Effect of an Extract of *Withania somnifera* Root on Estrogen Receptor-positive Mammary Carcinomas

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Abstract. *The chemopreventive activity of an extract of Withania somnifera (WS) roots was examined in female Sprague-Dawley rats that received the mammary carcinogen methylnitrosourea (MNU). The dose of the extract, administered by gavage, was 150 mg/kg body weight daily for 155 days, after injection of MNU. Rats in the treated group (N=15) had an average of 3.47 tumors, and rats in the control group (N=15) had 4.53, a reduction of 23%. The average weights of tumors were 4.98 g for rats in the treated group and 6.30 g for the controls, a difference of 21%. Labeling indices for Ki67 and proliferating cell nuclear antigen (PCNA) markers in cancers of the treated group were 42% and 38% lower, respectively, than those of the corresponding indices for the control group. These results indicate that the root extract significantly reduced the rate of cell division in the mammary tumors.*

Breast cancer is associated with high morbidity and mortality. Each year, approximately 1.6 million new cases of cancer are diagnosed, and over 500,000 women die from this disease (1, 2). The annual incidence of breast cancer is increasing in both industrialized and developing countries (3). The morbidity from breast cancer has been only modestly reduced by current treatment modalities, which include surgery, radiotherapy, adjuvant chemotherapy, and hormone therapy (4). Since there is still no effective treatment for patients with advanced stages of the disease, there is an urgent need for discovering agents that will reduce the incidence of breast cancer in high-risk women. Such discoveries require a search for new approaches to deal with this disease. One such approach is chemoprevention, by which the disease can be prevented, slowed, or reversed through the administration of one or more naturally-occurring and/or synthetic compounds (5, 6).

Currently, there is considerable interest in the use of herbal extracts for cancer prevention, and several such extracts have activity against different kinds of cancer (7). These agents prevent cancer by various pathways, *e.g.* inhibition of cell proliferation, stimulation of apoptosis, or inhibition of the activity of free radicals (8). Some traditional herbs are considered to have safe, economically-feasible, and effective preventive activity for breast cancer. One such herb, *Withania somnifera* (WS), also known as ashwagandha, has been used for many years in traditional medicine, especially for the treatment of tumors, arthritis, and stress (9, 10). This small, woody shrub, which grows to about two feet in height, is found in Africa, India, and the Mediterranean area. It is also grown in the United States, where it is known as "winter cherry".

The present study was performed to determine if an alcoholic extract of WS roots would be effective in the prevention of mammary cancer induced in rats with the chemical carcinogen methylnitrosourea (MNU). Female Sprague-Dawley rats treated with MNU develop multiple hormonally-responsive mammary carcinomas, starting at 5-6 weeks after carcinogen administration (11). These carcinomas are histologically similar to estrogen-positive breast carcinomas in humans (12). Furthermore, gene expression profiling has shown significant similarities to well-differentiated ER^+ human breast cancers (13). In this model, previous studies have demonstrated that treatments that alter the hormonal axis (*e.g.* selective ER modulators and aromatase inhibitors) are strong chemopreventive agents (14, 15). This model has also predicted the chemopreventive efficacy of a variety of agents that may not act on the hormonal axis (such as retinoid × receptor agonists and farnesyltransferase inhibitors) (16, 17).

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Table I. *Efficacy of the Withania somnifera root extract in the prevention of methylnitrosourea (MNU)-induced mammary cancer.*

			Mammary cancer[c]		
Group	Carcinogen[a]	Treatment[b]	Incidence (%)	Multiplicity	Average weight (g)
1	MNU	Root extract, 150 mg/kg BW/day	100	3.47	4.98
2	MNU	None	93	4.53	6.30

[a]Female Sprague-Dawley rats received MNU at 50 days of age. N=15 rats/group. [b]Root extract was administered by gavage when the rats were 42 days of age and continued until the end of the study. [c]Mammary cancer at 155 days after treatment with MNU.

Materials and Methods

Preparation of the WS extract. Roots of WS were obtained from Iraq and ground to a paste. The preparation was extracted with five volumes of 70% ethanol by stirring for two days. The alcoholic extract was filtered, and the solvent was evaporated under a vacuum. The extract was dried to a powder and kept in a closed container until the time for use (18). To avoid variations in activity for different preparations, enough extract was obtained in one batch for use throughout the experiment.

Experimental animals. These studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Forty female Sprague-Dawley rats at 28 days of age were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA). They were housed in animal quarters at 22˚C with a 12-h light/dark cycle and were given free access to water and food. At 50 days of age, 30 rats received an intravenous injection of methylnitrosourea (MNU), *via* the jugular vein, at a dose of 75 mg/kg body weight. The animals were randomized to two equal groups: Group 1, dosed with the root extract (150 mg/kg BW/day); and group 2, dosed with the vehicle (ethanol: polyethylene glycol 400, 10:90, *v/v*). Root extract treatment by daily gavage (7 times per week) began one week prior to the MNU injection and continued for the duration of the study (155 days). Another 10 rats were not injected with MNU and were randomized into two equal groups. Group 3 rats received root extract, 150 mg/kg/day, and group 4 rats were dosed with the vehicle alone

All animals were palpated for mammary tumors twice each week. The rats were weighed weekly and observed daily for signs of toxicity. At the time of sacrifice, mammary tumors were removed for determination of their weights and for histopathological and immunohistochemical analyses. For each group of animals, the average number and average weight of the mammary tumors were determined.

Immunohistochemistry. Mammary tumors were fixed in 10% neutral-buffered formalin for 24 h. For immunohistochemical localization of Ki67 and proliferating cell nuclear antigen (PCNA), markers for cell proliferation, tissues were processed in an automatic tissue processor, embedded in paraffin, and sectioned at 5-μm thickness. The slides were de-paraffinized by three washes in xylene and rehydrated through a series of graded alcohol steps (100, 95, and 70%) and water, each for 5 min. The slides were washed three times for 5 min each in phosphate-buffered saline containing 0.05% Tween 80 (pH 7.4). Incubations were in a humid chamber at room temperature. Antigen retrieval was achieved by heating the slides in a microwave in a solution of 0.01 M sodium citrate (pH 6.0) and subsequent cooling for 30 min, followed by washing. Endogenous peroxidase activity was blocked by incubating the slides for 30 min in 1% hydrogen peroxide in methanol. Non-specific binding was blocked by incubating the slides for 1 h with horse serum (Vector Laboratories, Inc., Burlingame, CA, USA). Slides were then incubated with either an antibody against Ki67 or PCNA (Abcam, Cambridge, MA, USA) for 1.5 h at room temperature. After another wash, a complex of avidin-biotin-horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories) was added according to the manufacturer's instructions. 3,3'-Diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) was used as the chromogen substrate, and photomicrographs were taken using an Olympus B×73 microscope (Olympus, Center Valley, PA, USA) under bright-field illumination. The labeling indices for Ki67 and PCNA were determined by counting 300 cells from different sections (N=3) of the slides.

Statistical analysis. The Student's *t*-test was used to assess differences between values for the treated and control groups.

Results

Mammary tumors were not observed in control rats (no MNU). The carcinogen-exposed rats treated with the WS root extract developed 23% fewer tumors relative to rats dosed only with the vehicle; after 155 days of treatment, the rats receiving the root extract had an average of 3.47 tumors/rat, and those in the control group had 4.53 tumors/rat (p=0.266) (Table I and Figure 1). The average weight of the mammary tumors was 4.98 g for the treated group and 6.30 g for the controls (Table I), a difference of 21% (p=0.585). At termination of the study, rats in the WS extract treated group had an average body weight of 261±15 g, whereas the average weight of those in the untreated group was 276±12g (p=0.108). Representative samples of the mammary tumors were examined histologically by hematoxylin and eosin staining and by staining for Ki67 and PCNA (Figure 2). The labeling indices for Ki67 and PCNA markers in tumors of the treated group were 42% and 38% lower (p=0.0096 and p=0.0002), respectively, than the corresponding indices for the control group. At termination of the study, the livers of rats not receiving the carcinogen were weighed. There were

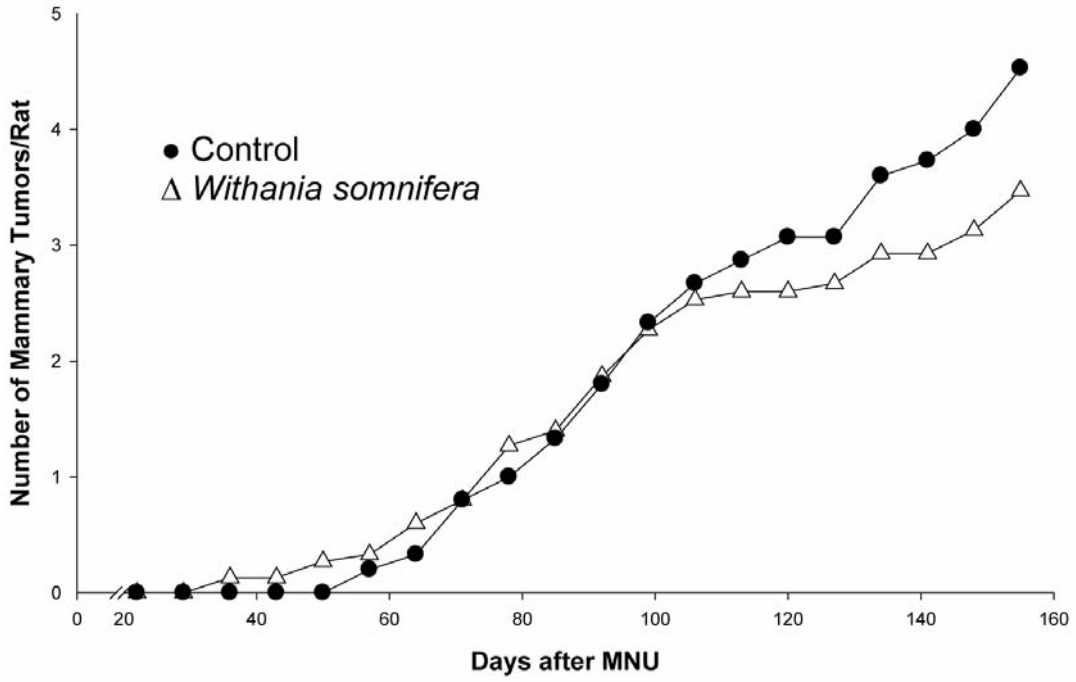

Figure 1. *Effect of Withania somnifera root extract in the prevention of methylnitorsourea-induced mammary tumors. Female Sprague-Dawley rats received Withania somnifera root extract (150 mg/kg body weight/day) one week prior to MNU and continually until the end of the study.*

no differences in the root extract-treated vs control livers; when expressed as liver weight per 100 g body weight, the value for the treated rats was 3.5, for the controls, the value was 3.4 (no significant difference).

Discussion

In the present study, the WS root extract was evaluated for chemoprevention of mammary cancer in female Sprague-Dawley rats. Use of the extract is practical since it is easy to obtain and relatively inexpensive. Moreover, the alcoholic extract allows for application of all active compounds present in the extract. Alcoholic extracts contain about twenty different withanaloids (12), which are steroidal compounds.

The MNU model for induction of mammary cancer is well-established for evaluating the preventive potential of various compounds (11-17). It is relevant to estrogen-dependent breast cancer in women, for whom the incidence of estrogen-dependent cancer is about 66% of the total. The WS root extract showed a 23% reduction in tumor development as compared to tumor development in untreated animals. Although this reduction was not statistically significant (p=0.266), a trend was observed. The weights of tumors in the treated group were less than those of tumors in untreated rats. Furthermore, the Ki67 and PCNA markers demonstrated less cell division in mammary cancer of treated rats (p<0.01) relative to those of the untreated group. It is possible that a higher dose of the root extract might be needed in order to observe significant differences in mammary cancer multiplicity. In addition, as the study progressed the differences between the groups became greater; thus, it is possible that the study was terminated too early.

This decrease in development of tumors and inhibition of tumor cell proliferation is attributed to the effects of the various compounds present in the alcoholic extract of WS roots. The withaniloid compounds present in the extract have effects on the development and proliferation of tumors (6, 19, 20). They act as antioxidant agents; have anti-inflammatory, cytotoxic, and immunostimulatory effects; cause cell-cycle arrest; and increase apoptosis. In other experiments, we have determined that the root extract inhibits the proliferation of MCF-7 and MDA-MB-231 cells both *in vitro* and *in vivo* (unpublished data).

In the present experiment, in which rats received WS root extract (150 mg/kg/day by oral gavage) for 155 days, the animals showed no visible signs of toxicity or significant reductions in body weights. These results provide a basis for further studies with higher doses for evaluation of the safety of the extract and for determination of its mechanism(s) of action. Currently, evaluations of WS root extract in MMTV-Neu mice (a model for estrogen-independent breast cancer) and an assessment of the chronic toxicity of the extract in rodents are being performed.

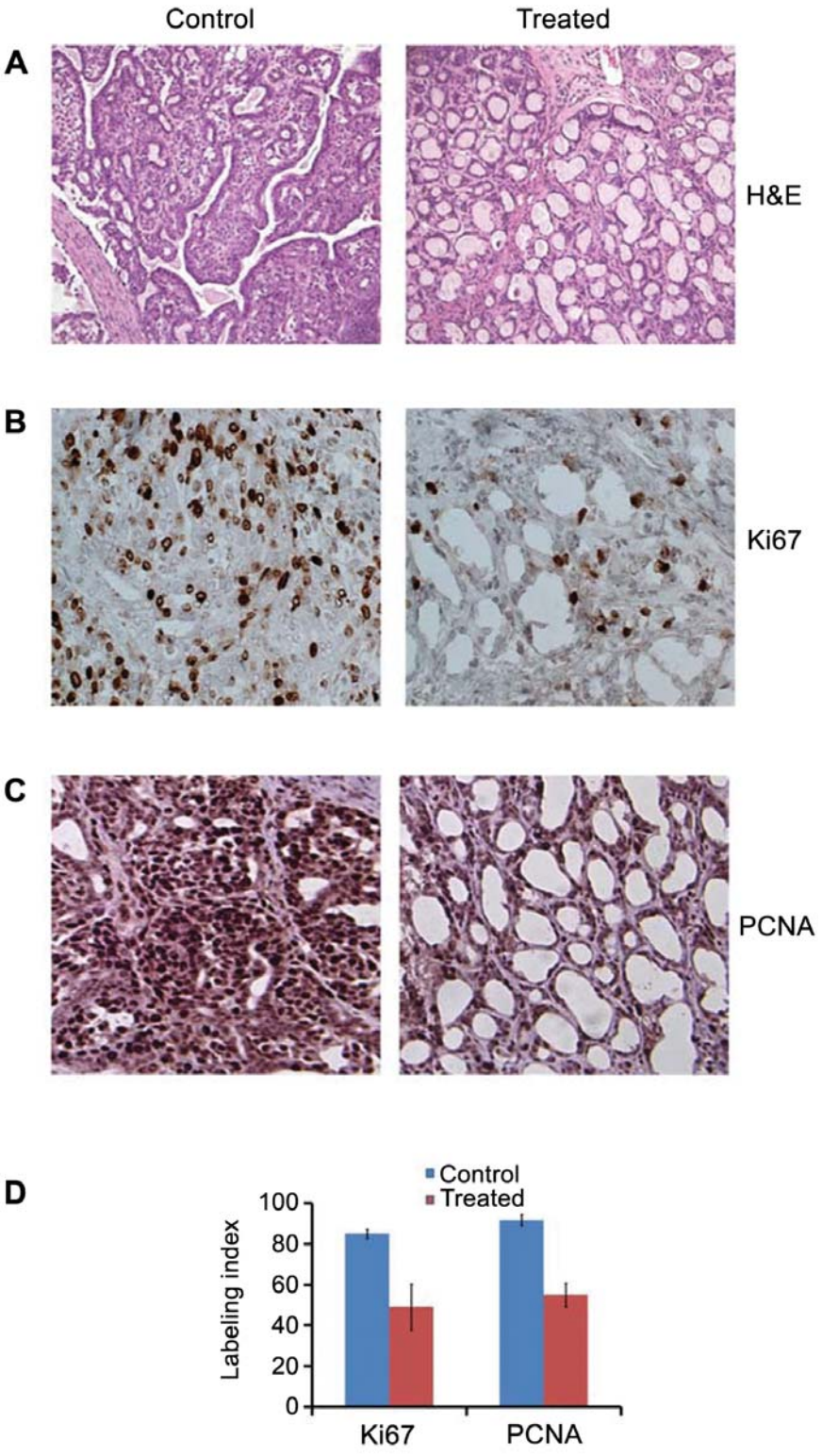

Figure 2. *Hematoxylin and eosin (H&E), Ki67, and Proliferating cell nuclear antigen (PCNA) staining of the mammary glands of control and Withania somnifera root extract-treated rats, (magnification ×20). A: H&E sections showed high mitotic activity in the mammary glands of control rats. B: The mammary glands of treated rats showed less Ki67 staining relative to controls. C: The mammary glands of treated rats showed less PCNA staining relative to controls. D: The labeling indices, as determined by Ki67 and PCNA staining, were 42% and 38% less, respectively, than those of the controls.*

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