Chemoprotective influence of *Zanthoxylum* spps. on hepatic carcinogen metabolizing and antioxidant enzymes and skin papillomagenesis in murine model

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In the present study, the putative potential of pericarp of dried fruit of *Zanthoxylum* (Rutaceae Family), a common spice additive in India’s west coast cuisines, in protecting against carcinogenesis has been reported. Extract from dried fruit of *Zanthoxylum* was orally administered to mice at two dose levels: 100 and 200 mg/kg body wt. for 14 days. Results reveal bifunctional nature of *Zanthoxylum* species as deduced from its potential to induce phase-I and phase-II enzyme activities associated with carcinogen activation and detoxification in the liver of mice. Hepatic glutathione S-transferase and DT-diaphorase were found significantly elevated by the treatment. *Zanthoxylum* was also effective in augmenting the antioxidant enzyme activities of glutathione peroxidase, superoxide dismutase and catalase albeit significantly by high dose of the extract ($P<0.05$; $P<0.01$). Reduced glutathione was also significantly elevated in the liver of treated animals ($P<0.05$).

The present study also investigated peri-initiation application of acetone extract of *Zanthoxylum* on initiated mouse skin. Results showed a significant reduction in tumor incidence from 68% to 36% ($P<0.05$); as well as, a reduction in tumor burden per effective mouse from 3.87 to 0.72 ($P<0.01$). Cumulatively, the findings strongly suggest cancer chemopreventive potential of *Zanthoxylum* spps.

**Keywords:** Antioxidant enzymes, Chemoprevention, Xenobiotic detoxification, *Zanthoxylum* spps

Experimental and epidemiological evidence strongly corroborate benefits of edible plant parts and phyto-products with disease prevention including carcinogenesis¹⁻⁴. The fruiting body of *Zanthoxylum* not only has anecdotal medicinal properties but is widely consumed as a seasoning agent and condiment in southwestern dietary regimen of India. Several species of *Zanthoxylum* with distinct morphological types have been identified and reported from different parts of world. Traditionally, different parts of the plant belonging to this genus have been ascribed in indigenous system of medicine (Ayurveda) to be beneficial against a plethora of diseases including cholera, helminthes and malarial infectivity, inflammation, nociception and consumed as an aromatic tonic for fever and gastroprotective effects⁵⁻¹¹. In addition, studies have reported antiproliferative and apoptosis inducing activities in various cancer cells attesting its chemotherapeutic potential¹²⁻¹⁴. Reports also indicate the plant harbors hepato-protective and anti-inflammatory properties⁷⁻¹⁵⁻¹⁸.

Steam distillation of dried ripe fruit of this plant yields essential oil that has been reported to possess antiseptic, chemotherapeutic and disinfectant properties and is also effective in treating inflammatory dermatitis⁶⁻¹⁹⁻²⁰. Previous studies from our laboratory documented *in vitro* modulation of carcinogen-DNA adduct by essential oil from this material source²¹. The present study report chemopreventive intervention by hydro-alcoholic extract of the pericarp of fruit of *Zanthoxylum limonene* spps. The inferences are based on relative induction of phase-I and II enzymes associated with carcinogen activation and detoxification and above basal antioxidant enzymes, which may restrict the carcinogen - DNA adduct formation²²⁻²⁵. Additionally, the chemoprotective influence of *Zanthoxylum* extract on two-stage mouse skin papillomagenesis was assessed for any alterations in tumor incidence resulting from treatment with the extract during the peri-initiation stage.

**Materials and Methods**

*Chemicals*—1-Chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced
glutathione (GSH), 7,12, dimethylbenz(a)anthracene (DMBA) and croton oil, were obtained from Sigma Chemical Co. (St.Louis, MO, USA). The rest of the chemicals utilized were obtained from local firms (India) and were of highest purity grade.

**Animals**—Random-bred male Swiss albino mice (8-9 weeks old) were used for this study. They were maintained in air-conditioned animal facility (Jawaharlal Nehru University, New Delhi) with a 12-h light/dark cycle, and provided (unless otherwise stated) with standard food pellets (Hindustan Lever Ltd., India) and tap water ad libitum.

**Experimental material and preparation of extract**—Dried fruit of *Zanthoxylum* was procured from Goa (India) and used only after proper verification and authentication from a qualified ethnomontanist. Voucher specimen has been preserved in author’s laboratory (PR) for future reference. An 80% ethanol - water (80:20) extract of the material was prepared using Soxhlet and the final material obtained (12% w/w from starting material) was concentrated in a lyophilizer and stored in a desiccator at 4°C. Two different concentrations-100 mg/kg body wt., and 200 mg/kg body wt. of the extract was prepared in 0.05 ml of olive oil for treatment to animals under Experiment – I. Prior to treatment, the mixture was vigorously vortexed to maintain the homogeneity and consistency of test material to be gavaged.

Acetone extract of this material was prepared for the elucidation of its chemopreventive potential in 2-stage mouse skin papillomagenesis model. Dried powdered material (50 gms.) was suspended in acetone (100 ml) and left at 4°C with intermittent shaking. After four days, acetone was filtered and stored in dark amber colored bottle at 4°C. Small volumes (0.1 ml) of this acetone extract, was applied topically over the shaved area of animals under Experiment-II.

**Experimental design**

Experiment-I was designed to evaluate the hepatic phase-I and II drug metabolizing and antioxidant enzymes. Experiment-II was designed to affirm the chemopreventive ability of *Zanthoxylum* on skin papillomagenesis.

**Experiment-I**—Animals were randomly assorted into following groups.

Group-I (n = 8): Animals were sham-treated with 50μl olive oil by oral gavage daily for 14 days; this group of animals served as negative control.

Group-II (n = 8): Animals were treated with 100 mg/kg body wt of the extract by oral gavage daily for 14 days.

Group-III (n = 8): Animals were treated with 200 mg/kg body wt of the extract by oral gavage daily for 14 days.

Group-IV (n = 8): Animals were put on pulverized diet containing 0.75% BHA for 14 days; this group of animals served as positive control.

Body weight of all mice was recorded initially, at weekly intervals and at the end of the experiment. Diets were withheld from the animals on the night prior to the day of termination of the experiment.

**Preparation of homogenates, cytosol and microsome fractions**—Animals were killed by cervical dislocation and the entire liver was then perfused with cold 0.9% NaCl and processed for separation of microsomal and cytosol fraction as described earlier.

**Microsomal enzyme assay**—Cytochrome P<sub>450</sub> and cytochrome b<sub>5</sub> were determined by the procedure of Omura and Sato<sup>27</sup>. NADPH-cytochrome P<sub>450</sub> reductase was done according to the method of Omura and Takesue<sup>28</sup> whereas, assay for NADH-cytochrome b<sub>5</sub> reductase was carried according to the method of Mihara and Sato<sup>29</sup>. Aminopyrine N-demethylase activity was assayed by estimating formaldehyde liberated during N-demethylation by the procedure of Nash<sup>29</sup>. Aniline hydroxylase assay was performed by determining p-aminophenol formation from aniline using the procedure described<sup>30</sup>.

**Phase-II and antioxidant enzyme assays**—The cytosolic glutathione S-transferase activity was determined spectrophotometrically at 37°C according to the procedure of Habig et al.<sup>31</sup>, whereas DT-diaphorase was measured as described by Ernster et al.<sup>32</sup>. Reduced glutathione was estimated as total non protein sulphydryl group by the method as described by Moron et al.<sup>33</sup>. Glutathione peroxidase activity was measured by the coupled assay method as described by Paglia and Valentine<sup>34</sup> while glutathione reductase was determined by the procedure of Carlberg and Mannervick<sup>35</sup>. Catalase was estimated at 240 nm by monitoring the disappearance of H<sub>2</sub>O<sub>2</sub> as described by Aebi<sup>36</sup>. Superoxide dismutase was assayed utilizing the protocol described by Marklund and Marklund<sup>37</sup> which involves inhibition of pyrogallol autoxidation at pH 8.0. Estimation of protein was carried out by the method of Lowry et al., using crystalline bovine serum albumin as the standard<sup>38</sup>.
Experiment-II—The hairs on the inter-scapular area (mean 1.5 cm diameter) were clipped off 3 days before the application of carcinogen and/or test material. Animals only in the resting phase of hair growth cycle were selected for the experiment. Animals were assorted randomly into following control and experimental groups:

Group-I (n=15): Animals of this group were topically treated in the shaved area with 0.1ml Zanthoxylum extract for 14 days. However, these mice were subsequently treated topically with croton oil twice a week until end of the experiment.

Group-II (n=25): Animals were treated topically in the shaved area with a single sub carcinogenic dose of DMBA (0.050 mg/0.1ml acetone). Two weeks after carcinogen application; croton oil (0.1ml of 1% acetone) was applied twice a week until the end of the experiment.

Group-III (n=25): Animals of this group were topically treated once a day within shaved area with 0.1ml Zanthoxylum extract in acetone for 14 days. DMBA was applied to these animals on 7th day (six hours after treatment with the modulator) followed by croton oil treatment as given to Group-I and II mice.

Animals were weighed initially and at autopsy. Papillomas appearing in the shaved area were recorded at weekly intervals and papillomas >1mm in diameter were included in data analysis only if they persisted for 2 weeks or more. Animals were sacrificed 6 weeks after commencement of the treatments.

Statistical analysis—Results are presented as mean ± S.E.M. For Experiment-I, statistical analysis was performed using student’s t-test. Value of P < 0.05 was considered to indicate a significant difference between groups. Chi-square test was employed to evaluate the modifying influence of Zanthoxylum extract on skin papillomagenesis (Experiment-II) by analyzing significance levels of the difference between the control and experimental group.

Results

Experiment-I Cytochrome P<sub>450</sub> and oxidoreductase enzymes—Compared to control mice, both doses of Zanthoxylum were effective in elevating the cytochrome P<sub>450</sub> specific activity significantly despite dose independent significant decrease in the specific activity of cytochrome b<sub>5</sub> (Table 1, Group-III) and cytochrome b<sub>5</sub> reductase (Table 1, Group-II & III). However, cytochrome P<sub>450</sub> reductase specific activity was unaffected by Zanthoxylum treatment at both dose levels currently investigated. In the positive control group of animals (Group IV, BHA treated), no

<table>
<thead>
<tr>
<th>Groups &amp; Treatment</th>
<th>Cyt. P&lt;sub&gt;450&lt;/sub&gt; (nmole/mg protein)</th>
<th>Cyt. b&lt;sub&gt;5&lt;/sub&gt; (nmole/mg protein)</th>
<th>Cyt. P&lt;sub&gt;450&lt;/sub&gt; reductase (μmole NADPH oxidised/min/mg protein)</th>
<th>Cyt. b&lt;sub&gt;5&lt;/sub&gt; reductase (μmole NADH oxidised/min/mg protein)</th>
<th>Aminopyrine demethylase (n moles product formed/min/mg protein)</th>
<th>Aniline hydroxylase (n moles product formed/min/mg protein)</th>
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<tbody>
<tr>
<td>I. Control (saline; negative control)</td>
<td>0.98 ± 0.01 (1.00)</td>
<td>0.53 ± 0.02 (1.00)</td>
<td>0.121 ± 0.00 (1.00)</td>
<td>1.46 ± 0.14 (1.00)</td>
<td>4.94 ± 0.58 (1.00)</td>
<td>1.49 ± 0.21 (1.00)</td>
</tr>
<tr>
<td>II. Zanthoxylum extract (Dose-I: 100 mg/kg, b.wt)</td>
<td>1.28 ± 0.04 * (1.30)</td>
<td>0.55 ± 0.03 (1.03)</td>
<td>0.125 ± 0.01 (1.19)</td>
<td>1.22 ± 0.17 * (0.83)</td>
<td>6.07 ± 0.91 * (1.22)</td>
<td>1.52 ± 0.14 (1.02)</td>
</tr>
<tr>
<td>III. Zanthoxylum extract (Dose-II: 200 mg/kg, b.wt)</td>
<td>1.32 ± 0.07 ** (1.36)</td>
<td>0.42 ± 0.07* (0.79)</td>
<td>0.119 ± 0.01 (0.98)</td>
<td>0.98 ± 0.07 * (0.67)</td>
<td>7.20 ± 0.84 ** (1.45)</td>
<td>1.50 ± 0.17 (1.00)</td>
</tr>
<tr>
<td>IV. BHA (0.75 % diet; positive control)</td>
<td>0.92 ± 0.03 (0.93)</td>
<td>0.60 ± 0.05 (1.13)</td>
<td>0.125 ± 0.00 (1.03)</td>
<td>1.33 ± 0.06 (0.91)</td>
<td>5.00 ± 0.86 (0.84)</td>
<td>1.52 ± 0.11 (1.02)</td>
</tr>
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</table>

Zanthoxylum extract was given daily for 14 days at the specified dose levels.

Values in parenthesis denote relative changes in parameters assessed i.e., level of activity in the liver of mice receiving test substance relative to that of control.

Statistical significance (against control, Group-I) are as follows: *P<0.05; **P<0.01;
significant difference with reference to these microsomal haemproteins and the associated reductases was discernable.

Aniline hydroxylase and aminopyrene demethylase—Aminopyrene demethylase specific activity was found significantly induced above basal level (Table 1; Group-I vs. Group-II & III) by treatment with Zanthoxylum extract at both dose levels currently examined. In contrast, aniline hydroxylase specific activity remained comparable to the negative control group in all the treated animals. Similarly, BHA treated animals also did not reveal any significant alterations in their levels of activity reference to these enzymes.

Glutathione S-transferase and DT-diaphorase—These enzymes evaluated in the cytosolic fraction of liver homogenate clearly revealed significant increased specific activity in almost all treated animals compared to the control group. The enzyme activity was seen significantly induced in the high dose treated group (cf. Group-I vs. Group-III, Table 2); 2.53 folds increase in glutathione S-transferase and 1.52 fold increase in DT-diaphorase activity relative to negative control (Group-I). In the positive control group of animals, glutathione S-transferase specific enzyme activity was seen maximally elevated, approximately 3.50 folds relative to the negative control group (cf. Group-I vs. Group-IV; Table 2).

Antioxidant enzymes - Glutathione peroxidase and glutathione reductase—Only high dose of Zanthoxylum extract was effective in significantly elevating the specific activity of antioxidant enzymes—glutathione peroxidase, superoxide dismutase and catalase. In contrast, glutathione reductase activity was found elevated in animals treated with high as well as low doses of the extract (cf. Group-I vs. Group- II & III, Table 2). Furthermore, in positive control group (Group-IV) specific activity of glutathione reductase was induced approximately 1.5 folds whereas, glutathione peroxidase specific activity was only 1.3 folds elevated.

Reduced glutathione—Measured as non-protein sulphydryl, was found significantly elevated over that of the control basal level by approximately 1.30 and 1.21 folds in liver of animals upon treatment with Zanthoxylum extract at the currently investigated dose levels. In BHA treated positive control group (Group-IV, Table 2) it was elevated by approximately 1.44 folds.

<table>
<thead>
<tr>
<th>Groups and Treatment</th>
<th>Glutathione S-transferase</th>
<th>DT-diaphorase</th>
<th>Glutathione peroxidase</th>
<th>Glutathione reductase</th>
<th>Superoxide dismutase</th>
<th>Catalase</th>
<th>Reduced glutathione</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Control (saline; negative control)</td>
<td>0.60 ± 0.02 (1.00)</td>
<td>0.052 ± 0.00 (1.00)</td>
<td>228.1 ± 19.1 (1.00)</td>
<td>51.4 ± 1.99 (1.00)</td>
<td>5.87 ± 0.23 (1.00)</td>
<td>44.90 ± 4.01 (1.00)</td>
<td>5.64 ± 0.05 (1.00)</td>
</tr>
<tr>
<td>II. Zanthoxylum extract (Dose-I: 100 mg/kg, b.wt)</td>
<td>1.18 ± 0.07 * (1.96)</td>
<td>0.068 ± 0.00 ** (1.30)</td>
<td>238.30 ± 16.4 (1.26)</td>
<td>56.03 ± 2.25 * (1.23)</td>
<td>6.01 ± 0.44 (1.00)</td>
<td>44.10 ± 2.05 (0.98)</td>
<td>7.34 ± 0.51 * (1.30)</td>
</tr>
<tr>
<td>III. Zanthoxylum extract (Dose-II: 200 mg/kg, b.wt)</td>
<td>1.52 ± 0.10 * (2.53)</td>
<td>0.079 ± 0.00 *** (1.52)</td>
<td>298.50 ± 7.7* (1.30)</td>
<td>66.01 ± 2.25 ** (1.31)</td>
<td>6.94 ± 0.58* (1.18)</td>
<td>64.60 ± 4.4 ** (1.43)</td>
<td>6.88 ± 0.04 * (1.21)</td>
</tr>
<tr>
<td>IV. Butylated hydroxyanisole (0.75% diet) (positive control)</td>
<td>2.10 ± 0.03 *** (3.50)</td>
<td>0.104 ± 0.01 ** (2.00)</td>
<td>298.53 ± 7.7* (1.30)</td>
<td>77.10 ± 3.25 ** (1.50)</td>
<td>7.56 ± 0.44 (1.00)</td>
<td>74.52 ± 3.56 (1.65)</td>
<td>8.12 ± 0.11 ** (1.44)</td>
</tr>
</tbody>
</table>

Zanthoxylum extract/BHA was given daily for 14 days at specified dose levels. Values in parenthesis denote relative changes in parameters assessed i.e., level of activity in liver of mice receiving the test substance relative to control. Statistical significance are as follows (against control, Group-I); *P<0.05; **P<0.01.

Units are as follows: Glutathione S-transferase, (µmole CDNB-GSH conjugated/min/mg protein); DT-diaphorase (µ mole of DCPIP reduced/min/mg protein); Glutathione peroxidase and glutathione reductase, n mole of NADPH consumed/min/mg protein; Superoxide dismutase, (units/mg protein); Catalase, (µ mole H2O2 consumed/ min/mg protein); Reduced glutathione, (nmole/gm tissue).
Experiment-II—Results of skin papillomagenesis obtained from application of test material during peri-initiation period has been presented in Table 3. No adverse effect on body weight gain during the observation period was noticeable (data not shown). Furthermore, no evidence of development of spontaneous tumors in the colony of Swiss mice has been encountered. It was observed that Zanthoxylum extract, when applied alone also did not show any evidence of papilloma during the observation period (Group-I). In Group-II, 17 out of 25 effective animals treated with DMBA and croton oil developed papillomas (68%) and the average number of tumors per tumor bearing animals was 3.87. Mice of Group-III which received, in addition to DMBA and croton oil, topical application of Zanthoxylum extract during peri-initiation phase of papillomagenesis revealed only 36% tumor incidence and the average number of papillomas per tumor bearing mice was significantly reduced to 0.72.

**Discussion**

Efficacy of plant derived products including spices and condiments in the human diet are excellent candidates for chemoprevention in high-risk population\(^9\). The liver plays a pivotal role in activation and elimination of carcinogenic xenobiotics; therefore, in the present investigation, modulation of Zanthoxylum induced hepatic biotransformation and antioxidant enzymes were evaluated from a ‘chemopreventive’ point of view. The findings indicated that Zanthoxylum induces hepatic phase-I and phase-II enzymes as a bifunctional inducer implying its potency in ‘blocking’ and ‘suppressing’ the initiation stages of carcinogenicity. Several isoforms of the phase-I cytochrome P\(_{450}\) (CYP) system representing different gene products have been identified in microsomes from human, as well as several mammalian species\(^{40,41}\). Despite their overlapping substrate specificity, almost all catalyze functionalization/activation of most chemical carcinogens through insertion of one atom of molecular oxygen into the xenobiotic or carcinogenic substrate\(^{41}\). Zanthoxylum mediated selective modulation of CYP isoenzyme profile was evident in this study since aniline hydroxylase, an epoxide producing enzyme and representative of CYP 2E1 class was not affected by the treatment. In contrast, aminopyrine demethylase (representative of CYP 1A, 2B, 2D and 3A) was seen significantly induced by the extract treatment. Other components of microsomal haemproteins such as cytochrome b\(_5\), cytochrome P\(_{450}\) and cytochrome b\(_5\) reductase that facilitate the flow of electrons from NADPH to terminal electron acceptor to augment xenobiotic substrate oxidation was differentially affected by treatment with Zanthoxylum. Notwithstanding the generation of reactive forms of carcinogens derived from phase-I induction and prooxidants, xenobiotic detoxification requires the concerted action of phase-II enzymes. Using CDNB as a nonspecific substrate, significant induction of the phase-II enzyme glutathione S-transferase has been observed. In addition, another phase-II prototype enzyme-DT-diaphorase, which facilitates the bio-reductive metabolism of quinones, was altered by Zanthoxylum. Moreover, Zanthoxylum effectively augmented the intracellular concentration of reduced glutathione maintaining the intracellular reducing milieu in addition to its conjugating ability. This favors solubilisation and detoxification of potentially toxic and carcinogenic xenobiotic substrate, which may otherwise become an etiological cause towards the ‘initiation’ of carcinogenesis. Moreover, antioxidant enzymes-glutathione peroxidase and superoxide dismutase were also elevated in liver of Zanthoxylum treated animals. These enzymes protect the cells from detrimental effects of oxygen radical and hydrogen peroxide ubiquitously generated under duress of normal oxidative metabolism. Recent work
has revealed that the consequential effect of reactive oxygen species may be severe with major impact for diseases such as cancer. Thus, in this context, the overall effect of induction of phase-I and II, antioxidant enzymes and reduced glutathione by *Zanthoxylum* in liver augment xenobiotic (including carcinogen) detoxification preventing binding of metabolically activate indirectly acting carcinogens and pro-oxidants to genomic DNA of host.

In a previous communication from our laboratory, *in vitro* inhibition of aflatoxin-DNA adduct by *Zanthoxylum* derived essential oil has been documented\(^2\). Furthermore, as corollary to the present observations, it was convincingly documented that the pericarp extract of *Zanthoxylum* is associated with anti-initiation and chemopreventive potential in DMBA induced two stage skin papillomagenesis in mouse model. The findings reveal not only a decline in tumour burden per effective mouse following exposure of initiated skin to topical application of pericarp extract during peri-initiational phase alone. The inhibition of protein kinase C phosphorylation by an alkaloid component of *Zanthoxylum* might be an additional factor contributing to underlying mechanism of action during the promotion stages of carcinogenesis in addition to carcinogen detoxification within cutaneous tissue.

In conclusion, based on aforementioned observation *Zanthoxylum* sps. shows promise as a chemopreventive agent through ‘blocking’ and ‘suppressing’ the initiation stages of carcinogenesis. Further studies are underway to isolate its active compound and explore molecular pathways associated with chemoprotection.

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References


