CHAPTER 5

Hibiscus rosa sinensis prevents two-stage skin carcinogenesis: Possible role in the inhibition of tumor promotion related events
The skin being the largest organ of the body is constantly exposed to a variety of agents including physical and chemical agents. It is especially at a threat due to the reactive oxygen generating species, which these agents produce and also to those produced in vivo by various metabolic processes. The exposure to various free radical generating compounds is unavoidable as we come in contact with them knowingly or unknowingly through various sources. For example through environmental pollutants, fried and spicy food, processed meat (Sugimura 2000), pesticides, radiations, occupation such as chromium exposure in cement workers (Slaga et al 1981), medication like cyclophosphamide and drug abuse (Lu and Kacew 2002), contaminated water and physical stress (Zhao et al 2000). The fact that free radicals are the causative factors of cancer (Sun 1990) is supported by various studies, which show that tumor promoters increase the generation and decrease the degradation of free radicals, and that the scavengers of these free radicals are the inhibitors of various biochemical events that occur during tumor promotion (Perchellet and Perchellet 1989). Major factors for the causation of cancer in human beings are cigarette smoking, infection and inflammation (Suginiura 2000). The mouse skin model of multistage carcinogenesis continues to serve as a major in vivo model for studying the sequential and stepwise evolution of the process of cancer formation. It is of utmost importance as it provides the basis for the investigation of the effects of the factors linked with the disease that in turn provide a platform for confronting with those causative factors. Besides, the effects of anti-initiating and anti-promoting drugs/agents can be distinguished from one another. BPO, UVR and TPA have been used in different studies as tumor promoters in two-stage carcinogenesis (Athar et al 1990, Kapadia et al 2003, Wei et al 1998). All of them have been shown to act via the generation of reactive oxygen species (ROS). BPO is a moderately effective tumor promoter and is involved in the generation of free
Hibiscus rasa sinensis

radicals (Kawanishi et al. 1999). Ultraviolet radiations, as opposed to TPA and BPO are also capable of acting as a complete carcinogen. UVB region of the solar radiations is the main waveband that is responsible for the induction of skin cancer. However, both UVA and UVB regions of the UVR play the role of a promoter in the melanoma stimulation and lymphoma development in mice (Husain et al. 1991).

Extensive research is being carried out on cancer chemoprevention that emphasizes the use of antioxidants, which may be naturally occurring or synthetically prepared compounds (Morse and Stoner 1993). A large number of plant extracts (Nakamura et al. 1999, Tanaka et al. 2000, Saleem et al. 2001), single compounds and dietary components including fruits and vegetables (Wei et al. 1995, Singh and Shukla 1998, Zhao et al. 1999) are reported to act as protective agents against cancer. Example, Beta vulgaris (beetroot) extract has a potent cancer chemopreventive property both in vitro assay and in vivo system in two-stage mouse lung and skin carcinogenesis (Kapadia et al. 2003). Vitamin C is obtained by natural sources like lemon, oranges and guavas. It also has pronounced effects in delaying the process of carcinogenesis. It exerts its effects by quenching reactive oxygen species like peroxyl radical and the singlet oxygen (Sies et al. 1992). It also protects against UVA-generated singlet oxygen (Linetsky et al. 1998), inhibiting lipid peroxidation, ODC activity and DNA synthesis (Pinnell 2003).

In this study, we have used a plant that has a range of medicinal properties, is available worldwide and is a household plant as well. Hibiscus rosa sinensis L. (Malvaceae), commonly called as China rose, is a part of traditional medicines that are used for the treatment of cough, fever, dysentery, venereal diseases, as an abortifacient and is also applied topically to cancerous swellings (Ross 1999). The plant also possesses various pharmacological properties like radical
scavenging effect (Masaki et al 1995), anti-pyretic and anti-inflammatory (Singh et al 1978) activities. The extract of *H. rosa sinensis* contains various compounds including quercetin, carotene, niacin, riboflavin, malval acid, gentisic acid, margaric acid and lauric acid (Ross 1999). Based on these facts we speculated the role of *H. rosa sinensis* extract against TPA, BPO and UVB induced tumor promotion response and oxidative stress; and DMBA/ croton oil induced tumorigenesis in mice.

**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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>I</td>
<td>Acetone</td>
</tr>
<tr>
<td>II</td>
<td>Acetone</td>
</tr>
<tr>
<td>III</td>
<td><em>H. rosa sinensis</em> (D1)</td>
</tr>
<tr>
<td>IV</td>
<td><em>H. rosa sinensis</em> (D2)</td>
</tr>
<tr>
<td>V</td>
<td><em>H. rosa sinensis</em> (D2)</td>
</tr>
</tbody>
</table>

Acetone = 0.2ml/animal, *H. rosa sinensis* (D1)= 3.5mg/Kg body wt., *H. rosa sinensis* (D2) = 7.0mg/Kg body wt. Doses of BPO and UVB; the age, sequence of treatments and the time of sacrifice of all the animals were similar to that described in Chapter 4.

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

<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>Acetone</td>
</tr>
<tr>
<td>II</td>
<td>Acetone</td>
</tr>
<tr>
<td>III</td>
<td><em>H. rosa sinensis</em> (D1)</td>
</tr>
<tr>
<td>IV</td>
<td><em>H. rosa sinensis</em> (D2)</td>
</tr>
<tr>
<td>V</td>
<td><em>H. rosa sinensis</em> (D2)</td>
</tr>
</tbody>
</table>
Acetone, *H. rosa sinensis* (D1) and *H. rosa sinensis* (D2) = same as described above.

TPA = 20nmol/0.2ml/animal. Sequence of treatments on the third day and the time of sacrifice of the animals were same as that described in Chapter 4.

**Long-term study with the plant extract against chemically induced skin carcinogenesis**

**Group I**

![Diagram](image)

**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

**Depicts Promoter:** Croton oil (0.5%/0.2ml/animal) was applied twice weekly for 20 weeks
Chapter 5

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Depicts Modulator: H. rosa sinensis D1 (3.5mg/Kg body wt.) was applied twice weekly for 20 weeks (One hour before each promoter treatment)

Depicts Modulator: H. rosa sinensis D2 (7.0mg/Kg body wt.) was also applied twice weekly for 20 weeks (One hour before promoter treatment)

The number of animals, treatment scheduled, observation criteria, termination time of the experiment and data representation were done in a manner similar as that described in Chapter 4.

RESULTS

Effect of H. rosa sinensis on BPO and UVB mediated oxidative stress and hyperproliferation response

Table 1 depicts prophylactic effect of H. rosa sinensis extract on BPO treated and UVB mediated depletion in reduced glutathione, its metabolizing enzymes and phase II detoxifying enzymes in murine skin. BPO and UVB caused 60, 65, 66 and 76% depletion in reduced glutathione, glutathione reductase, glutathione S-transferase and quinone reductase respectively. On the other hand, pretreatment with H. rosa sinensis extract caused 9-18% recovery in case of low dose and 17-28% recovery in case of high dose.

Table 2 shows effect of pretreatment of H. rosa sinensis extract on BPO treated and UVB-irradiated reduction in antioxidant enzymes in murine skin. There was 65, 75 and 62% reduction in the activities of catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase respectively. While pretreatment with H. rosa sinensis extract resulted in 6-14% recovery at low dose and 12-22% recovery at high dose in the activities of the above said enzymes.

Table 3 shows the effect of H. rosa sinensis extract pretreatment on BPO treated and UVB
induced lipid peroxidation and hydrogen peroxide content in murine skin. The levels of lipid peroxidation and hydrogen peroxide formation increased to 228 and 164% in BPO treated and UVB irradiated group. On the other hand, in the *H. rosa sinensis* extract pretreated group there was 29-53% suppression in lipid peroxidation and 21-50% inhibition in hydrogen peroxide formation.

Figure 1 depicts the increase in cutaneous ornithine decarboxylase activity and unscheduled DNA synthesis in BPO and UVB treated group and its inhibition by *H. rosa sinensis* 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 *H. rosa sinensis* extract resulted in 79-117% inhibition of the elevated ODC activity and 59-82% inhibition of DNA synthesis at two different doses.

**Effect of *H. rosa sinensis* on two-stage skin carcinogenesis and TPA mediated oxidative stress and hyperproliferation response in mice**

The effect of pretreatment of animals with *H. rosa sinensis* extract against TPA induced depletion in the levels of reduced glutathione and glutathione metabolizing enzymes is shown in Table 4. Topical application of TPA significantly depleted the reduced glutathione content and glutathione metabolizing enzymes viz., glutathione reductase and glutathione-S-transferase to 51, 45 and 55% respectively. On the other hand, pretreatment of animals with *H. rosa sinensis* extract resulted in 10-24% recovery at low dose and caused 24-36% recovery at high dose in the levels of reduced glutathione and its metabolizing enzymes.

Table 5 depicts the prophylactic effect of *H. rosa sinensis* extract on TPA-mediated depletion of the antioxidant enzyme armory and elevation in lipid peroxidation and hydrogen peroxide content. The activities of catalase (35%), GPX (59%) and G6PD (51%) were significantly
depleted on application of TPA to the dorsal shaven portions of the mice skins. On the contrary, pretreatment with *H. rosa sinensis* extract at two different doses caused recovery in the activities of the primary and secondary antioxidant enzymes. The percentage of recovery at low dose was 9-17 and 21-30 at high dose, in case of *H. rosa sinensis* pretreatment. Malondialdehyde formation and H$_2$O$_2$ content were significantly elevated in TPA treated group to 190% and 156% respectively. Pretreatment of animals with *H. rosa sinensis* resulted in 31-50% decrease in MDA formation, and 29-45% recovery in H$_2$O$_2$ content respectively in a dose dependent manner. Thus, an overall dose dependent response with two different doses of *H. rosa sinensis* extract was observed which offered protection against TPA induced tumor promotion and oxidative stress in murine skin.

Figure 2 shows the effects of prophylactic treatment of *H. rosa sinensis* extract on TPA induced ODC activity and thymidine incorporation in DNA. There was 5.76-fold increase in ODC activity and 2.3-fold increase in thymidine incorporation in DNA on treatment of animals with single topical application of TPA on mice skins. The activity of ODC was also statistically significantly restored in case of pretreatment with *H. rosa sinensis* extract in a dose dependent manner. Besides, the incorporation of [³H] thymidine into DNA was suppressed by 98-122% dose dependently.

Figure 3 (a & b) shows the effect of pretreatment of *H. rosa sinensis* extract on two-stage skin carcinogenesis. The first tumor appeared in the 7th week following which the number of tumors per mouse were recorded each week for 25 weeks. In anti-promotion study groups, the appearance of first tumor was delayed from 10 to 11 weeks dose dependently. The percentage of tumor bearing mice decreased from 50 & 40% and the total number of tumors/mouse at 25 weeks decreased from 3.5 & 2.9 in *H. rosa sinensis* extract treated group in a dose dependent
manner. As regards the morphology, the lesions that were darker in appearance, polypoidal and were considered as squamous cell carcinoma.

Figure 4 depicts the histopathological changes of the effect of pretreatment of *H. rosa sinensis* extract on two-stage skin carcinogenesis in mice. The photographs of DMBA and croton oil treated group revealed the development of squamous cell carcinomas. Histologically, intradermal proliferation with keratin pearls, that are characteristic feature of squamous cell carcinoma, were observed. While, in case of anti-promotion study with *H. rosa sinensis*, the occurrence of squamous cell carcinoma was very less, rather commonly seen was the formation of verruca with hyperkeratosis and papillomatosis. In the higher dose group most common was the occurrence of mild hyperplasia as against squamous cell carcinoma in case of DMBA and croton oil treated group. Morphologically also, the size of the tumors were drastically decreased as noticed by personal observation and the occurrence of tumors in the anti-tumor promotion group was minimum.

DISCUSSION

Carcinogenesis is basically a multi-step process involving three stages - initiation, promotion and progression. There is a substantial amount of evidence that indicates a key role of free radicals in all these stages of cancer (Sun 1990, DiGiovanni 1992, Cerutti 1994). To combat the free radicals, body has its own line of defense, which includes naturally occurring, reduced glutathione, antioxidant enzyme armory and detoxifying enzymes (Chaudière and Ferrari-Iliou 1999). Nevertheless, there are innumerable reports, which show that exposure to exogenous agents and to those, which generate free radicals result in a sharp decrease in the level and the activities of biochemical parameters of oxidative damage (Nakamura et al 1999, Saleem et al 1999, Sharma et al 2002) and that this damage may be implicated in one of the many etiologies

Since prevention is better than cure, therefore, it is advisable to bring in the use of those agents/compounds that have the ability to intervene or delay the process of disease formation. In recent years, ample evidence has been gathered which emphasizes the importance of chemoprevention (Wattenberg 1992a and b) to reduce the risk of any disease. Based on animal experimentations and epidemiological data new chemopreventive agents have been used to prevent different malignancies (Morse and Stoner 1993, Birt et al 1997, Goodman 1997, Lipkin M). These chemopreventive agents belong to any of the categories ranging from fruits, vegetables, beverages and single compounds to medicinal plants (Block et al 1992, Singh and Shukla 1998, Tanaka et al 2000). Plants, in particular, have been a source of medicine for hundreds of years, and their products continue to play an essential role in medicine. The use of different medicinal plants in the prevention of cancer has been very well elucidated (Tanaka et al 2000, Saleem et al 2001). Many effective anticancer agents that are currently in use are derived from plant sources. Vinblastine, vincristine, etoposide, teniposide, and paclitaxel are a few phytochemicals that are important botanically obtained commercial anticancer drugs (Cragg et al 1997). Podophyllotoxin, an antineoplastic agent, obtained from Podophyllum is highly effective against a number of tumors like lung metastatic tumors, malignant lymphoma, and carcinoma of nasopharynx (Utsugi et al 1996). Ipomeanol, a furan derivative produced by sweet potatoes (Ipomoea batatas, Convolvulaceae) has been in clinical trials for treatment of lung cancer (Kinghorn and Balan-drin 1993).

H. rosa sinensis extract possesses numerous compounds which include quercetin, its glycosides, riboflavin, niacin, carotene, malvalic acid gentisic acid, margaric acid and lauric
Chapter 5  Hibiscus rosa sinensis

acid (Ross 1999). Many of these compounds have proven their potential as antioxidants and potent protective agents against cancer of different organs (Makita et al. 1996, Hennekens et al. 1984, Weisburger 1991, Woutersen et al. 1999, Block et al. 1992). All these components appear to have biological activities that might play a crucial role in the chemopreventive activity of the plant. Flavonoids have been shown to exert antioxidant, anti-inflammatory, antitumor, antiproliferative and antiviral effects. For example, quercetin prevents against gastrointestinal cancer (Van Der Logt et al 2003), cervical neoplasia induced by 20-methyl cholanthrene in virgin Swiss albino mice possibly by boosting the antioxidant enzyme status (De et al 2000). It has been shown to inhibit several important steps of angiogenesis including proliferation (Tan et al 2003). β-carotene and lauric acid are also believed to possess antioxidant activities (Lee and Park 2003, Henry et al 2002). Beta-carotene exhibits the fastest singlet oxygen quenching in membrane system (Cantrell et al 2003).

Gentisic acid also possesses similar type of properties like anti-oxidant and anti-inflammatory activities, besides it has been studied at length and is described in one of the subsequent chapters. The results of the present study show that pretreatment with H. rosa sinensis extract restored intracellular antioxidant, reduced glutathione and antioxidant enzymes together with inhibition of lipid peroxidation and hydrogen peroxide generation in a dose dependent manner, in both the cases i.e. with BPO plus UVB, and TPA. This indicates the role of modulatory activity of H. rosa sinensis extract since antioxidant enzymes like catalase and glutathione peroxidase, which have a direct role in scavenging free radicals are also restored by the plant extract. Parallel to these effects, there occurred reduction in the peroxidation of lipids and formation of hydrogen peroxide content. Inhibition of hydrogen peroxide implies that further generation of hydroxyl radicals is also reduced which protects the cells by making them less
prone to the attack by these damaging moieties.

Further confirmation of its protective effect comes from the long-term studies carried out on Swiss mice. In this experiment, *H. rosa sinensis* extract protected against tumorigenic response of DMBA initiated and croton oil promoted mice skin since it decreased the number of tumors/mouse and percentage of mouse with tumors in anti-promotion studies at two different doses. Also, the occurrence of first tumor was delayed in the plant treated group. Besides, the protective effect of *H. rosa sinensis* extract was seen and verified histologically as shown in Figure 4. Although, the anti-initiation study with the plant extract was not very successful (data not shown) the extract of *H. rosa sinensis* showed convincing results in anti-tumor promotion study (Sharma et al 2003). The traditional system of medicine has been using this plant for quite some time now for the treatment of cancerous swellings even though the exact mechanism of its action is not very clear (Ross 1999). The data of the present study gives an insight into the mechanism of action of the anti-tumor activity of *H. rosa sinensis* extract against cancer since *H. rosa sinensis* was able to inhibit not only BPO plus UVB induced but also TPA induced unscheduled DNA synthesis, ODC activity and oxidative challenge in mouse skin model system. ODC catalyses the conversion of ornithine to putrescine that is involved in the DNA synthesis. Putrescine, in plants and microorganisms can be produced by decarboxylation of arginine while all the mammalian cells lack arginine decarboxylase and produce putrescine exclusively from ODC (Pegg 1988). Thus, ODC activity is required for permitting the synthesis of polyamines that in turn are required for cell division. Tumor promoters like TPA cause a transient increase in ODC activity, which is mediated by activation of protein kinase C leading to an increased ODC mRNA and protein (Pegg 1988). TPA mediated mechanism of ODC induction depends to a large extent on the accumulation of
prostaglandins, lipoxygenase products and free radical production (Pegg 1988). And inhibitors of ODC induction are also the inhibitors of carcinogenesis, for example tamoxifen (Sporn and Su 2000).

Therefore, we conclude that part of the antitumor activity of \textit{H. rosa sinensis} extract may be derived from its strong antioxidant properties and its ability to exert modulatory influence on the marker parameters of tumor promotion. Since \textit{H. rosa sinensis} extract exhibits excellent power of protection against tumor promotion other mechanisms might also be involved simultaneously. Thus, we suggest that it should be tested elaborately against cancer of different organs.
Table 1. Prophylactic effect of *Hibiscus rosa sinensis* extract on BPO treated and UVB-irradiated 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 (nmols dichloroindophenol reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>32.35 ± 1.59</td>
<td>93.34 ± 9.07</td>
<td>202.72 ± 24.27</td>
<td>226.99 ± 12.08</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>19.48 ± 0.79*</td>
<td>60.52 ± 1.85**</td>
<td>133.30 ± 6.14*</td>
<td>172.06 ± 2.84**</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>24.63 ± 1.92*</td>
<td>76.98 ± 6.56*</td>
<td>161.85 ± 8.26*</td>
<td>202.44 ± 10.27*</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>28.67 ± 1.88**</td>
<td>80.72 ± 6.11*</td>
<td>173.05 ± 12.35*</td>
<td>210.94 ± 8.37**</td>
</tr>
<tr>
<td>Only <em>H. rosa sinensis</em> (D2)</td>
<td>36.39 ± 1.37</td>
<td>84.11 ± 16.32</td>
<td>188.78 ± 16.62</td>
<td>223.39 ± 6.12</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. **P < 0.01 and *P < 0.05 compared with the corresponding value for acetone treated control group. ***P < 0.01 and *P < 0.05 compared with corresponding value for BPO and UVB treated group.
Table 2. Depicts effect of pretreatment of *Hibiscus rosa sinensis* extract on BPO treated/ UVB-irradiated reduction in cutaneous antioxidant enzymes in Swiss mice

<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>93.37 ± 7.71</td>
<td>154.35 ± 14.30</td>
<td>91.28 ± 10.77</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>60.75 ± 1.88**</td>
<td>88.36 ± 5.26**</td>
<td>56.45 ± 5.32*</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>72.42 ± 4.31*</td>
<td>97.99 ± 8.09NS</td>
<td>69.35 ± 2.03*</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m²/s)</td>
<td>81.31 ± 7.21*</td>
<td>106.53 ± 6.17*</td>
<td>74.22 ± 3.95*</td>
</tr>
<tr>
<td>Only <em>H. rosa sinensis</em> (D2)</td>
<td>102.41 ± 11.33</td>
<td>127.42 ± 2.97</td>
<td>112.83 ± 7.66</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E., n = 6. **P < 0.01 and *P < 0.05 compared with the corresponding value for acetone treated control group. *P < 0.05 compared with corresponding value for BPO and UVB treated group.
Table 3. Study on effect of *Hibiscus rosa sinensis* extract pretreatment on BPO treated/ UVB-irradiated elevation of lipid peroxidation and hydrogen peroxide content 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 H$_2$O$_2$/gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>2.38 ± 0.33</td>
<td>251.50 ± 24.36</td>
</tr>
<tr>
<td>BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m$^2$/s)</td>
<td>5.42 ± 0.51***</td>
<td>412.39 ± 13.79***</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D1) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m$^2$/s)</td>
<td>4.72 ± 0.26 NS</td>
<td>359.29 ± 12.18*</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D2) + BPO (20mg / 0.2ml acetone / animal) + UVB (0.42 J/m$^2$/s)</td>
<td>4.16 ± 0.24*</td>
<td>287.42 ± 15.83**</td>
</tr>
<tr>
<td>Only <em>H. rosa sinensis</em> (D2)</td>
<td>2.64 ± 0.41</td>
<td>240.56 ± 11.13</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. **$p < 0.001$ and $p < 0.05$ compared with corresponding value for BPO and UVB treated group.
Table 4. The effects of pretreatment with *Hibiscus rosa sinensis* extract on TPA mediated depletion in reduced glutathione content and glutathione metabolizing 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>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (0.2ml / animal)</td>
<td>27.02 ± 1.56</td>
<td>60.21 ± 1.17</td>
<td>237.61 ± 17.98</td>
</tr>
<tr>
<td>Only TPA (20nmol / 0.2ml acetone / animal)</td>
<td>13.78 ± 0.36***</td>
<td>27.33 ± 1.05***</td>
<td>130.05 ± 11.08***</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D1) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>20.22 ± 0.63***</td>
<td>33.43 ± 2.06 NS</td>
<td>162.79 ± 9.4*</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D2) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>23.53 ± 0.99***</td>
<td>46.68 ± 1.04***</td>
<td>186.64 ± 12.69**</td>
</tr>
<tr>
<td>Only <em>H. rosa sinensis</em> (D2)</td>
<td>28.49 ± 0.60</td>
<td>61.19 ± 3.23</td>
<td>255.22 ± 4.54</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 <0.01, *P < 0.05 and *P < 0.05 compared with corresponding value for treatment with TPA alone.
Table 5. Prophylactic effect of *Hibiscus rosa sinensis* extract on TPA mediated reduction 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>73.45 ± 5.40</td>
<td>196.41 ± 11.11</td>
<td>58.00 ± 1.44</td>
<td>1.73 ± 0.26</td>
<td>290.55 ± 27.1</td>
</tr>
<tr>
<td>Only TPA (20nmol / 0.2ml acetone / animal)</td>
<td>25.89 ± 1.06***</td>
<td>116.74 ± 7.83***</td>
<td>29.75 ± 0.70**</td>
<td>3.29 ± 0.13**</td>
<td>454.57 ± 30.3***</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D1) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>38.62 ± 3.49**</td>
<td>148.54 ± 10.4*</td>
<td>34.95 ± 1.08**</td>
<td>2.75 ± 0.09*</td>
<td>368.65 ± 19.94*</td>
</tr>
<tr>
<td><em>H. rosa sinensis</em> (D2) + TPA (20nmol / 0.2ml acetone / animal)</td>
<td>44.57 ± 1.89***</td>
<td>158.89 ± 9.20**</td>
<td>47.40 ± 3.74***</td>
<td>2.42 ± 0.11***</td>
<td>323.36 ± 13.81***</td>
</tr>
<tr>
<td>Only <em>H. rosa sinensis</em> (D2)</td>
<td>80.83 ± 7.93</td>
<td>186.88 ± 8.27</td>
<td>58.40 ± 5.75</td>
<td>1.48 ± 0.18</td>
<td>224.94 ± 16.62</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 TPA alone.
FOOTNOTES AND FIGURE LEGENDS

Figure 1. Effect of pretreatment of animals with *H. rosa sinensis* extract on BPO and UVB induced ODC activity and $[^3]H$ thymidine incorporation in mice skin

Each value represents mean ± SE of six animals. ### $p<0.001$ as compared to acetone treated negative control group. ### $p<0.001$ when compared with BPO treated and UVB irradiated group. D-1 and D-2 represent topical applications of 3.5mg and 7.0mg *H. rosa sinensis*/Kg/body weight.

Figure 2. Role of *H. rosa sinensis* extract pretreatment on 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 acetone treated control group. ### $p<0.001$ when compared with TPA treated group. D1 and D2 represent topical applications of 3.5mg and 7mg of *H. rosa sinensis*/Kg/body weight.

Figure 3 (a & b) shows the effect of pretreatment of *Hibiscus rosa sinensis* extract on number of tumors per mouse and percentage of mice with tumors

Figure 4 depicts the histopathological changes of the effect of pretreatment of *Hibiscus rosa sinensis* extract on two-stage skin carcinogenesis in mice

Fig. 4a – Section showing histology of acetone treated normal skin tissue

Fig. 4b – Section showing histopathology of DMBA/croton oil treated mice skin depicting squamous cell carcinoma with characteristic cell nests (epithelial pearls) depicting hyperkeratinization in the center

Fig. 4c – Depicting verruca formation with hyperplasia and keratinization at 3.5 mg/Kg body weight of *H. rosa sinensis* extract.

Fig. 4d – Depicting verruca formation with hyperkeratinization at 3.5 mg/Kg body weight of *H. rosa sinensis* extract.

Fig. 4e – Showing hyperplastic epidermis and inflamed dermis in skin tissue of anti-tumor promotion group animals at 7.0 mg/Kg body weight of *H. rosa sinensis* extract.

Fig. 4f – Showing marked hyperplasia of the epidermis in anti-tumor promotion group animals pretreated with 7.0 mg/Kg body weight of *H. rosa sinensis* extract.
Figure 1

Effect of pretreatment of animals with *H. rosa sinensis* extract on BPO and UVB induced ODC activity and [³H] thymidine incorporation in mice skin.

**ODC activity (pmol ¹⁴CO₂ released/hr/mg protein)**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>ODC activity (pmol ¹⁴CO₂ released/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>2500</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>1500</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
</tr>
</tbody>
</table>

**[^H] thymidine incorporation DPM/µg DNA**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>[³H] thymidine incorporation DPM/µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>350</td>
</tr>
<tr>
<td>4</td>
<td>300</td>
</tr>
<tr>
<td>5</td>
<td>250</td>
</tr>
</tbody>
</table>

- Only Acetone
- BPO+UVB
- *H. rosa sinensis* (D1)+BPO+UVB
- *H. rosa sinensis* (D2)+BPO+UVB
- Only *H. rosa sinensis* (D2)
Figure 2
Role of *H. rosa sinensis* extract pretreatment on TPA induced ODC activity and $[^3H]$ thymidine incorporation in Swiss mice

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

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

**Treatment groups**
1. Only Acetone
2. Only TPA
3. *H. rosa sinensis* (D1) + TPA
4. *H. rosa sinensis* (D2) + TPA
5. Only *H. rosa sinensis* (D2)

Significance Levels:
- # # #: Significant at the 0.001 level
- ***: Significant at the 0.001 level
- **: Significant at the 0.01 level
Chapter 5

Hibiscus rosa sinensis

Figure 3 (a & b)
Effect of pretreatment of animals with Hibiscus rosa sinensis extract on croton oil mediated skin tumor promotion in DMBA initiated mice

(a)

(b)

Percentage of mice with tumors

Weeks on test

Number of tumors/mouse

Weeks on test

- DMBA + Croton oil
- DMBA + H. rosa sinensis (D1) + Croton oil
- DMBA + H. rosa sinensis (D2) + Croton oil
Figure 4 Depicts the histopathological changes of the effect of pretreatment of *Hibiscus rosa sinensis* extract on two-stage skin carcinogenesis in mice.

**Fig. 4a** – Section showing histology of acetone treated normal skin (20X)

**Fig. 4b** – Section showing histopathology of DMBA and croton oil treated mice skin depicting squamous cell carcinoma (10X)
Fig. 4c – Depicting verruca formation at lower dose of *H. rosa sinensis* extract (20X).

Fig. 4d – Depicting verruca formation at lower dose of *H. rosa sinensis* extract (40X).
Fig. 4e – Showing hyperplastic epidermis and inflamed dermis in skin tissue of anti-tumor promotion group animals at the higher dose of *H. rosa sinensis* extract (20X).

Fig. 4f – Showing marked hyperplasia of the epidermis in anti-tumor promotion group animals pretreated with the higher dose of *H. rosa sinensis* extract (40X).