# Effect of *Withania somnifera* Root Extract on Spontaneous Estrogen Receptor-negative Mammary Cancer in MMTV/*Neu* Mice

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**Abstract.** *The cancer-preventive activity of an extract of Withania somnifera (WS) roots was examined in female transgenic (MMTV/Neu) mice that received a diet containing the extract (750 mg/kg of diet) for 10 months. Mice in the treated group (n=35) had an average of 1.66 mammary carcinomas, and mice in the control group (n=33) had 2.48, showing a reduction of 33%. The average weights of the carcinomas were 2.36 g for mice in the treated group and 2.63 g for the controls, a difference of 10%. Labeling indices for Ki67 and proliferating cell nuclear antigen marker in mammary carcinomas of the treated group were 35% and 30% lower, respectively, than those of the corresponding control group. Expression of the chemokine was reduced by 50%. These results indicate that the root extract reduced the number of mammary carcinomas that developed and reduced the rate of cell division in the carcinomas.*

Breast cancer is the most common cancer in women, with about 1.7 million new cases accounting for 25% of all female cancers (1). The incidence of this disease is increasing in both industrialized and developing countries (2). Considerable progress has been made in treating breast cancer through surgery, radiotherapy, chemotherapy, and hormone therapy (3). However, those carcinomas that do not express the estrogen receptor [and, in particular, the triple-negative type that lacks both estrogen and progesterone receptors and shows no overexpression of human epidermal growth factor receptor 2 (HER2)] remain generally resistant to therapy (4). Of all human breast carcinomas, 15-20% are triple-negative (5). Thus, 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 dealing with this disease. A prominent approach is chemoprevention, by which the disease can be prevented, slowed, or reversed through the administration of one or more naturally-occurring or synthetic compounds (6, 7).

Currently, there is substantial interest in the use of herbal extracts for prevention of cancer, and several such extracts have demonstrated activity against different kinds of cancers (8). Some traditional herbs are considered to provide safe, economically-feasible, and effective activity for breast cancer prevention. These agents act by various pathways; *e.g.* inhibition of cell proliferation, stimulation of apoptosis, or inhibition of the activity of free radicals (9). 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 (10, 11). This small, woody shrub, which reaches 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. We previously showed that an alcoholic extract of WS had a modest activity in preventing the development of carcinogen-induced, estrogen-receptor positive mammary carcinomas in rats (12).

In MMTV/*Neu* mice with activated Her2/*Neu*, expression of the *c-neu* oncogene leads to uncontrolled cell growth and is sufficient to induce a high incidence and multiplicity of mammary carcinomas (13). The tumors that develop in these mice, similar to hormone-resistant human breast carcinomas, have low levels of estrogen receptor-α and express no progesterone receptor (14). The present study was performed to determine if an extract of WS roots prevents spontaneous development of estrogen receptor-negative mammary carcinomas in transgenic mice.

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*Key Words:* Withania, chemoprevention, mammary cancer, mice.

Table I. *Effect of Withania somnifera in the prevention of mammary cancers in female MMTV/Neu mice. Female MMTV/Neu mice were generated in the Chemoprevention Center at the University of Alabama at Birmingham. Beginning at 52 days of age, mice were treated with the root extract. The study was terminated at 10 months after initial diet supplementation with the root extract.*

| Group | No. of mice | Treatment | Mammary carcinomas | | |
|---|---|---|---|---|---|
| | | | Incidence | Multiplicity | Weight (g) |
| 1 | 35 | Root extract,750 mg/kg diet | 71% | 1.66 (33%↓) | 2.36 10%↓) |
| 2 | 33 | None | 70% | 2.48 | 2.63 |

## Materials and Methods

*Preparation of 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 use. 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 (UAB) Institutional Animal Care and Use Committee (approval number 131109528). Female FVB CD-1 transgenic mice, into which the *Her2/Neu* had been introduced, were generated in the Chemoprevention Center at UAB. The mice were maintained in UAB facilities where they were housed in animal quarters at 22˚C with a 12-h light/dark cycle, and were given free access to water and Teklad (4%) mash diet. (Harlan Laboratories, Madison, Wisconsin, USA). These facilities are accredited by the American Association of Laboratory Animal Care. The experiments followed guidelines for the care and use of animals.

At 52 days of age, the animals were randomized into two groups: Group 1 mice (N=35) were allowed to consume diet containing the root extract (750 mg/kg of diet); and Group 2 mice (N=33) were fed only the unsupplemented diet. The diet containing the root extract was prepared using a Patterson-Kelly liquid solid blender with an intensifier bar. The prepared diet was kept at 5˚C until administered to the mice. Fresh food was placed in food cups three times per week. Consumption of the diets continued for the duration of the study (until mice were 12 months of age).

All animals were palpated for mammary tumors twice each week. The mice 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 weight of the mammary carcinomas were determined.

*Immunohistochemistry*. Mammary carcinomas were fixed in 10% neutral-buffered formalin for 24 h for immunohistochemical localization of Ki67 and proliferating cell nuclear antigen (PCNA), markers of cell proliferation. They were placed in an automatic tissue processor, embedded in paraffin, and sectioned at 4-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 carried out 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). For detection of Ki67 and PCNA, slides were incubated with antibodies to Ki67 and PCNA (Abcam, Cambridge, MA, USA) for 1.5 h at room temperature. After washing, 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 with 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. For determination of the chemokine CCL2, which is the cytokine considered to be most responsible for the metastasis of breast cancer cells, slides were incubated with anti-mouse CCL2 (MCP-1; eBioscience) overnight at 4˚C. The slides were rinsed three times with phosphate buffered saline at room temperature and then incubated with goat anti-mouse IgG- (Santa Cruz Biotechnology, Dallas, Texas, USA). for 1 h at room temperature. The fluorescence was read using a widefield fluorescent microscope (Olympus). Stained slides were scored according to the intensity of staining (0, +1, +2, or +3), and percentage of tumor cells staining positively for CCL2 (0-30% +1; 31-70%, +2; or >70%, 3). The score for the intensity of immunostaining was multiplied by the score for percentage of cells staining positively to obtain the final score.

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

## Results and Discussion

In the present study, the WS root extract was evaluated for chemoprevention of mammary cancer in MMTV/*Neu* mice. Use of the extract is practical since it is easy to obtain and is inexpensive. Moreover, the preparation used allows

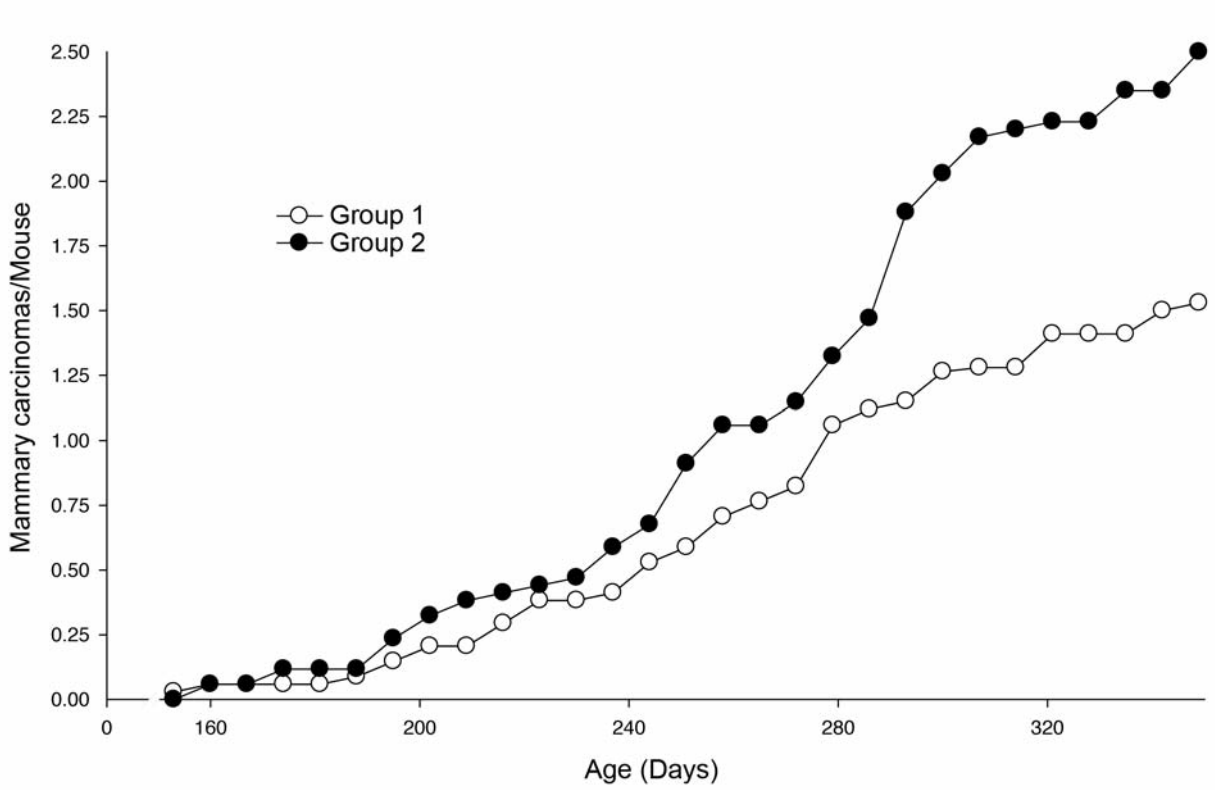


Figure 1. *Effect of the WS extract on development of mammary carcinomas in transgenic MMTV/Neu mice. Group 1 mice received WS root extract (750 mg/kg diet) for 10 months and Group 2 standard diet only.*

application of all active compounds present in the extract. Such extracts contain about 20 withaniloids (9), which are steroidal compounds.

For mice dosed for approximately 10 months, there was no appreciable difference in the weights of the mice in the two groups (33 g for controls *vs*. 32 g for the treated mice, $p$=0.1) and no difference in survival (all mice survived). The tumor incidence was similar (70% for untreated mice and 71% for treated mice), but the tumor multiplicity (Table I, Figure 1) was different (2.48 for untreated mice and 1.66 for treated mice; $p$=0.1). This represented a 33% reduction in multiplicity. Although this reduction was not statistically significant, a trend was evident. The average weights of the tumors in control and treated mice were 2.63 and 2.36 g, respectively. In carcinomas of treated mice, expression of proliferative markers Ki67 and PCNA were 35% less ($p$=0.001) and 30% less ($p$=0.002), respectively; the expression of CCL2, a marker of inflammation, was 50% less ($p$=0.0012) (Figure 2). This decrease in development of mammary carcinomas and inhibition of tumor cell proliferation is attributed to the effects of the various compounds present in the alcoholic extract of WS roots.

These data add to the considerable evidence indicating that extracts of Withania and isolated withaniloids have activity in treating and preventing cancers of various types. The withaniloid compounds have effects on the development and proliferation of tumors (7, 15, 16). Withaferin A was found to prevent the epithelial–mesenchymal transition of non-malignant MC-10A cells (17) and inhibit growth and metastasis of malignant MDA-MB-231 xenografts in mice (18). It also induced apoptosis, mediated by reactive oxygen species, in various types of human breast cancer cells (19-21). Possible mechanisms of action of withaferin A in the prevention and therapy of cancer are through induction of vimentin disassembly in breast cancer cells (22), and activation of NOTCH signaling (17). Disassembly of vimentin is associated with reduced epithelial-to-mesenchymal transition and metastasis, and enhanced NOTCH signaling promotes cell–cell communication and enhances processes associated with differentiation. The inhibition of cell growth is expressed as a blockade at the $G_2$-M phase of the cell cycle (23). Withaferin A also has anti-inflammatory (24) and immunostimulatory (25) effects. In breast cancer cells that express estrogen receptors, withaferin A suppressed estrogen receptor-$\alpha$, but not estrogen receptor-$\beta$ expression (26). In recent studies, withaferin A and WS inhibited the proliferation of both estrogen receptor-positive and -negative breast cancer cells

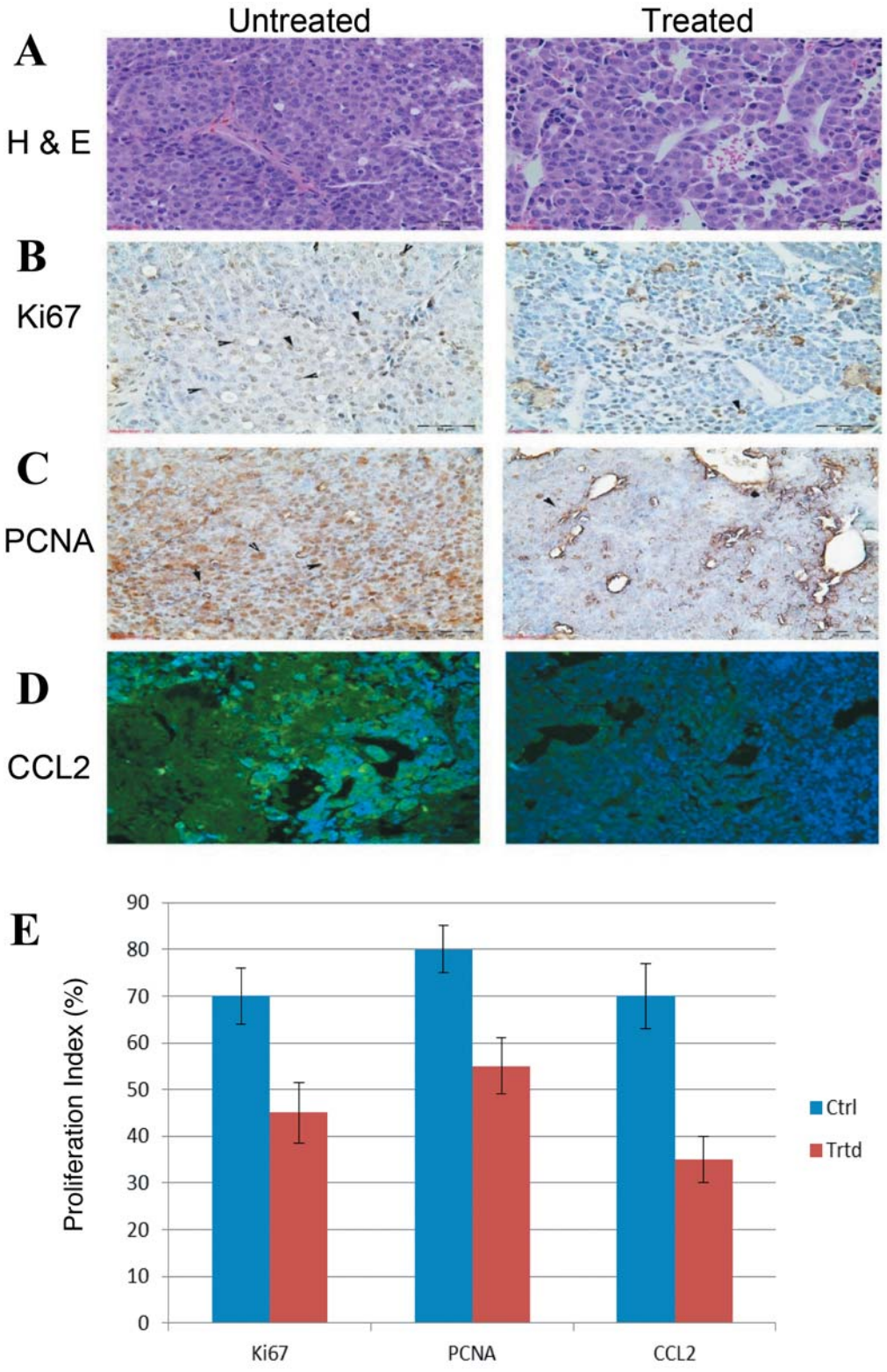


Figure 2. *Hematoxylin and Eosin (H&E), Ki67, and PCNA staining of the mammary glands of control and WS-treated mice (magnification ×20). (A): H&E-stained sections of mammary carcinomas in WS-treated and untreated mice. Mammary carcinomas of treated mice showed decreased staining for Ki67(B), PCNA (C) and cytokine CCL2 (D). (E): Labeling indices, as determined by Ki67 and PCNA staining, and CCL2 were 42%, 38% and 50% less, respectively, in treated (Trtd) mice than in those of controls (Crtl).*

and estrogen receptor-positive mammary tumors (12, 27). This indicates that WS inhibits breast carcinomas independently of estrogen receptor status. Furthermore, in a limited study, a root extract of WS alleviated chemotherapy-induced fatigue and enhanced the quality of life of patients with breast cancer (28).

In other experiments, we determined that WS root extract inhibits the proliferation of MCF-7 and MDA-MB-231 cells *in vitro* and *in vivo*, and in these cells causes a reduction in expression of the cytokines CXCL1, -2, and -3; CCL2; (unpublished data). In the present study, WS caused a significant decrease in CCL2 levels in mammary tumors. Inhibition of CCL2 inhibits the invasiveness of breast cancer cells (29).

Since no apparent toxicity was noted for mice used in this experiment, it is possible that higher doses of the root extract of WS would reveal a greater difference in mammary cancer multiplicity and possibly a difference in incidence. The present results provide the basis for further studies with higher doses for evaluation of the efficacy and safety of the WS extract, and for determination of its mechanism(s) of action.

## Acknowledgements

This effort was supported by NIH grant U54 CA118948.

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*Received June 16, 2014*
*Revised August 20, 2014*
*Accepted August 25, 2014*