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ORIGINAL ARTICLE **OPEN ACCESS**

Modulation of DMBA induced development of breast cancer and chemopreventive effects of *Madhuca longifolia* ethanolic seeds extract in Sprague Dawley female rat model: An Enzymatic Study

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ABSTRACT
The aim of the research was to explore the chemopreventive activity of Madhuca longifolia ethanolic seeds extract in a 7,12 dimethylbenz (a) anthracene (DMBA) carcinogenesis model. Chemical carcinogenesis DMBA was used. Five groups were designed for the study and Sprague Dawley female rat were intraperitoneally injected with vincristine 50mg/kg b.w. at a standardized dose. Mahua extract was given orally at an optimum dose of 15 mg/kg b.w. and 30 mg/kg b.w. as per experimental protocol. The parameters LPO, GST, SOD, CAT and GSH have been evaluated. The Gr. V results showed significant elevation of glutathione-s–transferase (124.01 ± 1.02%)), superoxide dismutase (96.94 ± 2.93%), catalase (97.14 ± 2.27%), reduced glutathione (143.78 ± 2.51%) and inhibition of lipid peroxidation (82.1 ± 3.81%) in breast than Gr. II, Gr. III and Gr. IV. Thus, the present data strongly indicated that the plant seeds have potential to act as anti-breast cancer promoting agent in Sprague Dawley female rat model. Further studies need to be done to understand the mechanism of action.
Keywords: *Carcinogenesis, Chemoprevention, Breast, Cancer, Madhuca longifolia.*

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INTRODUCTION

Cancer is the leading cause of mortality worldwide, and in its advanced stages, it is extremely difficult to treat. According to an epidemiological study, one of the most common causes of cancer's aetiology is DNA damage caused by oxidants and free radicals. [1] Cancer chemoprevention is the use of natural and synthetic agents to suppress, prevent or delay tumorigenesis by blocking the initiation stage of carcinogenesis, or by curtailing the promotion stage wherein the initiated cells proliferate to give rise to a tumor. [2] Phytochemicals are chemicals which are found in plants also include indoles, lignans, phytoestrogens, stanols, saponins, terpenes, flavonoids, carotenoids, anthocyanidins and phenolic acids. [3] The evidence suggested that prolonged oxidative and nitrosative stress can result in cell injury and that they play roles in various stages of carcinogenesis. [4] Tumor cells present de-regulated cell proliferation and differentiation, and acquire autonomous and unlimited growth properties together with resistance to apoptosis. [5] Their growth also stimulated by reactive oxygen species. Free radicals, generally composed of reactive oxygen species (ROS) and reactive nitrogen species (RNS), are generated in the body by various endogenous and exogenous systems. [6] The overproduction of free radicals also known to cause several chronic diseases including cancer. Xenobiotics explored the chemical substances that are foreign to animal life and thus includes such examples as plant constituents, drugs, pesticides, cosmetics, flavorings, fragrances, food additives, industrial chemicals and environmental pollutants. [7] The role of metabolic activation in carcinogenesis and the importance of DNA damage and mutation have led to additional avenues of research regarding mechanisms of carcinogenesis and influences on the carcinogenic process. The first of these is DNA repair. [8] Xenobiotics also considered as chemical substances from natural or synthetic sources found within an organism that are not naturally produced by the organism or expected to be present. [9] Oxidative stress (OS) expressed as a state of excess ROS production and/or the reduction in savaging antioxidants, which results in pathophysiological changes similar to the general adaption syndrome of cellular stressors. [10] Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (PUFA).

[11] GST proteins are crucial antioxidant enzymes that regulate stress-induced signaling pathways. Glutathione (GSH) is an antioxidant that acts as a free radical scavenger and a detoxifying agent in cells. It is useful in a multitude of processes, cellular proliferation, cell division, and differentiation, and is the most commonly elevated metabolite detected during oxidative stress. [12] Superoxide dismutase (SOD) constitute a very important antioxidant defense against oxidative stress in the body. The enzyme acts as a good therapeutic agent against cancer. Catalase also plays an important role in cancer. [13] Antioxidants are secondary constituents or metabolites found naturally in the plants and they produce array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate. Breast cancer is one of the most common types of cancer, and even though there has been a lot of molecular and cellular research, the death rate is still high. [14] Appropriate in vivo models are needed for preclinical testing of new treatments for diseases that already exist and to stop the spread of diseases that are hard to treat. [15]Species differences in mammary gland development and sensitivity to carcinogens, as well as the molecular heterogeneity of human breast cancer, present difficulties in the modelling of breast cancer in animals. [16] In the glandular tissue of the breast, breast cancer develops in the lining cells (epithelium) of the ducts (85%) or lobules (15%). The malignant development is initially contained within the duct or lobule ("in situ"), where it often exhibits no symptoms and has a low risk of spreading (metastasis). *Madhuca longifolia* (J. Koenig Ex L.) J. F. Macbr. (Family: Sapotaceae) have pharmaceutical, nutraceutical, ethno medicinal and ethnopharmacological value such as anti- inflammatory, antipyretic, antihyperglycemic, antifertility, antiulcer, antimicrobial, antioxidant, cardio protective, anti-carcinogenic, immuno-modulant, anti-rheumatic, oxytocic, anti-estrogenic, uterotonic, antiepileptic, demulcents as well as many other useful pharmacological activities. The parts include flowers, fruits, seeds, leaves, barks etc. This plant-based foods alsoeffective against the various diseases i.e., ulcer, snakebite, scabies, rheumatism etc. In this experiment, chemical carcinogen was used by inducing breast cancer namely 7,12- Dimethylbenz[a]anthracene (DMBA) which occurred chronic inflammation, create oxidative stress and damaged DNA. As best of knowledge, no cancer chemoprevention activity of this plant seeds previously reported. The aim of the research was to find out the chemo preventive efficacy of Madhuca longifolia seeds to prevent breast cancer in the animal model.

MATERIAL AND METHODS

Chemicals

DMBA, ethylene diamine tetra acetic acid (EDTA), pyrogallol, thiobarbituric acid (TBA), bovine serum albumin(BSA), vincristine, 1-chloro-2, 4-dinitrobenzene (CDNB), sodium dodecyl sulphate (SDS), reduced glutathione (GSH), Hydrogen peroxide 30% etc. were purchased. All the above mentioned & other reagents and chemicalsused for this research were of analytical grade.

Animals

Adult female Sprague Dawley rats weighing 100–150 gm, were taken for this study and maintained at twelve hours light/dark cycle, humidity (55% to 65%), temperature (21^0C to 25^0C). Six rat per cage were housed in wire-mesh cages. Standard food pellets diet (rat/mice feed) and drinking water was given ad libitum. The animals in each group had their backs shaved two days before the experiment started.

Preparation of Drug

Ethanolic extract of *Madhuca longifolia* seeds (15mg/30 mg) was dissolved in 200 µl phosphate buffer saline. Each day of the experiment, just before the treatment, it was prepared.

Preparation of Carcinogenesis DMBA Preparation

100 mg DMBA were taken in a measuring tube using micropipette. Thereafter 9 ml 900 µl acetone was given in a tube and the total volume was 10 ml. The preparation was given tropically and the ratio was 100 µl per rat. So, the DMBA were dissolved of 100 µg / 100 µl per acetone concentration. Each day of the experiment, just before the treatment, it was prepared.

Experimental Design

Vehicle control group (Group-I)

This group received PBS orally (200 µl/rat) and acetone (100 µl/rat) topically over the skin shaved area for 16 weeks. Carcinogen control group (Group-II)

This group received one topical application (skin shaved area) of DMBA at an interval of 72 h, at a dose of 50mg/kg body weight in acetone (100 µl/rat), followed by twice a week for 9 weeks starting from day 8 of first DMBA application.

Positive control group (Group III)

This group received the same treatment as same as group II and also administered intraperitoneally with 50 mg/kg standard drug of Vincristine from the day (on day 8th after the 1st DMBA application) of croton

oil treatment.
Lower dose test group (Group-IV)
This group received the same treatment as for group II and also received the Mahua Extract (ME) at a dose of 15 mg/kg body weight/day orally from the day (on day 8th after the 1st DMBA application) of croton oil treatment.
Higher dose test group (Group-V)
This group received the same treatment as for group II and also received the Mahua Extract (ME) at a dose of
30 mg/kg body weight/day orally from the day (on day 8th after the 1st DMBA application) of croton oil treatment. As per guidelines, the rats in groups I, II, III, IV and V were sacrificed after sixteen weeks of treatment with the first application of DMBA.

Estimation of Antioxidant and oxidative enzymatic parameters Assay of Lipid per oxidation (LPO)

Thiobarbituric acid was employed to quantify the lipid peroxidation in microsomes, and the results were computed using the nanomoles of TBARS produced per milligram of protein and an extinction coefficient of 1.56×105 M–1cm– 1 [17].

Assay of Glutathione - S - Transferase (GST)

Utilizing 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate, glutathione-S-transferase (GST) activity was assessed by observing a rise in absorbance at 340 nm. Nanomoles of the CDNB-GSH conjugate were believed to constitute the enzyme's specific activity per milligram of protein [18].

Assay of Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) activity in the cytosol was determined and represented as units per milligram of protein. Pyrogallol auto oxidation inhibition is quantified as a proxy for SOD activity. An "enzyme activity unit" is the amount of enzyme required for 50% inhibition. An increase in absorbance at 420 nm was used to measure the amount of pyrogallol auto-oxidation occurring in Tris-HCl buffer (50 mM, pH 7.5) [19].

Assay of Catalase (CAT)

Catalase (CAT) activity was measured in the cytosol and expressed as units per milligram of protein, where a unit is the amount of enzyme required to release half the peroxide oxygen from H2O2 in 100 seconds at 25°C [20].

Assay of Reduced Gluthathione (GSH)

The amount of GSH was determined as nanomoles per milligram of protein in the cytosol [20].

Statistical Analysis

Each experimental data was presented as MEAN ± SEM. All of the groups were compared and analysed using a one- way ANOVA and then Dunnett's multiple comparison tests were performed. In this case, the values were deemed statistically significant if the p-value was less than 0.05. [21, 22]

RESULTS

Estimation of Antioxidant and oxidative enzymatic parameters Lipid per oxidation (LPO)

The significant increase was shown of lipid per oxidation in carcinogen control group II (158.3 ± 3.03%) compared to the vehicle control group I. The positive control group III (131.5 ± 3.19%) showed inhibition of lipid peroxidation. However, simultaneous treatment of group IV (105.6 ± 2.73%) showed better result than group III. The highest inhibition of lipid peroxidation showed by higher dose test group V (82.1 ± 3.81%) than group III & IVafter 16 weeks treatment (Figure 1.).

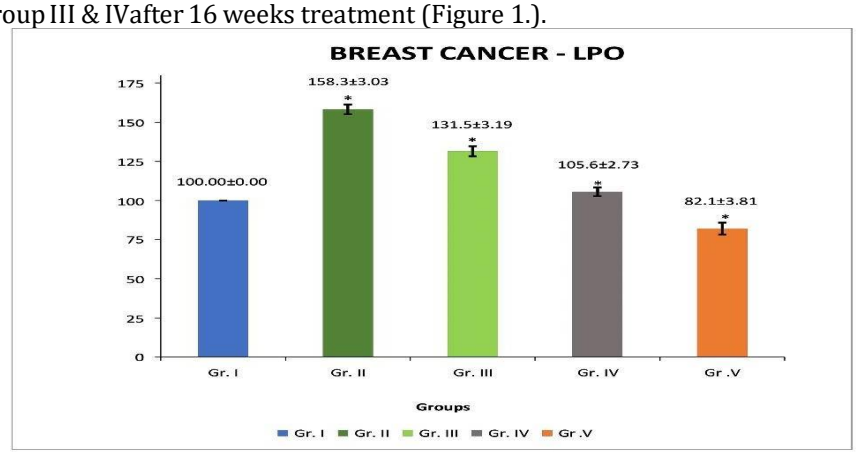

Figure 1. Breast Cancer-LPO

Glutathione - S - Transferase (GST)

The significant decrease was shown of glutathione-S-Transferase (GST) in carcinogen control group II (47.38 ± 1.46%) compared to the vehicle control group I. The positive control group III (65.53 ± 0.75%) showed significant increase of GST activity. However, simultaneous treatment of group IV (86.88 ± 2.02%) showed better result than group III. The highest increase of GST showed by higher dose test group V(124.01 ± 1.02%) than group III & IV after 16 weeks treatment (Figure2.).

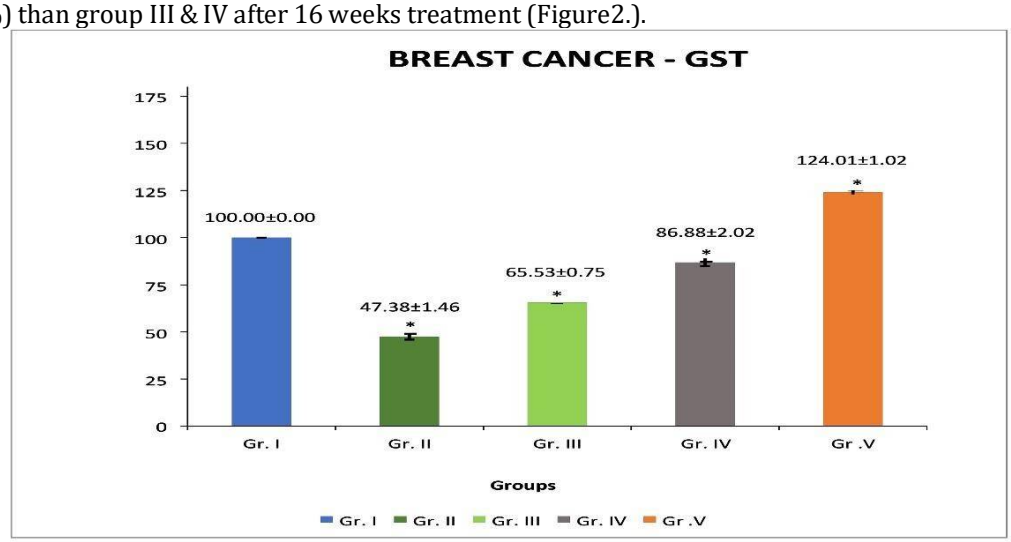

Figure 2. Breast Cancer-GST

Reduced Gluthathione (GSH)

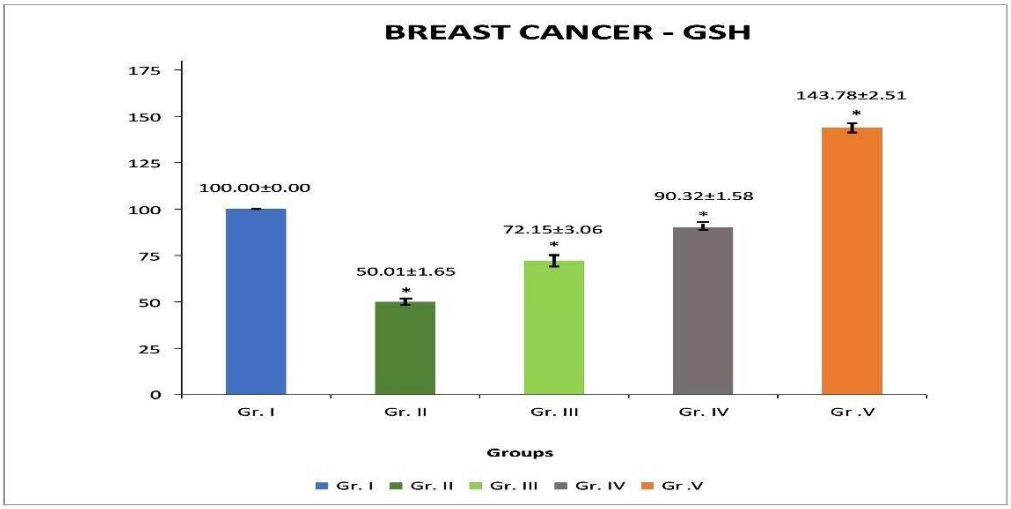

Figure 3. Breast Cancer-GSH

The significant decrease was shown of reduced Gluthathione (GSH) in carcinogen control group II (50.01 ± 1.65%) compared to the vehicle control group I. The positive control group III (72.15± 3.06%) showed significant increase of GSH activity. However, simultaneous treatment of group IV (90.32 ± 1.58%) showed better result than group III. The highest increase of GSH showed by higher dose test group V (143.78 ± 2.51%) than group III & IV after 16 weeks treatment (Figure 3.).

Superoxide Dismutase (SOD)

The significant decrease was shown of superoxide dismutase (SOD) in carcinogen control group II (39.58 ± 0.76%) compared to the vehicle control group I. The positive control group III (45.77 ± 2.15%) showed significant increase of SOD activity. However, simultaneous treatment of group IV (77.24 ± 1.91%) showed better result than group III. The highest increase of SOD showed by higher dose test group V (96.94 ± 2.93%) than group III & IV after 16 weeks treatment (Figure 4.).

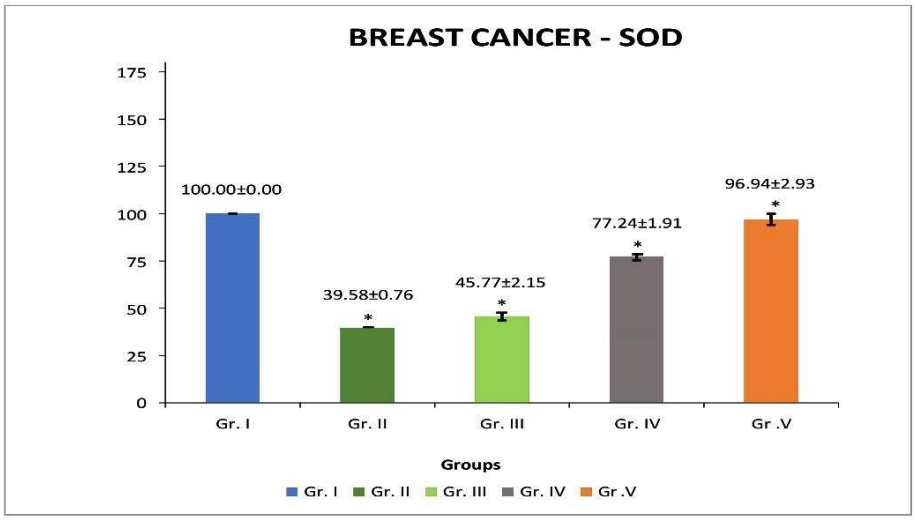

Figure 4. Breast Cancer-SOD

Catalase (CAT)

The significant decrease was shown of catalase (CAT) in carcinogen control group II (24.99 ± 2.11%) compared to the vehicle control group I. The positive control group III (44.01 ± 1.37%) showed significant increase of CAT activity. However, simultaneous treatment of group IV (68.09 ± 0.81%) showed better result than group III. The highest increase of CAT showed by higher dose test group V (97.14 ± 2.27%) than group III & IV after 16 weeks treatment (Figure 5.).

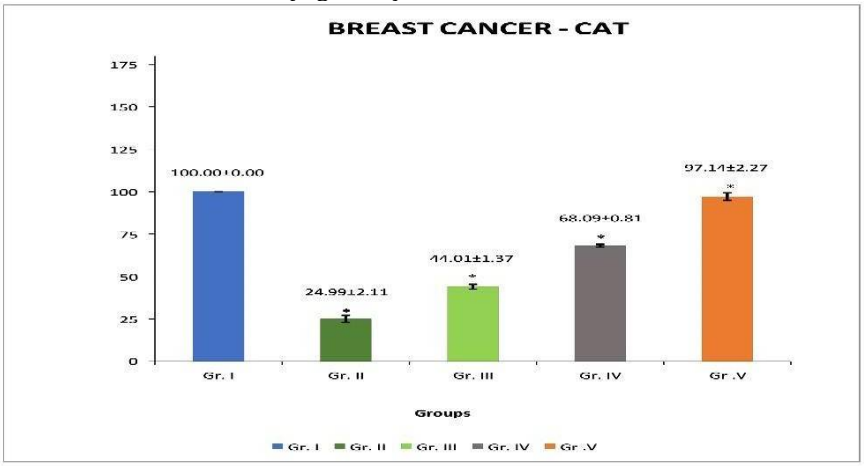

Figure 5. Breast Cancer-CAT

DISCUSSION

Reactive oxygen species (ROS) have been related to the pathophysiology and aetiology of many human diseases and they have been proposed as causal agents in tumour promotion, carcinogenesis and mutagenesis. The occurrence of cancer and reports of tissue degradation have been linked to free radicals and ROS. They cause DNA strand breakage and DNA base oxidative alteration, which have mutagenic and carcinogenic effects. Additionally, they regulate gene expression via an epigenetic mechanism. Numerous chemicals with antioxidant activity have been discovered to impede the growth of tumours, whereas numerous substances with tumour promoter activity have been found to commonly overproduce ROS. Lipid peroxidation is a chain reaction initiatedby the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (PUFA) [23]. GST proteins are essential anti-oxidant enzymes that control signalling pathways brought on by stress. Glutathione (GSH) is the most abundant antioxidant found in living organisms and has multiple functions, most of which maintain cellular redox homeostasis. GSH preserves sufficient levels of cysteine and detoxifies xenobiotics while also conferring therapeutic resistance to cancer cells. [24] However, to counteract the harmful consequences of oxidative damage brought on by ROS, the human body is outfitted with a number of highly sophisticated antioxidant defence systems. SOD is the only enzyme that has been found to utilize a free radical as a substratetoinactivate superoxide. Asurge in CAT activity must follow SOD's free radical scavenging activity because hydrogen peroxide, a by-product of SOD, is more tissue- damaging than oxygen radicals and has to

be scavenged by CAT. By eliminating hydrogen peroxide, CAT is essential for both cellular defence and the protection of cellular membranes against oxidative damage from free radicals. Therefore, an increase in CAT activity is also necessary for an increase in SOD activity to have a positive impact. Due to their capacity to decrease ROS, phytochemicals have an anti-cancer impact by shielding vital cellular components from oxidative stress. The ability of phytochemicals to inhibit the proliferation of cancer cells and trigger apoptosis in cancer cells is just one of many significant anti-cancer strategies. In the present study, significant DNA damage was observed in carcinogen control group treated animals as compared to the vehicle control group. Mahua seeds can effectively suppress the proliferation of the cancer cells that were the subject of the study in an animal model. However, the DNA damage was reduced significantly when the animals were treated with the positive control and ME treated test groups as compared with the carcinogen control treated rat. Based on animal protocol, lipid peroxidation was significantly higher in the carcinogen control group II compared to the vehicle control group I for breast cancer. The group III, IV and V were significantly reduced. The GST, GSH, SOD, CAT were significantly lower in the carcinogen control group II compared to the vehicle control group I for breast cancer. The group III, IV and V were significantly increased. Breast cancer is the most common diseases in mostly international scenario and national aspects as well. The present results showed that ethanolic extract of Mahua longifolia seeds can actively inhibit the growth of breast cancer cells and also emphasize the potent anticancer activity in vivo. Therefore, the plant seed extract can be utilized as a chemopreventive or therapeutic agent in breast cancer.

CONFLICT OF INTEREST

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

AUTHOR'S CONTRIBUTION

DS: Concept and design of the work, Data collection, Data analysis and Interpretation, Drafting of the article.SKS: Concept and design of the work, Drafting of the article.

ETHICS STATEMENT

This study was performed in accordance with the institute guidelines for the care and purpose of laboratory animals.

INFORMED CONSENT

All the experiments were carried out in accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

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