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Potent chemopreventive/antioxidant activity detected in common spices of the Apiaceae family

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Abstract

Spices are used worldwide, particularly, in the Asian and Middle-Eastern countries and considered protective against degenerative diseases, including cancer. Here, we report the efficacy of aqueous and non-aqueous extracts of eleven Apiaceae spices for free radical-scavenging activity and to inhibit cytochrome P450s in two separate reactions involving: i) 4-hydroxy-17β-estradiol ($4E_2$), DNA and $CuCl_2$ and ii) 17β-estradiol, rat liver microsomes, co-factors, DNA and $CuCl_2$. Oxidative DNA adducts resulting from redox cycling of $4E_2$ were analyzed by ^{32}P-postlabeling. Aqueous (5 mg/ml) and non-aqueous extracts (6 mg/ml) substantially inhibited (83% – 98%) formation of DNA adducts in the microsomal reaction. However, in non-microsomal reaction, only aqueous extracts showed the inhibitory activity (83% – 96%). Adduct inhibition was also observed at 5-fold lower concentrations of aqueous extracts of cumin (60%) and caraway (90%), and 10-fold lower concentrations of carrot seeds (76%) and ajowan (90%). These results suggests the presence of two groups of phytochemicals - polar compounds that have free radical-scavenging activity, and lipophilic compounds that selectively inhibit P450 activity associated with estrogen metabolism. Because most of these Apiaceae spices are used widely with no known toxicity, the phytochemicals from the Apiaceae spices used in foods may be potentially protective against estrogen-mediated breast cancer.

Keywords

Apiaceae spices; Antioxidant activity; Free radical scavenging activity; Redox activity; Oxidative DNA adducts

Introduction

Epidemiological data suggest that an elevated level of free radicals is associated with various diseases including cancer (1). Free radicals formed in cell metabolism and through

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various environmental factors like pollution, radiation or cigarette smoke are highly reactive and cause DNA damage which possibly leads to aging, cardiac diseases and cancer (2). The examples of important reactive oxygen species (ROS) include super oxide, hydroxyl radical and nitric oxide radicals etc. (2, 3). The imbalance between the free radicals and antioxidants that quenches them leads to cellular damage termed as oxidative stress (4–6).

Antioxidants and enzymes such as catalase play an important role in balancing biological systems by suppressing the formation of active oxygen species (ROS) by reducing hydroperoxides ($ROO^{\bullet}$) and H_2O_2, and scavenging free radicals among others (7). Thus, it is important to maintain equilibrium between free radical generation and antioxidants supply. When endogenous antioxidants are insufficient to engage the highly reactive free radicals, it is beneficial to provide antioxidants, e.g. dietary to further mitigate damaging free radicals. The antioxidant potential of fruits and vegetables has been established by *in vitro* and *in vivo* studies (8, 9). Bioactive phytochemicals in fruits and vegetable are highly diverse yet commonly demonstrate similar bioactivities including antioxidant, antiproliferative, and anti-angiogenic properties as well as blocking of cell cycle and/or by induction of apoptosis that contribute to demonstrated anticancer properites (10, 11). Further, consumption of fruits and vegetables is associated with reduced risk of cancer, heart disease, and memory loss (12, 13).

Historically, herbs and spices have enjoyed a rich tradition of use for their flavor enhancement characteristics and for their medicinal properties. Spices provide a rich array of phytochemicals that may reduce the risk of certain cancers (14, 15). *In-vitro* studies indicate that bioactive components of herbs and spices can inhibit pathways that regulate cell division, cell proliferation, and detoxification as well as inflammatory and immune response (15). Moreover, spices are rich in phytochemicals like essential oils, polyphenolics and phenolic terpenes; all of which have been shown to inhibit or attenuate cancer initiation or progression (16).

DNA damage has been associated with cancer as well as aging processes (17). There are several pathways identified in the development of cancer. One of them is oxidative DNA damage contributed by redox activity of endogenous and exogenous species. To assess the DNA damage caused by ROS, ^{32}P-postlabeling followed by 2-dimensional polyethyleneimine-cellulose thin-layer chromatography (PEI-cellulose TLC) is commonly used (18). This method has an advantage of detecting a variety of polar adducts in addition to the oxidative DNA lesion 8-oxo-2’-deoxyguonosine (8-oxodG) (19) .

In this study, we compared 11 commercially available organic spices (Fig. 1) belonging to Apiaceae family for their antioxidant capacity using the redox activity of 4-hydroxy- 17β-estradiol ($4E_2$). Chemopreventive activity of the spices extracts was assessed by the inhibition of cytochrome P450s associated with metabolism of 17β-estradiol.

Materials and Methods

Chemicals and spices

17β-estradiol (E_2) and 4-hydroxy-17β-estradiol were purchased from Steraloids, Inc., (Newport, RI). Acetonitrile and HPLC-grade water were purchased from Sigma-Aldrich. Anise (*Pimpinella anisum*), coriander (*Coriandrum sativum* var. vulgare), cumin (*Cuminum cyminum*), dill (*Anethum graveolens*), fennel (*Foeniculum vulgare* var. vulgare), carrot seeds (*Daucus carota*), celery (*Apium graveolens*) and parsley (*Petroselinum crispum*) were purchased from local stores, angelica (*Angelica archangelica*) seeds from Mountain Rose Herbs (Eugene, OR), caraway (*Carum carvi*) from Frontier Natural Products Corp. (Norway, IA), and ajowan (*Carum copticum*) from Zamouri Spices (Olathe, KS). All enzymes, chemicals and solvents used for ^{32}P-postlabeling assay were as described elsewhere (20). All other chemicals were of analytical grade.

Preparation of the extracts

Test spices were powdered and sieved to obtain fine powder. Spices (0.5 g each) were extracted individually three times with 10 vol. of water or acetonitrile separately for 2 h in a shaking water bath at room temperature (22 °C). Samples were centrifuged at 3,000x *g* for 15 min and supernatant was collected. Pooled extracts were concentrated under reduced pressure in SpeedVac (Savant, Thermo Scientific) and reconstituted in 1 ml 50% dimethyl sulfoxide (DMSO) and passed through 0.22 μm filter. Ajowan, carrot seeds, cumin and dill extracts were further diluted to 2.5, 1, 0.5, 0.25, 0.01 mg/ml for dose-response studies. The effect of temperature and time on the extraction efficiency was investigated by extracting 1 g of fennel with 10 vol of water at 22, 40, 60, 80 and 100 °C for 6, 4, 2, 1 and 0.5 h, respectively, and extracts were concentrated and reconstituted in 1 ml 50% DMSO.

Effect of the spice extracts on redox activity of $4E_2/CuCl_2$

Salmon testis (st)-DNA (300 μg/ml) in 10 mM Tris-HCl (pH 7.4) was pre-incubated with either vehicle alone (DMSO) or aqueous (0.01 to 5 mg/ml) and non-aqueous extracts (0.012 to 6 mg/ml) for 15 min at 37 ºC. After adding $4E_2$ (50 μM; 2% ethanol) and $CuCl_2$ (50 μM), the reaction mixture was further incubated at 37 °C for 4 h, and the DNA was isolated by solvent- extraction and ethanol precipitation as described (18, 20). The isolated DNA was tested for oxidative DNA adducts.

Effect of the spice extracts on microsomal activation of E_2

st-DNA (300 μg/ml) was pre-incubated in 1 ml reactions containing 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 2.5 mM glucose-6-phosphate, 1 U/ml G6PDH, 0.5 mM NADP+, and α-naphthoflavone- induced rat liver microsomal proteins (1 mg/ml) in the presence of vehicle alone or aqueous and non-aqueous extracts (0.01 to 5 mg/ml) in a shaker water bath at 37 °C for 15 min. E_2 (50 μM; 2% ethanol) and $CuCl_2$ (50 μM) were added to the reaction mixture and further incubated for 4 h. Reactions were terminated by the addition of EDTA followed by centrifugation (9,000x *g*; 10 min). DNA was isolated from the supernatant by removal of RNA and proteins by digesting with RNases A and T1 and proteinase K and a series of extractions with phenol, phenol: Sevag (chloroform:isoamyl alcohol, 24:1), and Sevag,

followed by precipitation of the DNA with ethanol (5). The DNA concentration was estimated spectrophotometrically.

Analysis of oxidative DNA adducts by ^{32}P-postlabeling

Oxidative DNA adducts were analyzed by nuclease P1-mediated ^{32}P-postlabeling/TLC (22). Briefly, 10 μg of DNA was digested with micrococcal nuclease and spleen phosphodiesterase (enzyme: substrate, 1:5, 37 °C, 5 h). Before further treatment with nuclease P1 (enzyme: substrate, 1:2.5; 37 °C; 1 h) to enrich DNA adducts, an aliquot was removed for evaluation of normal nucleotide levels. Enriched adducts and normal nucleotides were labeled with [γ-^{32}P] ATP (60 μCi, 0.4 μM; 6,000 Ci/mmol) and T4 polynucleotide kinase. Labeled oxidative adducts were resolved by 2-D PEI-cellulose TLC (D1 = 1 M formic acid/40 mM sodium phosphate, pH 6; and D2 = isopropanol: 4 M ammonium hydroxide, 1:1). Normal nucleotides were labeled in parallel and resolved in 180 mM sodium phosphate, pH 6.0, by 1-D PEI-cellulose TLC. Adduct and normal nucleotides were detected and quantitated by Packard InstantImager. Adduct levels were expressed as adducts/10^6 nucleotides.

Statistical analysis

All calculations were performed with GraphPad Prizm statistical software (version 4.03; La Jolla, CA). One way analysis of variance (ANOVA) was done to compare the dose response. Student's *t*-test was used to compare for parameters between treatment groups. We chose a p-value of 0.05 to declare significant results.

Results

Preparation of aqueous and non-aqueous extracts

The dry spices were powdered and extracted with 10 vol. of water or acetonitrile separately in order to extract polar or lipophilic compounds, respectively. Water is preferred as a solvent to determine its ability to extract bioactives as most of the folklore formulations or household consumption of spices are water based. Both aqueous and organic extracts were concentrated to dryness and stored at −80 °C until use and then reconstituted in 50% DMSO prior to use.

Inhibition of redox activity of $4E_2$

The antioxidant activity of the spice extracts was determined by incubating the aqueous or non-aqueous extracts with st-DNA damaged with oxygen free radicals from redox cycling of $4E_2$ by $CuCl_2$. Analysis of oxidative DNA adducts by ^{32}P-postlabeling assay revealed several oxidative DNA adducts (Fig. 2). These adducts have been assigned as polar DNA adducts formed by attack of hydroxyl radicals (23).

Aqueous extracts of fennel prepared under various conditions (22 °C for 6h, 40°C for 4 h, 60°C for 2 h, and 80°C for 1 h) showed essentially the same degree of adduct inhibition (94–97%) indicating that the length of extraction can be offset by increase in temperature. However, extracts prepared at 100 °C for 0.5 h showed somewhat lower adduct inhibition

(81%) indicating some bioactives were lost at this temperature. Subsequent extractions for the remaining studies employed three extractions at room temperature for 2 h each.

Aqueous extracts from fennel, cumin, parsley, anise, ajowan, angelica, carrot seeds, celery, caraway, coriander and dill exhibited 83–97% inhibition of the oxidative DNA adducts at a concentration of 0.4 mg/ml (Fig. 3A). Aqueous extracts of ajowan, carrot seeds and cumin were also tested at lower doses. Ajowan extract showed the highest antioxidant activity, with 40% and 75% inhibition at 0.01 and 0.05 mg/ml, respectively, followed by carrot seed extract which showed about 70% inhibition at 0.2 mg/ml, and cumin extract which showed about 20% inhibition at 0.2 mg/ml. With the exception of carrot seeds (85%), angelica (35%) and caraway (22%) (Fig. 3B) the non-aqueous extracts of these spices failed to show any significant protection. .

Inhibition of P450 activity by aqueous extracts

$4E_2$ is formed by P450-mediated activation of E_2 and represents a presumptive carcinogenic metabolite. In this study we used microsomal activation of E_2, Cu^{2+}-mediated activation of the resulting $2E_2/4E_2$, and oxidative DNA adduct formation to determine effects of the spice extracts on metabolic activation of E_2. Analysis of the purified DNA by ^{32}P-postlabeling showed essentially the same adduct profile (Fig. 4) as found by Cu^{2+}-$4E_2$ reaction (Fig. 2). No qualitative differences were found in the absence or presence of the spice extracts, however, significant quantitative differences occurred (Fig. 4). Compared with the vehicle treatment (358 ± 58 adducts/10^6 nucleotides), the aqueous extracts of anise, fennel, cumin, caraway and dill inhibited the adduct formation almost completely (85–95%); coriander extract showed over 70% inhibition (Fig. 5A).

The non-aqueous extracts of all these six spices also showed essentially the same degree of adduct inhibition as observed with the aqueous extracts, i.e., 90–96% inhibition with extracts of anise, fennel, cumin, caraway and dill, and 83% inhibition with coriander extract (Fig. 5B).

Discussion

Antioxidants have attracted much interest for their protective effect against free radical damage associated with many degenerative diseases including cancer. We previously demonstrated by co-chromatography that the profile of polar oxidative adducts generated *in vitro* using either $4E_2/CuCl_2$ or $H_2O_2/CuCl_2$ were similar (23), indicating that DNA adducts resulting from redox activity of $4E_2$ in the presence of $CuCl_2$ originated from oxidative mechanisms.

The potential of test spices (fennel, cumin, parsley, anise, ajowan, angelica, carrot seeds, celery, caraway, coriander and dill) to inhibit $4E_2$/Cu(II)-induced oxidative DNA adducts was evaluated in this study. Aqueous extracts of these spices showed high antioxidant potency by inhibiting the redox activity of $4E_2$, whereas non-aqueous extracts were essentially ineffective, except for carrot seeds, angelica and caraway which showed modest protection. It is evident that phytochemicals in aqueous extracts are more potent antioxidant than in non-aqueous extracts and likely to represent different group(s) of compounds. Our

results corroborate the finding of Goswami and Chatterjee (24) who demonstrated high antioxidant activity of ajowan (*Trachyspermum ammi*) and fennel (*Foeniculum vulgare*) extracts. In another study aqueous extract of dill has been shown to possess higher hydroxyl radical scavenging activity compared to onion, pepper and garlic (25). Jayakumar and Kanthimathi (26) have also reported significant protection of hydrogen peroxide-induced DNA damage analyzing by comet assay with cumin, fennel and caraway extracts.

Several polyphenolics from these spices have been reported to possess antioxidant properties. Anethole is one of the chief components of anise and fennel and its derivative anethole dithiolethione showed antioxidant activity with their ability to suppress tumor necrosis factor and ability to reduce oxidative stress by acting as scavengers of hydroxyl radicals (27, 28). In another study, anethole induced apoptosis, and combinations of anethole with cisplatin and oxaliplatin were highly synergistic (29). There are several other examples of phenolics such as p-cynene (ajowan and cumin), limonene (celery and caraway), thymol (ajowan) and β-pinene (cumin and celery) that have been shown to possess high antioxidant properties (30–32). In addition, the biological properties of polar and non-polar compounds from spices have been reviewed (33–35). A good correlation of phenolic content with high antioxidant activity of has been established by Kim et al (25). However, the concentration of the phenolics and flavonoids in various herbs and spices varies depending on their cultivar and climate (25).

E_2 treatment has been reported to enhance polar oxidative DNA adducts (36); however, the exact mechanism for this is unclear. The effect of E_2 treatment on the expression of various P450 enzymes involved in its metabolism has been studied. Diethylstilbesterol, an E_2 analogue, can induce CYP1A2 levels in mouse liver (37). E_2 itself has been shown to increase CYP1A2 expression in hamster liver (38) and it is known that E_2 induces the expression of several P450s involved in its metabolism (38). Thus, E_2 treatment may induce an environment conducive to the production of its carcinogenic metabolites in the liver.

In this study we also examined the capacity of E_2 to induce oxidative DNA adducts in a cell-free system and its modulation by the various spice extracts. Rat liver microsome/Cu(II)-mediated activation of E_2 resulted in the formation of oxidative DNA adducts, as analyzed by ^{32}P-postlabeling, and the adduct pattern was identical to the oxidative DNA adducts resulting from $CuCl_2$-$4E_2$ reaction. Further evidence in support of the microsomal E_2 adducts being hydroxylated DNA adducts and not related to quinone derivatives of 2-/4-E_2 is based on chromatographic properties (39).

Both aqueous and non-aqueous extracts of all the test spices showed significant inhibition of microsomal E_2 DNA adducts. These DNA adducts originate from a two-step process: First, E_2 is metabolized by microsomal P450s to $2E_2$/$4E_2$. Second, the resulting $2E_2$-/$4E_2$ then produce ROS in the presence of $CuCl_2$. Since the aqueous, not the non-aqueous extracts of the spices inhibited $4E_2$/$CuCl_2$-generated oxidative adducts, the inhibition of the microsomal DNA adducts by the non-aqueous extracts may be attributed to the inhibition of the P450s, while the inhibition of the microsomal DNA adducts by the aqueous extracts likely resulted from the scavenging of the ROS (Fig. 6).

Some of the spices investigated in this study such as ajowan, caraway, fennel, cumin and anise are common in the Asian and Middle-Eastern cuisines as well as in American eateries and are used to varying degrees in home recipes. It is estimated that daily intake of each of these spices may be 2–20 g in Indian subcontinent. Thus, human consumption of these spices in some parts of the world is already established at relatively high levels. Establishing *in vivo* efficacy of the aqueous and non-aqueous extracts of these spices will provide a simple and effective strategy for preventing breast and perhaps other cancers.

Acknowledgments

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Figure 1.
Photographs of selected spices used in the study.

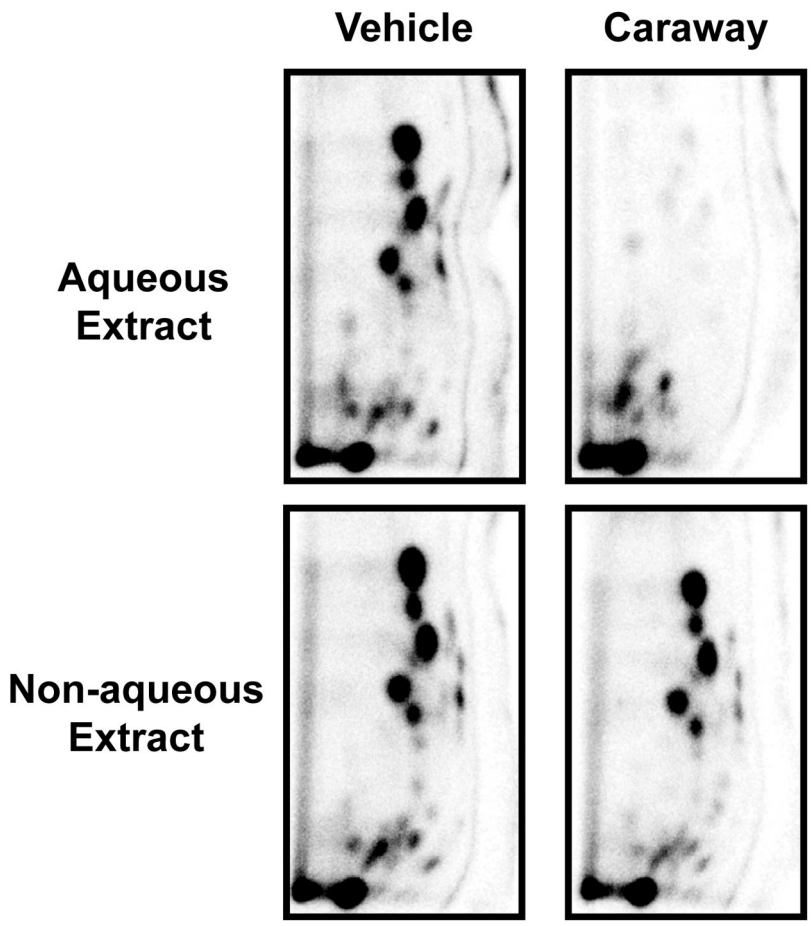

Figure 2.

Representative autoradiographs of ^{32}P-labeled oxidative DNA adducts resulting from Cu(II)-mediated activation of $4E_2$ in the presence of either vehicle (2% ethanol) or aqueous and non-aqueous spice of caraway extracts. Labeled oxidative DNA adducts were resolved by two-directional PEI-cellulose TLC (D1 = 1 M formic acid/40 mM sodium phosphate, pH 6.0 onto a 6 cm Whatman no. 17 paper wick; D2 = isopropanol: 4 M ammonium hydroxide, 1:1) and detected by Packard InstantImager.

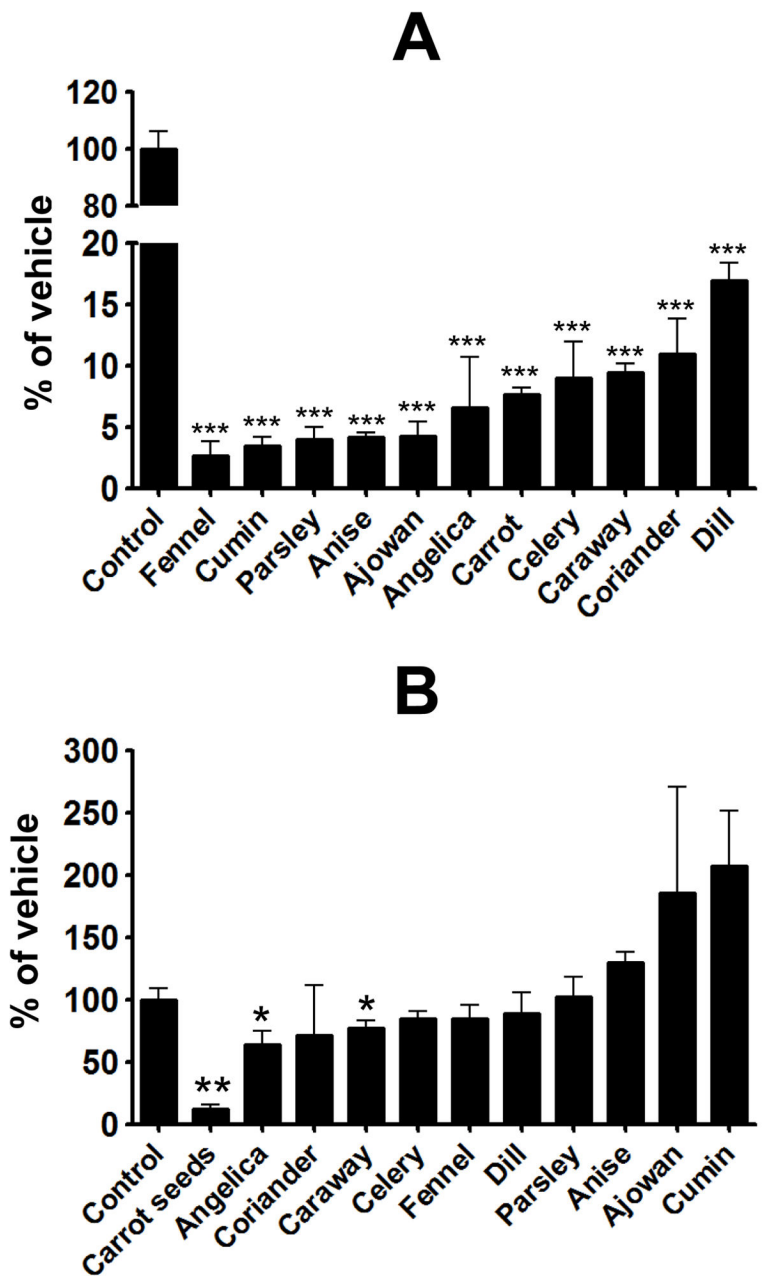

Figure 3.

Effect of aqueous extracts (A) and non-aqueous extracts of indicated spices on Cu(II)-mediated oxidatively generated DNA damage by $4E_2$. Experimental conditions are described in Materials and Methods. Levels of DNA adducts were determined as adducts/10^6 nucleotides and represented as % of control. Data represent average ± SD of four samples and a p-value of <0.05 was considered significant.

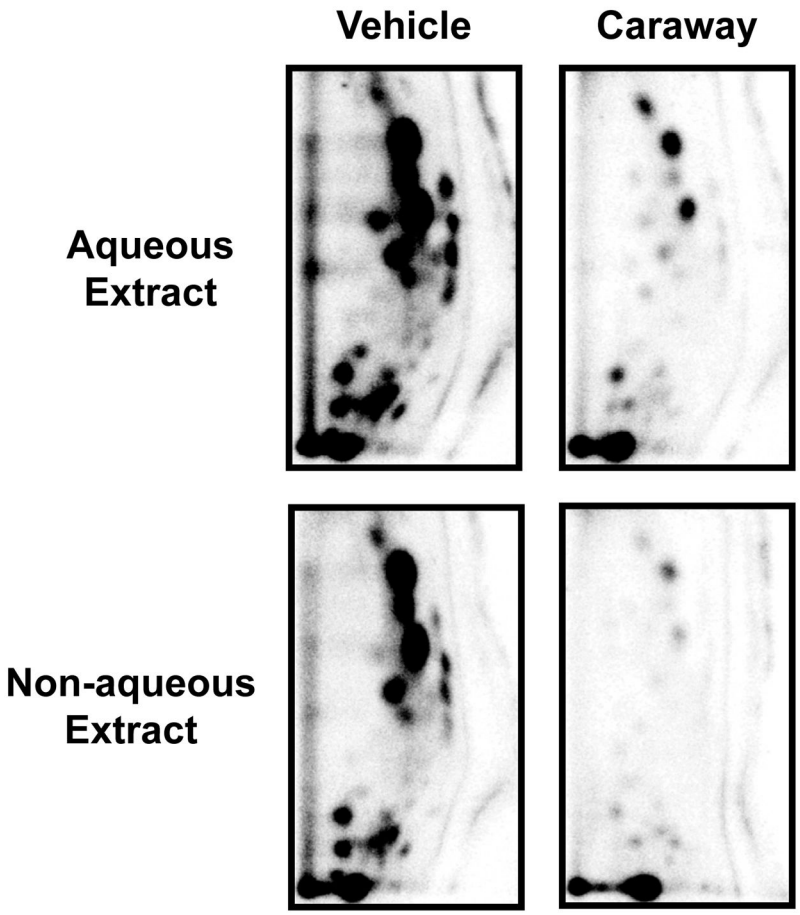

Figure 4.

Representative autoradiographs of ^{32}P-labeled oxidative DNA adducts resulting from rat liver microsomes/Cu(II)-mediated activation of E_2 in the presence of vehicle (2% ethanol) or caraway extracts. Labeled adducts were resolved by two-directional PEI-cellulose TLC (D1 = 1 M formic acid/40 mM sodium phosphate, pH 6.0 onto a 6 cm Whatman no. 17 paper wick; D2 = isopropanol: 4 M ammonium hydroxide, 1:1) and detected by Packard InstantImager.

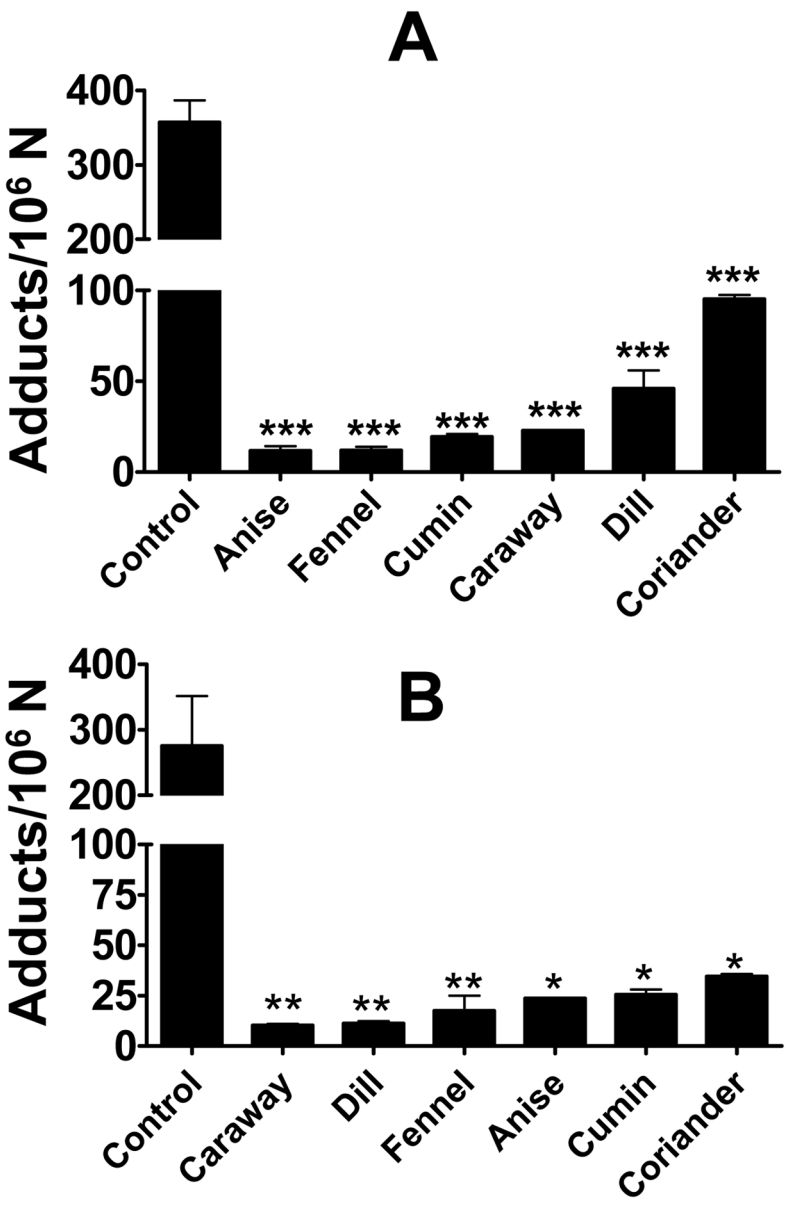

Figure 5.

Effect of aqueous (A) and non- aqueous extracts (B) on rat liver microsome/Cu(II)-mediated oxidatively generated DNA damage by E_2. Data represent average ± SD of four replicates, and a p-value of <0.05 was considered significant. Levels of oxidative DNA adducts were determined as adducts/10^6 nucleotides.

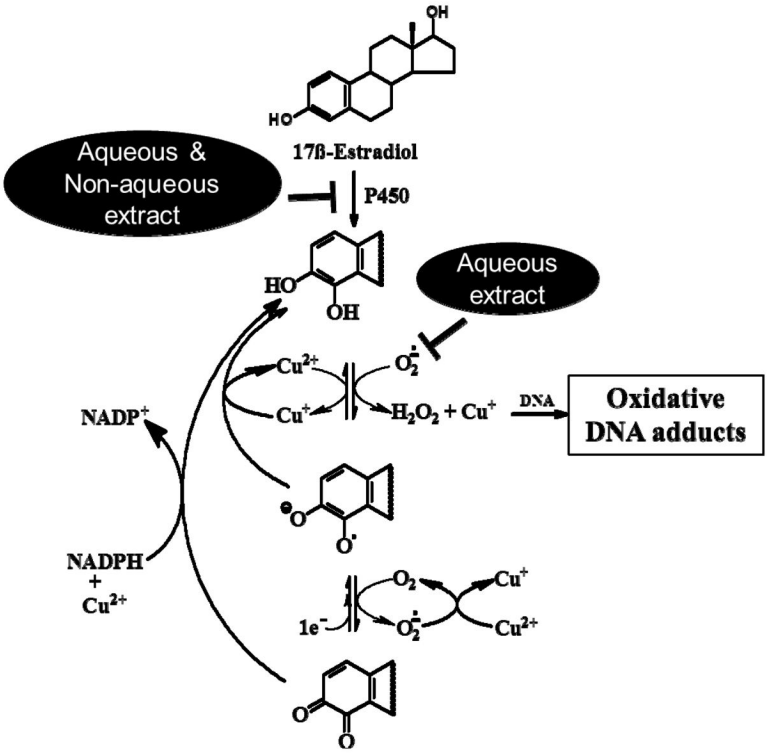

Figure 6.

Proposed mechanism of inhibition of estrogen mediated oxidative DNA adducts by aqueous and non-aqueous extracts. Partially adapted from Spencer *et. al.* (39).