

Chemotherapeutic Efficacy of *Shemamruthaa*-an Herbal Formulation on Oxidative Stress Markers and Apoptotic Genes Expression in 7,12-dimethylbenz(α)anthracene Induced Breast Tumour in Rats

Ayyakkannu Purushothaman^{1,2}, Panchanatham Sachdanandam^{2,3*}

¹Department of Biochemistry, Mohamed Sathak College of Arts and Science (Affiliated to University of Madras), Chennai, Tamil Nadu, INDIA.

²Department of Medical Biochemistry, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, Tamil Nadu, INDIA.

³Department of Pathology, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, Tamil Nadu, INDIA.

Submission Date: 22-02-2020; Revision Date: 24-03-2020; Accepted Date: 04-04-2020

ABSTRACT

Plants have proved to be an important source of anti-cancer drugs. Here we have investigated the anticancer activity of the herbal formulation *Shemamruthaa* in rat models. SM contains multiple phenolic compounds and secondary metabolites that contribute to their biological properties. Female Sprague Dawley rats of 180 ± 10 g were categorized into four groups. Two groups were administered DMBA (25 mg/rat, orally) dissolved in olive oil to induce mammary carcinoma. One of these groups received *Shemamruthaa* (SM) (400 mg/ kg b.wt, orally) for 14 days after 90 days of DMBA induction. A vehicle treated control and drug control groups were also included. The status of oxidative stress markers, DNA fragmentation and western blot analysis of PCNA, p53, Bax, Bcl-2 and caspases were carried out in control and experimental rats. Our findings revealed that the SM formulation induced cell death was considered to be apoptotic by observing the typical DNA ladder formation on electrophoresis. The results of western blot analysis established the down-regulation of cell proliferative PCNA, anti-apoptotic protein Bcl-2 and up-regulation of pro-apoptotic proteins like p53, Bax and caspases expressions in mammary tissues of SM treated cancer bearing animals. Our results demonstrate that SM can inhibit cancer cell proliferation and induce apoptosis via p53-dependent mechanism. Our findings indicate that SM contains potential anti-cancer agents acting either singly or in combination against breast cancer cell proliferation.

Key words: *Shemamruthaa* (SM), Apoptosis, Hydroxymethylfurfural (HMF), Trilinolein, Flavonoids, Mammary carcinoma.

Correspondence:

Dr. P. Sachdanandam,
Professor, Department of
Medical Biochemistry, Dr.
ALM Post-Graduate Institute
of Basic Medical Sciences,
University of
Madras, Taramani
Campus, Chennai-113, Tamil
Nadu, INDIA.
Phone no: +91-044-24501578

Email: psachdanandam2000@yahoo.co.in

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide,

accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008. About half the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries.^[1,2] Current cancer therapies, *viz.* chemotherapy, γ -irradiation, immunotherapy or suicide gene therapy, primarily exert their anticancer effect by triggering apoptosis in cancer cells.^[3] The term 'apoptosis' was coined by Kerr, Wyllie and Currie in 1972 to describe a mode of cell death associated with fragmentation of genomic DNA.^[4] In addition

SCAN QR CODE TO VIEW ONLINE



www.ajbls.org

DOI :
10.5530/ajbls.2020.9.8

to DNA fragmentation, apoptosis is morphologically characterized by the cytoplasmic condensation, nuclear pyknosis, chromatin condensation, cell rounding, membrane blebbing, cytoskeletal collapse and the formation of membrane-bound apoptotic bodies that are rapidly phagocytosed and digested by macrophages or neighboring cells without activating immune response.^[5,6] Apoptosis is delicately regulated and balanced in a physiological context. Failure of this regulation results in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration, AIDS and other viral or bacterial infections or cancer.^[7,8]

In the last decades, phytochemicals have attracted a growing attention as anticancer agents due to their ability to modulate apoptosis signalling pathways.^[9,10] It has been shown that the phytochemicals present in herbal formulations are more effective than their individual constituents in preventing cancer through both additive and synergetic effects.^[11,12] In this perspective, the study of herbal formulations from traditional medicine represents a challenging research field, since it has been applied for the treatment of cancers for many years.^[13,14]

***Hibiscus rosa-sinensis* Linn.** (Family: Malvaceae) commonly known as China rose is a potent herb in traditional system of medicine.^[15] It is being used against cough, fever, dysentery, venereal diseases and cancerous swellings.^[16] In South Asian traditional medicine, various parts of the plant are used in the preparation of a variety of foods.^[17] The ethanol extract of *Hibiscus rosa-sinensis* flowers showed a dose dependent increase in radical scavenging ability against various free radicals and also exhibited a significant inhibition of lipid peroxidation *in vitro*. The extract rendered significant protection against cyclophosphamide induced genotoxicity.^[18] Phytochemical studies revealed the presence of several chemicals, including flavonoids, flavonoid glycosides, hibiscetin, cyanidine, cyanidin glucosides, taraxeryl acetate, β -sitosterol, campesterol, stigmasterol, ergosterol, citric, tartaric and oxalic acids, cyclopropenoids and anthocyanin pigments.^[17,19-22]

***Phyllanthus emblica* Linn.** (*Emblica officinalis* Gaertn. [EO], Family: Euphorbiaceae), also known as Amla, has been used in Ayurveda, the ancient Indian system of medicine. According to the main classic texts on Ayurveda, *Charak Samhita* and *Susbrut Samhita*, amla is regarded as the “best among rejuvenative herbs”.^[23] Previous studies suggest that consumption of Amla can have beneficial effects against diseases such as cancer.^[24,25] It has been suggested that bioactive components of EO invoke anti-cancer effects through various complementary and overlapping mechanisms of

action including the induction of metabolizing enzymes, modulation of gene expression etc. A recent study showed that EO extracts exhibit a higher level of antioxidant capacity against free radical species.^[26] It is known that *Emblica* is a good source of vitamin C, polyphenols, flavones, tannins, Gallic acid, Ellagic acid, Chebulinic acid, Quercetin, Chebulagic acid, Emblicanin-A, Emblicanin-B, Punigluconin, Pedunculagin, isocorilagin, mallotusin and other bioactive substances,^[26,27] while these substances are strong antioxidants and might contribute to the anticancer effects of *Emblica*.

The use of traditional medicine to treat infection has been practiced since the origin of mankind and honey produced by *Apis mellifera* is one of the oldest traditional medicines considered to be important in the treatment of several human ailments.^[28] This natural product exhibits antioxidant, chemopreventive, antiatherogenic, immunoregulatory, antimicrobial and wound healing properties. Various signalling pathways including stimulation of tumour necrosis factor-alpha (TNF- α) release, inhibition of cell proliferation, induction of apoptosis and cell cycle arrest, as well as inhibition of lipoprotein oxidation mediate the beneficial effects exerted by honey.^[29-36] Honeys were very rich in compounds known to possess anticancer properties, such as polyphenols and phenolic acids and exhibit significant biological effects in human cancer cells. Therefore, honey was also incorporated in the formulation with the aim to potentiate cancer treatment related processes.

Hence, *Shemamruthaa*, a phytochemical combination constituting dried flowers of *Hibiscus rosasinensis*, fruits of *Phyllanthus emblica* and honey in a definite ratio was formulated and evaluated for the first time with a view to potentiate more intense anticancer effect. In our previous studies, we have reported that SM exerts profound anticancer activity through its role in reinstating the normal levels of glycoprotein components, revitalizing the membrane stability,^[37] restoring antioxidant status^[38] and deranged energy metabolism in DMBA-induced mammary carcinoma bearing rats.^[39] The present study was designed to evaluate the therapeutic efficacy of SM on DMBA-induced mammary carcinoma bearing rats. The status oxidative markers (prooxidants), activities of phase I and phase II xenobiotic-metabolizing enzymes, the expression of marker of proliferation (proliferating cell nuclear antigen (PCNA) and apoptosis (Bcl-2, Bax, caspase 3 and caspase 9) were analyzed in the mammary tissues of control and experimental rats.

MATERIALS AND METHODS

Chemicals

DMBA (7,12-Dimethylbenz[a]anthracene) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a RT kit (New England Biolabs, UK). DNA markers, protein markers, normal melting agarose, acrylamide, nitrocellulose membrane and N, N'-methylene bisacrylamide were purchased from Amersham Pharmacia, Germany. All other chemicals and reagents used were of analytical grade and purchased from Glaxo Laboratories, BDH division, Mumbai, India, SISCO Research Laboratories, SD fine chemicals, Mumbai, Sarabhai Chemicals, Baroda, India.

Preparation of *Shemamruthaa* (SM)

The flowers of *Hibiscus rosa-sinensis* (Family: Malvaceae; red flowered variety, single petals) were collected from a local garden in southern part of India (Kancheepuram District, Tamil Nadu, India) and the pharmacognostic authentication was done by Dr. Sasikala Ethirajulu, Research Officer (Pharmacognosy) and Dr. S.Jega Jothi Pandian, Research Officer In-charge, Siddha Central Research Institute (Central Council for Research in Siddha, Department of AYUSH, Ministry of Health and Family Welfare, Government of India) Chennai-600 106. The fruits of *Emblica officinalis* Gaertn. (Family: Euphorbiaceae; Synonym: *Phyllanthus emblica* Linn.), at the mature stage, were purchased commercially from the local market and the rinds were carefully removed from the seeds. The flowers of *Hibiscus rosa-sinensis* and *Emblica* rinds were air dried under shade, pulverized to fine powder using a laboratory scale cutting mill and mixed with pure honey in a definite ratio.

Animals

Adult female albino rats of Sprague-Dawley strain weighing 180 ± 10 g were provided from Central Animal House facility, University of Madras, Taramani Campus, Chennai-600113, Tamil Nadu, India. The animals were maintained under standard conditions of humidity, temperature ($25 \pm 2^\circ\text{C}$) and light (12 h light/dark). They were fed with standard rat pellet diet manufactured by M/s. Pranav Agro Industries Ltd., India under the trade name "Amrut" rat/mice feed 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 (CPCSEA), Ministry of Environment and Forests (Animal Welfare Division), Government of India and Institutional Animal Ethics Committee (IEAC) Guidelines, (IAEC.No.01/030/2011).

Experimental Design

Adult female Sprague-Dawley rats (8 weeks old) were divided into four Groups having six animals in each Group and given the following dose regimen:

- Group I Normal control animals received a single oral dose of olive oil (1 ml) at the age of eight weeks (Control).
- Group II Animals induced for mammary carcinoma with a single oral dose of 25 mg of DMBA dissolved in one ml of olive oil after overnight fasting (Welsch, 1985).^[40] After two months, the rats were palpated regularly to find out the appearance of mammary tumour(s). After three months, mammary carcinoma was confirmed by histological examinations (DMBA-induced).
- Group III Mammary carcinoma was induced as in Group II, in addition, after three months; animals were treated with SM at the dose of 400 mg/ kg body weight/day and continued for fourteen days by gastric intubation (DMBA+ SM).
- Group IV Animals received 400 mg/kg body weight of SM alone and served as SM control

Gross observations and Tumour volume

The tumour incidence and changes in body weight, liver and kidney weights were measured and the weights were recorded in g. During the experimental period, i.e., Prior to sacrificing the animals, the animals were weighed, explored by inspection and palpation and the two major and perpendicular diameters of each tumour were measured with a Vanier calliper. The tumour volume (v) was measured using the formula:

$$v = 4/3 \pi (d_{1/2}) \times (d_{2/2})^2, \text{ where } d_1 \text{ and } d_2 \text{ are the two diameters of the tumour } (d_1 > d_2)$$

After sacrificing, the volume of each tumour was calculated using its three diameters:

$$v = 4/3 \pi (d_{1/2}) \times (d_{2/2}) \times (d_{3/2}); (d_1 > d_2 > d_3).$$

Oxidative stress markers: lipid and protein oxidation

The levels of lipid peroxides were measured as thiobarbituric acid reactive substances (TBARS) by the method described by Okhawa *et al.* (1979).^[41] Lipid hydroperoxides were measured with the method of ferrous oxidation in xylol orange (FOX) as described by Jiang *et al.*, (1992) and Wolff (1994).^[42,43] The protein carbonyl content was quantified by the method of Levine *et al.*, (1990).^[44]

DNA fragmentation analysis by Agarose gel electrophoresis

Agarose gel electrophoresis was carried out for the analysis of DNA fragmentation by the method of Yokozawa and Dong (2001).^[45] The DNA of mammary tissues of control and experimental animals were isolated following the manufacture's instructions provides by the kit (Genie, Bangalore, India) and dissolved in TE buffer. The DNA samples (1mg) were electrophoresed on 1.2% agarose gel using TBE buffer at 40 V for 3 h. Then, the gel was stained with Ethidium bromide (EtBr) and viewed under UV –Transilluminator and photographed.

Western blot analyses

The mammary tissue was homogenized in 20 mM Tris-HCl (pH 7.5), containing 150 mM NaCl, 1% Nonidet P-40, 1 mM Phenylmethylsulphonyl fluoride and 1 µg/ml Aprotinin. After incubation on ice for 30 min, the homogenate was centrifuged at 14,000 rpm for 10 min at 4°C. The protein concentration of the homogenate was estimated by the method of Lowry *et al.*, (1951).^[46] The samples were boiled with sample solubilizing buffer (SSB) for 5 min and separated on 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli, (1970).^[47] The gel was transferred onto a nitrocellulose membrane (Hybond C+, Amersham life sciences) at 30V for 3 h. Membrane was then washed thrice with PBS and blocking was done with Tris-Buffered Saline with Tween-20 (TBST buffer: 20 mM Tris, 500 mM NaCl and 0.1% Tween-20, pH 7.5) containing 5% non-fat dried milk. Then, the membrane was incubated with primary antibody (rabbit monoclonal antibody, Bax and Bcl₂ (Cell signaling Technology) and rabbit monoclonal antibody p53, PCNA, Caspase-3 and -9 (Sigma chemical company, St. Louis, USA) in TBST buffer containing 1% non-fat dried milk and agitated gently at room temperature for 3 h. After incubation with the primary antibody, the blots were washed thrice for 5 min with TBST buffer and incubated for 75 min at room temperature with goat rabbit anti-goat horseradish peroxidase (HRP) conjugated secondary antibody in phosphate-free TBST buffer containing 5% non-fat dried milk. The bands were detected using chemiluminescence (Pierce).

Histopathological analysis

Formalin-fixed liver and mammary tissue samples from control and experimental rats 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). Histopathological evaluation was performed according to Costa *et al.* (2002).^[48]

Statistical analysis

The results are expressed as mean±standard deviation (S.D.). Differences between groups were assessed by ANOVA using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (L.S.D.) Differences were considered significant at $p < 0.05$.

RESULTS

Gross Observations

Figure 1 and Figure 2 represents the changes in the whole body weight and tumour volume in control and experimental animals. The body weight and organ weights such as liver and kidneys were significantly decreased in DMBA-induced mammary carcinoma bearing rats when compared to control animals. Oral administration of SM significantly recouped the body weight and organ weights. The SM treated rats showed

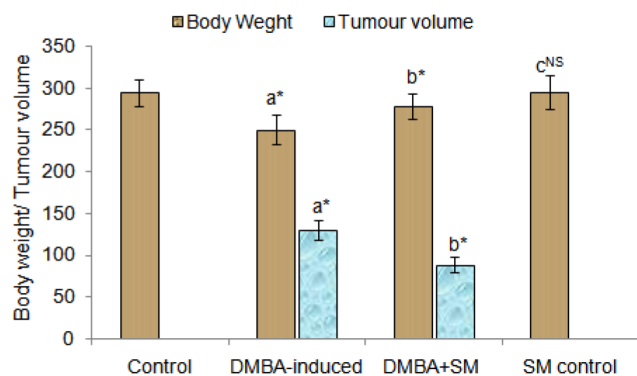


Figure 1: Effect of SM on changes in body weight and tumour volume in control and experimental animals.

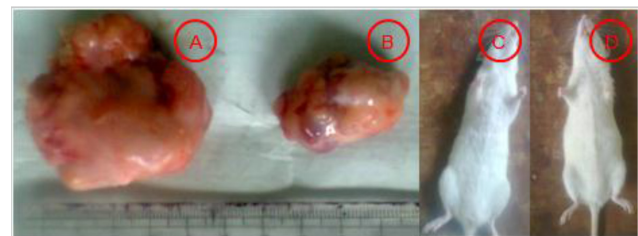


Figure 2: Representative photograph depicts the mammary tumours excised from DMBA-induced (A) and DMBA+SM treated (B) female Sprague-Dawley rats. The gross appearance of the normal mammary gland morphology in control (C) and SM control (D) rats.

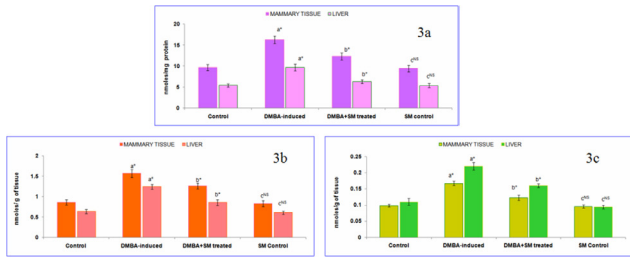


Figure 3: Oxidative stress markers 3a- levels of thiobarbituric acid reactive substances (TBARS) 3b- levels of lipid hydroperoxides (LOOH) 3c- levels of protein carbonyl (PC) in the mammary tissue and liver of control and experimental animals.

a significant reduction in tumour volume when compared to DMBA-induced mammary carcinoma rats. Values are expressed as mean \pm SD of six animals in each group.

Initial weights of the animals are more or less similar (180 ± 10 g).

Comparison is made as:

^aDMBA-induced vs. Control

^bDMBA-induced vs. DMBA+ SM treated

^cControl vs. SM control.

Statistical significance: * $p < 0.05$. ^{NS}Non-Significant.

Effect of SM on Oxidative stress markers

Figure 3a-3c show the extent of lipid and protein oxidation in the mammary gland and liver tissues of control and experimental animals. The extent of lipid and protein oxidation was significantly higher in the mammary gland and liver of DMBA-induced rats (Group II) when compared to control rats. SM treatment resulted in free radical scavenging and there by significantly ($p < 0.05$) decreasing lipid peroxidation (LPO), protein oxidation and restoring the enzymatic antioxidant activities to near normal levels in SM treated rats.

DNA fragmentation

Genomic DNA was isolated from control and experimental animals. Animals treated with SM exhibited typical DNA ladder formation, which is a hallmark of apoptosis (Figure 4). In untreated, control and SM control animals DNA ladder formation was not observed.

Effect of SM on expression of PCNA, pro-apoptotic and anti-apoptotic genes toward apoptosis

The protein expression levels of pro-apoptotic genes/anti-apoptotic genes were examined by western blot in mammary tissues of control and experimental animals. The results of Western blot were presented in Figure 5. As seen in Figure 5, SM treatment significantly induced protein levels of p53 and Bax. Meanwhile, the cell

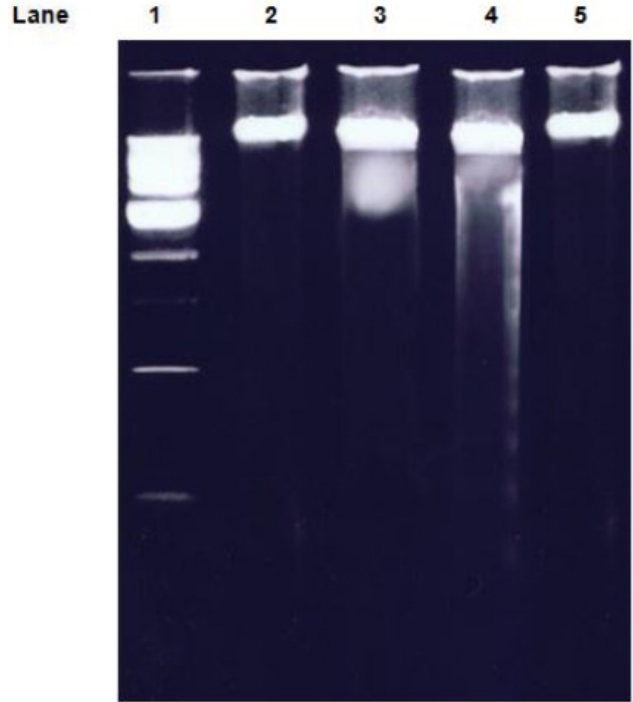


Figure 4: DNA fragmentation Lane 1, DNA marker. Control and SM control animals show the normal pattern of DNA (Lane 2 and 5). Lane 3, DMBA-induced. DNA ladder formation in SM treated animals (Lane 4).

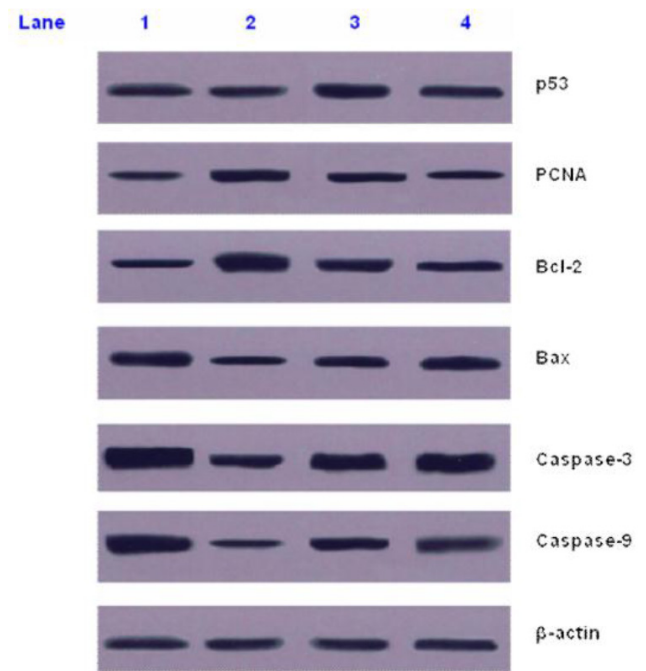


Figure 5: Western blots analyses showing the expression of p53, PCNA, Bcl-2, Bax, caspase-3 and caspase-9 in mammary tissue of control and experimental rats.

Lane 1: Control; Lane 2: DMBA-induced; Lane 3: DMBA+ SM treated; Lane 4: SM Control

proliferative (PCNA) and anti-apoptotic gene, Bcl-2 was down-regulated by SM treatment. Clearly, the balance between pro-apoptosis and anti-apoptosis genes in DMBA-induced cancer bearing rats was modulated by SM treatment and the new profile of gene expressions within the intrinsic pathway favours apoptosis.

DISCUSSION

There was a sharp fall in the body weight in mammary carcinoma induced animals. This may be due to tumour cachexia, characterized by weakness, lethargy, anorexia, depletion of host components, tissue wasting and a progressive waning of vital functions.^[49] The SM treated animals showed a gradual increase in their body weights and significant reduction in tumour volume when compared to DMBA-induced mammary carcinoma rats indicating the counteractive property of SM. This might be due to the protective effect of flavonoids present in the SM since, flavonoids are known to display a vast array of cellular events, they can control the overall process of carcinogenesis by several mechanisms including modulation of survival/proliferation pathways,^[50] activation of caspases,^[51] down-regulation of Bcl-2 and Bcl-xL expression and enhanced expression of Bax and Bak,^[52] and modulation of nuclear factor κ B^[53] might be responsible for tumour regression in SM treated animals.

The reactive oxygen species (ROS) that are formed during the metabolism of DMBA can diffuse from the site of generation to other targets within the cell or even propagate the injury outside to intact cells. The liver is especially vulnerable to such ROS-induced damage although endowed with a rich supply of antioxidants. Thus, ROS-induced lipid and protein oxidation with compromised antioxidant defences may constitute an underlying mechanism of carcinogenesis caused by DMBA. SM treatment resulted in free radical scavenging and there by significantly ($p < 0.05$) decreasing lipid peroxidation (LPO), protein oxidation and restoring the enzymatic antioxidant activities to near normal levels in SM treated rats.

The HPLC analysis of SM showed the presence of gallic acid (992.25 μ g/g), quercetin (335.75 μ g/g), caffeic acid (156.6 μ g/g), rutin (6.0 μ g/g) and ferulic acid (0.3 μ g/g).^[38] An *in vitro* oxidation model showed quercetin, myricetin and rutin being more powerful antioxidants than the traditional vitamins. Flavonols and flavones possess antioxidant and free radical scavenging activities in foods and have significant Vitamin C sparing activity with myricetin being one of the most active flavonoids.^[54] Thus, polyphenolic compounds of SM elicit oxidative

defence against ROS-induced oxidative stress by their potent antioxidant role and protect cells.^[55]

In previous reports from this laboratory, we have demonstrated that the anticancer effect of SM was accompanied by the restoration of deranged energy metabolism, antioxidant status and membrane stabilizing property.^[37-39] Considering the anticancer effect of SM *in vivo* and lack of toxicity, we further ventured to understand the mechanism of induction of cell death by studying the cytotoxic potential of SM. Apoptosis is a major form of cell death, characterized by a series of stereotypic morphological changes such as formation of apoptotic bodies, chromatin condensation, shrinkage of cells, bleb formation and inter-nucleosomal DNA fragmentation (hallmark of apoptosis). In the present study, we demonstrated that SM induced DNA fragmentation with DNA ladder formation characteristic of apoptosis.

In the present study, the marker of cell proliferation (PCNA) was over-expressed in the mammary gland of rats treated with DMBA alone. Over-expression of PCNA could be used as a reliable marker for evaluating tumour grade and assessment of tumour progression.^[56] The cell proliferative PCNA was down-regulated by SM treatment. This shows the antiproliferative effect of SM.

In contrast to frequent p53 mutations in human breast cancer, DMBA-induced breast cancer is rarely associated with p53 alterations,^[57] although such have been well documented. The reduced activity of p53 gene in DMBA-induced rats (Group II) may be due to over expression of Mouse double minute 2 homolog (Mdm2) and other cellular factors that down-regulate its level.^[58] It was also evidenced by Kim *et al.*^[59] that chloroform extract of *Caesalpinia sappan* increased the expression of p53 in head and neck cancer cells *in vitro*. It can be mentioned that the inhibition of mammary preneoplasia may be mediated through the elevated expression of p53 gene. Observed elevations in the expression of p53 upon SM treatment when compared to DMBA-induced rats are in line with the above findings.

Apoptosis, the programmed cell death, maintains a balance between cell death and cell renewal by removing excess, damaged or abnormal cells. Bcl-2 family is a 25-kDa integral membrane proteins that govern mitochondrial outer membrane permeabilization and can be either anti-apoptotic (Bcl-2) or pro-apoptotic (Bax) proteins. It has been reported that Bcl-2 prevents Bax/Bak oligomerization, which in turn leads to the release of several apoptogenic molecules from the mitochondria. The increased levels of anti-apoptotic Bcl-2 expression in cancer condition conferred cell survival by inhibiting apoptosis and interacting

directly with Bax and preventing activation of caspases.^[60] Downregulation of Bax proteins has been shown in metastatic breast adenocarcinoma and oral cancers.^[61] The Bax/Bcl-2 ratio determines the fate of a cell in response to stress and measurement of the expression of these proteins together may provide valuable predictive information about outcome of the cancer treatment. Abrogation of Bcl-2 expression as well as reinforcement of Bax expression not only cause tumour regression but also render them more sensitive to apoptosis-inducing treatment. Our finding of the present investigation is well in accordance with the earlier investigations. The results of the present study inevitably demonstrates that SM induced cell death by activating apoptotic pathway in DMBA-induced mammary cancer rats and also interferes with progression of tumours in rats without affecting other cellular functions and body weight. This effect is further confirmed by histopathological studies of mammary tissues of control and experimental animals.

Phenolic compounds such as flavonoids, di-hydroxy benzoic acid, caffeic acid, ferulic acid and cinnamic acid are found in various honeys and plant sources. High phenolic and hydroxymethylfurfural (HMF) contents of honey resulted in the growth inhibitory effects of cancer cells^[62] and flavonoids have been reported to induce cell death of various cancer cell lines. The presence of alkaloids, terpenoids, phenolics, glycosides, steroids and tannins (as determined by preliminary phytochemical screening); flavonoids gallic acid, caffeic acid, rutin, quercetin and ferulic acid (as determined using HPLC analysis) and HMF, pyrogallol and trilinolein (as determined using GC-MS analysis) could have synergistically contributed to the apoptotic effect of SM.

CONCLUSION

Based on the results, it might be concluded that “the herbal formulation, *Shemamruthaa* (SM), constituted of dried flowers of *Hibiscus rosasinensis*, fruits of *Emblica officinalis* and honey has been found to exhibit pronounced antiproliferative and apoptotic effect”. This enhanced anticancer effect of SM might be attributed to the synergistic action of polyphenols such as flavonoids, tannins, alkaloids, glycosides, saponins, steroids, terpenoids, vitamin C, niacin, pyrogallol, hydroxymethylfurfural, trilinolein and other compounds present in the SM formulation.

ACKNOWLEDGEMENT

The authors would like to thank Shri. S.Kumaravel, Senior Scientist, Food Testing Laboratory [NABL Accredited Laboratory as per ISO/IEC 17025:2005] Indian Institute of Crop Processing Technology (Ministry of Food Processing Industries, Government of India), Tanjavur-613 005, Tamil Nadu, India for providing GC-MS and HPLC facilities.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

α : alpha; β : beta; γ : gamma; π : pi; μ : micro; μg : microgram; μl : microlitre; μM : micromole; dl : decilitre; **DMBA**: 7,12-dimethylbenz(a)anthracene; **EO**: *Emblica officinalis* Gaertn; **HPLC**: High-performance liquid chromatography; **LOOH**: Lipid hydroperoxides; **LPO**: Lipid peroxidation; **NF- κ B**: Nuclear factor-kappa B **RT-PCR**: Reverse Transcriptase-Polymerase Chain Reaction; **SM**: *Shemamruthaa*; **SD**: Standard deviation; **PCNA**: Proliferating Cell Nuclear Antigen.

SUMMARY

This study reports the antiproliferative and apoptotic effects of an indigenous herbal formulation *Shemamruthaa*. The profound anticancer effect of *Shemamruthaa* might be attributed to the synergistic action of polyphenols, flavonoids, hydroxymethylfurfural, trilinolein, and other bioactive compounds present in the formulation. Collectively, these results demonstrate that *Shemamruthaa* holds potential for the treatment and development novel anticancer chemotherapeutics against mammary carcinoma.

REFERENCES

- Ahmedin JDVM, Freddie B, Melissa M, Center MPH, Jacques FME, *et al*. Global Cancer Statistics. CA Cancer J Clin. 2011;61(2):69-90.
- Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin D. GLOBOCAN. Cancer incidence and mortality worldwide. IARC cancer base No. 10. Lyon, France: International Agency for Research on Cancer. 2008. Available from: <http://globocan.iarc.fr>; 2008.
- Fulda S, Debatin KM. Targeting apoptosis pathways in cancer therapy. Curr Cancer Drug Targets. 2004;4(7):569-576.
- Kerr JF, Wyllie AH, Currie AR. Apoptosis. A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1974;26(4):239-57.
- Sato A, Satake A, Hiramoto A, Okamoto A, Nakama K, Kim H, *et al*. Association of nuclear-intermediate filament lamin B1 with necrotic and apoptotic-morphologies in cell death induced by 5-fluoro-2'-deoxyuridine. Nucleic Acids Symposium Series. 2009;53:293-4.

6. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. *Nature*. 2000;407(6805):784-8.
7. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science*. 1995;267(5203):1456-62.
8. Los M, Herr I, Friesen C, Fulda S, Schulze-Osthoff K, Debatin KM. Cross-resistance of CD95- and drug-induced apoptosis as a consequence of deficient activation of caspases (ICE/Ced-3 proteases). *Blood*. 1997;90(8):3118-29.
9. Aravindaram K, Yang NS. Anti-inflammatory plant natural products for cancer therapy. *Planta Medica*. 2010;76(11):1103-17.
10. Tosetti F, Noonan DM, Albini A. Metabolic regulation and redox activity as mechanisms for angioprevention by dietary phytochemicals. *International Journal of Cancer*. 2009;125(9):1997-2003.
11. Fulda S. Modulation of apoptosis by natural products for cancer therapy. *Planta Medica*. 2010;76(11):1075-9.
12. Liu RH. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. *J Nutr*. 2004;134(12):3479S-85S.
13. Boivin D, Blanchette M, Barrette S, Moghrabi A, Beliveau R. Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NF kappa B by edible berry juice. *Anticancer Res*. 2007;27(2):937-48.
14. Hsiao WLW, Liu L. The role of traditional Chinese herbal medicines in cancer therapy –from TCM theory to mechanistic insights. *Planta Medica*. 2010;76(11):1118-31.
15. Nade VS, Subhash D, Kawale LA, Upasani CD, Yadav AV. Effect of *Hibiscus rosasinensis* on reserpine-induced neurobehavioural and biochemical alterations in rats. *Indian J Exp Biol*. 2009;47:559-63.
16. Ross IA. *Hibiscus rosa sinensis*. Medicinal plants of the world. Chemical constituents, traditional and modern medicinal uses. Totowa, Humana Press. 1999;155-63.
17. Gilani AH, Bashir S, Janbaz KH, Shah AJ. Presence of cholinergic and calcium channel blocking activities explains the traditional use of *Hibiscus rosasinensis* in constipation and diarrhoea. *J Ethnopharmacol*. 2005;102(2):289-94.
18. Khatib NA, Gautam G, Hashikar N, Joshi RK, Taranalli AD. Effect of *Hibiscus rosasinensis* extract on modifying cyclophosphamide induced genotoxicity and scavenging free radicals in swiss albino mice. *Pharmacologyonline*. 2009;3:796-808.
19. Ajay M, Chai HJ, Mustafa AM, Gilani AH, Mustafa MR. Mechanisms of the antihypertensive effect of *Hibiscus sabbdariffa* L. calyces. *Journal Ethnopharmacology*. 2007;109(3):388-93.
20. Gauthaman KK, Saleem MT, Thanislas PT, Prabhu VV, Krishnamoorthy KK, Devaraj NS, et al. Cardioprotective effect of the *Hibiscus rosa-sinensis* flowers in an oxidative stress model of myocardial ischemic reperfusion injury in rat. *BMC Complementary and Alternative Medicine*. 2006;6(1):32.
21. Sachdewa A, Khemani LD. Effect of *Hibiscus rosa-sinensis* ethanol flower extract on blood glucose and lipid profile in streptozotocin induced diabetes in rats. *Journal of Ethnopharmacology*. 2003;89(1):61-6.
22. Sachdewa A, Raina D, Srivastava AK, Khemani LD. Effect of *Aegle marmelos* and *Hibiscus rosa sinensis* leaf extract on glucose tolerance in glucose induced hyperglycemic rats (Charles foster). *Journal of Environmental Biology*. 2001;22(1):53-7.
23. Scartezzini P, Antognoni F, Raggi MA, Poli F, Sabbioni C. Vitamin C content and antioxidant activity of the fruit and of the Ayurvedic preparation of *Emblca officinalis* Gaertn. *J Ethnopharmacol*. 2006;104(1-2):113-8.
24. Veena K, Shanthy P, Sachdanandam P. Anticancer Effect of Kalpaamruthaa on Mammary Carcinoma in Rats with Reference to Glycoprotein Components, Lysosomal and Marker Enzymes. *Biol Pharm Bull*. 2006;29(3):565-9.
25. Ngamkitidechakul C, Jaijoy K, Hansakul P, Soonthornchareonnon N, Sireeratawong S. Antitumour Effects of *Phyllanthus Emblica* L.: Induction of Cancer Cell Apoptosis and Inhibition of *in vivo* Tumour Promotion and *in vitro* Invasion of Human Cancer Cells. *Phytother Res*. 2010;24(9):1405-13.
26. Luo H, Rankin GO, Li Z, Depriest L, Chen YC. Kaempferol induces apoptosis in ovarian cancer cells through activating p53 in the intrinsic pathway. *Food Chem*. 2011;128(2):513-9.
27. Khan KH. Roles of *Emblca officinalis* in Medicine: A Review. *Botany Research International*. 2009;2(4):218-28.
28. Manisha DM, Shyamapada M. Honey: Its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine*. 2011;1(2):154-60. doi: 10.1016/S2221-1691(11)60016-6.
29. Al-Waili NS, Haq A. Effect of honey on antibody production against thymus-dependent and thymus-independent antigens in primary and secondary immune responses. *Journal of Medicinal Food*. 2004;7(4):491-4.
30. French VM, Cooper RA, Molan PC. The antibacterial activity of honey against coagulase-negative *Staphylococci*. *Journal of Antimicrobial Chemotherapy*. 2005;56(1):228-31.
31. Gheldof N, Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of *in vitro* lipoprotein oxidation in human serum samples. *Journal of Agricultural and Food Chemistry*. 2002;50(21):3050-5.
32. Molan PC. The evidence supporting the use of honey as a wound dressing. *International Journal of Lower Extremity Wounds*. 2006;5(1):122.
33. Mabrouk GM, Moselhy SS, Zohny SF, Ali EM, Helal TE, Amin AA, et al. Inhibition of methylnitrosourea (MNU) induced oxidative stress and carcinogenesis by orally administered bee honey and Nigella grains in Sprague Dawley rats. *Journal of Experimental and Clinical Cancer Research*. 2002;21(3):341-6.
34. Schramm DD, Karim M, Schrader HR, Holt RR, Cardetti M, Keen CL. Honey with high levels of antioxidants can provide protection to healthy human subjects. *Journal of Agricultural and Food Chemistry*. 2003;51(6):1732-5.
35. Swellam T, Miyanaga N, Onozawa M, Hattori K, Kawai K, Shimazui T, et al. Antineoplastic activity of honey in an experimental bladder cancer implantation model: *in vivo* and *in vitro* studies. *International Journal of Urology*. 2003;10(4) 213-9.
36. Tonks A, Cooper RA, Price AJ, Olan PC, Jones KP. Stimulation of TNF-alpha release in monocytes by honey. *Cytokine*. 2001;14(4):240-2.
37. Purushothaman A, Nandhakumar E, Sachdanandam P. Anticancer effect of *Shemamruthaa* (a phytochemical formulation) on 7, 12-dimethylbenz(a)anthracene induced mammary carcinoma in rats. *Asian J Pharm Clin Res*. 2012;5 (1):101-7.
38. Purushothaman A, Nandhakumar E, Sachdanandam P. Modulatory effect of *Shemamruthaa*, a phytochemical combinant against DMBA-induced oxidative damage in mammary carcinoma rats. *Asian pacific Journal of Tropical Biomedicine*. 2012;S (2):S765-71.
39. Purushothaman A, Nandhakumar E, Sachdanandam P. Phytochemical analysis and anticancer capacity of *Shemamruthaa*, an herbal formulation against DMBA- induced mammary carcinoma in rats. *Asian Pacific Journal of Tropical Medicine*. 2013;6(12):925-33.
40. Welsh CW. Factors affecting the growth of carcinogen induced rat mammary carcinomas: A review and tribute to Charles Brendon Huggins. *Cancer Res*. 1985;45(8):3415-43.
41. Okhawa H, Ohishi N, Yagi K. Assay of lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979;95(2):351-8.
42. Jiang ZY, Hunt JV, Wolff SP. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal Biochem*. 1992;202(2):384-7.
43. Wolff SP. Ferrous oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Meth Enzymol*. 1994;233:182-9.
44. Levine RL, Garland D, Olive CN, Amici A, Climent I, Lenz AG, et al. Determination of Carbonyl Content in Oxidatively Modified Proteins. *Methods Enzymol Academic Press, New York*. 1990;186:464-78.
45. Yokozawa T, Dong E. Role of ginsenoside-Rd in cisplatin-induced renal injury: Special reference to DNA fragmentation. *Nephron*. 2001;89:433-8.
46. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:265-75.
47. Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T₄. *Nature*. 1970;227(5259):680-5.
48. Costa I, Solanas M, Escrich E. Histopathologic characterization of mammary neoplastic lesions induced with 7,12 dimethylbenz(alpha)anthracene in the rat: A comparative analysis with human breast tumours. *Archives of Pathology and Laboratory Medicine*. 2002;126(8):915-27.
49. Argiles JM, Azcon-Bieto J. The metabolic environment of cancer. *Biol Cell Biochem*. 1988;81(1):3-17.

50. Selvendiran K, Koga H, Ueno T, Yoshida T, Maeyama M, Torimura T. *et al.* Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids. *Cancer Res.* 2006;66(9):4826-34.
51. Shimizu M, Deguchi A, Lim JTE, Moriwaki H, Kopelovich L, Weinstein I. Epigallocatechin gallate and polyphenon E inhibit growth and activation of epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin Cancer Res.* 2005;11(7):2735-46.
52. Kuo PL, Lin CC. Green tea constituent epigallocatechin-3-gallate inhibits HepG2 cell proliferation and induces apoptosis through p53-dependent and Fas-mediated pathways. *J Biomed Sci.* 2003;10(2):219-27.
53. Gong L, Li Y, Nedeljkovic-Kurepa A, Sarkar F. Inactivation of NF κ B by genistein is mediated via Akt signaling pathway in breast cancer cells. *Oncogene.* 2003;22(30):4702-9.
54. Koo HM, Suhaila M. Flavonoid (Myricetin, Quercetin, Kaempferol, Luteolin and Apigenin) Content of Edible Tropical Plants. *J Agric Food Chem.* 2001;49(6):3106-12.
55. Selvendiran K, Sakthisekaran D. Chemopreventive effect of piperine on modulating lipid peroxidation and membrane bound enzymes in benzo(a) pyrene induced lung carcinogenesis. *Biomed Pharmacother.* 2004;58(4):264-7.
56. Bishayee A, Dhir N. Resveratrol-mediated chemoprevention of diethylnitrosamine-initiated hepato carcinogenesis: Inhibition of cell proliferation and induction of apoptosis. *Chem Biol Interact.* 2009;179(2-3):131-44.
57. Todorova VK, Kaufmann Y, Luo S, Klimberg VS. Modulation of p⁵³ and c-myc in DMBA-Induced Mammary Tumors by Oral Glutamine. *Nutrition and Cancer.* 2006;54(2):263-273.
58. Tabakin-Fix Y, Azran I, Schavinsky-Khrapunsky Y, Levy O, Aboud M. Functional inactivation of p⁵³ by human T-cell leukemia virus type 1 Tax protein: Mechanisms and clinical implications. *Carcinogenesis.* 2006;27(4):673-81.
59. Kim EC, Hwang YS, Lee SK, Park MH, Jeon BH, Jeon CD, *et al.* *Caesalpinia sappan* induces cell death by increasing the expression of p⁵³ and p21WAF1 in head and neck cancer cells. *Am J Clin Med.* 2005;33(Suppl 3):405-14.
60. Marzo I, Naval J. Bcl-2 family members as molecular targets in cancer therapy. *Biochem Pharmacol.* 2008;76(8):939-46.
61. Pugalendhi P, Manoharan S, Suresh K, Baskaran N. Genistein and daidzein, in combination, protect cellular integrity during 7,12-dimethylbenz(a) anthracene (DMBA) induced mammary carcinogenesis in sprague-dawley rats. *Afr J Tradit Complement Altern Med.* 2011;8(2):91-7.
62. Tsiapara AV, Jaakkola M, Chinou I, Graikou K, Tolonen T, Virtanen V, *et al.* Bioactivity of Greek honey extracts on breast cancer (MCF-7), prostate cancer (PC-3) and endometrial cancer (Ishikawa) cells: profile analysis of extracts. *Food Chem.* 2009;116(3):702-8.

Cite this article: Purushothaman A, Sachdanandam P. Chemotherapeutic Efficacy of Shemamruthaa- an Herbal Formulation on Oxidative Stress Markers and Apoptotic Genes Expression in 7,12-dimethylbenz(α)anthracene Induced Breast Tumour in Rats. *Asian J Biol Life Sci.* 2020;9(1):51-9.