ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

CHEMOPREVENTIVE ACTION OF *SPHAERANTHUS INDICUS* ON DMBA-INDUCED SKIN PAPILLOMAGENESIS IN MICE

Shiksha Gadekar[1], Sadhna Goyal[1], Azaz Khan[2], Megha Jha*[2]

[1]*Department of Chemistry, Govt. M.V.M. College, Bhopal, Madhya Pradesh, India*

[2]*Pinnacle Biomedical Research Institute (PBRI), Near, Bharat Scout and Guides Campus, Shanti Marg, Shyamla Hills Road, Bhopal, Madhya Pradesh, India*

**Corresponding author: meghajhabtbpl@gmail.com*

ABSTRACT

Cancer has emerged as a major health problem globally as a consequence to the increased longevity of the population, changing the environment and life style. Chemoprevention is a new and promising strategy for reducing cancer burden. Recently, some natural products have been identified for their chemopreventive activity to reduce the cancer incidence. *Sphaeranthus indicus* (*S. indicus*) is known for its potential to treat various ailments in human beings.The present study was designed to explore the anticancer and antioxidative potential of *S. indicus* seed extract (SIE) against chemical-induced skin carcinogenesis in mammals. Skin tumors were induced in Swiss albino mice by a single topical application of 7, 12-dimethylbenz(a)anthracene (100µl acetone), followed by 1 % Croton oil was applied on skin 2 times a week up to 16 weeks. In contrast, mice treated with the *S. indicus* extract (200 and 400 mg/kg/b.wt./animal/day)- DMBA single application was given afterwards the 100 µl dose of *S. indicus* extract at the dose was given one hour before each application of 1% croton oil 2 times a week up to 16 weeks demonstrated significant reduction in tumor incidence, tumor yield and tumor burden, as compared to the 7,12-dimethylbenz(a) anthracenee croton oil-treated control group.Further, biochemical assays revealed a significant enhancement in the levels of reduced glutathione, superoxide dismutase and catalase but a significant reduction in lipid peroxidation levels in both the liver and skin with SIE treatment, as compared to carcinogen-treated control group. These results suggest that *S. indicus* has the potential to become a pivotal chemopreventive agent that can reduce cancer in mammals.

Keywords: Cancer, *Sphaeranthus indicus,* Chemical carcinogenesis, Chemoprevention, Oxidative stress

1. INTRODUCTION

Epithelial or skin carcinogenesis involves a multistep process proceeded by initiation, promotion and progression of carcinogens. The interaction of carcinogen and accumulation of genetic events within stem cells lead to a progressively dysplastic cellular appearance and deregulated cell growth or differentiation, activating the oncogenes and finally resulting in skin carcinogenesis [1]. Skin carcinogenesis is the most commonly diagnosed, surpassing lung, breasts, colorectal, prostate, etc. It is initiated as pre-cancerous lesions with environmental toxins playing a very crucial role in the development of skin carcinogenesis [2]. It is a major and growing public health-related problem among all new carcinogenic cases diagnosed annually in the world with almost one-third cases originating in the skin. In India, skin carcinogenesis accounts for 1-2% of all carcinogenesis as the majority of the population of the country receive high amounts of UV radiation [3]. Reactive oxygen species (ROS) are the by-products generated during the respiratory and metabolic reactions in our body. These species have both positive and negative impacts on our body depending on their concentrations: at low levels, these molecules have important roles in signal transduction, whereas higher levels of ROS can cause oxidative stress and tissue destruction. In cancer cases, the cells generate reactive molecules rapidly to accelerate their growth, so these molecules can act as a cancer marker. We need to discover products that can scavenge these free radicals without affecting our normal cells [4]. Besides the advancement in cancer treatment techniques, there should be a preventive approach that is effective and safer. Chemoprevention is the administration of natural or synthetic compounds to prevent, slow down, and reverse the occurrence of cancer. Various civilizations

across the world still rely on herbal medicines as the first line of treatment. Owing to their safety, low toxicity, antioxidant properties, cost effectiveness, and general acceptance (as dietary supplements, fruits, vegetables, phytochemicals, and minerals), these are being investigated for the prevention of cancer. Extensive research over the past few decades has identified numerous dietary and botanical natural compounds that have anticancerous properties [5-7]. DMBA (7, 12-dimethylbenz (a) anthracene) is a polycyclic aromatic hydrocarbon, acts as a pro-carcinogen and is an ultimate carcinogen after metabolic activation. It is widely used as an initiator as well as a promoter to induce skin carcinogenesis in rodents. Therefore, DMBA is commonly employed to study the chemopreventive potential of natural and synthetic entities [8].Liver is the primary site for biotransformation of xenobiotics and for detoxification process. These detoxifying agents perform a crucial role in the metabolic activation and excretion of carcinogenic metabolites. Measurement of the status of these agents in liver helps to test the chemopreventive efficacy of natural and synthetic entities. Studies have documented that when cells are exposed to carcinogens, the detoxification cascade is stimulated [9]. A large number of plant species used in folk medicine have been used since the earliest days of humanity and have considerable importance in international trade [10]. *Sphaeranthus indicus* Linn (Asteraceae) is a multi-branched aromatic herb 1-2 feet in height, distributed commonly in plains all over India and up to an altitude of 50 feet in hills [11]. In Ayurvedic system of medicine, the whole herb is used in insanity, anaemia, piles, asthma, leukoderma, indigestion, bronchitis, spleen diseases, elephantiasis dysentery, vomiting and urinary discharges [12]. The whole herb is used in Ayurvedic preparations to treat mental disorders and epilepsy [13-15]. Hot water extract of the herb is used as an anthelminitic, fish poison, diuretic, and as an aphrodisiac [16-18]. Flowers are used in conjunctivitis [17]. The external application of a paste of this herb is useful in treating pruritus and edema, gout, arthritis, filariasis and cervical adenopathy [19]. The plant is traditionally used for diarrhea [20]. The major constituents of *S. indicus* include methyl chavicol, δ-cadinene, α-ionone, para-methoxycinnamaldehyde, α-terpinene, citral, geraniol, geranyl acetate, β-ionone, oscimene, eugenol, sphaeranthene, sphaeranthol, estragole, and indicusene [21, 22]. Owing to the presence of a wide range of medicinal constituents in *S. indicus*, the present study was designed to evaluate the antioxidative and antitumorigenic potential of this plant extract in a mammalian model using a two-stage skin carcinogenesis protocol.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

DMBA, croton oil, reduced glutathione and nicotinamide adenine dinucleotide, and 1, 1', 3, 3'-tetramethoxypropane were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Heparin, thiobarbituric acid (TBA), trichloroacetic acid, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis (2-nitro benzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), nitro blue tetrazolium (NBT) and Phenazine methosulphate (PMS) were purchased from Hi-Media Laboratories, Mumbai, India. DMBA was dissolved at a concentration of 100μg/100μl in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.All other chemicals and solvents used were of analytical grade.

2.2. Plant material

Seeds of *S. indicus* were collected from Pinnacle Biomedical Research Institute (PBRI), Near, Bharat Scout and Guides Campus, Shanti Marg, Shyamla Hills Road, Depot Chouraha, Bhopal, Madhya Pradesh 462003, India. The identification and authentication of plant was done by Dr. Saba Naaz, Botanist, from the Department of Botany, Saifia College of Science and Bhopal. A voucher specimen number 189/Saif./Sci./Clg/Bpl. was kept in Department of Botany, Saifia College of Science, Bhopal for future reference.

2.3. Hot soxhlet extraction method

In this method, the seeds of *S. indicus* were collected, washed and rinsed properly. They were dried in shade and powdered mechanically. The whole or coarsely powdered plant material of *S. indicus* was successively extracted by solvent like petroleum ether, chloroform and methanol in increasing polarity order for different period of time (6h, 8h, and 10h). The powder was placed "thimble" in chamber of the Soxhlet apparatus. In flasks the extracting solvent was heated, and its vapours get condensed in condenser. The condensed extractant drops into the thimble containing the powder, and extracts it by contact. When in chamber the level of liquid reaches to the top of siphon tube, the liquid contents of chamber siphon drop into flask. This process was continuous and was carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The extract thus obtained were filtered and concentrated to dryness, weighed and stored

for further use [23]. On the basis of qualitative and quantitative phytochemical analysis methanolic extract was selected for antioxidative and antitumorigenic potential.

2.4. Animals

Random bred 30Swiss albino mice (3-5 weeks old, weighing 20-30 gm) were obtained from the animal house of Pinnacle Biomedical Research Institute (PBRI), Bhopal, India. They were maintained under standard laboratory conditions of temperature (22±2°C), humidity (55±5%) and 12:12 hours light and dark cyclewas maintained in the animal house and fed with standard pellets (Golden Feeds, New Delhi, India) and water was available *ad libitum*. The mice were divided into different treatment groups randomly and kept in propylene cage with sterile husk as bedding.Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1824/PO/ERe/S/15/CPCSEA). Protocol Approval Reference No. PBRI/IAEC/2019/12-21-008).

2.5. Acute oral toxicity

Acute toxicity study of the prepared seed extracts of *S. indicus* was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423 [24] the animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, The dose level to be used as the starting dose is selected from one of four fixed levels 5, 50, 300 and 2000 mg/kg body weight. Acute toxicity was determined as per reported method [25].

2.6. Experimental design

A total of 30 animals were unevenly divided into subsequent 5 groups to determine the anti-carcinogenic potential of SIE against DMBA-induced skin papillomagenesis in mice. Three days before the beginning of the experiment, hair on the interscapular region of the mice were clipped. For the study, only the mice in the resting phase of the hair cycle were considered. Until the termination of experiment body weights of the animals were recorded weekly.

Group-I: Drug (SIE) treated Control: Animals received SIE (200 mg/kg/b. wt./animal/day) by oral gavage alone during the experimental period. For tumor induction, the animals were not served with DMBA and croton oil protocol.

Group-II: Carcinogen treated (Positive Control): Mice of this group were treated with a single dose of DMBA (100 µg/ 100 µl of acetone) over the shaven area of the skin.

Group-III: Promoter: After two weeks, until the end of experiment croton oil (1% v/v in acetone) was applied three times per week. For 16 weeks this group acquire double distilled water (DDW) equivalent to SIE (100 µl/ mouse) by oral gavage.

Group-IV: SIE treated (Experimental 1): These experimental animals received the same treatment as in Group-II and also received SIE at a dose of 200 mg/ kg body wt. / animal/ day, orally for 7 days before and 7 days after DMBA application.

Group-V: SIE treated (Experimental 2): Animals of this group received the same treatment as in Group-II and were administered SIE (400 mg/kg b. wt. / animal/ day) by oral gavage, starting from the time of croton oil treatment till the end of experiment (i.e., 16 weeks).

The following morphological parameters were studied in Groups I -V:

1. Tumor incidence: The number of mice having at least one tumor expressed as a percentage incidence.
2. Tumor yield: The average number of papillomas per mouse.
3. Tumor burden: The average number of tumors per tumor bearing mouse.
4. Diameter: The diameter of each tumor was calculated.
5. Weight: The weight of the each tumor was measured which appeared in animals at the ending of each experiment.
6. Body weight: The weights of the mice were measured weekly.
7. Average latent period: The time period between the administration of the promoting agent and the appearance of 50% of tumors was evaluated. After the application of the promoting agent the average latent period was determined by multiplying the number of tumors appearing each week with the time in weeks and dividing the sum by total number of tumors.
8. Inhibition of tumor multiplicity = (Total no. of papillomas in carcinogen control) - (total no. Papillomasin treated) X 100/Total no of papillomas in carcinogen control [26].

2.7. Biochemical study

The animals from all the groups were sacrificed by cervical dislocation 16 wks after the commencement of treatment, and their liver and dorsal skin that were affected by tumors were quickly excised and washed thoroughly with chilled 0.9% NaCl (pH 7.4). Both of the tissues (liver and skin) were then weighed and blotted dry. A 10% tissue homogenate was prepared from the part of the tissue sample in 0.15M Tris-KCL (pH 7.4) to estimate the reduced glutathione and LPO levels.

2.7.1. Reduced glutathione

The level of GSH was estimated as total nonprotein sulfhydryl group by the method of Moron *et al.* [27]. Free endogenous-SH was assayed, and the absorbance was recorded at 412 nm using an UV-VIS Systronics spectrophotometer. Reduced GSH was used as a standard. The levels of GSH were expressed as mmol/g of tissue.

2.7.2. Lipid peroxidation

The LPO level was calculated spectrophotometrically by the thiobarbituric acid reactive substances method, as described by Ohkhawa *et al.* [28]. The optical density of LPO was observed at 532 nm, and the content of thiobarbituric acid reactive substances was expressed as nmol/mg of tissue.

2.7.3. Superoxide dismutase

Superoxide dismutase (SOD) level was determined according to the method of Marklund and Marklund [29] by quantification of pyrogallol auto-oxidation inhibition, and the results were expressed as units/mg protein. Auto-oxidation of pyrogallol in Tris- HCL buffer (50mM, pH 7.5) was measured by the increase in absorbance at 420 nm.

2.7.4. Catalase

Catalase activity was measured by the method of Aebi [30]. Phosphate buffer (50mM) was used for homogenate preparation and centrifuged at 4307 g for 10 min. The change in absorbance was observed spectrophotometrically at 240 nm. The activity of the enzyme was expressed as U/mg of tissue, where U was mmol of H_2O_2 disappearance/min.

2.8. Statistical analysis

All the data concerning anti-cancer study are expressed as mean ± SD. Statistical analysis was carried out by one-way ANOVA followed by Dunnett's using the "GraphPad-Prism" statistic computer program (GraphPad InStat; Version 3.05). A difference in the mean values of $p<0.05$ was considered statistically significant.

3. RESULTS

To determine the safety of *S. indicus*for human use, toxicological evaluation is carried out in experimental animals. In the acute toxicity study, no signs of toxicity were found upto the dose of 2000 mg/kg body weight. Hence1/10th and 1/5th doses *i.e.*200 mg/kg and 400 mg/kg have been fixed as ED50 for present study Table 1.

Table 1: Acute oral toxicity of *S. indicus* extract

Groups	Observations/ Mortality
5 mg/kg Bodyweight	0/3
50 mg/kg Bodyweight	0/3
300 mg/kg Bodyweight	0/3
2000 mg/kg Bodyweight	0/3

A gradual increase in body weight of mice was noted in both the experimental groups (Group IV & Group V), while the similar increase was not evident in carcinogen treated control animals (Group II) (Table 2). The gain in the body weight in mice might be induced due to the administration of *S. indicus* extract (SIE).Animals of both the groups survived throughout the experimental period. Oral administration of SIE during peri- (Group IV) and post- (Group V) initiational stages of DMBA-induced skin papillomagenesis, significantly reduced the tumor yield and tumor burden to 3.17 and 2.33 in both the experimental groups (positive control value 5.3), while the cumulative numbers of papillomas were reduced to 28 and 24 respectively (positive control value 68). Furthermore, the size of papillomas in both the positive control (Group II) and experimental mice (Groups IV &V) also varied significantly table 3. The mice assorted in Groups II-V and given two stage protocol for tumor inductionrevealed 100% and 83.33% (Groups IV & V) skin papillomas while the respective figure for positive control (Group II) was 100%. The average papilloma weight of the control was 115 mg, whereas it was only 62.3 and 48.0 mg for both the SIE treated group at 16 weeks. The maximum inhibition of multiplicity of papillomas was occurred in SIE treated groups. No tumor development was recorded throughout the experiment in the animals treated orally with *S. indicus* seed extract.

Biochemical parameters like LPO, SOD, GSH and CAT were calculated by the screening the liver and skin obtained from the treated animals. In the present study results of methanolic extract of *S. indicus* showed that lipid peroxidation level was found to be significantly reduced in DMBA induced animals in rats whereas superoxide dismutase, catalase, and GSH showed significant increasing levels of antioxidant effect Table 4.

Table 2: Effect of *S. indicus* on DMBA/croton oil-induced carcinogenesis in mice on change in body weight

S. No.	Treatment group	0th day	4th day	8th day	12th day	16th day
I	Control	24.16±1.80	27.87±2.432	30.36 ± 2.44	32.78±2.71	34.06±2.783
II	DMBA alone	25.24±2.013	28.32±1.901	30.11±1.952	30.85±2.091	29.00±2.363
III	DMBA + Croton	24.38±2.653	27.03±1.997	28.99±1.842	32.74±1.941	32.06±0.460
IV	DMBA + Croton oil + extract 200mg/kg	23.73±2.139^{ns}	25.53±2.969^{ns}	28.89±2.576^{ns}	31.45±2.55^{ns}	33.18±2.078^{ns}
V	DMBA + Croton oil + extract 400mg/kg	23.93±2.459^{ns}	28.21±2.499^{ns}	31.651±2.204^{ns}	34.80±1.868^{ns}	37.265±1.852**

*Values are expressed as MEAN±SD at n=6, One way ANOVA followed by Bonferroni test, *P<0.050, **P<0.001 and ^{ns}P>0.001 compared to the Group III*

Table 3: Effect of *S. indicus* extract on tumor yield, tumor burden and % incidence

Treatment Group	Tumor Yield	Tumor Burden	% Incidence
Control	0	0	0
DMBA alone	5.3	5.3	100%
DMBA + Croton	6.17	6.17	100%
DMBA + Croton oil + extract 200mg/kg	3.17	3.17	100%
DMBA + Croton oil + extract 400mg/kg	2.33	2.33	83.33%

Table 4: Effect of *S. indicus extract* on the anti-oxidant enzymes

Groups	LPO (nmol MDA/mg tissue)	SOD (unit/mg tissue)	GSH (mmol/mg tissue)	CAT (mg tissue / ml enzyme)
Control	12.166 ± 0.577	93.47 ± 9.083	7.086 ± 0.699	29.33 ± 0.126
DMBA alone	35.05 ± 1.645	30.11 ± 6.401	1.327 ± 0.04	15.34 ± 0.023
DMBA + Croton	40.11 ± 6.703	23.08 ± 3.961	0.802 ± 0.048	10.52 ± 0.561
DMBA + Croton oil + extract 200mg/kg	19.75 ± 2.026**	70.95 ± 4.991**	4.576 ± 0.054**	17.95 ± 0.510**
DMBA + Croton oil + extract 400mg/kg	15.36 ± 0.439**	82.45 ± 8.900**	6.554 ± 0.982**	23.63 ± 0.991**

*Values are expressed as MEAN±SD at n=6, One way ANOVA followed by Bonferroni test, *P<0.050, **P<0.001 and ^{ns}P>0.001 compared to the Group III*

4. DISCUSSION

Carcinogenesis is a multi-step process exemplified by initiation, promotion, and progression steps in which genetic and epigenetic events determine the neoplastic conversion of normal cells [31]. The polycyclic aromatic hydrocarbon 7, 12-dimethylbenz (a)-anthracene (DMBA) can act as a complete carcinogen or an initiator of mouse skin carcinogenesis [32, 33]. It is well

established that promotion with TPA produces oxidants and oxidatively damaged macromolecules [34, 35]. On the other hand, the activity of xanthine oxidase, an enzyme capable of generating superoxide radicals, was noted to be increased in mice treated with TPA [36]. Previous reports suggest that 12-Otetradecanoylphorbol- 13-acetate (TPA) promotes an enhanced release of reactive oxygen species (ROS), induction of epidermal ornithine decarboxylase (ODC), and over expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) proteins [37, 38]. Cancer chemoprevention is a new approach in cancer prevention, in which chemical agents are used to prevent cancer in normal and/or high-risk populations. Chemoprevention aims to halt or reverse the development and progression of precancerous cells through the use of noncytotoxic nutrients and: or pharmacological agents during the time period between tumor initiation and malignancy [39]. Over millions of years, plants have developed the capacity to synthesize a diverse array of chemicals. There are many families of phytochemicals and they help the human body in a variety of ways. Phytochemicals may protect human from a host of diseases. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases. There are many phytochemicals in fruits and herbs and each works differently. The different combinations and polymers of the aforementioned form the large, diverse group of compounds known as polyphenols, which show potent antioxidant capacity and possible protective effects on human health [40]. In the current study, the animals treated with DMBA/croton oil alone showed 100% tumor incidence and high tumor yield, tumor burden and short average latent period due to their carcinogenic potential in the absence of any treatment. After the administration of SIE, a significant reduction occurred in the cumulative number of tumors and the average latent period was also prolonged. The consumption of crude plant extracts also showed a significant improvement in all biochemical parameters by restoring them to normal levels. Free radicals generated by the carcinogen lead to the deterioration of membranes and proteins by the LPO reaction. Various aldehydes, e.g., acrolein, malondialdehyde, and 4-hydroxy- 2-nonenal, are formed as secondary metabolites during the peroxidation reaction [41, 42]. In the present study, the level of increased malondialdehyde in Group II was associated with the adverse effect of carcinogen, which was found to be reduced during the SIE treatment. Administration of SIE extract at the peri- post-initiation stage was found to be most effective in reducing malondialdehyde formation during carcinogenesis. The carcinogen-treated control group was deprived of antioxidants such as GSH, SOD and catalase because these are consumed during the oxidative stress, but the SIE administration, in the present experiment, normalized the antioxidant content of the cells. The results obtained from the present study suggest that SIE has the potential to reduce oxidative stress and tumorigenesis, by restoring the antioxidative enzymes, in mammals.

5. REFERENCES

1. Krueger H, McLean D, Williams D. *Prog Exp Tumor Res,* 2008; **40**:111-121.
2. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. *Cancer J Clin,* 2001; **51**:15-36.
3. Staples MP, Elwood M, Burton RC, Williams JL, et al. *Med J Aust,* 2006; **184**:6-10.
4. Sosa V, Moline T, Somora R, Paciucci R, et al. *Age Res Rev*, 2013; **12**:376-390.
5. Mukhtar H. *Cancer let,* 2012; **326**:123-127.
6. Steward WP, Brown K. *Br J cancer,* 2013; **109**:1-7.
7. Singh R, Sharma J, Goyal PK. *Adv Pharmacol Sci,* 2014; 158936.
8. Rastogi S, Shukla Y, Paul BN, Chowdhuri DK, et al. *Toxicol Appl Pharmacol,* 2007; **224**:228-240.
9. Sindhu G, Manoharan S. *Basic Clin Pharmacol Toxicol,* 2010; **107**:818-824.
10. Ebong PK, Atangwho IJ, Eyong EU, Egbung GE. *Am J Biochem Biotechnol*. 2008; **4**:239-244.
11. Ramachandran S. *Pharmacogn Rev,* 2013; **7**:157-169.
12. Kirtikar KR, Basu BD, ICS. Indian Medicinal Plants. In: Blatter E, Caius JF, Mhaskar KS, editors. 2nd ed. Allahabad, India: Lalit Mohan Basu; 1981; **2**:1346-8.
13. Nadkarni AK. Indian Materia Medica. 3rd ed. Bombay: Popular Parkashan Private Ltd; 2007; 1:1163.
14. Prajapati N, Purohit SS, Sharma AK, Kumare TA. 1st ed. Jodhpur: Agrobios. Handbook of Medicinal plants: A complete source book; 2003:484.
15. Agarwal VS. New Delhi: Kalyani Publishers. Drug plants of India; 1997; 2:656.
16. Paranjape P. Indian Medicinal Plants. In: Paranjape P, editor. Forgotten healer: A guide to Ayurvedic

herbal medicine. Delhi: Chaukhamba Sanskrit Pratisthan; 2001:148-149.
17. Chopra RN, Nayar SL, Chopra IC. 1st ed. New Delhi: National Institute of Science Communication; 1996. Glossary of Indian Medicinal Plants; p. 232.
18. Kapoor SL, Kapoor LD. *Bull Med Ethnobot Res*. 1980; **1:**120-144.
19. Sahu TR. *Ancient Sci Life,* 1984; **3**:245-9.
20. Girach RD, Aminuddin, Siddioui PA, Khan SA. *Int J Pharmacog*, 1994; **32**:274-283.
21. Baslas KK. *Perfum Essent Oil Rec,* 1959; **50**:765-768.
22. Lodha V. *Indian Perfumer*, 2003; **47**:29-30.
23. Handa SS, Khanuja SPS, Longo G, Rakesh DD. "Extraction Technologies for Medicinal and Aromatic Plants. International Centre for Science and High Technology," Trieste, 2008; 21-25.
24. Guideline Document on Acute oral Toxicity Testing, Series on Testing and Assessment No. 423. Paris: Organization for Economic Co-Operation and Development, OECD Environment, Health and Safety Publications; 1996. Available from: http//www.oecd.org/ehs.
25. Jonsson M, Jestoi M, Nathanail AV, Kokkonen UM, et al. *Food Chem Toxicol*. 2013; **53**:27-32.
26. Girit IC, Jure-Kunkel M, McIntyre KW. *Comp Med,* 2008; **58**:264-270.
27. Moron MA, Depierre JW, Mannervick B. *Acta Biochimi Biophys Sin.* 1979; **582**:67-78.
28. Ohkhawa H, Ohishi N, Yogi K. *Anal Biochem,* 1979; **95**:351-358.
29. Marklund S, Marklund G. *European J Biochem,* 1974; **47**:469-474.
30. Aebi H. Catalase: in vitro. In: Colowick SP, Kaplan NO, editors. Method in enzymology105. New York: Academic Press; 1984; 121-126.
31. Zoumpourlis V, Solakidi S, Papathoma A, Papaevangeliou D. *Carcinogenesis*, 2003; **24**: 1159-1165.
32. Slaga TJ, Fischer S M, Weeks C E, Klein-Szanto A J P, et al.. *J Cell Biochem*, 1982; **18**:99-119.
33. Brown K, Balmain A. *Cancer Metastasis Rev*, 1995; **14**:113-124.
34. Bowden GT, Finch J, Domann F, Krieg P. Molecular mechanisms involved in skin tumor initiation, promotion, and progression. In: Mukhtar H, editor. Skin Cancer: Mechanisms and Human Relevance. CRC Press, 1995; 99-111.
35. Bowden GT, Nelson MA, Levy JP, Finch J, Krieg P. Molecular mechanisms of skin carcinogenesis induced by chemicals and ionizing radiation. In: Hecker, Jung, Marks, Tilgen, editors. Skin Carcinogenesis in Man and in Experimental Models. New York: Springer- Verlag, 1993; **128**: 309-21.
36. Pence B, Reiners JJ. *Cancer Res*, 1987; **47**:6388-6392.
37. Murakami A, Nakamura Y, Torikai K, et al. *Cancer Res*, 2000; **60**:5059-5066.
38. Seo HJ, Park KK, Han SS, et al *Int J Cancer*, 2002; **100**:456-462.
39. Alberts DS, Garcia DJ. *J Nutr*, 1995; **125**:692-697.
40. Santos-Buelga C, Scalbert A. *J Sci Food Agric*, 2000; **80**:1094-1117.
41. Niki E. *Free Radic Biol Med.* 2009; **47:**469-484.
42. Yin H, Xu L,Porter NA.*Chem Rev,*2011;**111**:5944-5947.