ORIGINAL ARTICLE

Anticancer potential of nutraceutical formulation through antioxidant, anti-inflammatory, and antiproliferative mechanisms in N-methyl-N-nitrosourea-induced mammary cancer

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Abstract

Background: Previous investigation has shown the promising anticancer potential of nutraceutical formulations prepared using fine powders of amla, apple, garlic, onion, papaya, turmeric, wheatgrass, and cow urine distillate in N-methyl-N-nitrosourea (MNU)-induced mammary cancer. Combination of nutraceuticals is known to act by multiple mechanisms in the prevention of cancer. **Aim:** An evaluation was performed to study the possible mechanisms involved in the anticancer potential of self-fortified nutraceutical formulation (SFNF) in MNU-induced mammary cancer in Sprague Dawley rats. **Materials and Methods:** Mammary cancer was induced using MNU in female Sprague Dawley rats. At the end of experimental period, mammary tissues were analyzed for malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), myeloperoxidase (MPO), and tumor necrosis factor-alpha (TNF-α) levels. Similarly, serum samples were analyzed for Estradiol (E2) and insulin-like growth factor 1 (IGF-1) levels. Immunohistochemical analysis of Ki-67 antigen was performed in mammary tissues. **Results:** Findings from this study showed that the tissue MDA levels were decreased; SOD and CAT levels were increased in SFNF supplemented rats. SFNF supplemented rats also showed decrease in MPO and TNF-α level compared to MNU control rats. SFNF supplemented rats showed significant reduction in serum E2 ($P < 0.01$) and IGF-1 ($P < 0.001$) levels. Significant reduction in Ki-67 ($P < 0.01$) immunoreactive cells of tumors of SFNF supplemented rats was also observed. **Conclusion:** SFNF was effective in the prevention of MNU-induced mammary cancer through antioxidant, anti-inflammatory, and antiproliferative mechanisms in rats.

Key words: Anti-inflammatory, antioxidant, antiproliferative mammary cancer, nutraceuticals, N-methyl-N-nitrosourea

INTRODUCTION

Cancer is the second leading cause of death worldwide. Therefore, the fight against cancer is one of the most important areas of research in medicine, and one that possibly contributes to the increased interest in chemoprevention as an alternative approach to the control of cancer.[1] Breast cancer represents the most frequent of all cancer pathologies in the world, with more than 1 million newly diagnosed cases and about 370,000 cancer-related deaths in women each year, even with all the significant progress in its diagnosis and treatment.[2] The use of complementary and alternative medicine is increasing rapidly in developed countries, which is already in use as traditional medicines in various Asian countries.[3] Dr. Stephen DeFelice first coins the term nutraceuticals in 1989, and he defined nutraceuticals as "as foods, food ingredients, or dietary supplements that demonstrate specific health or medical benefits including the

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prevention and treatment of disease beyond basic nutritional functions."[4] A number of nutraceuticals serve as candidates for development of breast cancer chemopreventive agents because of promising epidemiological, preclinical, and pilot clinical findings. Their mechanisms of action may involve an ability to target multiple molecular pathways in carcinogenesis without eliciting toxic side effects.[5] In our previous investigation, we prepared four nutraceutical formulations (nutraceutical formulation [NF], NF fortified, self-fortified NF [SFNF], and self-fortified NF fortified cow urine distillate [SFNFCUD]) using fine powders of amla, apple, garlic, onion, turmeric, papaya, and cow urine distillate and studied for anticancer potential in N-methyl-N-nitrosourea (MNU)-induced mammary cancer in rats. All the formulations showed significant anticancer potential, but SFNF and SFNFCUD showed more and equipotent anticancer potential.[6] Ample of studies have shown that amla, apple, garlic, onion, turmeric, and papaya are enriched with many phytochemicals that can fight against cancer by multiple mechanisms. Hence, we evaluated mechanisms involved of anticancer potential of SFNF in MNU-induced mammary cancer.

MATERIALS AND METHODS

Drugs and Chemicals

MNU was purchased from Sigma Chemical Co. (St. Louis, O); tumor necrosis factor-α (TNF-α) rat ELISA kit (Assaypro, USA); chemiluminescence enzyme immunoassay (CLIA) estradiol (E2) kit (Immunospec corporation, USA); insulin-like growth factor 1 (IGF-1) rat ELISA kit (Bio Vendor, Germany) and; SP6 Ki-67 monoclonal antibody (Thermo Fisher Scientific Inc., USA). All other chemicals including solvents used were of high purity and of analytical grade marketed by Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Nutraceuticals

All the nutraceuticals (apple fruit, amla fruit, garlic bulbs, onion bulbs, papaya leaves, turmeric rhizomes, and wheatgrass) used in the preparation of SFNF were of fine grade and collected from the local market.

Animals

Virgin, female Sprague Dawley rats of 35 days of age were obtained from Teena Labs. Pvt. Ltd., Hyderabad, Andhra Pradesh, India. The animal house was well ventilated. The animals were housed in large spacious hygienic cages during the course of the experimental period. The animals were housed under standard laboratory conditions of temperature (21 ± 1°C); relative humidity 50 ± 15% with a 12-h light/dark schedule. They were provided with food (Nutrimix Std-1020, Nutrivet Laboraotories, Pune) and water *ad libitum*. Experimental protocol was approved by IAEC and study was carried out as per CPCSEA guidelines (Regd. No. 516/01/a/CPCSEA).

Preparation of SFNF

SFNF was prepared using fine powders of self-fortified amla, self-fortified papaya, self-fortified wheatgrass along with apple, garlic, onion, and turmeric powders. Self-fortification was done by deliberately fortifying the powder with their respective freshly prepared juice (100 g of powder fortified with 50 mL of juice) for 3 times before adding to the final formulation.[6]

Tumor Induction

MNU was used as a carcinogen for the tumor induction. Mammary cancer was induced by a single dose of 50 mg/kg body weight of MNU was dissolved in 0.9% saline adjusted with acetic acid (pH 4.0) and then administered intraperitoneally.[7]

Experimental Design

At the age of 43 days, female Sprague Dawley rats were randomly divided into three groups consisting of eight animals in each group and Group I (Normal control): No mammary cancer induction; no treatment; Group II (MNU control): At the age 50 days, animals received single dose of MNU (50 mg/kg body weight, i.p.). Group III (Test): Mammary carcinoma was induced (as in Group II) and treated with SFNF (500 mg/kg body weight, p.o.). SFNF treatment was started from 1 week before MNU administration once in a day for 24 weeks. At the end of the experiment, blood samples were collected; mammary tumors were excised for further analysis.

Determination of Mammary Gland Antioxidant Enzymes and Oxidants

Determination of antioxidant enzymes

Estimation of superoxide dismutase (SOD) was performed according to the standard protocols described by Kakkar *et al.* (1984).[8] Catalase (CAT) was assayed by the method of Sinha.[9]

Determination of tissue malondialdehyde (MDA)

MDA was estimated by the method of Ohkawa *et al.* (1979).[10] To 0.2 ml of tissue homogenate, 0.2 ml of 811% sodium dodecyl sulfate and 1.5 ml of glacial acetic acid (20%) were added. pH was adjusted to 3.5 with NaOH, and then, 1.5 ml of 0.8% thiobarbituric acid was added to the mixture and volume made up to 4 ml. Reaction mixture was heated in

oil bath at 95°C for 60 min. 1 ml distilled water added after cooling with 5 ml N-butanol pyridine and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the supernatant was removed and absorbance was read at 620 nm.

Analysis of Mammary Tissue Myeloperoxidase (MPO) and TNF-α

The activity of MPO was assessed using the method modified from that of Mullane 1985.[11] TNF-α concentration in rat mammary gland was measured using the "Assay Max Rat TNF-alpha ELISA kit of murine monoclonal antibody" (Assaypro, 41 Triad South Drive St. Charles, MO 63394, USA).

Analysis of Serum Estradiol (E2) and IGF-1

The serum E2 levels were quantified by CLIA according to the kit (Catalog No.C29-853) protocol. Quantitative estimation of IGF-1 in serum samples was done using IGF-1 rat ELISA kit according to the manufacturer instructions (Cat. No.: RMEE25R).

Immunohistochemical (IHC) Analysis of Ki-67

At the end of the treatment or experiment, animals were sacrificed, and tumors were harvested and weighed. Fresh tumor tissue was immediately placed in 4% paraformaldehyde for IHC analysis.[12] For IHC evaluation, we used the monoclonal primary antibody Ki-67 (Clone SP6, Thermo Scientific™). The antibody was incubated at a dilution of 1:50, overnight at 4°C. To evaluate the cellular Ki-67 proliferation index (PI), it was considered the brown labelled nuclei by the Ki-67 antigen, independently of the invasiveness of the neoplasia. With a magnification of 100× were located the more labeled areas, then under the magnification of 400× were counted 1000 neoplastic cells in each section identifying the brown labeled nuclei.

Statistical Analysis

Data were represented as mean ± standard error of the mean and analyzed by one-way analysis of variance followed by Tukey's *post hoc* test using GraphPad Prism version 5.0. The results were considered statistically significant when $*P \leq 0.005$, $**P < 0.01$, $***P < 0.001$.

RESULTS

Effect on Mammary Gland Oxidative Stress and Inflammation

Mammary tissue SOD, CAT levels were decreased and MDA levels were increased significantly in MNU control rats as compared to normal control. In SFNF treated group, significant increase in mammary tissue SOD and CAT levels with concomitant decrease in MDA was observed when compared to MNU control group. Similarly, MPO and TNF-α levels were significantly increased in MNU control group as compared to healthy rats. In SFNF treated group, both MPO and TNF-α levels were significantly decreased as compared to MNU control group [Table 1].

Effect on serum E2 and IGF-1

E2 and IGF-1 levels were increased significantly in MNU control group as compared to normal group. In SFNF treated group, both E2 and IGF-1 levels were reduced significantly as compared to MNU control group [Table 2].

IHC Analysis of Ki-67

Ki-67 expression was localized in the nucleus of ductal epithelial cells in the mammary gland. More Ki-67 expression was observed in mammary tumors of control rats [Figure 1]. Following 6 months treatment, significant reduction in Ki-67 immunoreactive cells in terms of Ki-67 proliferative index was observed in mammary tumors of SFNF treated rats [Figure 2] as compared to MNU control rats [Table 3].

DISCUSSION

A number of risk factors such as reproductive and hormonal factors, alcohol consumption, cigarette smoking, dietary factors, and chronic inflammation have been identified for breast cancer, whose mechanisms by which they increased risk of the disease are not always clear. It has been proposed that the production of reactive oxygen species, leading to an oxidative stress is the linking factor between these carcinogens and development of breast cancer. Free radicals oxidize lipids, proteins, and DNA and they are involved in the initiation and promotion/progression of carcinogenesis.[13]

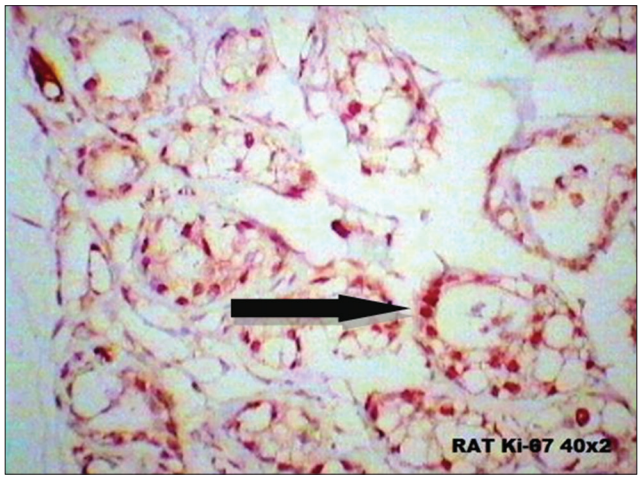

Figure 1: Mammary tumors of control rat showing high proliferative index (Ki-67 nuclear antigen)

Table 1: Effect of SFNF on mammary tissue oxidative stress and inflammation

Group	Oxidative stress parameters			Inflammatory markers	
	SOD (units/mg protein)	CAT (units/mg protein)	MDA (nmol/g of tissue)	MPO (units/g of tissue)	TNF-α (pg/ml)
Normal control	12.10±0.68	38.62±2.04	112.1±6.41	3.08±0.60	26.14±4.30
MNU control	5.15±0.23	12.93±2.33	482.8±12.25	81.37±7.63	162.1±9.10
SFNF	9.86±0.73**	27.00±3.90**	194.1±14.62***	19.47±2.86***	56±9.17***

All the values were expressed as mean±SEM. n=8.0. **P<0.01, ***P<0.001 when compared with MNU control. MPO: Myeloperoxidase, TNF-α: Tumor necrosis factor-alpha, MDA: Malondialdehyde, CAT: Catalase, SOD: Superoxide dismutase, MNU: N-methyl-N-nitrosourea, SFNF: Self-fortified nutraceutical formulation, SEM: Standard error of the mean

Table 2: Effect of SFNF treatment on serum E2 and IGF-1 levels

Group	E2 (pg/ml)	IGF-1 (ng/ml)
Normal control	8.06±0.98	203±16.22
MNU control	37±4.05	885.1±15.67
SFNF	19.87±1.91**	424±26.86***

All the values were expressed as mean±SEM. **P<0.01, ***P<0.001 compared to the MNU control. n=8. E2: Estradiol, SFNF: Self-fortified nutraceutical formulation, IGF-1: Insulin-like growth factor-1, MNU: N-methyl-N-nitrosourea, SEM: Standard error of the mean

Table 3: Effect of SFNF treatment on Ki-67 PI of mammary tumors

Group	Ki-67 PI (%)
MNU control	19.67±4.28
SFNF	9.33±3.76**

All the values were expressed as mean±SEM. **P<0.01 when compared with MNU control. Ki-67 PI: Ki-67 proliferative index, SFNF: Self-fortified nutraceutical formulation, MNU: N-methyl-N-nitrosourea, SEM: Standard error of the mean

MNU, a mutagen and genotoxic substance is a potent inducer of cellular oxidative stress either by increased free radical generation and/or depletion of antioxidant system, leading to chromosomal aberrations, point mutations, cell death, and DNA damage resulting in the development of mammary cancer supported by previous studies.[14]

Significant reduction in mammary tissue SOD and CAT levels with concomitant increase in MDA levels was observed in MNU-treated rats compared to healthy rats suggesting the depletion of antioxidant system and increased free radical generation. Whereas SFNF treated rats showed significant increase in mammary tissue SOD and CAT levels with concomitant decrease in MDA levels suggesting the antioxidant potential of nutraceutical formulation.

MPO is a well-known enzyme, mainly released by activated neutrophils, characterized by powerful prooxidative, and proinflammatory properties. MPO has been detected by immunohistochemistry in breast tissue from women with

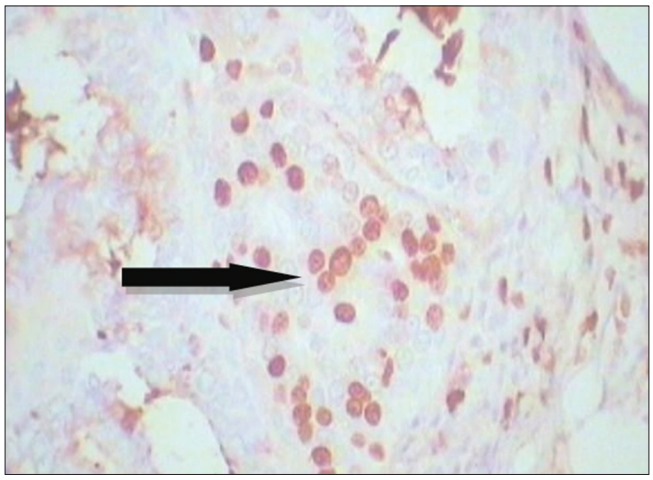

Figure 2: Mammary tumor of self-fortified nutraceutical formulation treated rat showing low proliferative index (Ki-67 nuclear antigen)

cancer.[15] A growing body of laboratory research has shown that proinflammatory cytokines can facilitate tumor initiation, tumor growth, and metastasis by damage to DNA, altering tumor cell biology and activating stromal cells in the tumor microenvironment, such as vascular endothelial cells, tumor-associated macrophages, and fibroblasts.[16] TNF-α is a major proinflammatory cytokine generally produced by macrophages in response to proinflammatory signals is a master regulator of tumor-associated inflammation and tumorigenesis and shown to be highly expressed in breast carcinomas. The multifunctional cytokine, TNF-α, is involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of malignant diseases such as breast cancer.[17,18] Thus, it is plausible to hypothesize that inflammation and inflammatory markers such as MPO and TNF-α could play an important role in breast carcinogenesis.

MNU-treated rats showed significant increase in mammary tissue MPO and TNF-α level as compared with healthy rats suggesting the role inflammation in MNU-induced mammary cancer. Treatment with SFNF showed significant reduction in MPO and TNF-α level in mammary tissues as compared to MNU-treated rats suggesting the anti-inflammatory potential of SFNF in MNU-induced mammary cancer.

Breast cancer is a classical model of hormone-dependent malignancy. It is known that estrogens are involved in the growth and differentiation of the normal mammary gland and they intervene in the growth of the ductal branching. However, there is considerable evidence that estrogens are also mammary carcinogens.[19] The Sprague Dawley rat exposed to MNU is one of the most commonly used, thoroughly characterized models for human breast cancer, particularly with regard to the ability of hormones to regulate tumor growth and is also considered to be an excellent model of human breast cancer.[20] The most widely acknowledged mechanism of estrogen carcinogenicity is its binding to its specific nuclear estrogen receptor alpha for exerting a potent stimulus on breast cell proliferation through its direct and/or indirect actions on the enhanced production of growth factors.[21,22] Supporting with this, in our study, we observed significant increase in IGF-1 levels along with significant increase in E2 levels in MNU-treated rats as compared with healthy rats suggestive of that MNU-induced mammary tumors are hormonal dependent. IGF-1 is a more potent mitogen than E2 for breast cancer cells. Antiestrogens are widely used in the management of hormonally responsive breast cancer in both adjuvant and palliative settings and are currently being evaluated as chemopreventive agents.[23]

It was also observed that significant decrease in serum E2 levels along with significant decrease in serum IGF-1 levels in SFNF-treated rats as compared to MNU control rats suggesting the antiestrogenic action of SFNF. Epidemiological studies suggest an inverse association between a higher intake of flavonoids and breast cancer risk. Flavonoids, which are widely distributed in the plant kingdom, have been recently reported as candidate compounds that can exert chemopreventive effects in estrogen-dependent or independent breast cancer.[24]

Uncontrolled proliferation is a hallmark of malignancy and may be assessed by a variety of methods including estimation of Ki-67 antigen. In normal breast tissue, Ki-67 is expressed at low levels (<3% of cells) in ER-negative cells, but not in ER-positive cells population.[25] In the present investigation, we observed significant increase in mean percent of Ki-67 positive cells in tumors of MNU control rats suggesting the hyperproliferation of tumors. Further, we observed significant reduction in mean percent of Ki-67 positive cells in tumors of SFNF-treated rats as compared to MNU control rats representing the antiproliferative activity of SFNF.

Results from several studies had shown that flavonoids can inhibit the growth of cancer cells with an ability to act as "chemopreventers."[26] All the nutraceuticals used in the preparation of SFNF are enriched with several phytoconstituents including different flavonoids as major part. These flavonoids may be acted synergistically in the prevention of MNU-induced mammary cancer through antioxidant, anti-inflammatory, and antiproliferative mechanisms along with other phytoconstituents.

Nutraceutical has potential benefits of becoming the most popular sector in the near future. Using them in combination could be beneficial as many them can acts synergistically helps in reducing dose needed and toxicity. Being instrumental in the prevention and cure of breast cancer is just one aspect of nutraceuticals, it has much more such cures which are under stringent study and can be implemented in the future bringing commendable benefit to women and all humankind in general in a nutritious and organic way.

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