Chemopreventive effect of Balsamodendron mukul on mouse skin carcinogenesis, hyperproliferation response and oxidative stress
BPO is one of the bonafide oxidant compounds that have an array of uses. It is used in cosmetic and pharmacological industries particularly in acne creams, as a bleaching agent in food and as a free radical initiator in plastic industries (Rudra and Singh 1997). Therefore, exposure to this free radical generating compound becomes rather unavoidable. It is also known for its adverse effects such as its ability to promote skin tumors (Slaga et al 1981). It is capable of causing tumors on DMBA initiated mouse skin (Zhao et al 2000).

Different UV absorbers and sunscreens are widely available but protection by these means is not sufficient because of inadequate use, incomplete protection against different spectral range and toxicity. Therefore, exposure to UV radiations through sunlight becomes inevitable. In many countries UV irradiation has been used for the treatment of various skin related problems such as eczema and acne (Kennedy et al 1995). Ultraviolet light, which can act both as an initiator and a promoter, is considered to be a complete carcinogen (Blum 1959). Most of the human nonmelanoma skin cancers crop up from the exposure to these radiations (Husain et al 1991). It has been estimated that every year out of 9-12 lakhs of new cases of squamous and basal cell carcinomas in the US, nearly 90% are attributed to UV exposure (Buckman et al 1998). One possible mechanism for both initiational and promotional ability of UV light is photooxidation while induction of DNA synthesis and ODC activity can be correlated exclusively to its promotional ability. There are various factors that accelerate the formation of skin cancer, one of them includes unsaturated lipids. It has been suggested that unsaturated lipids are required for photocarcinogenesis of skin just as they are required in certain cancers (Black 1983). The chromophores present in skin absorb the UVR, get excited and then transfer their excitation energy to oxygen thereby producing free
radicals that have the ability to attack the double bonds of unsaturated lipids and DNA (Black et al 1997).

Chemoprevention has been recognized as the science that deals with the intervention of the process of any disease so that the manifestation of the disease is delayed or inhibited (Boone et al 1990). Numerous chemopreventive agents have come into light over the past few decades. The present study elucidates the effectiveness of one of the medicinal plants that has a wide range of beneficial effects but has not been studied elaborately against skin cancer.

*Balsamodendron mukul*, an indigenous plant, is used against a wide range of disorders like: cardiac disorders, skin diseases, pectoral and hepatic disorders, urinary disorders and nervous diseases. It is also beneficial in case of obesity, fever, wounds, abscesses, ulcers, leucoderma, tumors and is also used in extra dural haematomas in some parts of the world (Kirtikar and Basu 1935, Asolkar et al 1992).

The phytochemical analysis of the plant reveals the presence of ellagic acid, ferulic acid, diterpenes like α-comphorene A and cembrane alcohol, allylcebrol, flavones like quercetin, amino acids like arginine, cystine, histidine and serine. Most of these phytochemicals have pronounced anti-oxidant (Khanduja et al 1999, Huang et al 1988), anti-inflammatory (Theoharides et al 2001) and anti-tumor (Tanaka et al 1993, Khanduja et al 1999) activities in different studies. The compounds present in gum of guggul include linoleic acid, Z-gugglosterol and Z & E-gugglosterones (Chatterjee and Pakashi 1994).

The selection of the plant was based on its anti-oxidant, anti-inflammatory and hypolipidemic properties (Asolkar et al 1992).

A lot of studies have been carried out to prevent BPO and UV irradiation induced toxicities separately (Saleem et al 1999, Fuchs et al 1989) but to the best of our knowledge there is barely
one such study that has stressed upon the chemoprevention of the combination of BPO and UVB induced toxicity (Sultana et al 2003). Considering the above facts, the current study was designed to find out the effect of B. mukul, in the intervention of the additive effects of BPO and UV radiations. BPO has been used for a long time now as a component of acne creams and other cosmetics. Therefore, its exposure is widespread. UVR is component of the sunlight to which every individual is exposed. Since both these components act as tumor promoters, the additive effects of these compounds were studied. Besides, combination of BPO and UVB was employed in order to simulate the adverse effects of UVR on the BPO treated cutaneous portion. A confirmation of the results of B. mukul pretreatment obtained against BPO + UVB was done by checking the efficacy of the plant extract against a well-known tumor promoter (TPA) and two-stage carcinogenesis protocol. For experimentally studying the process of carcinogenesis, the toxic and chemopreventive effects of different agents, two-stage protocols of tumor induction in mice skin are extremely helpful. Therefore, to study the long-term effect of the plant extract, DMBA and croton oil were employed as they are used extensively for induction of tumors in two-stage mouse skin carcinogenesis protocols. The assessment of antioxidant activity was done using the assay of primary and secondary antioxidant enzymes, microsomal lipid peroxidation and estimation of hydrogen peroxide content while the index of anti-promotion activity was the estimation of the inhibition of ODC activity and radiolabelled thymidine incorporation in mouse skin DNA.
**TREATMENT PROTOCOL**

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>BPO + UVB</td>
</tr>
<tr>
<td>III</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>BPO</td>
</tr>
<tr>
<td>IV</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>UVB</td>
</tr>
<tr>
<td>V</td>
<td>B. mukul (D1)</td>
<td>B. mukul (D1)</td>
<td>B. mukul (D1) + BPO + UVB</td>
</tr>
<tr>
<td>VI</td>
<td>B. mukul (D2)</td>
<td>B. mukul (D2)</td>
<td>B. mukul (D2) + BPO + UVB</td>
</tr>
<tr>
<td>VII</td>
<td>B. mukul (D2)</td>
<td>B. mukul (D2)</td>
<td>B. mukul (D2)</td>
</tr>
</tbody>
</table>

Vehicle [Acetone: Methanol (9: 1)] = 0.2ml/animal, BPO = 20mg/0.2ml/animal, UVB = 0.42J/m²/s, B. mukul (D1) = 10mg/Kg body wt., B. mukul (D2) = 15mg/Kg body wt.

Each group contained six mice (6-8 week old). On the third day, pretreatment with methanolic extract of *B. mukul* was done half an hour prior to BPO application. After BPO application, the animals were irradiated with UVB radiations for two hours.

*For oxidative stress & ODC activity estimation:* Animals of all the groups were sacrificed after 16hrs of BPO treatment and were processed for cytosol preparation.

*For the estimation of incorporation of tritiated thymidine in DNA:* Sixteen hours after the treatment with BPO/vehicle, the animals of all the groups were injected with [³H] thymidine (20μCi/0.2ml saline/animal) intra-peritoneally and were sacrificed by cervical dislocation after 2hrs. The skins of mice were quickly removed, cleaned free of extraneous material and homogenized in cool distilled water for further processing.
Short-term studies of the plant extract against TPA induced biochemical alterations

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>I</td>
<td>Vehicle</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle</td>
</tr>
<tr>
<td>V</td>
<td>B. mukul (D1)</td>
</tr>
<tr>
<td>VI</td>
<td>B. mukul (D2)</td>
</tr>
<tr>
<td>VII</td>
<td>B. mukul (D2)</td>
</tr>
</tbody>
</table>

Vehicle [Acetone: Methanol (9:1)] = 0.2ml/animal, TPA = 20nmol/0.2ml/animal, B. mukul (D1) = 10mg/Kg body wt., B. mukul (D2) = 15mg/Kg body wt.

Each group contained (6-8 week old) six mice. On the third day, pretreatment with methanolic extract of B. mukul was done half an hour prior to TPA application.

For oxidative stress estimation: The animals of all the groups were sacrificed after 12hrs of TPA treatment and were processed for subcellular fractionation.

For ODC activity: All the animals were sacrificed after 6hrs of TPA treatment and were processed for cytosol preparation.

For the estimation of incorporation of tritiated thymidine in DNA: Eighteen hours after the treatment with TPA/vehicle, the animals of all the groups were injected [³H] thymidine (20μCi/animal/0.2ml saline) intra-peritoneally and were sacrificed by cervical dislocation after 2hrs. The skins of mice were quickly removed, cleaned free of extraneous material and homogenized in cool distilled water for further processing.
Study of the plant extract against two-stage skin carcinogenesis

**Group I**

Vehicle

(Throughout the study period)

**Group II**

Croton oil

**Group III**

*B. mukul* (D1)

**Group IV**

*B. mukul* (D2)

**Depicts Vehicle:** [Acetone: Methanol (9: 1)] /0.2ml/ animal, was applied throughout the study period

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

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

**Depicts Modulator:** *B. mukul* (D1 = 10mg/Kg body wt.), was applied twice weekly for 20 weeks (One hour before each promoter treatment)
Depicts Modulator: *B. mukul* (D2 = 15mg/Kg body wt.), was also applied twice weekly for 20 weeks (One hour before each promoter treatment).

Four groups of 20 mice (4-6 week old) each were used. After ten days of single topical DMBA application, croton oil treatments (twice weekly) were started that continued for 20 weeks. One hour before each croton oil application, the mice were pretreated with methanolic extract of *B. mukul*. Animals in all the groups were observed for apparent signs of toxicity if any and mortality during the entire period of study. The data is represented in terms of percentage of mice with tumors and number of tumors per mouse and is plotted as a function of weeks on test. After 25th week, the animals were sacrificed and the histopathology of their tissue was done.

**RESULTS**

Table 1 shows *B. mukul* extract pretreatment on BPO and UVB radiation induced depletion in the levels of reduced glutathione, its metabolizing and phase II enzymes in murine skin. Single topical application of BPO followed by UVB, only BPO treatment and only UVB irradiation significantly depleted the reduced glutathione content to 63, 72 and 76%, glutathione reductase to 72, 66 and 79%, glutathione-S-transferase to 68, 75 and 81% and quinone reductase to 68, 85 and 83% respectively in the treatment groups. While pretreatment of mice skins with *B. mukul* extract followed by BPO treatment and subsequent UVB irradiation resulted in 7-18% and 12-24% recovery at low dose and high dose respectively.

Table 2 shows the effects of *B. mukul* on BPO and UVB mediated decrease in the antioxidant enzyme activities. The activities of glutathione peroxidase and glucose-6-phosphate dehydrogenase decreased to 66 and 70% with both BPO treatment and UVB irradiation; 62 and 61% on only BPO treatment; and 76 and 81% on only UVB irradiation respectively in the levels of the enzymes. Pretreatment with *B. mukul* extract, on the other hand, caused 10-11% and 20-
23% recovery in the enzyme activities in a dose dependent manner. Lipid peroxidation and hydrogen peroxide formation increased to 246 and 120% with both BPO treatment and UVB irradiation, 264 and 113% with only BPO treatment while 182 and 110% with only UVB irradiation respectively in the two parameters elevated. Concomitantly, pretreatment with B. mukul extract prevented the levels of MDA formation from 34 -77% and H$_2$O$_2$ formation from 9-14% respectively in a dose dependent manner.

Figure 1 depicts the effect of B. mukul extract pretreatment on BPO and UVB induced ODC activity and radiolabelled thymidine incorporation in DNA. The ODC activity and thymidine incorporation in DNA showed 2.6 and 2.0 -fold increase in case of the combination of BPO treatment and UVB irradiation, 2.3 and 1.8 -fold increase in case of only BPO treatment and in case of only UVB irradiation the ODC activity and thymidine incorporation into the DNA increased by 1.5 and 1.3 -fold respectively. Application of B. mukul extract followed by BPO treatment and subsequent UVB irradiation restored ODC activity up to 103% and up to 44.5% in case of unscheduled DNA synthesis in a dose dependent manner.

The effect of pretreatment of dorsal portions of mice skin with B. mukul extract against TPA induced depletion in the levels of reduced glutathione, its metabolizing and phase II enzymes is shown in Table 3. Table 4 shows the effects of B. mukul against TPA mediated decrease in the antioxidant enzyme activities, lipid peroxidation and hydrogen peroxide formation. Single topical application of TPA caused significant depletion in the reduced glutathione content (57%), glutathione reductase (61%), glutathione-S-transferase (54%) and quinone reductase (55%). While pretreatment of mice skins with B. mukul extract resulted in 7-25% and 18-38% recovery at low dose and high dose respectively. TPA also caused down regulation of the antioxidant enzyme activities like catalase (39%), glutathione peroxidase (63%) and glucose-6-
phosphate dehydrogenase (57%). Parallel to these changes, it increased the level of lipid peroxidation (205%) and hydrogen peroxide content (142%). The activities of catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase, however, were significantly brought back to their respective near normal values on B. mukul extract pretreatment especially at the higher dose. The percentage of recovery in case of antioxidant enzyme activities was 13-20% and 21-37% at low and high doses respectively. The decrease caused by B. mukul in the formation of MDA was 70-84% and in case of H$_2$O$_2$ content was 16-27% respectively in a dose dependent manner.

Figure 2 represents the prophylactic effect of B. mukul extract pretreatment on TPA mediated rise in ODC activity and thymidine incorporation in DNA. The ODC activity and thymidine incorporation in DNA showed 5.8 and 2.4-fold increase respectively on treatment of mice skins with single topical application of TPA. Upon pretreatment with B. mukul extract, ODC activity was restored from 53–134% and 33–54% in case of unscheduled DNA synthesis in a dose dependent manner.

Figure 3 depicts the effect of B. mukul extract pretreatment on DMBA and croton oil mediated skin carcinogenesis in mice. The only vehicle treated group did not show any signs of toxicity or tumor formation even at the end of 25 weeks. Single topical application of DMBA was used to initiate tumors in mice that were ten days later promoted by croton oil (0.5%) twice weekly for 20 weeks. The appearance of first tumor was noticed at the end of 7th week. All the animals were observed for 25 weeks. The number of tumors at the 10th week in DMBA and croton oil treated group was 4 with 45% incidence of tumor bearing mice while the number of tumors at the end of 25 weeks was 11 with 100% incidence of tumor bearing mice. In the prophylaxis groups where the animals were given topical applications of B. mukul extract prior to the
application of croton oil twice every week, the appearance of first tumor was prolonged to 10 weeks at both the doses. At the 10th week the percentage of tumor bearing mice was 10 & 5% and 60 & 50% at 25th week with lower and higher dose respectively. The total number of tumors/mouse at 25 weeks dropped to 5.6 & 4.4 in B. mukul treated group dose dependently at 10 and 15 mg/Kg body weight.

Morphologically, tumors of the animals of DMBA/Croton oil treated group were larger in size, more in number and darker in appearance as compared to B. mukul extract pretreated mice. The tumors of DMBA and croton oil treated group were polypoidal and pedunculated with the inner vascularized fibrotic core showing squamous epithelial cells that were arranged in an arboreal pattern shown histologically. As regards the body weight, no significant difference was observed in any of the groups during the entire period of study (data not shown).

Figure 4 depicts the histological changes of the effect of pretreatment of B. mukul extract on two-stage skin carcinogenesis in mice. The pictures of DMBA and croton oil treated group represented squamous cell carcinoma, which possess certain characteristics identical to those observed in human squamous cell carcinomas like hyperchromatism, intradermal infiltration and hyper keratinization (Stoll 1979). Pleomorphism with anaplasia and carcinomatous changes like keratin pearls were a common feature of the positive control group tumors. While, in case of B. mukul extract pretreated group, the formation of squamous cell carcinoma was significantly reduced. Occurrence of papillomas, hyperkeratinization and hyperplasia was more common in anti-tumor promotion groups as opposed to squamous cell carcinoma in group 2 animals.
DISCUSSION

Tumor develops only after initiation is followed by promotion, and if the causative factors continue to persist without any preventive measures being employed then the probability of further progression of papillomas into carcinomas becomes more. Though the probability of such conversions is otherwise less but once carcinomas develop, regression becomes rather difficult. Since the diagnosis of tumor at an early stage is difficult, therefore, its treatment is often delayed that causes a lot of suffering.

Exposure to certain compounds such as BPO is unavoidable and even more unavoidable is the exposure to sunlight, particularly, UV radiations. There are several reports elucidating oxidative stress and toxicity profile of BPO in mice skin (Saleem et al 1999, Zhao et al 2000). Likewise, UV treatment has been shown to deplete the glutathione content in skin (Connor and Wheeler 1987). It has been reported to induce epidermal ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase activities, incorporation of tritiated thymidine into DNA (Verma et al 1979a and b) and prostaglandin synthesis (William and Weinstein 1975), all of which play a significant role in the promotion stage of tumor formation.

Our observations from present study indicate that glutathione and its metabolism are adversely affected by BPO and UVB radiations per se and also by their combination. On the other hand, the data also reveals that B. mukul has some compensating effect on the impairment of glutathione metabolism. Like most chemopreventive agents, B. mukul modulated GST (significantly at the higher dose), the enzyme involved in the biotransformation system and has a prominent role in conjugation and excretion of the carcinogens. It also augmented the levels of reduced glutathione thus rendering protection to the cell from the foreign agent/ carcinogen i.e. against TPA, BPO and UVB.
The role of free radicals during inflammatory response has been reported since inflammation also occurs on contact with tumor promoters (Sun 1990). The plant extract is known to possess potent anti-inflammatory activity (Sosa et al 1993). Also, since free radicals play a significant role in the biotransformation of chemical carcinogens like activation of DMBA and B(a)P (Prashar et al 1994) and promotion stage of cancer (Sun 1990), it implies that if any agent is potent enough to prevent the generation of these radicals it will also be capable of delaying/preventing the occurrence of initiation and promotion and ultimately the development of cancer.

The plant used in the present study, decreases the formation of MDA and prevents the formation of reactive moieties like hydrogen peroxide, which can further produce even more dangerous radicals such as hydroxyl radicals (Sun 1990). The higher dose of the plant extract proves to be significantly effective against the oxidative damage induced not only by the chemical agent but also the by the combination of chemical and physical agents i.e. TPA, BPO & UVB respectively. It also restores the cellular antioxidant enzyme activities especially at the higher dose, therefore we can suggest that B. mukul might play a role in chemopreventing two-stage skin carcinogenesis by modulating oxidative stress induced initiation and promotion events.

Furthermore, another mode of action of B. mukul comes into light with the study of ODC activity and DNA synthesis. BPO treatment and UVB radiation per se and also their combination causes induction of ODC activity and radiolabelled thymidine uptake. However, on pretreatment with B. mukul there was significant reduction of ODC activity and DNA synthesis as compared to BPO + UVB treated group. Similar trend was also observed against TPA treatment induced oxidative stress and hyperproliferation. The elevation of ODC activity and DNA synthesis in case of tumor promotion has well been documented (Machishi et al 1995). The increase in ODC activity leads to increased polyamine synthesis that plays a role in
hyperplasia. Therefore, if *B. mukul* has the capacity to inhibit ODC activity and unscheduled DNA synthesis, it may also delay the process of tumor promotion by preventing hyperproliferative response. This fact is further strengthened in the long-term tumor studies. In the chronic study, the data obtained unveils the fact that the plant protects against tumorigenesis as it decreased the latency period of tumor development, the number and incidence of tumor in Swiss mice.

Therefore, it can be suggested that ultimately the protection against two-stage skin carcinogenesis might be mediated by multiple actions, which include restoration of cellular antioxidant enzymes, detoxifying enzymes, -SH group containing natural antioxidant, ODC activity and DNA synthesis as evidenced against adverse effects of TPA, BPO and UVB. *B. mukul* pretreatment also exhibited the protective trend in elevating the detoxifying enzymes. Though the lower dose did not significantly recover the protective enzymes, the higher dose showed significant recovery. Other mechanisms in the modulation of carcinogenesis by the plant may also be simultaneously operative.

There are reports that suggest a link between UV-induced skin cancer and unsaturated lipids (Black 1983). In fact, Watson and Mellenby showed in as early as 1930 that the incidence of coal tar induced skin tumors was enhanced by dietary fat (Watson and Mellenby 1930). Diets rich in fat content have been shown to enhance colon (Newberne and Nauss 1986), pancreas (Roebuck et al 1989) and breast (Welsch 1987) cancers. The plant used in this study, *B. mukul* is known to exert hypocholesterolemic and hypolipidemic effects. It is also used in the treatment of obesity (Asolkar et al 1992). Owing to the fact that the plant extract also inhibits skin tumorigenesis initiated by DMBA and promoted by croton oil, we speculate the role of its
hypcholesterolemic and hypolipidemic activities on the abrogation of skin tumorigenesis. However, to establish any such correlation, elaborate studies need to be designed.

*B. mukul* contains a wide array of compounds such as ellagic acid, ferulic acid, diterpenes and flavones like quercetin (Kris-Etherton et al 2002). The presence of these chemopreventive phytochemicals can be associated with the protective effect of the plant. Polyphenols and flavones like ellagic acid, ferulic acid and quercetin have proven their worth as potent chemopreventive agents. Different plant polyphenols and flavones have been extensively studied and found extremely effective against different cancer models and have been shown to act by different mechanisms. Ellagic acid is found abundantly in various fruits, nuts and vegetables that exhibit antioxidant and anticarcinogenic activities. It is protective against oxidative stress and tumorigenesis in mice (Khanduja et al 1999) and raises the activities of phase II metabolizing enzymes (Uda et al 1997). It is known to inhibit chemically induced cancer in the skin, lung, esophagus and liver in animal models, and tumor promotion induced by TPA in mouse skin (Stoner and Mukhtar 1995). Quercetin shows a wide range of effects in biological systems. It is also protective against oxidative damage and tumorigenesis in animal model system and is supposed to inhibit carcinogenesis by affecting the molecular events related to carcinogenesis (Yang et al 2001). It has been shown to exert various beneficial effects not just by its antioxidant nature but also by its other properties like enhancement of programmed cell death as shown in HPB-acute lymphoblastic leukemia (ALL) cell line (Russo et al 2003), targeting pathways of signal transduction and acting as anti-inflammatory and immunomodulatory agent (Cho et al 2003). Both quercetin and ellagic acid, have been shown to enhance the phase II metabolizing enzymes (Horvathova et al 2003). Ferulic acid has been found to protect against gastrointestinal cancer
by the induction of hepatic UDP-glucuronosyltransferase enzyme activity (Van Der Logt et al 2003). Ellagic acid and ferulic acid have been shown to significantly reduce the incidence of tongue neoplasms (squamous cell papilloma and carcinoma) and preneoplastic lesions (hyperplasia and dysplasia) when fed to rats (Tanaka et al 1993). The results shown by the plants could be the additive effects of these phytochemicals, some of which also have a similar mechanism of action as shown by the plant extract in the studies described here. Furthermore, the diterpenes present in the plant might also have some protective action as some diterpenes tested recently have exhibited anticancer effects (Cavin et al 2002). Other labdane diterpenoids obtained from medicinally important plant, *Thuja* have already been reported to possess cancer chemopreventive activity (Tanaka et al 2000). Diterpenes, cafestol and kahweol have been associated with the anticarcinogenic effect of coffee in human epidemiological and animal experiments (Cavin et al 2002). Besides, certain diterpenes like paclitaxel are used for the treatment of some cancerous as well as noncancerous conditions (Cragg et al 1977, Hartwall 1982).

In addition to preventing tumor formation, the plant extract also suppressed oxidative damage, tumor promotion marker enzyme, ODC and inhibited unscheduled DNA synthesis. Therefore, we suggest a positive role of the plant, *B. mukul* against the combination of BPO & UVB, and TPA induced tumor promotion related alterations and two-stage skin carcinogenesis in Swiss albino mice.
Table 1. Prophylactic effect of *Balsamodendron mukul* extract on BPO treatment and UVB-irradiation mediated depletion in reduced glutathione, its metabolizing enzymes and phase II detoxifying enzymes in murine skin

<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>Vehicle (0.2ml / animal)</td>
<td>24.04 ± 0.29</td>
<td>112.69 ± 7.19</td>
<td>235.37 ± 18.06</td>
<td>186.19 ± 7.74</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>15.08 ± 1.02<em>a,b,</em></td>
<td>81.29 ± 3.48**</td>
<td>159.33 ± 7.90*a</td>
<td>126.76 ± 4.64<em>a,b,</em></td>
</tr>
<tr>
<td>Only BPO (20mg / 0.2ml acetone / animal)</td>
<td>17.34 ±1.66<em>a,b,</em></td>
<td>74.53 ± 1.54*b</td>
<td>177.56 ± 11.71</td>
<td>158.19 ± 6.42*b</td>
</tr>
<tr>
<td>Only UVB (0.42 J/m²/s)</td>
<td>18.24 ± 2.32*</td>
<td>89.14 ± 7.07*a</td>
<td>191.08 ± 21.89NS</td>
<td>154.19 ± 10.55*b</td>
</tr>
<tr>
<td><em>B. mukul</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>19.33 ± 0.64**</td>
<td>96.93 ± 1.22**</td>
<td>176.45 ± 7.39NS</td>
<td>151.56 ± 12.68NS</td>
</tr>
<tr>
<td><em>B. mukul</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>20.92 ± 1.16**</td>
<td>99.73 ± 2.24**</td>
<td>188.08 ± 4.59*</td>
<td>160.66 ± 13.72*</td>
</tr>
<tr>
<td>Only <em>B. mukul</em> (D2)</td>
<td>23.26 ± 0.63</td>
<td>97.21 ± 4.19</td>
<td>236.20 ± 6.56</td>
<td>201.20 ± 9.28</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 vehicle treated control group. **P < 0.01 and *P < 0.05 compared with corresponding value for BPO treated + UVB irradiated group.
Table 2. Shows effect of pretreatment of *Balsamodendron mukul* extract on BPO treatment and UVB-irradiation mediated reduction in antioxidant enzymes and elevation of lipid peroxidation and hydrogen peroxide content in murine skin

<table>
<thead>
<tr>
<th>Treatment groups</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>Vehicle (0.2ml / animal)</td>
<td>160.66 ± 8.83</td>
<td>80.41 ± 5.49</td>
<td>3.03 ± 0.24</td>
<td>328.04 ± 13.74</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>105.99 ± 7.83***</td>
<td>56.27 ± 3.46**</td>
<td>7.46 ± 0.97***</td>
<td>395.26 ± 11.43***</td>
</tr>
<tr>
<td>Only BPO (20mg / 0.2ml acetone / animal)</td>
<td>99.44 ± 3.78***</td>
<td>49.12 ± 4.97***</td>
<td>7.99 ± 0.78***</td>
<td>371.25 ± 10.44***</td>
</tr>
<tr>
<td>Only UVB (0.42 J/m²/s)</td>
<td>121.97 ± 8.57**</td>
<td>65.59 ± 2.88**</td>
<td>5.53 ± 0.35***</td>
<td>362.16 ± 8.74**</td>
</tr>
<tr>
<td><em>B. mukul</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>123.08 ± 6.46 NS</td>
<td>65.43 ± 7.67 NS</td>
<td>6.43 ± 0.124 NS</td>
<td>366.78 ± 23.79 NS</td>
</tr>
<tr>
<td><em>B. mukul</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>138.83 ± 8.37*</td>
<td>74.83 ± 8.95 NS</td>
<td>5.11 ± 0.20*</td>
<td>347.70 ± 12.64*</td>
</tr>
<tr>
<td>Only <em>B. mukul</em> (D2)</td>
<td>155.33 ± 5.85</td>
<td>76.82 ± 3.69</td>
<td>4.09 ± 0.245</td>
<td>324.89 ± 12.95</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 vehicle treated group. *P < 0.05 when compared with corresponding value for BPO & UVB treatment.
Table 3. Effect of *Balsamodendron mukul* extract pretreatment on TPA mediated reduction in reduced glutathione, its metabolizing enzymes and phase II detoxifying enzymes in murine skin

<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>Vehicle (0.2ml / animal)</td>
<td>31.62 ± 2.67</td>
<td>100.49 ± 11.96</td>
<td>206.20 ± 14.35</td>
<td>198.89 ± 4.22</td>
</tr>
<tr>
<td>Only TPA (20nmol / 0.2ml acetone / animal)</td>
<td>18.19 ± 0.75&lt;sup&gt;***&lt;/sup&gt;</td>
<td>61.68 ± 5.33&lt;sup&gt;*&lt;/sup&gt;</td>
<td>112.11 ± 8.49&lt;sup&gt;***&lt;/sup&gt;</td>
<td>109.85 ± 15.59&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. mukul</em> (D1) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>20.29 ± 0.52&lt;sup&gt;*&lt;/sup&gt;</td>
<td>74.19 ± 1.58&lt;sup&gt;*&lt;/sup&gt;</td>
<td>156.96 ± 18.68&lt;sup&gt;NS&lt;/sup&gt;</td>
<td>160.55 ± 6.10&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. mukul</em> (D2) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>24.08 ± 1.84&lt;sup&gt;*&lt;/sup&gt;</td>
<td>82.99 ± 4.27&lt;sup&gt;*&lt;/sup&gt;</td>
<td>191.20 ± 9.98&lt;sup&gt;***&lt;/sup&gt;</td>
<td>177.37 ± 8.45&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Only <em>B. mukul</em> (D2)</td>
<td>27.57 ± 2.81</td>
<td>92.43 ± 5.64</td>
<td>223.71 ± 5.59</td>
<td>206.24 ± 26.7</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. <sup>***</sup><sup>P</sup> < 0.001 and <sup>*</sup><sup>P</sup> < 0.05 compared with the corresponding value for vehicle treated control group. <sup>***</sup><sup>P</sup> < 0.001, <sup>**</sup><sup>P</sup> < 0.01 and <sup>*</sup><sup>P</sup> < 0.05 compared with corresponding value for treatment with TPA alone.
Table 4. Effect of prophylactic treatment of *Balsamodendron mukul* extract on TPA mediated reduction in antioxidant enzymes and induction of 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>Vehicle (0.2ml / animal)</td>
<td>71.38 ± 1.55</td>
<td>182.46 ± 6.37</td>
<td>68.93 ± 1.53</td>
<td>1.84 ± 0.133</td>
<td>307.74 ± 15.69</td>
</tr>
<tr>
<td>Only TPA (20nmol / 0.2ml acetone / animal)</td>
<td>27.72 ± 5.36***</td>
<td>114.67 ± 7.79***</td>
<td>39.54 ± 1.03***</td>
<td>3.77 ± 0.464**</td>
<td>438.17 ± 30.95**</td>
</tr>
<tr>
<td><em>B. mukul</em> (D1) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>41.96 ± 6.37NS</td>
<td>138.47 ± 10.4NS</td>
<td>50.83 ± 5.46NS</td>
<td>2.48 ± 0.261*</td>
<td>390.05 ± 20.98NS</td>
</tr>
<tr>
<td><em>B. mukul</em> (D2) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>54.48 ± 7.09*</td>
<td>153.09 ± 10.12*</td>
<td>59.70 ± 3.83***</td>
<td>2.23 ± 0.215*</td>
<td>355.53 ± 16.75*</td>
</tr>
<tr>
<td>Only <em>B. mukul</em> (D2)</td>
<td>56.55 ± 8.87</td>
<td>188.66 ± 9.34</td>
<td>73.20 ± 4.59</td>
<td>1.08 ± 0.199</td>
<td>331.17 ± 28.37</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 vehicle treated control group. ***P <0.001 and *P < 0.05 compared with corresponding value for treatment with TPA alone.
FOOTNOTES AND FIGURE LEGENDS

Figure 1. *Balsamodendron mukul* pretreatment modulates BPO treated/ UVB-irradiated induction of ODC activity and $[^{3}H]$ thymidine incorporation in murine skin

Each value represents mean ± SE of six animals, ### p<0.001 and # p<0.05 as compared to vehicle treated negative control group. ** 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 10mg and 15mg *B. mukul*/Kg/body weight.

Figure 2. Effect of pretreatment with *Balsamodendron mukul* against TPA induced ODC activity and $[^{3}H]$ thymidine incorporation in Swiss mice

Each value represents mean ± SE of six animals. ### p<0.001 when compared with vehicle treated control group. ** p<0.001, * p<0.01 and * p<0.05 when compared with TPA treated group. D1 and D2 represent topical applications of 10mg and 15mg of *B. mukul*/Kg/body weight.

Figure 3. Effect of pretreatment with *Balsamodendron mukul* extract on DMBA and Croton oil mediated skin carcinogenesis in mice

Figure 4. Shows the histopathological alterations of *Balsamodendron mukul* extract pretreatment on two-stage skin carcinogenesis in mice

**Fig. 4a** - histology of vehicle treated control mice skin depicting normal tissue

**Fig. 4b** - histopathology of DMBA/croton oil treated mice skin depicting squamous cell carcinoma. It shows infiltration of malignant squamous cells depicting pleomorphism and hyperchromatism.

**Fig. 4c** - histopathological alterations in skin tissue of anti-tumor promotion group animals depicting verruca formation (low grade variant of squamous cell carcinoma) with 10mg/Kg body weight of *B. mukul* extract. It depicts marked hyperkeratinization and infiltration of inflammatory cells.

**Fig. 4d** - histopathological alterations in skin tissue of anti-tumor promotion group animals depicting verruca formation at 10mg/Kg body weight of *B. mukul* extract. It depicts marked hyperkeratinization.

**Fig. 4e** - histopathological alterations in skin tissue of anti-tumor promotion group animals depicting hyperplasia at 15mg/Kg body weight of *B. mukul* extract.

**Fig. 4f** - histopathological alterations in skin tissue of anti-tumor promotion group animals depicting hyperplasia at 15mg/Kg body weight of *B. mukul* extract.
Figure 1

*Balsamodendron mukul* pretreatment modulates BPO treated/UVB-irradiated induction of ODC activity and \(^{[3]}\text{H}\) thymidine incorporation in murine skin.

![Bar graph showing ODC activity and thymidine incorporation](image)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>ODC activity (pmol \text{^{14}}\text{CO}_2\text{ released/hr/mg protein})</th>
<th>(^{[3]}\text{H})Thymidine incorporation (DPM/\mu g DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>270</td>
</tr>
<tr>
<td>2</td>
<td>2000 ###</td>
<td>320 ###</td>
</tr>
<tr>
<td>3</td>
<td>1500 ###</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>1200</td>
<td>200 #</td>
</tr>
<tr>
<td>5</td>
<td>1800 *</td>
<td>300 NS</td>
</tr>
<tr>
<td>6</td>
<td>1500 **</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td>1000 NS</td>
<td>200</td>
</tr>
</tbody>
</table>

Legend:
- □ Only Vehicle
- □ Only BPO
- □ *B. mukul (D1) + BPO + UVB
- □ Only UVB
- □ *B. mukul (D2) + BPO + UVB
- □ Only *B. mukul (D2)
Figure 2
Effect of pretreatment with *Balsamodendron mukul* against TPA induced ODC activity and [³H] thymidine incorporation in Swiss mice

![Graph showing oxygen radicals (µmol αCO₂ released/hr/mg protein) across different treatment groups.](image)

![Graph showing thymidine incorporation (DPM/µg DNA) across different treatment groups.](image)

Legend:
- Only Vehicle
- Only TPA
- B.mukul (D1) + TPA
- B.mukul (D2) + TPA
- Only B.mukul (D2)
Figure 2
Effect of pretreatment with *Balsamodendron mukul* against TPA induced ODC activity and [³H] thymidine incorporation in Swiss mice.

![Graph showing the effect of pretreatment with *Balsamodendron mukul* on ODC activity and thymidine incorporation.](image)

- **ODC activity (pmol CO₂ released/ hr/mg protein):**
  - Treatment groups:
    - 1: Only Vehicle
    - 2: Only TPA
    - 3: B. mukul (D1) + TPA
    - 4: Only B. mukul (D2)
    - 5: B. mukul (D2) + TPA

- **[³H] thymidine incorporation (DPM/μg DNA):**
  - Treatment groups:
    - 1: Only Vehicle
    - 2: Only TPA
    - 3: B. mukul (D1) + TPA
    - 4: Only B. mukul (D2)
    - 5: B. mukul (D2) + TPA
Figure 3
Effect of pretreatment with *Balsamodendron mukul* extract on DMBA and Croton oil mediated skin carcinogenesis in mice

- DMBA/Croton oil
- DMBA + Balsamodendron (12) + Croton oil
- DMBA + Balsamodendron (24) + Croton oil

Percentage of mice with tumors vs. Weeks on test

Number of tumors/mouse vs. Weeks on test
Figure 4. Shows alterations in the histopathology of two-stage skin carcinogenesis on pretreatment of *Balsamodendron mukul* extract in mice.

**Fig. 4a** – histology of vehicle treated control mice skin depicting normal tissue (20X).

**Fig. 4b** – histopathology of DMBA and croton oil treated mice skin depicting squamous cell carcinoma (20X).
Fig. 4 c – histopathological alterations in skin tissue of anti-tumor promotion group animals depicting verruca formation (low grade variant of squamous cell carcinoma) at 10mg/Kg body weight of B. mukul extract. (40X).

Fig. 4 d – histopathological alterations in skin tissue of anti-tumor promotion group animals depicting verruca formation at 10mg/Kg body weight of B. mukul extract. (20X).
Fig. 4e – histopathological alterations in skin tissue of anti-tumor promotion group animals depicting hyperplasia at 15mg/Kg body weight of *B. mukul* extract (40X).

Fig. 4f – histopathological alterations in skin tissue of anti-tumor promotion group animals depicting hyperplasia at 15mg/Kg body weight of *B. mukul* extract (40X).