CHEMICAL INDUCED OXIDATIVE STRESS DURING SKIN CARCINOGENESIS AND ITS DEBILITATION BY *AEGLE MARMELOS* FRUIT EXTRACT

Annapurna Agrawal*- M.Sc. E mail: annapurna85.agrawal@gmail.com
Swafiya Jahan*- M.Sc. E mail: swafiajahan@gmail.com
Preeti Verma*- M.Sc. E mail: preetu.verma@gmail.com
P. K. Goyal*- M.Sc, Ph. D. E mail: pkgoyal2002@gmail.com

*Affiliation - Radiation & Cancer Biology Laboratory
Department of Zoology
University of Rajasthan
Jaipur-302 004, India

Corresponding Author: Dr. P.K.Goyal
Professor & Principal Investigator
M.Sc., Ph.D.
Radiation & Cancer Biology Laboratory, Department of Zoology
University of Rajasthan
Jaipur-302 004 (India)
Phone: 09829134133
Fax: 0091 141-2656273
E-mail: pkgoyal2002@gmail.com
pkgoyal2002@rediffmail.com,

Summary

The principal aim of the present work was to evaluate the chemopreventive effect of hydro-alcoholic extract of the whole plant of *Aegle marmelos*, in 6-7 weeks old male Swiss albino mice, on two stage process of skin carcinogenesis induced by a single topical application of 7, 12-dimethylbenz (a) anthracene (DMBA) and two weeks later promoted by repeated application of croton oil till the end of experiment (i.e. 16 weeks).The oral administration of *A. marmelos* at 50 mg/kg/b.wt./day during peri- initiational and post-initiational phases of papillomagenesis significantly inhibited the decrease in reduced glutathione ($p \leq 0.001$), catalase ($p \leq 0.001$) and proteins ($p \leq 0.001$) as well as the increase in lipid peroxidation ($p \leq 0.001$) levels in skin and liver of experimental mice while compared to carcinogen treated control. The results from the present study suggest the chemopreventive effect of *Aegle marmelos* fruit extract in DMBA induced skin papillomagnesis.

Key Words: Chemoprevention**,** *Aegle marmelos,* Lipid peroxidation (LPO), Reduced Glutathione (GSH), Oxidative stress, Antioxidant defense system

Introduction

Despite a remarkable progress in the development of anticancer therapies, cancer still remains as a major global health burden. The number of cancer related deaths is expected to increase by two-fold in the next 50 years. Since many types of cancers are preventable, the current cancer control strategy involves a paradigm shift from chemotherapy to chemoprevention.

Chemoprevention refers to the use of non-toxic chemical substances of either natural or synthetic origin to prevent carcinogenesis by stimulating detoxification of carcinogens and their potentially reactive metabolites or by halting, delaying or reversing the proliferation and subsequent malignant transformation of damaged cells. In fact, the promising results from numerous preclinical and limited clinical studies highlight the chemoprevention strategy as a realistic approach to fight cancer [1,2]. According to the report from the World Cancer Research Fund (WCRF), about 30–40% of cancers can be prevented by appropriate food and nutrition, physical activity and avoidance of obesity [3].

Antioxidants are emerging as prophylactic and therapeutic agents, which scavenge free radicals and prevent the damage caused by them [4]. Experimental investigations provide evidence supporting the role of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) in the etiology of cancer [5]. Human body is equipped with various antioxidants visualizing superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH) etc., which can counteract the deleterious action of ROS and protect from cellular and molecular damage. Antioxidants act as radical scavengers inhibiting LPO and other free radical–mediated processes thereby protecting the human body from various diseases [6].

Aegle marmelos, commonly known as bael, is a spinous tree belonging to the family Rutaceae. It is widely found in India, Bangladesh, Burma and Sri Lanka. It is distributed mainly within the sub-himalayan forest, in dry hilly regions. It is called "Shivadume" the tree of lord Shiva. *Aegle marmelos* has an important place in indigenous system of medicine. Its edible leaf, root, bark, seed and fruit are valued highly in Ayurvedic medicine in India [7]. In fact, as per Charaka (1500 BC) no drug has been longer or better known or appreciated by the inhabitants of India than the bael [8].The fruit is bitter, acrid , sour, astringent, aids digestion and stomach irritation, and is useful in treating diarrhea, dysentery and stomachalgia. Aqueous a*egle marmelos* fruit extract exhibits an anti-hyperlipidaemic [9] and hypoglycemic [10] effects in streptozotocin-induced diabetic rats. The ripe fruit used in different formulation for treatment of chronic diarrhea [11].

Looking towards the medicinal properties of this plant, the present study is undertaken to obtain insight into the possible anti-oxidant activity of *A. marmelos* against DMBA-induced skin tumorigenesis in mice.

Materials and Methods

Animal care and handling: The study was conducted on random-bred, 6-7 weeks old and 24 ± 2 gm body weight bearing, male Swiss albino mice. Animals were maintained under controlled conditions of temperature (25°C ± 2°C) and light (14hrs. light: 10hrs. dark).The animals were fed a standard mouse fed procured from Aashirwad Industries, Chandigarh (India), and water *ad libitum.* These animals were housed in polypropylene cages containing saw dust (procured locally) as bedding material. As a precaution against infection tetracycline hydrochloride water was given to these animals once each fortnight. The Departmental Animal Ethical Committee approved this study.

Chemicals: The initiator 7, 12-Dimethyl Benz (a) anthracene (DMBA) and the promoter croton oil were procured from Sigma Chemical Co., USA. DMBA was dissolved at a concentration of 100 µg/ 100µl in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

Preparation of *Aegle marmelos* Extract (AME): The fruits of *Aegle marmelos* L. were collected locally after their proper identification by a competent botanist (Voucher Specimen no: RUBL-20438) from the herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan (India). The pulp was removed from the fruit and shade dried, after that pulp was powdered in a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) for 36 (12 x 3) hrs at 40ºC. The liquid extract was cooled and concentrated by evaporating its liquid contents. The prepared *Aegle marmelos* extract (AME) was stored at low temperature until its further use. Such extract was redissloved in DDW prior for the oral administration in mice.

Experimental Design

Mice selected from the above mentioned random-bred colony, were assorted in to control and experimental groups; each group (I- V) comprised of 10 animals. The hair on the interscapular region of the mice was shaved 3 days prior to the experiment. Only the mice showing no hair growth were considered for the study.

Group-I: Vehicle treated (Negative control)

Animals belonging to this group received topical application of acetone (100 µl/ mouse) on the shaven dorsal skin, and double distilled water (DDW) equivalent to AME (100 µl/ mouse) by oral gavage for 16 weeks.

Group-II: AME alone treated (Drug treated control)

Animals of this group were administered AME orally at a dose of 50 mg/kg b. wt, dissolved in 100 µl of DDW as vehicle to each mouse, once in a day for the 16 weeks study period.

Group-III: Carcinogen treated (Positive Control)

These animals were applied topically a single dose of DMBA (100 µg/ 100 µl of acetone) over the shaven area of the skin of the mice. Two weeks later, croton oil (1% v/v in acetone) was applied three times per week until the end of experiment. This group received double distilled water (DDW) equivalent to AME (100 µl/ mouse) by oral gavage for 16 weeks.

Group –IV: AME treated (Experimental -1)

These mice were given the same treatment as in Group-III and also received AME at a dose of 50 mg/ kg b. wt. / animal/ day, orally for 7 days before and 7 days after DMBA application.

Group –V: AME treated (Experimental- 2)

Animals in this group received the same treatment as for Group-III and were administered AME (50 mg/kg b. wt. / animal/ day) by oral gavage, starting from the time of croton oil treatment until the end of the experiment (i.e. 16 weeks).

Biochemical Study

Biochemical alterations were studied in the animals of all the groups at the time of the termination of the experiment (i.e. the 16th week). At the end of the 16th week, the animals were killed by cervical dislocation. The dorsal skin affected by tumors was quickly excised and washed thoroughly with chilled saline (pH 7.4). It was then weighed and blotted dry. A 10% tissue homogenate was prepared from part of the sample (skin) in 0.15 M Tris-KCl (pH 7.4), and the homogenate was then centrifuged at 2000 rpm for 10 minutes. The supernatant thus obtained was taken for estimation of lipid peroxidation (LPO) and reduced glutathione (GSH). The following biochemical parameters were estimated in the liver and skin of mice.

(a) Lipid peroxidation (LPO) estimation

The level of LPO was estimated spectrophotometrecally by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkhawa *et al.* (1979)[12]. Briefly, thiobarbituric acid (0.6%), sodium dodecyl sulphate (0.1%), and trichloroacetic acid (20%) were added to 200 µl of the tissue homogenate (10%) prepared as described above. This mixture was heated for 60 minutes, cooled, and extracted with N butanol- pyridine (15:1), the optical density (OD) was recorded at 532 nm and the contents were expressed as nmol/mg of tissue.

(b) Glutathione (GSH) estimation

The level of reduced GSH was estimated as total nonprotein sulphahydryl group by the method of Moron *et al.* (1979) [13]. The homogenate was immediately precipitated with 100 µl of 25% trichloroacetic acid (TCA) and the precipitate was removed after centrifugation. Free endogenous-SH was assayed in a total volume of 3 ml by the addition of 200 µl of 0.6 mM 5, 5′ dithio-bis (2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer (pH 8.0) to 100 µL of the supernatant and the absorbance was recorded at 412 nm using a UV-VIS Systronics spectrophotometer. Reduced GSH was used as a standard. The levels of GSH were expressed as µmol/gm of tissue.

(c) Catalase (CAT)

The catalase activity was assayed by the method of Aebi (1984) [14]. The change in absorbance was followed spectrophotometrically at 240 nm after the addition of H_2O_2 (30 mM) to 100 µL of the supernatant (10% of skin homogenate was prepared in 50 mM phosphate buffer and centrifuged for 10 min.) in 50 mM phosphate buffer (pH 7).The activity of the enzyme is expressed as U/mg of tissue, where U is µmol of H_2O_2 disappearance/min.

(d) Total Proteins

Total proteins were estimated by the method of Lowery *et al.* (1951) [15]. using bovine serum albumin as a standard and the level was expressed as mg/ ml.

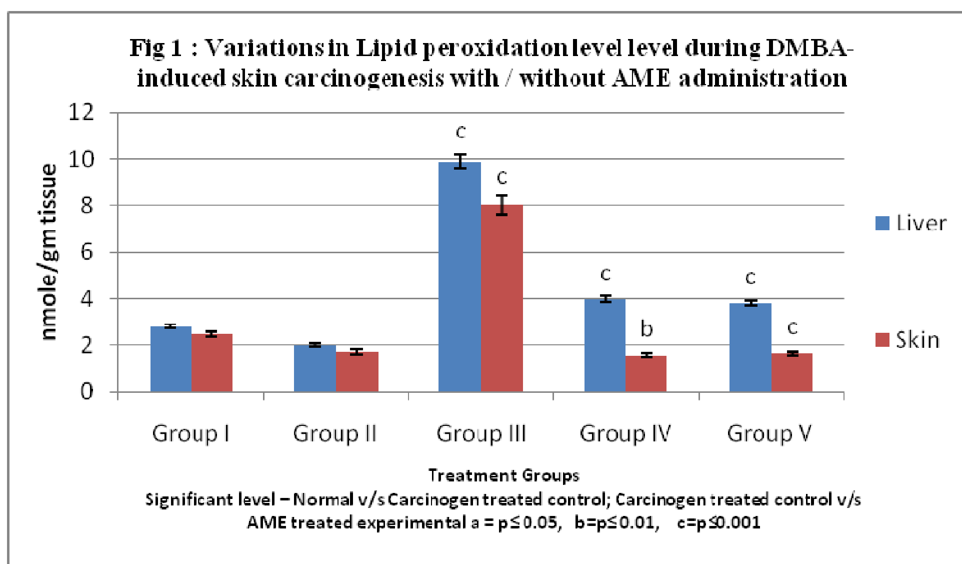
Statistical Analysis

The results are expressed as the mean ± standard error of the mean. The data of biochemical determinants from different groups were analyzed using the Student's *t* test.

Results

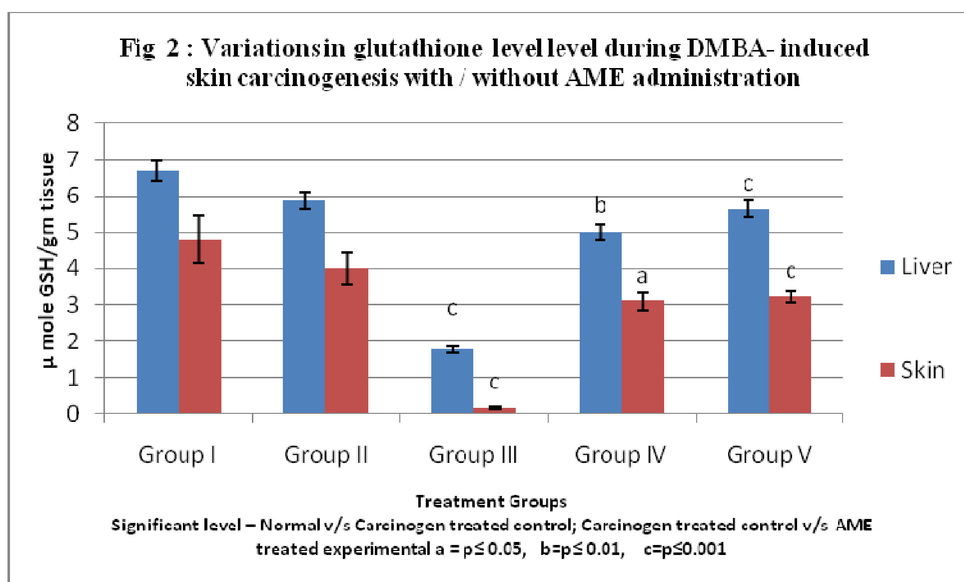
Lipid peroxidation (LPO)

A considerable elevation in LPO level was demonstrated in the liver and skin of Group III animals. Whereas, administration of AME significantly ($p \leq 0.001$) reduced the level of LPO in both the experimental groups (IV & V) as compared with the carcinogen treated control (Group III). However, no significant alteration in LPO level was observed in Group I and II as compared to Group III (Fig. 1).



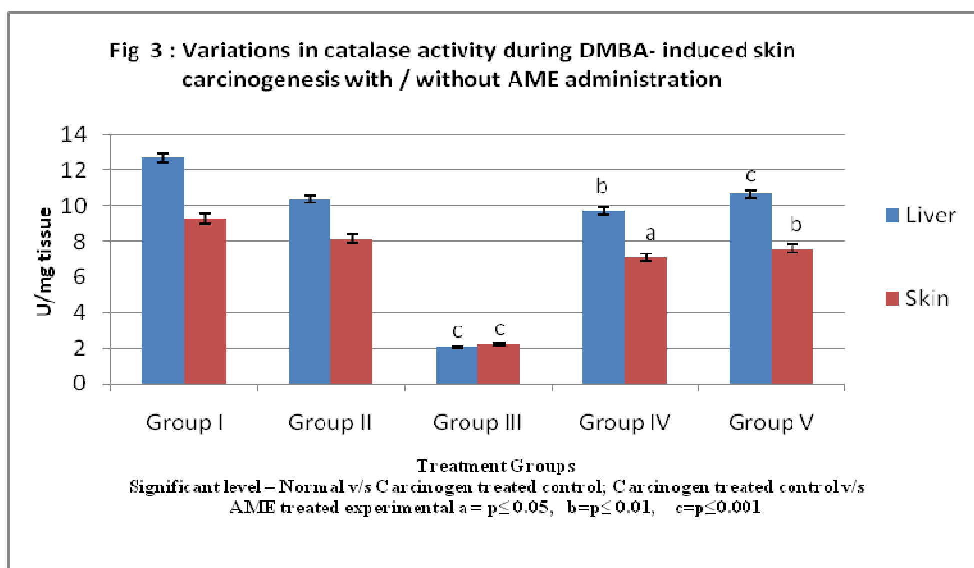
Reduced Glutathione (GSH)

Treatment with *A. marmelos* extract resulted in significantly enhanced level of the non-enzymatic antioxidant protein GSH in liver and skin of animals belonging to experimental groups IV & V as compared with the carcinogen treated control (Group III). On the other hand, a significant decrease ($p \leq 0.001$) from the normal level of GSH was recorded in Group III as compared with Groups I and II (Fig. 2).



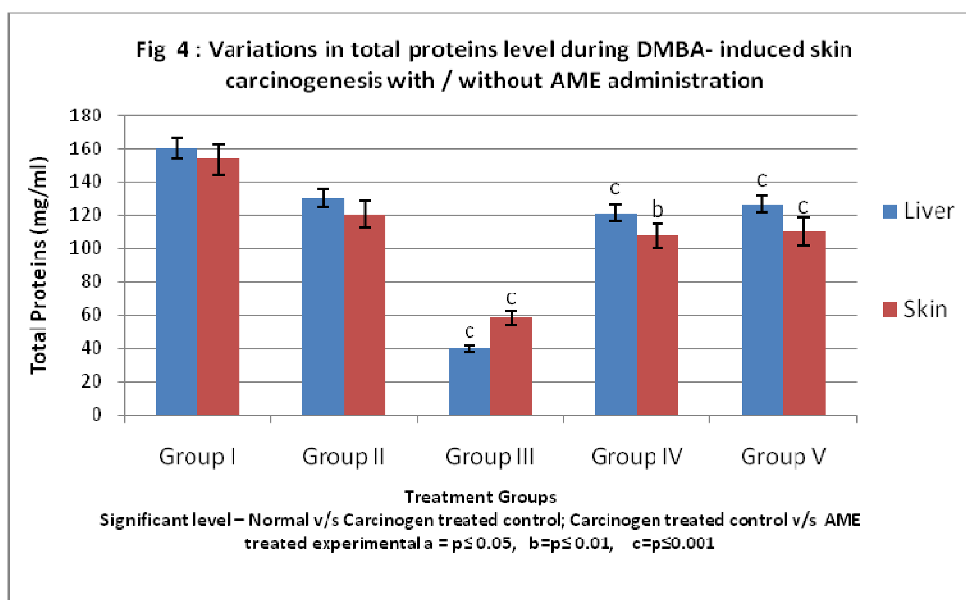
Catalase activity

Administration of DMBA/Croton oil (Group III) significantly reduced ($p \leq 0.001$) the Catalase activity in liver and skin while compared with the Group I and II. Oral administration of AME in Group IV and V increased Catalase levels significantly ($p \leq 0.001$) in both the tissues as compared to the carcinogen treated control (Group –III; Fig.3).



Total Proteins

A. marmelos treatment during peri- and post- initiational stage significantly ($p \leq 0.001$) elevated the level of total proteins in the liver and skin when compared with positive control (Group- III; Fig. 4).



Discussion

Chemoprevention, involving the use of synthetic or natural products to inhibit or reverse the carcinogenic process, is an effective approach to control cancer [16,17]. 7,12-dimethylbenz (a) anthracene, a polycyclic aromatic hydrocarbon (PAH) is widely present in our environment and is implicated in various types of cancer. Sources of PAHs include industrial and domestic oil furnaces, gasoline and diesel engines [18].

Reactive oxygen species generation is a major factor involved in all steps of carcinogenesis, i.e. initiation, promotion and progression[19].Oxidant-antioxidant balance impacts the rate of cell proliferation and tumor cells generally display low levels of lipid peroxidation which in turn can stimulate cell division and promote tumor growth [20] . Oxidative stress induced due to the generation of free radicals and/or decreased antioxidant level in the target cell and tissues has been suggested to play an important role in carcinogenesis [21] .

Free radicals are involved both in the initiation as well as promotion stage of tumorigenesis, and their biochemical reactions in each stage of the metabolic process is associated with cancer development [22] . The end products of lipid peroxidation are known to induce cellular damage and have been responsible for oxidative free radical induced human disease [23]. It is evident from the results that lipid peroxidation level was found to be significantly increased in positive control (Group III) animal when compared to vehicle treated animals (Group I). On contrary, reduced level of LPO was observed in experimental animals indicating that it is a potent free radical scavenger, which indicates that AME acts as a potent free radical scavenger. The similar findings have been reported while using *Emblica officinalis* and *Tinospora cordifolia* extract against chemical induced skin carcinogenesis [24, 25].

Reactive oxygen and/or nitrogen oxide species-induced stress (RONOSS) and its downstream events are clearly important for carcinogenesis. RONOSS can be induced by exposure to carcinogenic xenobiotics and microorganisms [26], and the various cellular alterations induced by RONOSS play a crucial role in carcinogenesis [27]. Antioxidants are expected to inhibit RONOSS because of an alteration in relevant enzyme profiles and quenching [28, 29].

GSH is major antioxidant that is found in large amount in all cells. GSH has many functions in metabolism, free radical scavenging, and regeneration of other antioxidants. The most important function of GSH is to provide protection against oxidative damage induced by reactive oxygen species, many of which are generated during normal cellular metabolic processes[30]. Glutathione acts as most important antioxidants in living system because it is a remover of H_2O_2 lipid peroxides and their products like 4-hydroxinonenal [31]. In the present study, GSH level was found to be significantly ($p \leq 0.001$) lower in liver and skin of positive control animals (Group III). On the other hand, significantly increased level of GSH was observed in *A. marmelos* treated experimental group (IV& V).

Superoxide dismutase is a metalloprotein, widely distributed in all the cells and plays a protective role against ROS-induced oxidative damage. It converts superoxide radical into hydrogen peroxide whereas glutathione peroxidase (GPXs) and catalase (CATs) convert H_2O_2 into water and oxygen [32]. In the present report, the catalase activity (CAT) was found to be significantly ($p \leq 0.001$) lower in liver and skin of positive control animals than that observed in the AME treated experimental mice.

In addition to lipids, proteins are also affected by ROS [33]. Protein oxidation is also common in ROS-induced carcinogenesis. Significantly decreased level of total proteins in liver and skin of animals belonging to Group III (carcinogen treated control) clearly reflects the overproduction of free radicals or inability of antioxidant defense system. In contrast, *A. marmelos* administration significantly ($p \leq 0.001$) increased such antioxidants levels, which might be responsible for the decrease in the activity of tumor promoter used in the present study.
Aegle marmelos, used in various medicinal systems, a rich source of proteins, polysaccharides, calcium, phosphorous, potassium, vitamin C & B, has been found to have anti-inflammation, anti-ulcer, anti-dyslipidemic, anti-initiating and anti-promoting properties. The current study demonstrates that *A. marmelos* extract can activate the defense system, following exposure to the carcinogen, by elevating the level of antioxidant enzymes.

When administration at different stages of tumorigenesis, *A. marmelos* not only lowers the carcinogenic activity of DMBA but also modulates the effect of the promoter, i.e. croton oil. The major mechanism for its action and efficacy seems to be the effectiveness of the fruit extract to intercept the free radicals and protect cellular molecules from oxidative damage. Further, it modulates the glutathione level and inhibits lipid peroxidation in liver and skin. Since *Aegle marmelos* has shown no toxic effect at the tested doses, it could well be applied in cancer chemoprevention to reduce the risk of cancer.

References

1. Kundu JK, SurhY-J. Inflammation: gearing the journey to cancer. *Mutat. Res.* 2008, 659:15–30.
2. Surh Y-J. Cancer chemoprevention with dietary phyto chemicals. *Nat Rev Cancer* 2003, 3:768–780.
3. Aggarwal S, Ichikawa H, Takada Y et al. Curcumin (diferuloylmethane) down-regulates expression of cell proliferation and antiapoptotic and metastatic gene products through suppression of IκBα kinase and Akt activation. *Mol Pharmacol.* 2006, 69:195–206.
4. Ratnam, DV, Ankola, DD, Bhardwaj, V, Sahana, DK, Ravi Kumar, MNV Review. Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. *J. Controlled Release* 2006, 113, 189-207.
5. Cheeseman, KL and Slater, TF. An introduction to free radical biochemistry. *Br. Med. Bull.* 1993, 49:481-483.
6. Mirunalini S, Kumaraguruparan R, Subapriya R, Nagini S. Garlic oil enhances hepatic and blood antioxidants during Hamster buccal pouch carcinogenesis. *Pharm Biol* 2004, 42(3):240-245.
7. Sharma, RK, Bhagwan, D. "Agnivesa's Charaka Samhita," 1988, Vol.3, Chaukhambha Orientalia, Varanasi.
8. CHEMEXCIL, "Selected Medicinal Plant of India," Basic Chemicals, pharmaceutical and cosmetic Export promotion Council, Bombay, 1992, 205-207.
9. Miyazaki, S, Ranganathan, D, and Kadarkariswami, M. Elucidation of toxicity of the *A. marmelos. Phytomedicine*, 2007, 4(2, 3), 204-205.
10. Kamalakkanan, N, Rajadurai, M, and Prince, PS. Effect of *Aegle marmelos* fruits on normal and streptozotocin-diabetic Wistar rats. J. Med. Food, 2003, 6(2), 93-98.
11. Citarasu, T, Sivaram, V, Immanuel, G, Rout, N and Murugan, V. Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp, Penaeus monodon with reference to haematological, biochemical and immunological changes. *Fish Shellfish Immunol.* 2006, 4, 372-384.
12. Ohkhawa H, Ohishi N, Yogi K. Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Analyt. Biochem*. 1979, 5: 351-358.
13. Moron MA, Depierre JW, Mannervick, B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochem Biophys Acta*. 1979, 582: 67-78.
14. Aebi, H. Catalase: in vitro. In: S.P.Colowick, N.O.Kaplan (eds). Method in Enzymology, Academic press, New York. 1984, 105: 121-126.
15. Lowry OH, Rosenbrough NJ, Farr AL, Landall AJ. Protein estimation with Folin phenol reagent. *J. Biol. Chem.* 1951, 193:265.
16. Metlin C, Chemoprevention: will it work? *Int. J. Cancer*, 1997; 10: 18-21.
17. Mirunalini, S and P. Subramanian. Influences of chronic administration of melatonin on hormonal rhythms in rats. *Biol. Rhythm Res.*, 2008; 39: 123-29.

18. Bishayee, A, Oinam, S and Chatterjee M B, Vanadium chemoprevention of 7, 12 dimethylbenz(a)anthracene–induced rat mammary carcinogenesis ; probable involvement of representative hepatic phase I and II xenobiotics metabolizing enzymes, *Breast Cancer Res Treat*, 2000; 63(2): 133-145.
19. Canuto, R A, Muzio, G, Biocca M E and Dianzani, M U. Lipid peroxidation in rat AH-130 hepatoma cells enriched in vitro with arachidonic acid. *Cancer Res*., 1991, 51: 4603-4608.
20. Huang, YL, Sheu J Y and Lint, H. Association between oxidative stress and changes of trace elements in patients with breast cancer. *Cancer Clin Biochem*, 1999, 32: 31-36.
21. Sharma, S N, Khan and Sultana, S. Effectof Onosma echiodes on DMBA/croton oil mediated carcinogenic response, hyperproliferation and oxidative damage in murine skin. *Life Sci.*, 2004, 75: 2391-410.
22. Weydert, CJ, Waugh, T A and Ritchie, J M. Over expression of manganese or copper-zinc superoxide dismutase inhibits breast cancer growth. *Free Radic. Biol. Med*., 2006, 41: 226-37.
23. Halliwell, B and Gutteridge, JMC. (1989) Free radical in Biology and medicine, 2nd ed., (Clarendon Press, Oxford).
24. Sancheti G, Jindal A, Kumari R, Goyal PK. Chemoprotective action of *Emblica officinalis* on skin carcinogenesis in mice. *Asia Pac J Cancer Prev.* 2005, 6: 197-201.
25. Chaudhary R, Jahan S, Goyal PK. Chemopreventive potential of an Indian medicinal plant (*Tinospora cordifolia*) on skin carcinogenesis. *J Environ Pathol Toxicol Oncol.* 2008, 27:233-243.
26. Kohen, R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions and methods for their quantification. *Toxicol Pathol.* 2002, 30:620-650.
27. Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. *Biochem Pharmacol.* 2002, 64:765-770.
28. Nakae D, Umemura T, Kurokawa Y. Reactive oxygen and nitrogen oxide species-induced stress, a major intrinsic factor involved on carcinogenic processes and a possible target for cancer prevention. *Asian Pac J Cancer Prev.* 2002, 3:313-318.
29. Ohshima H. Genetic and epigenetic damage induced by reactive oxygen species: implications in carcinogenesis .*Toxicol. Lett.* 2003, 140-141:99-104.
30. Bystrianyk R. Aloe helps blocks cancer. *J. Toxicol Environ health*. 2005,17:45-49.
31. Duffy S, So A, Murphy T H. Activation of endogenous antioxidants defense in neuronal cell prevents free-radical-mediated damage. *J Neurochem*. 1998; 71: 69-77.
32. Le C T, Hollor L, Vandervalk E J, Franken N A, Van Ravels F J, Wondergem J, Vander Laarse A. Protection of myocytes against free radical induced damage by accelerated turnover of glutathione redox cycle. *Eur Heart J.*1995; 16:553–562.
33. Hanimoglu H, Tanriverdi, T, Kacira, T, Sanus, GZ, Atukeren, P, Aydin S, Tunali Y, Gumustas K, Kaynar, MY. Relationship between DNA damage and total antioxidant capacity in patients with transitional meningioma. *Clin. Neurol. Neurosurg.* 109: 561-566.