CHAPTER 6

Effect of Onosma echioides on DMBA/Croton oil mediated carcinogenic response: Modulation of hyperproliferation and oxidative damage in murine skin
In today's developed world the problem of cancer is increasing and is now second only to cardiovascular diseases as a cause of death. In the year 2002, an estimate indicated that in the United States alone 1,284,900 new cases of cancer would be diagnosed and 555,500 people would die from cancer (Jemal et al 2002). In other parts of the world however if the mortality rates are not high, the sufferings of the people are pronounced.

The involvement of free radicals in both the initiation as well as the promotion stage, the biochemical events related in each stage and the metabolic alterations associated with cancer development (Boutwell 1974) have been highlighted with the help of two-stage skin carcinogenesis. A lot of emphasis has been laid down on the prevention of tumor promotion because the second stage of tumor promotion - the 'propagation' stage is believed to be reversible (DiGiovanni 1992). Various naturally occurring agents like vitamins, polyphenols, terpenes; plants with healing properties; plants commonly used for flavoring of food stuff; fruits and beverages have shown wonderful results in delaying or inhibiting tumor formation of different organs (Wattenberg 1992a, Wattenberg 1992b, Singh and Lippman 1998). After the dietary factors, it is the category of the medicinal plants that has been on the focus of chemoprevention research. In fact, in certain disciplines of Indian system of medicines like Ayurveda and Homeopathy, the plants are the main sources of attention and the cure of different diseases is based entirely on the dose and duration of the treatment with these botanical agents. While the Ayurvedic system makes use of different parts of the plants in more or less natural/crude form, the Homeopathic system works largely by the use of alcoholic decoctions prepared from plants.
In an effort to identify new chemopreventive agent, the present study was conducted to investigate the role of *O. echioides* (Boraginaceae) that is commonly called as Ratanjyot in India, in prevention of skin tumorigenesis. The leaves of the plant are used as purgative, the flowers are effective in case of rheumatism and palpitation of heart while the roots are applied in case of bruises and eruptions (Chopra et al 1996). *O. echioides* var. *hispidum* has been reported to be used in the treatment of burns and wounds (Koul et al 1993). The plant is valued in Tibetan and Spanish systems of medicine and is widely used in inflammations, rheumatism, palpitations of heart, derangements of blood, leucoderma, itch, fever, wounds and piles. It is also used as antihelmintic and alexipharmic. The main components present in the plant include shikonins and alkannins and their derivatives like shikonin acetate (Koul et al 1993). Chemopreventive effects of these two compounds have been examined by various researchers.

Inflammation is closely associated with the tumor promotion stage of cancer (Balkwill and Mantovani 2001). It has also been shown that the non-steroidal anti-inflammatory drugs (NSAIDs) are among the most potent and most promising agents employed for cancer chemoprevention probably because of their ability to inhibit prostaglandin biosynthesis, particularly at the level of the 'pro-inflammatory' enzyme cyclooxygenase-2 (Marks and Furstenberger 2000). Owing to the beneficial effects of its components and of the plant per se in inflammation, skin disorders and other ailments, the role of the plant extract was speculated in the amelioration of cutaneous carcinogenesis and thus the present study was designed.

*O. echioides* was tested against oxidative effects of single topical application of BPO followed by UVB irradiation. It is already known that both BPO and UVR act by
generation of oxidative stress. Therefore, the combined effects of BPO and UV light especially on the anti-oxidant enzyme profile were studied. Besides, in a similarly designed experiment, the effect of the plant on the parameters of oxidative stress and tumor promotion induced by a single topical application of TPA and two-stage carcinogenesis was studied following promising results in the former experiment.

TREATMENT PROTOCOL

Short-term studies of the plant extract against BPO & UVB induced biochemical changes

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment Regimen</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>I</td>
<td>Acetone</td>
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<tr>
<td>II</td>
<td>Acetone</td>
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<tr>
<td>III</td>
<td>O. echioides (D1)</td>
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<tr>
<td>IV</td>
<td>O. echioides (D2)</td>
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<tr>
<td>V</td>
<td>O. echioides (D2)</td>
</tr>
</tbody>
</table>

Acetone = 0.2ml/animal, O. echioides (D1) = 5mg/Kg body wt., O. echioides (D2) = 10mg/Kg body wt. Doses of BPO and UVB; the age, sequence of treatments and time of sacrifice of all the animals were similar to that described in Chapter 4 for all parameters.

Short-term studies of the plant extract against TPA induced biochemical changes

<table>
<thead>
<tr>
<th>Groups</th>
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</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>III</td>
<td>O. echioides (D1)</td>
</tr>
<tr>
<td>IV</td>
<td>O. echioides (D2)</td>
</tr>
<tr>
<td>V</td>
<td>O. echioides (D2)</td>
</tr>
</tbody>
</table>
Acetone, *O. echioides* (D1) and *O. echioides* (D2) = same as described above, TPA = 20nmol/0.2ml/animal. Each group contained 7-8 week old six mice. The time period between *O. echioides* pretreatment and TPA application, and the time of sacrifice of all the animals for the estimation of various parameters was same as that described in Chapter 4.

**Long-term effect of the plant extract on DMBA/ Croton oil induced skin carcinogenesis**

**Group I**

![Diagram](image)

(Throughout the study period)

**Group II**

![Diagram](image)

**Group III**

![Diagram](image)

**Group IV**

![Diagram](image)

**Depicts Acetone:** 0.2ml/animal was applied throughout the study period

**Depicts Initiator:** DMBA (25μg/0.2ml/animal) was applied topically only once
Chapter 6

Onosma echioides

**Depicts Promoter:** Croton oil (0.5%/0.2ml/animal) was applied twice weekly for 20 weeks

**Depicts Modulator:** *O. echioides* D1 (5mg/Kg body wt.) was applied twice weekly for 20 weeks (One hour before each promoter treatment)

**Depicts Modulator:** *O. echioides* D2 (10mg/Kg body wt.) was also applied twice weekly for 20 weeks (One hour before promoter treatment)

Number of animals in each group, treatments given to animals, data representation and time of sacrifice were similar to that described in Chapter 4.

**RESULTS**

**Effect of *O. echioides* on BPO and UVB mediated oxidative stress**

Table 1 shows the effect of prophylactic treatment of *O. echioides* extract on BPO and UVB irradiation mediated depletion in reduced glutathione, its metabolizing enzymes and phase II detoxifying enzymes in murine skin. Combination of BPO and UVB depleted the level of reduced glutathione to 68%, the activities of glutathione reductase, glutathione S-transferase and quinone reductase to 61, 59 and 65% respectively. On the other hand, pretreatment with *O. echioides* extract caused 8-23% recovery in case of low dose and 23-28% recovery in case of high dose in the above said parameters.

Table 2 depicts the effect of *O. echioides* extract pretreatment on BPO treatment and UVB irradiation mediated depletion in antioxidant enzymes in murine skin. The activities of catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase reduced to 59, 58 and 53% respectively in the BPO treated and UVB irradiated group. While pretreatment with *O. echioides* extract resulted in 20-27% recovery at low dose and 28-36% recovery at high dose in the activities of the above said enzymes.
Table 3 shows the effect of *O. echioides* extract pretreatment on BPO and UVB mediated induction of lipid peroxidation and hydrogen peroxide content in murine skin. Lipid peroxidation and hydrogen peroxide formation increased to 249 and 157% in BPO treated and UVB irradiated group. On the other hand, in the *O. echioides* extract pretreated groups there was 52-58% suppression in peroxidation of lipids and 31-44% inhibition in hydrogen peroxide formation.

**Effect of *O. echioides* on TPA induced oxidative stress**

*O. echioides* extract pretreatment on TPA mediated depletion of the levels of reduced glutathione, its metabolizing and phase II enzymes is shown in Table 4. Single topical application of TPA caused significant depletion in the reduced glutathione content (52%), glutathione reductase (65%) glutathione-S-transferase (48%) and quinone reductase (48.5%). While, pretreatment of mice skins with *O. echioides* extract resulted in 19-20% and 25-31% recovery at low dose and high dose respectively.

Table 5 shows the effects of *O. echioides* against TPA mediated decrease in the antioxidant enzyme activities, lipid peroxidation and hydrogen peroxide formation. TPA alone resulted in the down regulation of the antioxidant enzyme activities like catalase (36%), GPX (49%) and G6PD (47%), parallel to these changes, it increased the level of lipid peroxidation (213%) and hydrogen peroxide content (178%). The activities of catalase, GPX and G6PD were significantly recovered on *O. echioides* extract pretreatment especially at the higher dose. The percentage of recovery in case of antioxidant enzyme activities was 5-19% and 14-33% at low and high doses respectively. *O. echioides* caused suppression in the formation of MDA (32-59%) and H$_2$O$_2$ (20-38%) in a dose dependent manner.
Effect of *O. echinoides* on BPO and UVB induced hyperproliferation response

Figure 1 depicts the elevation in cutaneous ornithine decarboxylase activity and unscheduled DNA synthesis in BPO and UVB treated group and its inhibition by *O. echinoides* extract pretreatment. The activity of ODC and DNA synthesis increased to 265% and 219% in BPO treated and UVB irradiated group. On the other hand, pretreatment with *O. echinoides* extract resulted in 63-92% inhibition of the elevated ODC activity and 35-64% inhibition of DNA synthesis at two different doses.

Effect of *O. echinoides* on TPA induced hyperproliferation response

Figure 2 represents the prophylactic effect of *O. echinoides* extract pretreatment on TPA mediated rise in ODC activity and thymidine incorporation in DNA. The ODC activity and thymidine incorporation in DNA showed 5.76 and 2.3 -fold increase respectively on treatment of mice skins with single topical application of TPA on mice skins. Upon pretreatment with *O. echinoides* extract ODC activity was restored from 71-120% and 42-57% in case of unscheduled DNA synthesis in a dose dependent manner.

Effect of *O. echinoides* on DMBA & Croton oil induced carcinogenesis

Figure 3(a & b) shows the effect of pretreatment of *O. echinoides* extract on DMBA/ croton oil mediated mouse skin carcinogenesis. The first tumor was observed at the 8th week in the DMBA/ croton oil treated group. All the animals were observed for 25 weeks. The number of tumors at the 10th week in DMBA/ croton oil treated group was 4.8 with 45% mice bearing tumors while the number of tumors at the end of 25 weeks was 11.2 with 100% incidence of tumor bearing mice. In the prophylaxis groups, where the animals were given topical applications of *O. echinoides* extract prior to the application of croton oil twice weekly, the appearance of first tumor was prolonged to 10 and 11 weeks respectively at low and high
doses. At the 11th week the percentage of tumor bearing mice was 20-10% and it was 55-50% at 25th week in a dose dependent manner. The total number of tumors/mouse at 25 weeks dropped to 6.1 & 4.5 in *O. echioideae* treated group at 5 and 10 mg/Kg body wt respectively. Morphologically, tumors of the animals of the DMBA/Croton oil group were larger in size, darker in colour and more in number as compared to *O. echioideae* extract pretreated mice. The appearance of tumors in this (DMBA/Croton oil) group was mostly pedunculated and had polypoidal appearance. On the contrary, there was no significant difference in the body weight of the animals of all the groups during the entire period of study (data not shown).

Figure 4 depicts the histology of the changes of effect of pretreatment with *O. echioideae* extract on two-stage skin carcinogenesis in mice. The acetone treated mouse skin exhibited normal skin layer and appendages. The epidermis was seen with an approximately single layer of basal cells laid over by flattened squamous cells and the stratum corneum. DMBA and croton oil treated positive control group revealed squamous cell carcinoma type of tumors. Carcinomatous changes like anaplasia with hyperchromasia, intradermal proliferation and keratin pearls were commonly observed in the DMBA/croton oil treated group. While, in case of *O. echioideae* extract pretreated group, the formation of squamous cell carcinoma was significantly reduced.

**DISCUSSION**

The onset of tumor promotion is marked by numerous biochemical alterations (Boutwell 1974) including over expression of the activities of PKC and ODC, triggering unscheduled DNA synthesis and generation of ROS (Perchellet and Perchellet 1989).

Since the pathological development of tumors in mankind takes a long time to pass through the preneoplastic and premalignant stages to actually become malignant. Therefore,
opportunity to reverse the development of tumor is always present. Resultantly, in recent years extensive research on cancer prevention is being encouraged. The chemopreventive strategy encompasses the use of agents with certain effects that diminish the carcinogenic process.

In the present study, anti-tumor promotion ability of a plant extract was analyzed against the toxicities of BPO plus UVB, and TPA in mouse skin. It was found that the plant extract prevented the depletion of the cellular antioxidant, reduced glutathione and its associated enzymes along with the elevation of enzymes involved in the suppression of oxidation processes. *O. echinoides* has the ability to detoxify the foreign agents that pose threat to the integrity and proper functioning of the cell since it restored the levels of endogenous thiol group containing moiety, the reduced glutathione. It also modulated xenobiotic detoxification system enzymes i.e. it elevated the activities of phase II detoxifying enzyme, GST and quinone reductase. Quinone reductase is an important line of defense against superoxide generation by redox cycling of quinones (Perchellet et al. 1995). Elevation of GST in itself is an indicator of a chemopreventive agent (Boone et al. 1990). Besides, different agents are known to prevent against the skin papillomagenesis by modulating the detoxification enzyme system (Singh and Shukla 1999). Chemoprotectors like some phenolic antioxidants, azo dyes, aromatic diamines and aminophenols act by inducing the activity of quinone reductase (Prochaska et al. 1985). Besides glucosinolates, constituents of green vegetables and fruits, have been shown to act as chemoprotectors via induction of quinone reductase (Tawfiq et al. 1995). Additionally, the oxidative damage induced by single topical application of TPA and combined exposure to BPO and UVB was significantly decreased by *O.echinoides*, and this inhibition is thought to play a role in the tumor inhibitory activity of the plant because the
role of oxidants and free radicals in the process of carcinogenesis is inevitable. Induction of ODC activity is a marker for tumor promotion as it is invariably induced on application with tumor promoters (Verma 1992). Agents that are hyperplastic but are not tumor promoters might not necessarily induce ODC activity but tumor promoters do induce the activity of this enzyme (Verma et al 1979a). These facts lay emphasis that any agent which causes inhibition of hyperproliferative response (ODC and thymidine incorporation in DNA), will also inhibit the tumor formation process. That the plant is capable of preventing promotion stage of carcinogenesis became evident from the fact that the activity of ODC, the enzyme responsible for polyamine biosynthesis was down regulated and the synthesis of DNA was inhibited on O. echioides pretreatment, dose dependently. Any agent that is capable of preventing oxidative insult and promotion related changes would be eligible enough to be thought of as, also capable of preventing tumor formation.

Two-stage carcinogenesis was accomplished by topical applications of DMBA and croton oil that resulted in squamous cell carcinomas. Commonly observed in DMBA and croton oil treated group were malignant squamous cells invading the deeper layers of the skin, necrotic areas, keratin pearls, hyperchromatism, intradermal infiltration and hyper keratinization. With the pretreatment of the plant extract to the mice skin, on the contrary, not only the incidence of tumor was less but also the numbers of tumors per mouse were fairly reduced alongwith modulation in the histology of the skin.

The above-mentioned effects of O. echioides can be attributed to the presence of two potentially strong chemopreventive agents present in the extract- alkannins and shikonins. These agents have been reported earlier to have anti-tumor activity (Papageorgiou et al 1999). The current study further shows that the plant extract also suppressed the promotional
changes thereby prolonging the promotion stage by delaying tumor formation and reducing the number of tumors in mouse skin as compared to the positive control group animals treated with DMBA and croton oil. Also, the inflammatory changes are considered to be a part of promotion. Conversely, malignant transformation of epithelial cells is associated with chronic inflammation whereby the infiltrated and activated polymorphonuclear leukocytes may generate ROS that may interact with biomolecules and induce the process of cancer formation in the inflamed tissues. During the process of inflammation the NO⁻ may be released following the activity of inducible nitric oxide synthase. Additional changes may also take place like release of iron from hemoproteins, lipid peroxidation and DNA synthesis. Further generation of hydroxyl radicals may also take place (Perchellet et al 1995).

Inflammatory macrophages obtained from SENCAR mice have shown 4-times more hydrogen peroxide secretion as compared to promotion resistant C57 BL/6 mice (Lewis and Adams 1986).

Following exposure with chemical carcinogen in experimental studies, wounding and inflammation have been shown to augment the formation of tumors (DiGiovanni 1992). Various studies have been undertaken to check the effectiveness of shikonins in different aspects of wound healing. Shikonin and its derivative acetylshikonin have shown to possess anti-inflammatory effects. A derivative of shikonin, 2- acetylshikonin is reported to exhibit anti-inflammatory effects against acute edema induced by histamines, heat and UV radiations when applied topically. They have been shown to inhibit the biosynthesis of leukotriene B and 5-hydroxyeicosatetraenoic acid, which Wang et al (1994) suggest may play a major role in the mechanism of its anti-inflammatory action. Therefore, we cannot rule out the possibility of the anti-inflammatory effect of the components of O. echioides in the
amelioration of skin carcinogenesis although we did not carry out experiments to establish/confirm this speculation.

Acetylshikonin also show mild anti-pyretic and anti-analgesic effects. Hayashi concluded in 1977 that the derivative of shikonin was effective for the treatment of cutaneous injuries. Shikonin derivatives are known to possess antimicrobial and anti-thrombotic activities as well. Alkannins also in addition to shikonins and their derivatives are known to have bactericidal effect (Papageorgiou et al. 1999). Recently, shikonin has been shown to inhibit the early activation of TPA and reduce the incidence of azoxymethane-induced intestinal tumors in rats (Papageorgiou et al. 1999). The derivative of shikonin, acetylshikonin is known to inhibit arachidonic acid induced platelet aggregation (Ko et al. 1995).

Although the insights made in the present study provide a very small aspect of the modulation of the carcinogenesis process it paves way for further investigations so that substantial progress can be made in the field of chemoprevention.

It can be concluded that *O. echioides* acts as a modulator of two-stage skin carcinogenesis in Swiss albino mice since it prolongs the formation of tumors in skin, decreases the hyperproliferative response elicited not only by BPO and UVB but also by TPA. Besides, it prevents oxidative cell damage that is invariably associated with cancer formation. Since shikonin and alkannins are two major constituents of the extract, it can be suggested that their cumulative effects may be the underlying principle behind the protective potential of *O. echioides*. 
Table 1. Effect of prophylactic treatment of *Onosma echoides* extract on BPO treated and UVB mediated depletion in cutaneous reduced glutathione, its metabolizing enzymes and phase II detoxifying enzymes in mice

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Reduced glutathione (nmol GSH/gm tissue)</th>
<th>Glutathione reductase (nmol NADPH oxidized/min/mg protein)</th>
<th>Glutathione S-transferase (nmol CDNB conjugate formed/min/mg protein)</th>
<th>Quinone reductase (nmol dichloroindophenol reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>35.65 ± 1.05</td>
<td>127.99 ± 9.20</td>
<td>195.14 ± 8.04</td>
<td>170.48 ± 9.85</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>24.26 ± 0.52***</td>
<td>78.00 ± 4.21***</td>
<td>114.61 ± 15.09***</td>
<td>110.68 ± 11.51***</td>
</tr>
<tr>
<td><em>O. echoides</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>27.19 ± 0.60**</td>
<td>98.36 ± 7.03*</td>
<td>159.16 ± 6.96*</td>
<td>147.42 ± 8.96*</td>
</tr>
<tr>
<td><em>O. echoides</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>32.71 ± 0.79***</td>
<td>108.04 ± 11.62*</td>
<td>169.81 ± 5.56**</td>
<td>157.03 ± 8.34**</td>
</tr>
<tr>
<td>Only <em>O. echoides</em> (D2)</td>
<td>38.96 ± 2.17</td>
<td>136.74 ± 14.24</td>
<td>170.84 ± 11.74</td>
<td>223.42 ± 13.19</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. ***P < 0.001 and **P < 0.01 compared with the corresponding value for acetone treated control group. ***P <0.001, **P < 0.01 and *P < 0.05 compared with corresponding value for treatment with BPO and UVB treated group.
Table 2. Effect of pretreatment of mice skins with *Onosma echioides* extract on BPO treated and UVB-irradiated reduction in antioxidant enzyme profile

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Catalase (nmol H₂O₂ consumed / min / mg protein)</th>
<th>Glutathione peroxidase (nmol NADPH oxidized / min / mg protein)</th>
<th>Glucose-6-phosphate dehydrogenase (nmol NADP reduced / min / mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>82.47 ± 1.47</td>
<td>190.09 ± 20.95</td>
<td>71.82 ± 5.14</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>48.73 ± 4.72***</td>
<td>110.65 ± 15.41*</td>
<td>38.11 ± 2.16***</td>
</tr>
<tr>
<td><em>O. echioides</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>69.35 ± 5.20*</td>
<td>162.06 ± 14.39*</td>
<td>52.67 ± 4.39*</td>
</tr>
<tr>
<td><em>O. echioides</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>74.58 ± 2.88***</td>
<td>178.87 ± 12.19**</td>
<td>58.28 ± 4.63**</td>
</tr>
<tr>
<td>Only <em>O. echioides</em> (D2)</td>
<td>103.77 ± 7.04</td>
<td>202.78 ± 19.50</td>
<td>68.61 ± 8.00</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. ***P < 0.001 and *P < 0.05 compared with the corresponding value for acetone treated control group. ***P <0.001, **P < 0.01 and *P < 0.05 compared with corresponding value for treatment with BPO and UVB treated group.
Table 3. Prophylactic treatment of *Onosma echioides* extract on BPO and UVB induced lipid peroxidation and hydrogen peroxide formation in murine skin

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Lipid Peroxidation (nmol malondialdehyde formed / hr / gm tissue)</th>
<th>Hydrogen peroxide formation (nmol of H₂O₂ / gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>2.18 ± 0.68</td>
<td>311.54 ± 8.85</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>5.42 ± 0.49**</td>
<td>489.12 ± 27.35**</td>
</tr>
<tr>
<td><em>O. echioides</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>4.29 ± 0.11*</td>
<td>392.26 ± 15.17*</td>
</tr>
<tr>
<td><em>O. echioides</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>4.16 ± 0.24*</td>
<td>352.34 ± 11.42***</td>
</tr>
<tr>
<td>Only <em>O. echioides</em> (D2)</td>
<td>3.57 ± 0.82</td>
<td>223.33 ± 19.77</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. **P < 0.001 and *P < 0.01 compared with the corresponding value for acetone treated control group. ***P <0.001 and *P < 0.05 compared with corresponding value for treatment with BPO and UVB treated group.
Table 4. Pretreatment effect of *Onosma echioides* extract on TPA mediated reduction in reduced glutathione level and glutathione metabolizing enzymes in Swiss mice

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<tr>
<th>Treatment groups</th>
<th>Reduced glutathione (nmol GSH/gm tissue)</th>
<th>Glutathione reductase (nmol NADPH oxidized/min/mg protein)</th>
<th>Glutathione S-transferase (nmol CDNB conjugate formed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>29.41 ± 1.59</td>
<td>93.82 ± 8.02</td>
<td>243.74 ± 37.67</td>
</tr>
<tr>
<td>Only TPA (20nmol / 0.2ml acetone / animal)</td>
<td>15.44 ± 1.95***</td>
<td>60.72 ± 3.24**</td>
<td>118.12 ± 15.55*</td>
</tr>
<tr>
<td><em>O. echioides</em> (D1) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>20.99 ± 1.40*</td>
<td>79.90 ± 3.13**</td>
<td>165.19 ± 6.59*</td>
</tr>
<tr>
<td><em>O. echioides</em> (D2) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>23.16 ± 0.62**</td>
<td>84.60 ± 9.72*</td>
<td>194.88 ± 7.85**</td>
</tr>
<tr>
<td>Only <em>O. echioides</em> (D2)</td>
<td>26.47 ± 2.27</td>
<td>83.23 ± 1.73</td>
<td>228.77 ± 18.99</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. ***P < 0.001, **P < 0.01 and *P < 0.05 compared with the corresponding value for acetone treated control group. ***P <0.001, **P < 0.01 and *P < 0.05 compared with corresponding value for treatment with TPA alone.
Table 5. Prophylactic effect of *Onosma echioides* extract on TPA mediated depletion in antioxidant enzymes and elevation in lipid peroxidation and hydrogen peroxide content in murine skin

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Catalase (nmol H₂O₂ consumed / min / mg protein)</th>
<th>Glutathione peroxidase (nmol NADPH oxidized / min / mg protein)</th>
<th>Glucose-6-phosphate dehydrogenase (nmol NADP reduced / min / mg protein)</th>
<th>Lipid Peroxidation (nmol malondialdehyde formed / hr / gm tissue)</th>
<th>Hydrogen peroxide formation (nmol of H₂O₂ /gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>92.31 ± 10.03</td>
<td>218.03 ± 10.18</td>
<td>49.55 ± 2.96</td>
<td>2.12 ± 0.14</td>
<td>276.49 ± 12.31</td>
</tr>
<tr>
<td>Only TPA (20nmol / 0.2ml acetone / animal)</td>
<td>32.91 ± 1.69***</td>
<td>107.47 ± 11.86***</td>
<td>23.16 ± 1.58***</td>
<td>4.51 ± 0.198***</td>
<td>493.63 ± 20.91***</td>
</tr>
<tr>
<td><em>O. echioides</em> (D1) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>50.60 ± 6.15*</td>
<td>147.49 ± 12.42*</td>
<td>25.77 ± 4.73NS</td>
<td>3.84 ± 0.21*</td>
<td>437.76 ± 12.59*</td>
</tr>
<tr>
<td><em>O. echioides</em> (D2) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>59.97 ± 2.28***</td>
<td>180.14 ± 7.28***</td>
<td>29.99 ± 0.88**</td>
<td>3.26 ± 0.33**</td>
<td>388.97 ± 21.27**</td>
</tr>
<tr>
<td>Only <em>O. echioides</em> (D2)</td>
<td>80.45 ± 5.41</td>
<td>190.49 ± 13.01</td>
<td>54.88 ± 1.97</td>
<td>2.38 ± 0.30</td>
<td>343.66 ± 56.58</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. ***P < 0.001 compared with the corresponding value for acetone treated control group. **P <0.001, *P < 0.01 and *P < 0.05 compared with corresponding value for treatment with TPA alone.
FOOTNOTES AND FIGURE LEGENDS

Figure 1. Role of *Onosma echioides* pretreatment on BPO treated and UVB mediated induction in ODC activity and [³H] thymidine incorporation in murine skin

Each value represents mean ± SE of six animals. ## p< 0.001 as compared to acetone treated negative control group. *** p< 0.001, ** p< 0.01 and * p< 0.05 when compared with BPO treated and UVB irradiated group. D-1 and D-2 represent topical applications of 5mg and 10mg *O. echioides*/Kg/body weight.

Figure 2. Effect of *Onosma echioides* pretreatment on TPA induced ODC activity and DNA synthesis in mice skin

Each value represents mean ± SE of six animals. ### p< 0.001 as compared to acetone treated negative control group. ** p< 0.01 and * p< 0.05 when compared with TPA treated control group. D-1 and D-2 represent topical applications of 5 and 10mg *O. echioides*/Kg/body weight.

Figure 3 (a & b) Effect of *Onosma echioides* extract pretreatment on DMBA initiated and croton oil promoted mouse skin

Figure 4. Shows the histological alterations of *Onosma echioides* extract pretreatment on two-stage skin carcinogenesis in mice

Fig. 4a – histology of acetone treated control mice depicting normal skin tissue

Fig. 4b – histopathology of DMBA/croton oil treated positive control mice skin depicting squamous cell carcinoma. The malignant squamous cells have infiltrated in deeper region and also seen is a central area of necrosis

Fig. 4c– Section showing histopathological alterations in skin tissue of anti-tumor promotion group animals depicting verruca formation with marked hyperkeratinization at 5mg/Kg body weight of *O. echioides* extract.

Fig. 4d– Section showing histopathological alterations in skin tissue of anti-tumor promotion group animals depicting hyperplasia and infiltration of inflammatory cells at 5mg/Kg body weight of *O. echioides* extract.

Fig. 4e – Section showing mild hyperplasia in skin tissue of anti-tumor promotion group animals pretreated with 10mg/Kg body weight of *O. echioides* extract.

Fig. 4f– Section showing hyperplasia and keratinization of the epidermis in skin tissue of anti-tumor promotion group animals pretreated with 10mg/Kg body weight of *O. echioides* extract.
Figure 1

![Graph showing ODC activity and $[^3]$H thymidine incorporation across different treatment groups.]

- **ODC activity (pmol $^{14}$CO$_2$ released/h/mg protein)**
- **$[^3]$H thymidine incorporation DPM/ug DNA**

- **Treatment Groups**
  1. Only Acetone
  2. BPO + UVB
  3. *O.echioides* (D1) + BPO + UVB
  4. *O.echioides* (D2) + BPO + UVB
  5. Only *O.echioides* (D2)

**Significance Levels**
- $^*$
- $^{	ext{**}}$
- $^{	ext{***}}$
- $^{	ext{###}}$
Figure 2
Effect of *Onosma echioides* pretreatment on TPA induced ODC activity and DNA synthesis in mice skin

![Graph showing ODC activity and DNA synthesis](image)

**ODC activity**
- pmol[^1^CO₂] released/hr/mg protein
- Treatment groups: 1. Only Acetone, 2. O.echioides (D1) + TPA, 3. Only O.echioides (D2), 4. Only TPA, 5. O.echioides (D2) + TPA

**[^1^H]thymidine incorporation**
- DPM/µg DNA
- Treatment groups: 1. Only Acetone, 2. O.echioides (D1) + TPA, 3. Only O.echioides (D2), 4. Only TPA, 5. O.echioides (D2) + TPA
Figure 3 (a & b)
Effect of *Onosma echioides* extract pretreatment on DMBA initiated and croton oil promoted mouse skin

**a.)**
Percentage of mice with tumors

**b.)**
Number of tumors/mouse

- DMBA + Croton oil
- DMBA + *O. echioides* (D1) + Croton oil
- DMBA + *O. echioides* (D2) + Croton oil
Figure 4. Shows the histopathological alterations of *Onosma echoides* extract pretreatment on two-stage skin carcinogenesis in mice

**Fig. 4a** – histology of acetone treated control group mice depicting normal skin section (40X)

**Fig. 4b** – histopathology of DMBA/croton oil treated mice skin depicting squamous cell carcinoma (20X)
Fig. 4c – Section showing histopathological alterations in skin tissue of anti-tumor promotion group animals depicting verruca formation at lower dose of *O. echioides* extract (20X).

Fig. 4d – Section showing hyperplasia in skin tissue of anti-tumor promotion group animals pretreated with lower dose of *O. echioides* extract (40X).
Fig. 4e – Section showing mild hyperplasia in skin tissue of anti-tumor promotion group animals pretreated with higher dose of *O. echioides* extract (20X).

Fig. 4f – Section showing histopathological alterations in skin tissue of anti-tumor promotion group animals depicting hyperplasia and keratinization of the epidermis at higher dose of *O. echioides* extract (40X).