A Natural Antioxidant Mixture from Spinach Does Not Have Estrogenic or Antiestrogenic Activity in Immature CD-1 Mice

ABSTRACT The use of natural antioxidants and flavonoids in nutritional and pharmaceutical applications is increasing. Because some phytochemicals such as genistein, found in soy products, have estrogenic activity, we investigated the estrogenic potential of a natural antioxidant mixture (NAO) isolated from spinach leaves, using an in vivo uterotrophic bioassay and an in vitro transcriptional activation assay for the estrogen receptor (ER). Outbred female CD-1 mice (17 d old) were given subcutaneous injections of 17β-estradiol or genistein [500 and 500,000 μg/(kg · d), respectively] as positive controls or NAO [1000 to 1,000,000 μg/(kg · d)] for 3 d. Uterine wet weight/body weight ratios were determined. Both 17β-estradiol and genistein significantly increased uterine wet weight ratios compared with untreated controls, but NAO did not. Histological examination of the uterus showed that 17β-estradiol and genistein increased epithelial cell height, number and gland development, but NAO did not. Estrogenic activity of NAO was investigated in vitro using the ER transcriptional activation assay. BG1Luc4E2 cells expressing ER were stably transfected with a luciferase reporter gene responsive to estrogens. 17β-estradiol dose dependently increased luciferase activity; NAO had no effect. When NAO was tested for antiestrogenic activity, it did not lessen the effects of 17β-estradiol. These data suggest that NAO does not have estrogenic or antiestrogenic activity. Thus, an antioxidant mixture has been identified that does not have potentially adverse estrogenic activity. J. Nutr. 133: 3584–3587, 2003.

KEY WORDS: phytochemicals • uterotrophic bioassay • antioxidants • flavonoids • environmental estrogens

In recent years, the naturally occurring polyphenolic antioxidants have received increased attention as cancer-prevent-

ing agents (1). Natural antioxidants such as epigallocatechin gallate (green tea polyphenol) are being tested for their efficacy in chemoprevention of skin and prostate cancers (2,3). Studies suggest that factors such as structural variation and number of polyphenolic groups may influence the effectiveness of these chemicals in impairing signaling pathways associated with tumor promotion (4). However, considerable evidence exists for the role of antioxidants in fruits and vegetables in the maintenance of health and in disease prevention (5).

Spinach is an important dietary vegetable often associated with beneficial health effects. Fresh spinach contains ~1 g/kg of total flavonoids and other phenolic constituents that act as antioxidants due to the free radical scavenging properties of their hydroxyl groups. (6).

Natural antioxidant mixture (NAO) is a water-soluble extract obtained from spinach leaves that has been shown to have antioxidantitive (7), antiproliferative (8) and anti-inflammatory properties (9) in biologic systems. Further, NAO has been reported to show protective effects in various animal models of disease such as doxorubicin-induced cardiotoxicity (10), skin papilloma (11), prostate cancer (8) and LPS-induced septic shock (12,13). Based on NMR spectroscopy, the major active components of NAO have been identified as glucuronic acid derivatives of flavonoids, trans and cis isomers of p-coumaric acid and meso-tartarate derivatives of coumaric acid, and uridine (7). Each component showed antioxidant activity when the rate and extent of linoleic acid autooxidation was tested (7). Although each isolated fraction exhibited antioxidant activity, the combination of the fractions had a synergistic effect; NAO is a powerful antioxidant that is extremely effective in protecting against oxidation damage (7).

Many properties of NAO have been elucidated. It, as well as a glucurinated flavonoid recently isolated and purified from NAO and having the chemical structure of 6-(3,4-dihydroxyphenyl)-9-hydroxy-7-methoxy-dioxolo [4,5-c] chromen-8-one 4′-β-glucuronid (1,3), shows scavenging activity for reactive oxygen species, such as superoxide, OH anion radical and singlet oxygen (14). It is water-soluble, highly stable at high temperature and has a long shelf life (6). It is not genotoxic and lacks toxicity; the lethal dose at which 50% die (LD-50) of NAO in mice is 1500 mg/kg (6).

Because other phytochemicals with antioxidantive properties such as genistein, found in soy products, show estrogenic activity (15) and bind preferentially to estrogen receptor beta (16), we tested the estrogenic potential of NAO in established in vivo and in vitro bioassays. Knowledge of the estrogenic activity of NAO is important because the treatment of developing organisms with estrogenic compounds has long-term deleterious effects (17,18).

MATERIALS AND METHODS

Chemicals. 17β estradiol and genistein (Sigma Chemicals Co., St. Louis, MO) were dissolved in saline and propylene glycol (1:1). NAO, a water-soluble antioxidant, is composed of a mixture of...
natural molecules extracted and purified from spinach leaves as described previously (7). Briefly, NAO was prepared as follows: spinach leaves were homogenized with an equal amount of water. The resulting supernatant was collected and purified by ultrafiltration using a 3000-pmol membrane. The filtered fraction was collected, lyophilized, and stored at 4°C until diluted and further tested.

**Standardization of NAO.** Recovery of NAO comprised 3 g from 1 kg of wet spinach leaves. Every NAO batch was characterized by three assays. Each batch was tested for antioxidant activity using thiobarbituric acid and xylene orange assays (7) to monitor malondialdehyde and hydroperoxide levels. Each batch was analyzed by HPLC to determine the amount and concentration of active components such as cumaric acid derivatives and specific flavonoids. The actual concentration of active ingredients in each preparation of NAO stock solution was determined spectrophotometrically using a typical spectrum of NAO with a maximal absorption peak of cis- and trans-cumaric acid at 310 nm and two absorption peaks of flavonoids at 275 and 350 nm (7).

**Composition of NAO.** Flavonoids and p-coumaric acid derivatives were active components of the aqueous extract of spinach leaves (7). Based on 1H and 13C NMR spectroscopy, four of the seven hydrophobic fractions isolated from spinach extract were identified as glucuronide derivatives of flavonoids, three additional fractions as trans and cis isomers of p-coumaric acid and others as meso-tartaric derivatives of p-coumaric acid. The molecular weight of NAO, based on NMR analysis, was 500 to 1000. Active compounds that were identified by NMR can be separated into three categories of chemicals: flavonoid derivatives; cumaric acid derivatives; and hydrophilic components, one identified as uridine. The flavonoids (14) are similar in structure to genisteen, which was previously reported to show estrogenic activity (15).

For dosing of mice, 500 mg of NAO was dissolved in 2.5 mL saline and added to 2.5 mL propylene glycol (saline:propylene glycol, 1:1) to make a stock solution (100 g/L); this concentration was the highest dose tested (1,000,000 μg/kg) and was serially diluted in saline: propylene glycol (1:1) for the lower doses (1000, 10,000 and 100,000 μg/kg). Higher concentrations of NAO could not be tested due to lack of solubility.

**Immature uterotrophic bioassay.** Timed pregnant female CD-1 mice [Crl:CD-I(ICR)], obtained from the breeding colony at the National Institutes of Environmental Health Sciences (NIEHS; Research Triangle Park, NC), delivered their young on day 19 of gestation. All litters were standardized to 10 female pups per dam and housed in a temperature-controlled room (21 to 22°C, light and 12-h dark cycle. Mice consumed ad libitum fresh reverse osmosis/deionized water and NIH-31 feed (19). Animal procedures complied with NIEHS/NIH animal care guidelines. As previously described (20), pups were weaned on day 17, housed 5 per cage, and injected subcutaneously with varying doses (a minimum of 5 mice per group) of NAO dissolved in saline:propylene glycol at 275 and 350 nm (7).

**Statistical analysis.** Statistical analysis was performed by ANOVA and Dunnett’s test (Stat View SE+ Graphics; Abacus Concepts, Berkeley, CA). Differences were considered significant at *P < 0.05* using Dunnett’s test.

**RESULTS**

**Immature uterotrophic bioassay.** Estradiol and genistein (500 and 500,000 μg/kg, respectively) significantly increased the uterine wet weight/body weight ratio compared with untreated controls. The complete dose response for these two compounds has been previously reported (15). However, none of the NAO doses affected the uterine wet weight (data not shown) or the ratio (Fig. 1). Further, none of the NAO dose groups differed histologically from the control group (data not shown), whereas estradiol and genistein both increased uterine cell height, cell number and gland formation (15).

**ER transcriptional activation assay.** Estrogen activity. Microscopic examination of the cells following 24 h exposure to NAO did not indicate cell toxicity (Fig. 2A). The NAO did not induce luciferase activity relative to untreated controls, whereas estradiol increased it ∼50-fold (Fig. 2A). This indicates that NAO does not show estrogenic activity in this assay.

**Antiestrogen activity.** When tested alone, 0.0125 μg/L of 17β-estradiol caused a 50% maximal estrogenic response (Fig. 2B). When various doses of NAO were tested in combination with 17β-estradiol, the effects of estradiol were not affected, indicating that NAO was not antiestrogenic in this assay.

**DISCUSSION**

The beneficial effects of spinach consumption on human health are supported by epidemiological and preclinical data; however, any demonstrable estrogenic or antiestrogenic function of, media were removed and the cells were microscopically observed for viability. Luciferase activity induction was measured (20). Microscopic examination of the cells following exposure to the sample extracts did not indicate cellular toxicity. Antiestrogenic activity. Various concentrations of NAO were added to a known amount of 17β-estradiol (0.0125 μg/L) and results were compared with those for 17β-estradiol alone.

![FIGURE 1](https://academic.oup.com/jn/article-abstract/133/11/3584/4817956) Relative wet uterine weight of control immature CD-1 mice and those treated for 3 d with natural antioxidant mixture (NAO), estradiol, or genistein. Values (plotted on a log scale) are means ± SEM, n = 5. *Different from untested controls, *P < 0.05. The dose response curve for estradiol and genistein is included for reference of a positive control and a comparison of NAO to another phytoestrogen, respectively; the complete dose response curve for estradiol and genistein has been previously published (15).
tion would give cause for concern. Spinach is composed of various active compounds, such as flavonoids and other polyphenolic active ingredients, acting synergistically as anti-inflammatory, antioxidative, and anticancer agents (7). No estrogenic or antiestrogenic activity was found by the assays utilized in our study.

We have previously shown that NAO isolated from spinach leaves can prevent lipid peroxidation in both plant and animal systems (6,7). Investigators have demonstrated beneficial effects of NAO in animal models of cardiotoxicity (10), LPS-induced septic shock (9,12,13), skin papilloma (11) and prostate cancer (8). We plan to test its potential beneficial use in our animal model of estrogen-induced uterine adenocarcinoma (23). However, before initiating the study and to further characterize the properties of NAO, the current investigation was conducted to determine the estrogenic activity of NAO.

The results of the present study indicate that NAO, at doses comparable to genistein (15), does not show estrogenic activity; the possibility that it may show other hormonal activity continues to be tested.

Current literature highlights continuing interest in the protective biological effects of some natural antioxidants, especially from edible plants. For example, genistein isolated from soy products has been reported to have antioxidant effects, and therefore has been proposed as a preventative therapy for some disease processes. Genistein has other properties including estrogenic activity (15). We have shown that exposure to genistein during development causes long-term problems including reproductive tract neoplasia (24). Because estrogens and other environmental endocrine disrupting chemicals may be involved in fertility problems, early puberty, and cancers of the prostate, breast, ovary and uterus, it was important to determine whether NAO shows estrogenic activity before continuing with future experiments.

Because oxidative stress has been reported to play a key role in inflammatory processes, aging and carcinogenesis (25), interest continues in the protective biological effects of some natural antioxidants that may be candidates for cancer prevention therapy and extension of the human life span. Several studies have been conducted to elucidate the therapeutic potential of spinach antioxidants (6). Dietary intake of spinach extracts has been reported to beneficially affect subjects with various cancers, such as those of the ovary (26), lung (27), prostate (28), breast (29,30) and colon (30). During screening for inhibitors of lipid peroxidation, NAO was identified as a specific inhibitor of the lipoxygenase enzyme (6,7); it was subsequently studied for its efficacy as an antioxidant and found to be superior to other antioxidants such as vitamin E and green tea (6–8). The mechanism of its pharmacological effects is not understood, but it may involve direct effects such as inhibition of cellular proliferation and interference with the cell cycle, or it may be indirectly related to the scavenging of free radicals released in various organs stressed by oxidants, such as the liver, the spleen, the thymus and the reproductive tract. Elucidation of these mechanisms remains an important area of future study.

In summary, NAO does not show estrogenic or antiestrogenic activity. If no other hormonal or endocrine disrupting activity can be identified, NAO may be added to a growing list of dietary antioxidants that may be useful treatments in protecting against the toxic effects of environmental mutagens and carcinogens.

**LITERATURE CITED**