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis and reversal of multidrug resistance or a combination of these mechanisms<sup>[66]</sup>.

In summary, this study confirms that the formulation SM proved to be more effective in mitigating the mitochondrial dysfunction during mammary carcinogenesis and this is further supported in the preservation of membrane integrity by altering the levels of membrane-bound respiratory chain complexes, antioxidant enzymes and citric acid cycle enzymes to near normal levels. Restoration of cellular normal levels of different mitochondrial enzymes strongly suggests the cytoprotective role of the drug. Further approaches on elucidating the mechanism of chemopreventive action with reference to mitochondrial mediated intrinsic apoptotic pathway by SM are under investigation.

### Conflict of interest statement

We declare that we have no conflict of interest.

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