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Anti-nephrotoxic effect of administration of *Moringa oleifera* Lam in amelioration of DMBA-induced renal carcinogenesis in Swiss albino mice

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Abstract
Moringa oleifera Lam. (Moringaceae) is highly valued plant reported to have various biological activities. Considering the antioxidant properties of *Moringa oleifera* (MO), the chemopreventive property of hydro-ethanolic extract of drumsticks was evaluated on DMBA induced renal carcinogenicity. Groups of 10 male mice were pre-administered with MO (200 and 400mg/kg body weight) and standard (0.5%BHA) for 14 days prior to a single dose of DMBA (15mg/kg; p.o). The therapeutic efficacy of drumstick extract was observed in terms of normalization of altered renal oxidative stress parameters like LPO, SOD and CAT in kidney of mice. DMBA exposure elicited a significant escalation in LPO level and depletion in antioxidant enzymes namely superoxide dismutase and catalase. Investigated parameters were restored, nearly to the normal values, after MO extract treatment. These results suggested that MO extract could act against DMBA-induced kidney injury in mice by a mechanism related to its antioxidant properties.

Keywords: *Moringa oleifera*; oxidative stress; chemoprevention; antioxidant property.

Introduction

Medicinal plants have been used by all civilizations as a source of medicines since ancient times. In the recent times, there has been growing interest in exploiting the biological activities of different ayurvedic medicinal herbs, due to their natural origin, cost effectiveness and lesser side effects (Naik *et al*., 2003). Interest in medicinal plants as a re-emerging health aid in the maintenance of personal health and well-being has been fuelled by rising costs of prescription drugs, and the bioprospecting of new plant-derived drugs (Sharma *et al*., 2010).

Active oxygen species and free radicals play an important role in the pathogenesis of several human diseases, such as rheumatoid arthritis, and cardiovascular diseases (Hertog *et al*., 1997) including cancer. The antioxidant defense enzymes have been suggestive of playing an important role in maintaining physiological levels of oxygen and hydrogen peroxide and eliminating peroxides generated from inadvertent exposure to xenobiotics and drugs. Any natural compound with antioxidant properties may help in maintaining health when continuously taken as components of dietary foods, spices or drugs (Singh, 2000).

Among myriad of plants, *Moringa oleifera* Lam is one of the best known and most distributed species of Moringaceae family. Moringa is an important tropical crop that is used as human food, medicine and in oil production (Anwer *et al*., 2007). Leaves of this plant are traditionally known for or reported to have various biological activities, including hypocholesterolemic agent (Ghasi *et al*., 2000), regulation of thyroid hormone status (Tahiliani and Kar, 2000), antidiabetic agent (Makonnen *et al*., 1997), gastric ulcers (Pal *et al*., 1995), antitumor agent (Bharali *et al*., 2003), antihyperglycemic (Anwar *et al*., 2007) and hypotensive agent (Faizi *et al*., 1995). The leaves as well as the flowers, roots, gums, fruits and seeds are extensively used for treating inflammation (Mahajan and Mehta, 2008), cardiovascular action, liver disease (Rao and Misra,1998) and hematological, hepatic and renal function (Mazumder *et al*., 1999). It is generally known in the developing world as a vegetable, a medicinal plant and a source of vegetable oil (Bennet *et al*., 2003).

Epidemiological studies suggest that specific pharmacologically active agents present in the diet might reduce the relative risk of cancer development (Tanaka *et al*., 2001). A remarkable surge of interest in chemoprevention research has led to the identification of many phytochemicals of dietary origin as effective potential chemopreventive agents (Bharali *et al*., 2003). The synthetic polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) is a

potent carcinogen which is selectively active in sites such as mammary glands, skin, kidney and liver, and has been widely used as a prototype carcinogen in experimental animal models. After exposure to DMBA, stable DNA-carcinogen adducts may be found in various tissues including the kidney, liver and mammary gland (Song *et al*., 2000) and protein adducts of serum albumin and haemoglobin are formed as well.

Antioxidant systems are being shown to play an increasing role in the protection against exogenous oxidative stress. There are two basic categories of antioxidant namely synthetic and natural ones. Restriction on the use of synthetic anti-oxidants is being imposed because of their carcinogenicity (Mishra *et al*., 2009). Thus, there is an urgent need to develop potent anti-nephrotoxic and antioxidant especially among natural product agents against DMBA-induced renal disorders. DMBA exposure induces clinicopathological changes through toxicity occurred to skin, mammary glands, liver and kidney characterized by tubular, interstitial and glomerular damages including renal lesions, tumors and cancer risks. Emerging evidences suggest that DMBA induces the production of reactive oxygen species (ROS) that result in lipid peroxidation, DNA damage, and depletion of cell antioxidant defense systems (Bharali *et al*., 2003). Change in lipid peroxidation production reactions and antioxidant defense systems were associated with changes in a variety of biochemical pathways.

A positive correlation has been established between dietary supplementation with certain vegetables and plants and the reduction of toxic effects of various toxicants, environmental agents including carcinogens and heavy metals (Nandi *et al*., 1997; Sharma *et al*., 2010). In the light of aforementioned medicinal properties of *Moringa oleifera*, the present investigation was conducted to determine whether the treatment of DMBA exposed mice with *M.oleifera* hydro-ethanolic pod extract could protect the vital organ kidney from DMBA associated oxidative stress.

Materials and Methods

Chemicals

All chemicals used in the study were of analytical reagent grade and of highest quality available and were purchased from reliable firms like SRL (India), MERCK, RANBAXY, HIMEDIA, QUALIGENS (Mumbai) and SUYOG. DMBA was purchased from SIGMA. Standard kits for LPO, SOD and CAT were obtained from Cayman Chemicals, USA.

Experimental plant

The experimental plant *Moringa oleifera* was collected from Krishi Vigyan Kendra, Banasthali University, Banasthali, Tonk district, Rajasthan, India. The plant material was taxonomically identified by botanist of Krishi Vigyan Kendra, Banasthali, Tonk district.

Hydro-ethanolic extraction of plant material

Dried powdered pods were placed in the Soxhlet thimble with 80% ethanol in 250 ml flat bottom flask and further refluxed for 18 hours at 80^0C for two days. Collected solvent was cooled at room temperature and poured in a glass plate. The extract was concentrated under vacuum at 40°C to yield a semisolid mass, dried in hot air oven below 50ºC for 48 hours and stored in a dessicator. The percentage yield of extract was found to be 15.6% and stored at 25^0C in airtight containers. Suspensions of the extract was prepared in distilled water and used to assess renoprotective and antioxidant activity.

Experimental animals

Male Swiss albino mice (*Mus musculus)* weighing 15-30 g were obtained from Haryana Agricultural University, Hissar (India) for experimental purpose. The animals were acclimatized for a month prior to experiment. The Institutional Animal Ethical Committee approved the animal studies. All experiments were conducted on adult male albino mice when they weighed 25-35g (3-4 months old). Colony bred adult male albino mice were maintained under standard laboratory conditions at a temperature of 22 ± 3ºC, relative humidity of 50±5 % and photoperiod of 12h (12h-dark and 12h-light cycle). The mice were housed in polypropylene cages. In order to avoid diurnal variation all the experiments were carried out at same time of the day. Animals lead free access to standard food pellet diet (Hindustan Lever Limited: metal contents in parts per million dry weight: Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0) and drinking water *ad libitum* throughout the study. Essential cleanliness and, to the best extent, sterile condition were also adopted according to SPF facilities.

Acute toxicity studies

Acute oral toxicity was performed as per OECD-423 guidelines (Ecobichon, 1997). The albino mice were fasted overnight provided only water, after which the hydro-ethanolic extract of the pods of MO was administered by

gastric intubation to the relevant animals at the single dose of 15mg/kg body weight. The animals were then observed for 14 days. However, mortality was not observed, the procedure was repeated for further higher doses such as 50, 100, 150, 200, 300, 400, 800, 1600 mg/kg body weight. Mortality was not noticed up to 400 mg/kg, whereas, the LD_{50} of the extract was found to be 1800 mg/kg body weight. Toxic symptoms for which the animals were observed for 72 h include behavioral changes, locomotion, convulsions and mortality.

Experimental design

Adult Swiss albino male mice divided into six groups of 10 mice each were treated by oral gavage. Treatment consisted of pretreatment phase of MO in distilled water followed by the second phase in which the animals were given 15 mg/kg DMBA on day 15. The animals were then euthanized 4 days after DMBA administration. The groups were as follows:

Group 1: served as control (normal untreated mice), and received 1ml distilled water daily by oral gavage

Group 2: received pretreatment with distilled water for 14 days prior to a single dose of DMBA (15 mg/kg body weight: p.o.) served as DMBA control group.

Group 3 and 4: were administered with hydro-ethanolic extract of pods of MO (200 and 400 mg/kg body weight: p.o.) daily for 14 days, served as MO treated control group.

Group 5: received BHA (0.5 % mg/kg body weight: p.o.) daily for 14 days, dissolved in 0.5% acetone and served as standard treated control group.

Group 6 and 7: were treated with hydro-ethanolic extract of pods of MO (200 and 400 mg/kg body weight; p.o.) daily for 14 days, before being intoxicated with DMBA (15 mg/kg body weight; p.o, once) dissolved in olive oil.

Group 8: received BHA (0.5 % mg/kg body weight: p.o.) daily for 14 days, before being intoxicated with DMBA (15 mg/kg body weight; p.o, once) dissolved in olive oil.

The dose for DMBA, standard antioxidant, and plant was decided and selected on the basis of LD_{50} calculated in the laboratory and other published reports (Song *et al*., 2000; Bharali *et al*., 2003).

Nephroprotective activity

After 19 days of duration, the mice were fasted overnight and then sacrificed under light ether anaesthesia. Kidney were dissected out, washed immediately with ice-cold saline to remove blood, and the wet weight noted and then stored at -80°C for various biochemical assays, and histological studies. Half of each kidney was processed for biochemical analysis and the other half was used for histological examination. The enzyme levels were assayed using standard CAYMAN Chemicals assay kits, U.S.A.

Preparation of kidney homogenate

Kidney homogenate was prepared in cold 50 mM potassium phosphate buffer (pH 7.4), for LPO but for SOD and CAT, 1mM EDTA was added in it, using REMI homogenizer. The unbroken cells and debris were removed by centrifugation at 10,000 rpm for 15 min at 4°C using a REMI cooling centrifuge and the supernatant was used for the estimation of LPO, SOD, and CAT.

Renal oxidative stress parameters

Estimation of lipid peroxidation (LPO)

Cayman's Lipid Hydroperoxide Assay Kit measures the hydroperoxides directly utilizing the redox reactions with ferrous ions (Mihaljevic *et al*., 1996). In brief, 0.5 ml of sample was treated with 1 ml of chloroform and centrifuged at 1500g for 5 min. The supernatant was collected and used for assay. The chloroform-methanol solvent (degassed, 2:1, 0.45 ml) was added to 0.5 ml of supernatant followed by addition of 50ul chromagen and incubation for 5 min. Lipid hydroperoxide standards (50 µM ethanolic solution of 13-hydroperoxy octadecadienoic acid) were prepared at different concentrations (0-5 nmol) in chloroform-methanol solvent. The absorbance was measured at 500 nm against reference blank (chloroform-methanol solvent). The LPO was expressed as µM/gm of protein.

Superoxide dismutase (SOD)

The tissue SOD activity was assayed following the procedure of Cayman's Superoxide Dismutase Kit, which utilizes a tetrazolium salt for detection of superoxide radicals (Marklund, 1980). Kidney homogenate (10 µl) was taken, and 200 µl of dilute radical detector (24µm NBT) were added. The reaction was initiated by adding 20 µl of xanthine oxidase (50 µl in 1.95 ml 50 mM Tris-HCl, pH 8.0) to all the wells. The control was simultaneously run without kidney homogenate. SOD standard (bovine erythrocyte SOD) was prepared at different concentrations (0-0.25 U/ml) in 50 mM Tris-HCl, pH 8.0. The plate was incubated for 20 min and the absorbance was measured at 440-460 nm using plate reader. The enzyme activity was expressed as unit ml^{-1} and 1 unit of enzyme is defined as amount of enzyme

needed to exhibit 50% dismutation of the superoxide radical.

Catalase (CAT)
Catalase activity in the kidney was assayed following the procedure of Cayman Catalase Assay Kit, which utilizes the peroxidative function of CAT for determination of enzyme activity (Johansson *et al*., 1988; Wheeler *et al*., 1990). Kidney homogenate (20 µl) was taken with 30 µl of methanol and 100 µl of assay buffer (100 mM potassium phosphate, pH 7.0) in two wells. Formaldehyde standards were prepared by addition of 20 µl formaldehyde (4.25 mM) at different concentrations instead of samples. The reaction was initiated by the addition of 20 µl of H_2O_2 (30 mM). Blank, without kidney, homogenate was prepared with 100 µl of phosphate buffer and 20 µl of H_2O_2. The plate was incubated on shaker for 20 min. The reaction was terminated by adding 30 µl of potassium hydroxide (10 M) to each well and then 30 µl of purpled (chromogen) was added to each well. This was followed by incubation for 10 min and addition of 10 µl potassium periodate to each well. The decrease in optical density due to decomposition of H_2O_2 was measured at the end of 5 min against the blank at 540 nm using plate reader. One unit of activity is equal to the mol of H_2O_2 degraded min^{-1} protein at 25° C. The specific activity expressed in terms of units per mg of proteins.

Statistical analysis
The experimental results obtained are expressed as mean ± standard deviation (SD) of three replicates. The data was subjected to one-way analysis of variance (ANOVA) and differences between samples were determined by Tukey multiple comparison test using the SPSS 16.0 (Statistical Program for Social Sciences) program. The level of significance was set at $p<0.001$.

Results
Findings of the present investigations are summarized in Table 1. There were no adverse effects of MO on the animals at the given dose levels (200 and 400mg/ kg body weight/day for 14 days respectively). The hydro-ethanolic extract of *Moringa oleifera* pods was found to be non-toxic up to dose of 900mg/kg body weight. The renoprotective effect offered by 400mg/ kg body weight was found to be greater than that of 200mg/kg body weight. BHA was used as a positive control in the present study. Table 1 illustrates effect of hydro-ethanolic extract of *Moringa oleifera* pods and BHA on lipid peroxidation, activity of antioxidant enzymes (SOD and CAT) in control and treated groups against DMBA induced nephrotoxicity in mice.

Table1: Effect of hydro-alcoholic extract of the pods of *Moringa oleifera* on lipid peroxidation and antioxidant enzymes against DMBA-induced nephrotoxicity in mice.

Groups	Treatments (mg/kg)	LPO (µM/mg protein)	CAT (nmol/min/ml)	SOD (U/ml)
Control	-	82.28±1.07[a]	8.24±0.023[a]	7.36±0.017
DMBA	15	101.80±1.27*/a	5.07±0.030*/a	5.59±0.012
MO	200	71.44±0.96*/a	9.15±0.015*/a	8.51±0.004
	400	63.73±0.90*/a	9.52±0.016*/a	8.58±0.006
BHA	0.5%	60.44±0.87*/a	8.24±0.021**/a	8.11±0.008
MO+DMBA	200 + 15	86.66±1.12**/a	8.11±0.036[a]	6.68±0.014
MO + DMBA	400 + 15	85.01±1.10[a]	8.17±0.014[a]	6.95±0.014
BHA+ DMBA	0.5% + 15	84.32±1.10[a]	7.73±0.036**/a	6.54±0.015

Values are expressed as mean ± SD (n=10)
*p<0.001, **p<0.01 vs. control group
[a]p<0.001, [b]p<0.01 vs. Treated (DMBA) group

DMBA at a dose of 15mg/ kg body weight caused significant ($p<0.001$) increase in the LPO level and significant ($p<0.001$) decrease in CAT and insignificant ($p>0.01$) decrease in SOD activity, in comparison to control group. Oral administration of hydro-ethanolic extract of *Moringa oleifera* in dose of 200 and 400mg/ kg body weight and BHA (0.5%) significantly ($p<0.001$) decreased LPO level and significantly ($p<0.001$ and $p<0.01$) increased the CAT activity and insignificant elevation in SOD activity as compared to

control group. In comparison to DMBA treated group, pre-administration of hydro-ethanolic extract of *Moringa oleifera* pods at low dose (200 mg/ kg body weight), high dose (400mg/kg body weight) and BHA (0.5%) significantly ($p<0.001$) decreased the LPO level and significantly ($p<0.001$) enhanced the CAT activity. The SOD activity increased insignificantly ($p>0.01$) at low and high dose of MO and BHA, protecting kidney from renocellular damage due to DMBA. The renoprotective effect of the extract was comparable to the effect seen with BHA treatment.

Intake of hydro-ethanolic extract of *Moringa oleifera* at dose of 200 and 400mg/kg body weight and BHA (0.5%) along with DMBA significantly ($p<0.001$) improved the LPO level and CAT activity while the administration of same dose insignificantly ($p>0.01$) elevated the SOD activity. Figure 1 shows the level of LPO in kidney of control and treated mice. Figure 2 and 3 depict the effect of *Moringa oleifera* extract and BHA on antioxidant marker enzymes (SOD and CAT) against DMBA induced nephrotoxicity in mice.

Discussion

Cancer chemoprevention involves the use of either natural or synthetic compounds to delay, inhibit or reverse the development of cancer in normal or pre-neoplastic conditions. Recent upsurge in identifying natural products of dietary origin associated with high degree of safety margins has been found to be beneficial as potent cancer chemopreventive agents (Bharali *et al*., 2003). Comprehensive reviews provide strong evidence that high intake of vegetables and fruits are associated with reduced cancer incidence (Block *et al*., 1992). It is known that balance between Phase I and Phase II enzymes can afford protection against numerous chemical carcinogens, and the induction of antioxidant enzyme facilitates their degradation from the body (Miller, 1998). *Moringa oleifera* is believed to possess numerous medical properties and is being used for the treatment of ascites, rheumatism (Anwar *et al*., 2007), venomous bites (Mishra *et al*., 2009), enhancing cardiac function (Limaye *et al*., 1995), inflammation (Ezeamuzle *et al*.,1996), liver disease (Rao and Misra 1998), cancer, hematological, hepatic, and renal function (Mazumder *et al*.,1999).

In the present study, we determined superoxide dismutase (SOD) and catalase (CAT) activities, as they are the key component of cellular defense system against oxidative stress. SOD converts superoxide (O^{2-}) to hydrogen peroxide (H_2O_2) and is a major defense system for aerobic cells in combating the toxic effects of superoxide radical (Mishra *et al*., 2009). Catalase decomposes hydrogen peroxide (H_2O_2) into H_2O and O_2 and protects the tissue from highly reactive hydroxyl radicals. Lipid peroxidation, oxidative deterioration of lipid bilayer, has been measured as an index of production of excess ROS.

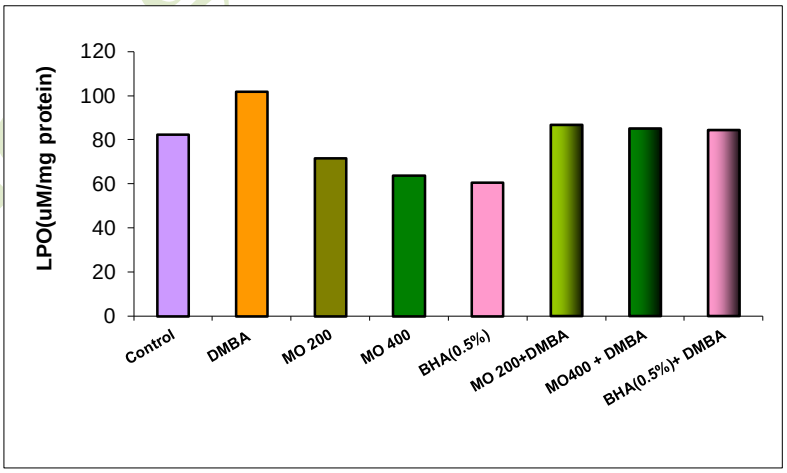

Figure 1: DMBA induced changes in renal lipid peroxidation and their response to administration of hydro-ethanolic extract of *Moringa oleifera* in male mice.

The toxicant induces a disturbance in the physiological state, which affects the enzyme activity. It then causes distortions in the cell organelles, which may lead to the elevation in the activity of various enzymes (Sharma *et al*., 2010). The DMBA toxicity

stimulates the oxidative stress and the antioxidant enzymes are induced as a defense mechanism. It has been discovered that DMBA toxicity leads to free radical damage via two different pathways: (1) the production of reactive oxygen species (ROS), including hydroperoxides, singlet oxygen, and hydrogen peroxide, and (2) the direct reduction of antioxidant reserves (Sharma *et al*., 2010). The balance between the production of oxidants and the scavenging of those oxidants by antioxidants determines the extent of lipid peroxidation. In the present study, the activities of SOD and CAT antioxidants were reduced by DMBA, thus rendering the tissues to the peroxidative damage.

Superoxide dismutase plays an important role in catalyzing the dismutation of superoxide radicals. SOD enzymes work in conjunction with H_2O_2 removing enzymes, such as catalase and glutathione peroxidase. These antioxidant enzymes depend on various essential trace elements and prosthetic groups for proper molecular organization and enzymatic action. Increase in SOD activity should accelerate the removal of the reactive oxygen species. Catalase, whose activity has also been augmented by MO, helps in removing the hydrogen peroxide produced by the action of SOD, when compared with both doses of MO. Induced SOD activity along with that of catalase explains the decrease in lipid peroxidation, which is an indicator of oxidative stress that persists in the cell. The decreased lipid peroxidation in the present study is in correlation with the induction of antioxidant enzymes above basal level by the *Moringa oleifera* extract.

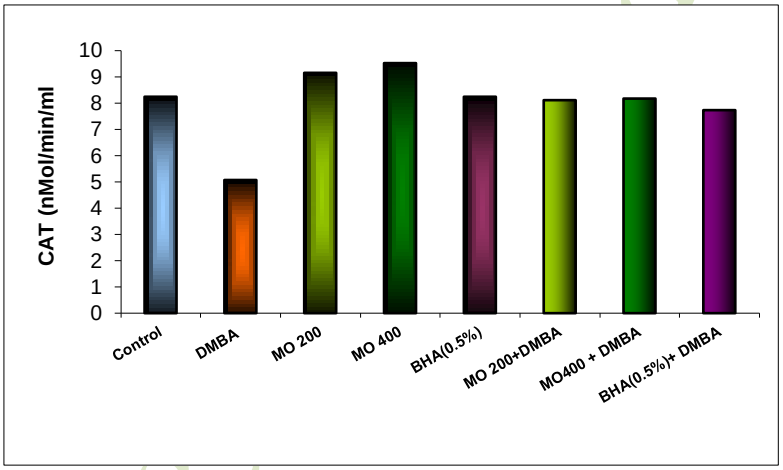

Figure 2: Effect of administration of MO extract and BHA on antioxidant enzyme Catalase against DMBA-induced nephrotoxicity in mice.

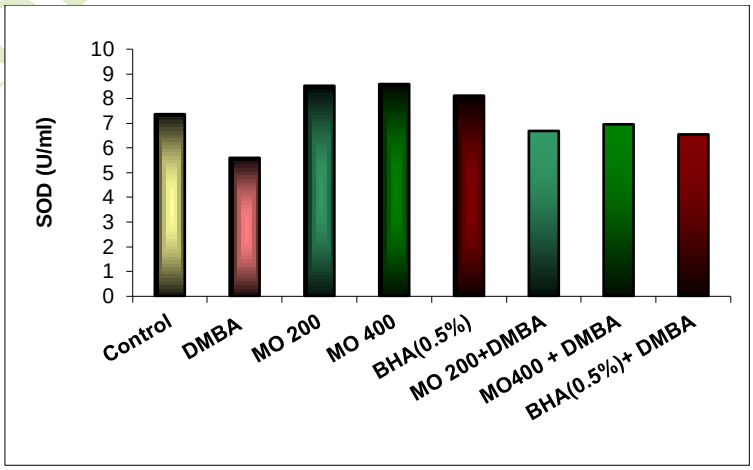

Figure 3: DMBA induced changes in renal SOD and their response to administration of hydro-ethanolic extract of *Moringa oleifera* in male mice.

Although the possible mechanism(s) of protection against DMBA induced nephrotoxicity was studied in the current study, it is possible that the protective effect of the extract is mediated through antioxidant and/or free radical scavenging activities. Literature has shown medicinal plants with nephroprotective properties to mediate their protection via antioxidant and/or free radical scavenging activities due to the high concentration of flavonoids and alkaloids they contain (Olagunju *et al*., 2009).

The antioxidant property of *Moringa* may be due to the presence of phenolic compounds that was confirmed in this study by phytochemical screening of the extract. In this respect, *Moringa* pods contain important bioactive compounds including glucosinolates, isothiocyanates, thiocarbamates, and flavonoids (Bharali *et al*., 2003). These compounds quench ROS, chelate metal ions and regenerate membrane-bound antioxidants. This finding is consistent with previous studies, which demonstrated the antioxidant activity of *Moringa* extract (Kumar and Pari, 2003; Arabshahi *et al*., 2007).

β-carotene, the major component reported from the drumsticks of the plant (Bharali *et al*., 2003) and vitamin A and C present in *Moringa oleifera* serve as an explanation for their mode of action in the induction of antioxidant profiles in the present investigation, however, the exact mechanism is yet to be elucidated. The biochemical basis of the chemopreventive potency of *Moringa oleifera* extract may be attributed to the synergistic action of the constituents of the extract and the induction of Phase-II enzymes (GSTs) and antioxidant enzymes, which might be implicated in the anticarcinogenic activity.

The induction of enzymes by the *Moringa oleifera* extract represents a promising chemopreventive strategy as a bifunctional inducer, along with the enhancement of antioxidant system enzymes which affords protection against cellular damage and inhibits cancer promotion. Since most of the phytochemicals and micronutrients having chemopreventive properties occur in low concentration in vegetables and fruits, it may be possible that selective interactions among these dietary constituents will have a long lasting, potent and effective modality for cancer chemoprevention.

Conclusion

Summing these facts, it is plausible for the alkaloid, flavonoid and phenolic components of MO to be responsible for the observed biological effects. These could constitute areas of future research. Again, the nephroprotection offered by the extract could be due to the presence of the phyto-principles contained in it. Hence, it is proposed that the nephroprotective activities of the hydro ethanolic extract of pods of MO in DMBA-induced nephrotoxicity may involve its antioxidant and/or oxidative free radical scavenging activities. Any natural compound with anti- oxidant properties may help in maintaining health when continuously taken as components of dietary foods, spices or drugs. The increase in the levels of antioxidant profiles i.e. SOD and Catalase by *Moringa oleifera* drumstick extract may be attributed to have biological significance in eliminating reactive free radicals that may affect the normal functioning of cells. Also, the results of this study have confirmed the rationale for the folkloric use of the hydro-ethanolic pod extract of MO in the treatment of renal disorders.

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