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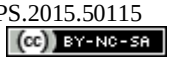
ISSN 2231-3354 

The Effects of *Cosmos caudatus* (Ulam Raja) on Detoxifying Enzymes in Extrahepatic Organs in Mice

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ABSTRACT

'Ulam Raja' or *Cosmos caudatus* is a common appetizer (ulam) consumed by the Malay community in Malaysia. However, *in vivo* studies pertaining to its antioxidant and chemoprotective properties are lacking. This study was done to determine the effects of *Cosmos caudatus* on detoxifying enzymes in extrahepatic organs (lungs, kidneys and stomach) in mice. Thirty adult male white mice were treated orally for 21 days with different doses of 'Ulam Raja' aqueous extract (UR) (100, 500, 1000mg/kg). The control group was given normal saline by oral gavage. Mice fed with diet containing 0.5% butylated hydroxyanisole (BHA) were used as positive control. After 21 days, the mice were sacrificed and extrahepatic organs were harvested. The activities of several detoxifying enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), DT-diaphorase (DTD)] were measured. Lipid peroxidation level was determined by measuring malondialdehyde (MDA) concentration. In lungs, 100, 500 & 1000 mg/kg UR oral supplementation resulted in significant increases in CAT, SOD and GST activities. DTD activity in lungs was significantly increased in mice treated with 1000mg/kg UR. MDA levels in lungs were significantly decreased in mice treated with 100mg/kg & 500 mg/kg UR but was significantly increased in mice treated with 1000mg/kg UR. In kidneys, DTD activity was significantly increased in mice treated with 1000mg/kg UR. In stomach, CAT activity was significantly increased in mice treated with 1000mg/kg UR. The results showed that *Cosmos caudatus* supplementation in mice could protect extrahepatic organs from xenobiotic and oxidative injury. This indicates that consumption of 'Ulam Raja' might be a useful chemoprotective measure.

INTRODUCTION

Aromatic herbs have long been an integral part of South East Asian culture as they are regularly consumed as part of diet. These herbs are eaten raw as salad, or used in cooking to enhance the flavour of food. In Malaysia, aromatic herbs are commonly eaten fresh as an appetizer (*ulam*) when eating rice, especially amongst the Malay community. Some of these plants have been used as folk remedies for the treatment of various ailments such as diabetes, high blood pressure, arthritis and fever, and are also consumed as health tonics (Ong and Norzalina, 1999). One of the local herbs, *Cosmos caudatus*, which is known locally as 'ulam raja', has the potential to be used in treating free radicals-associated diseases since it possess high antioxidant capacity (Shui *et al*., 2005). The major antioxidants in *Cosmos caudatus* were attributed to a number of proanthocyanidins that existed as dimers through hexamers, quercetin glycosides, chlorogenic, neo-chlorogenic, crypto-chlorogenic acid and (+)-catching (Shui *et al*., 2005). *Cosmos caudatus* is also rich in phenolic compounds such as flavonoids, flavones and flavanones and showed strong antioxidant activity (Mustafa *et al*., 2010). The major flavonoid components of *Cosmos caudatus* leaves are quercetin and rutin (Sukrasno *et al*., 2011). *Cosmos caudatus* exhibited stronger antioxidant activity compared to other herbs such as *Centella asiatica* and *Artemisia argyi* (Lee and Vairappan, 2011). Andarwulan *et al*. (2012) found that *Cosmos caudatus* has abundant ascorbic acid content. A recent study on commonly-utilized Malaysian herbs confirmed that *Cosmos caudatus* is a good source of antioxidants and phenolic compounds (Reihani and Azhar, 2012).

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Reactive oxygen species and free radicals are involved in a variety of pathological events, including cancer and aging process (Stohs, 1995). Compounds (natural or synthetic) with antioxidant properties that might contribute towards the alleviation of this damage may have significant roles in maintaining health when continuously taken as supplements. It is therefore suggested that the consumption of *Cosmos caudatus* might prevent the formation of free radicals and subsequently reduce the damage caused by these radicals. Although liver is the main organ responsible for drug and xenobiotic metabolism, significant activities of detoxifying enzymes are present in the extrahepatic tissues such as lungs, kidneys and stomach. These enzymes include endogenous antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), as well as phase II detoxification enzymes such as glutathione S-transferse (GST) and DT-diaphorase (DTD) (Singh *et al.*, 2000a, 2000b, 2001). The dysfunctioning of antioxidant enzymes has been implicated in several medical disorders including cancer (Gonzales *et al*., 1984; Saydam *et al*., 1997). Induction of detoxifying enzymes in extrahepatic organs will therefore ultimately help in alleviating pathologic events and drug/xenobiotic toxicity in living organisms. Extrahepatic enzyme induction depends not only on the nature of the inducing agent and the type of tissue but also on the particular test material under investigation (Singh *et al*. 2000a, 200b, 2001). So far, not much study has been done to see the effect of *Cosmos caudatus in vivo*. Therefore, the aim of the current study is to evaluate the effects of *Cosmos caudatus* on detoxifying enzymes (CAT, SOD, GST, DTD) and lipid peroxidation levels in murine extrahepatic tissues. BHA was used as a positive control since it has been proven to be chemoprotectant in previous animal studies (Hocman, 1988; Iverson, 1995; Iverson, 1999).

MATERIALS AND METHODS

Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), pyrogallol, 2,6 dichlorophenolindophenol (DCPIP), ethylenediamine tetracetic acid (EDTA), bovine serum albumin (BSA), tricholoroacetic acid (TCA), thiobarbituric acid (TBA), cacodylic acid, catalase, reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Plant materials

The fresh herbs (1 kg) were obtained from a local wet market in Chow Kit, Kuala Lumpur, Malaysia and were delivered to the Forest Research Institute of Malaysia (FRIM) in Kepong, Selangor, for aqueous extraction. At FRIM, the herbs were washed under running tap water to clear out dirt and the excess water was drained. The cleaned herbs were then oven-dried at 40 - 45^{0}C for 3 days, at which the humidity of the herbs is less than 10%. The dried herbs were ground to small particles using a grinder, in which 200 g of ground dried herbs was obtained. The ground dried *Cosmos caudatus* was then subjected to the extraction process using the 'reflux extraction' method using 1 L of water at 40 - 60^{0}C for 3 hours. The aqueous extract obtained was then thickened using a hot plate stirrer. The thickened extract was frozen at -80^{0}C for 24 hours and subsequently freeze-dried using the freeze-drier system for 5 days. After 5 days, 31.8 g of freeze-dried extract was obtained, giving a yield of 15.9%. The dried extract was kept in dark amber glass bottle wrapped in aluminium foil and stored in the freezer at -20^{0}C.

Animals

Experimental animals used in this study were adult male ICR white mice obtained from the Universiti Kebangsaan Malaysia (UKM) Animal House. Male mice aged 8-9 weeks and weighed between 25-30 g were used. The animals were treated after acclimatization period of seven days to room temperature and relative humidity of 28.5°C and 50%, respectively. They were housed in standard cages and put under 12 h-light/dark cycle and fed a standard rat chow diet with tap water given *ad libitum*. Food and water were not withheld before oral administration of the extracts to mice. Animals were maintained and handled according to the recommendations from the UKM animal ethics committee which had approved the study design of the experiment (Approval code: PP/FAR/2011/AZMAN/27-JANUARY/347-FEBRUARY-2011-FEBRUARY-2013).

Study design

A total of 30 mice were used, and they were divided into 5 groups (Control, 100UR, 500UR, 1000UR, BHA; n = 6 for each group). In the Control Group, mice were fed normal diet and sham-treated with normal saline through oral gavage daily for 21 days. Mice in the 100UR Group received normal diet and treated with 100 mg/kg body weight *Cosmos caudatus* extract, dissolved in 0.1 ml distilled water, through oral gavage daily for 21 days. Mice in the 500UR Group received normal diet and treated with 500 mg/kg body weight *Cosmos caudatus* extract, dissolved in 0.1 ml distilled water, through oral gavage daily for 21 days. Mice in the 1000UR Group received normal diet and treated with 1000 mg/kg body weight *Cosmos caudatus* extract, dissolved in 0.1 ml distilled water, through oral gavage daily for 21 days. In the BHA Group (positive control group), mice were fed normal diet fortified with 0.5 % BHA (w/w) for 21 days. All the mice were sacrificed after 21 days of treatment.

Preparation of homogenate and tissue supernatant

After the mice were sacrificed, their lungs, kidneys and stomach were harvested, trimmed free of extraneous tissue and homogenized in 0.1 M phosphate buffer (pH 7.4) to yield a 10% homogenate (w/v). 100 µl of the homogenate was used for estimation of lipid peroxidation level. The rest of the homogenate was centrifuged at 15000 x g for 30 min at 4^{0}C. The resulting supernatant obtained was used for assaying GST, DTD, SOD and catalase.

Determination of glutathione S-transferase

Glutathione S-transferase activity was determined spectrophotometrically according to the procedure of Habig et al. (1974). The specific activity of glutathione S-transferase was expressed as µmoles of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Determination of DT-diaphorase

DT-diaphorase activity was measured using the procedure described by Ernster et al. (1962). The activity was calculated using an extinction coefficient of 21 $mM^{-1}cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme required to produce one µmole of DCPIP per min.

Determination of catalase

Catalase was estimated using the method described by Aebi (1984). The specific activity of catalase was expressed as moles of H_2O_2 reduced/min/mg protein.

Determination of superoxide dismutase

Superoxide dismutase was assayed utilizing the technique of Marklund and Marklund (1974). A single unit of enzyme was defined as the quantity of superoxide dismutase required to produce 50% inhibition of autoxidation.

Estimation of lipid peroxidation

Lipid peroxidation was estimated using the thiobarbituric acid-reactive substances (TBARS) method as described by Ledwozyw et al. (1986) and was expressed in terms of malondialdehyde (MDA) formed per mg protein.

Statistical analysis

Data were expressed as Mean ± SEM and analyzed using ANOVA followed by Tukey's *post hoc* test. Results were considered significant at $p<0.05$.

RESULTS

Mortality

There was no mortality observed after 24 h and the following 21 days of administration of the *Cosmos caudatus* extract in all mice from both control and treated groups.

Body weight

There was an increase in daily body weight from day 0 until day 21 which corresponded to normal growth of mice (Table-1).

Table 1: Mean body weights of control and treated mice at the beginning and end of study period.

	Body weight (g)				
Day	**Control**	**100UR**	**500UR**	**1000UR**	**BHA**
1	25.17±1.70	24.33±1.87	23.83±1.63	28.17±1.29	27.53±1.56
21	30.83±1.92	27.00±1.98	28.83±2.09	32.17±1.67	31.28±1.35

100UR, 500UR, 1000UR: Groups of rats treated with oral *Cosmos cau*datus extracts at a daily dose of 100, 500 and 1000 mg/kg body weight, respectively. All values are expressed as Mean ± SEM (n=6/group). No statistical significance: control vs. treated groups (ANOVA)

Catalase activity

In lungs, catalase activity was increased in a dose dependent manner. There was a significant increase in all the groups studied compared to control (Figure 1). In kidneys, a significant increase was observed only in the positive control group (BHA group). In stomach, significant increase in activity was seen in 1000UR and BHA groups (Figure 2).

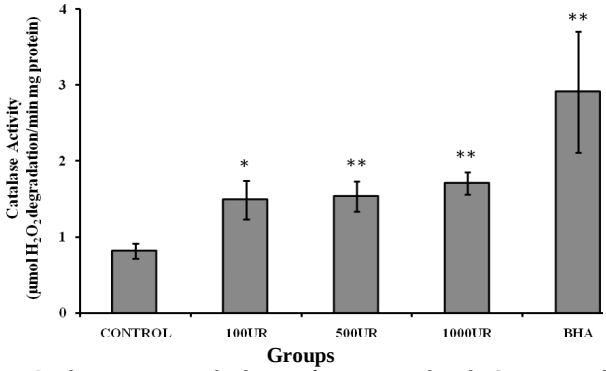

Fig. 1: Catalase activity in the lungs of mice treated with *Cosmos caudatus* extracts for 21 days. All values are expressed as Mean ± SEM (n=6/group). *p<0.05 compared with control group; **p<0.01 compared with control group

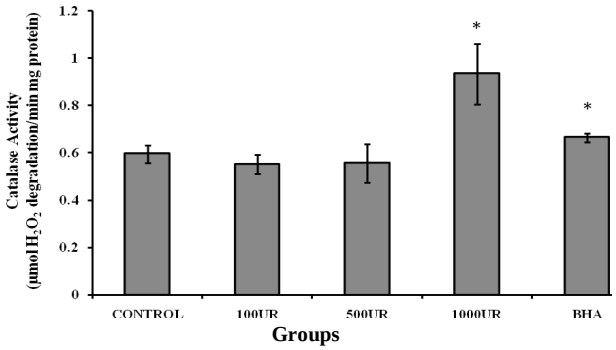

Fig. 2: Catalase activity in the stomach of mice treated with *Cosmos caudatus* extracts for 21 days. All values are expressed as Mean ± SEM (n=6/group). *p<0.05 compared with control group.

Superoxide dismutase (SOD) activity

In lungs, SOD activity showed significant increase in all groups compared to control (Figure 3). In kidneys and stomach, there was no significant result in all the groups studied.

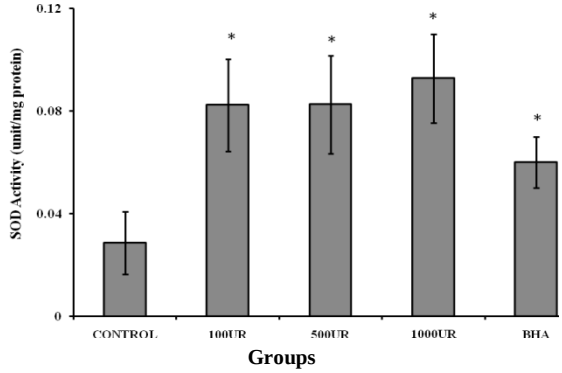

Fig.3: SOD activity in the lungs of mice treated with *Cosmos caudatus* extracts for 21 days. All values are expressed as Mean ± SEM (n=6/group). *p<0.05 compared with control group.

Glutathione S-transferase (GST) activity

In lungs, there was significant increase in GST activity in all the groups studied, the highest being in the 500UR group (Figure 4). In kidneys and stomach, there was no significant result in all the groups studied.

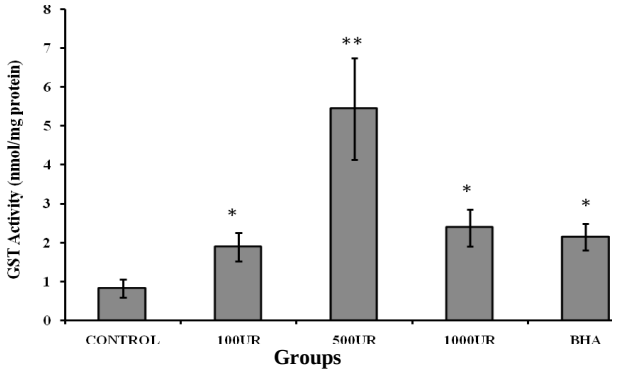

Fig. 4: GST activity in the lungs of mice treated with *Cosmos caudatus* extracts for 21 days. All values are expressed as Mean ± SEM (n=6/group). *p<0.05 compared with control group; **p<0.01 compared with control group

DT- diaphorase (DTD) activity

In lungs and kidneys, DTD activity was significantly increased in the 1000UR and BHA groups (Figure 5 & Figure 6). In stomach, no significant result was seen in all the groups studied.

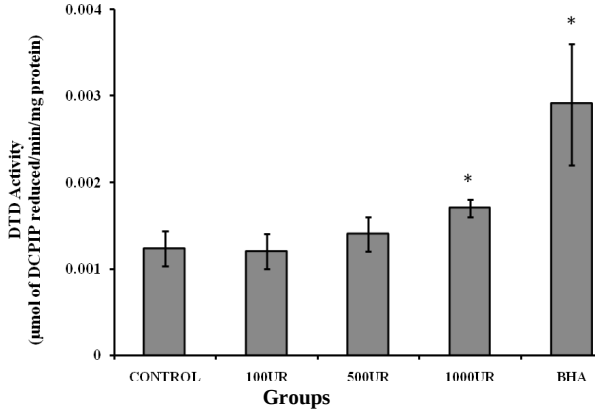

Fig. 5: DTD activity in the lungs of mice treated with *Cosmos caudatus* extracts for 21 days. All values are expressed as Mean ± SEM (n=6/group). *p<0.05 compared with control group.

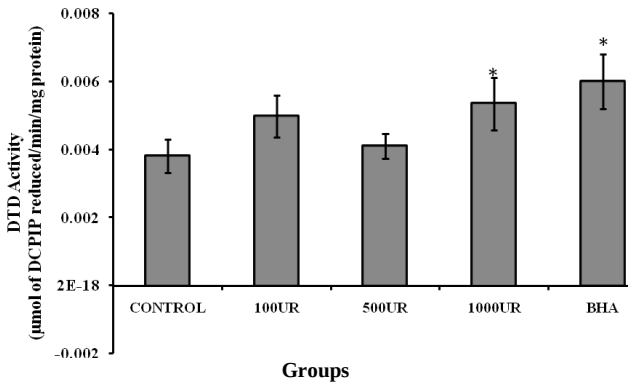

Fig. 6: DTD activity in the kidneys of mice treated with *Cosmos caudatus* extracts for 21 days. All values are expressed as Mean ± SEM (n=6/group). *p<0.05 compared with control group.

Malondialdehyde (MDA) concentration

In lungs, MDA concentration decreased significantly in the 100UR and 500UR groups. However, in the 1000UR and BHA groups, MDA concentration increased significantly compared to control (Figure 7). In kidneys, only the positive control (BHA group) showed significant decrease compared to control. In stomach, there was no significant result in all the groups studied.

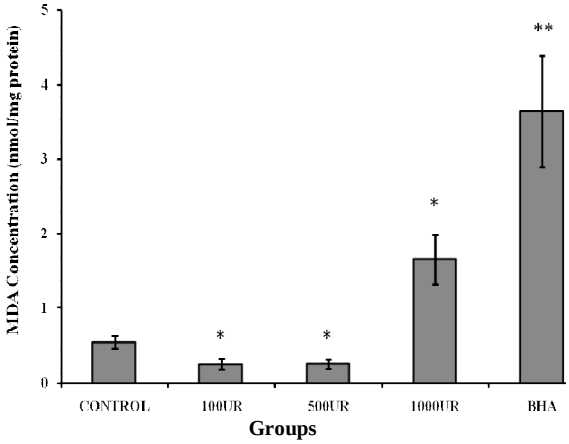

Fig. 7: MDA concentration in the lungs of mice treated with *Cosmos caudatus* extracts for 21 days. All values are expressed as Mean ± SEM (n=6/group). *p<0.05 compared with control group; **p<0.01 compared with control group

DISCUSSION

Damage from free radicals such as reactive oxygen species can lead to various medical disorders such as cancer. Antioxidants are substances which protect cells from free radicals. The antioxidant response mediated by certain herbal preparations may be anticipated to have biological significance in eliminating reactive free radicals that may otherwise affect the normal cell functioning. The dysfunction of antioxidant enzymes (e.g. SOD and catalase) has been implicated in several disorders such as cancer (Gonzales *et al*., 1984; Saydam *et al*., 1997). These enzymes have been suggested to play important roles in maintaining physiological levels of oxygen and hydrogen peroxide by hastening the dismutation of oxygen radicals and eliminating organic peroxides and hydroperoxides generated from inadvertent exposure to xenobiotics and drugs. SOD catalyzes the conversion of superoxide (O_2^-) into oxygen molecule and hydrogen peroxide, whereas catalase metabolizes hydrogen peroxide into oxygen and water. The augmented activity of SOD accelerates dismutation of superoxide radicals to hydrogen peroxide which is removed by catalase (Aebi, 1984).

On the other hand, GST and DTD are phase II drug/xenobiotic metabolizing enzymes. Glutathione S-transferase is a critical detoxification enzyme that primarily functions in conjugating 'functionalized P450 metabolites' with endogenous ligands (reduced glutathione) thus favouring their elimination from the body of living organisms (Hartman and Shankel, 1990). There is clear evidence to support the induction of glutathione S-transferase with protection against wide spectrum of cytotoxic, mutagenic and carcinogenic chemicals (Ketterer, 1988; Reed,

1990). DT-diaphorase protects against the toxicity of quinones and their metabolic precursors i.e. polycyclic aromatic hydrocarbon and benzene. Induction of DT-diaphorase facilitates bioreductive activation metabolism of quinones by two-electron oxido-reduction of quinone to hydroquinone, obliterating semiquinone radical and subsequent oxygen radical production. This is very important for maintaining homeostatic cellular environment (DeLong *et al*., 1986; Smart and Zinnoni, 1984).

Lipid peroxidation, a process initiated by free radicals, lead to oxidative deterioration of polyunsaturated fatty acids, which is a major component of cellular membrane. Under normal physiological conditions, only low levels of lipid peroxides occur in body tissues. The intense production of free radicals lead to peroxidative changes that ultimately result in increased lipid peroxidation. The first stage of lipid peroxidation is initiated by the presence of reactive oxygen species (ROS) that react with hydrogen atoms to form lipid radicals. Since lipid radicals are not stable, they readily react with oxygen molecules to form lipid peroxyl radicals (Rikans and Hornbrook, 1997). MDA, an end product of lipid peroxidation, is used as a marker of tissue damage (Ohkawa *et al*., 1979). Antioxidants are thought to be essential for preventing the formation of free radicals and they restrain some of the deleterious actions of ROS on lipids, DNA and proteins (Halliwell, 1996).

Xenobiotic detoxification is controlled by the liver as the main organ. The kidney, lung and stomach also have this detoxification system, but the magnitude of enzyme induction is lower than in the liver. Kidney, lungs and stomach are susceptible to developing tumours and other toxicities because they are frequently exposed to chemicals and their metabolites. Therefore, it is important to examine the inducibility of phase-II enzymes and antioxidant enzymes in the kidney, lungs and stomach (Singh *et al*., 2000a, 2000b, 2001).

In the lungs, there was significant increase in catalase, SOD and GST activities in all *Cosmos caudatus* treated groups. This indicated that there is an abundance of these enzymes in the lungs. Thus, their activities are easily induced even at lower doses of *Cosmos caudatus*. Catalase activity in the lungs increased in a dose dependant manner, indicating that the expression of this enzyme increased dose-dependently as well. The results for GST activity showed very significant increase ($p<0.01$) in mice treated with 500mg/kg of *Cosmos caudatus*, suggesting that this is the optimum dose for its expression. On the other hand, the highest dose of *Cosmos caudatus* (1000 mg/kg) is required to induce the expression of DTD. This indicated that at high doses of Cosmos caudatus, high amounts of quinone metabolites are produced which will subsequently induce significantly higher DTD expression.

Also in the lungs, there was significant decrease in MDA concentration in mice treated with lower doses of *Cosmos caudatus* (100 mg/kg and 500 mg/kg). This might possibly be due to the fact that some phenolic compounds in herbs have the capacities to quench lipid peroxidation, prevent DNA oxidative damage, and scavenge reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals (Cao and Cao, 1999; Kahkonen *et al*., 1999). However, there was significant increase in MDA concentration in mice treated with 1000 mg/kg of *Cosmos caudatus* and also in the BHA group. This indicated *Cosmos caudatus* protects against free radicals / oxidative stress at lower doses but acts as a pro-oxidant at higher dose. It could also be that at high doses, *Cosmos caudatus* is metabolized into a toxic metabolite (probably a quinone radical since DTD activity increased significantly as well) which could damage lung membranes. Lung perfusion might be sufficient to remove the toxic metabolites of *Cosmos caudatus* at lower doses; however at higher doses lung perfusion might not be adequate to remove all the toxic metabolites in the lungs, resulting in their accumulation. However, human consumption of *Cosmos caudatus* as a food appetizer in Malaysia is well below 1000 mg/kg body weight and is unlikely to reach toxic levels.

MDA is a marker of oxidative stress, an increase in its concentration indicates presence of higher level of free radicals. BHA can be oxidatively metabolized into quinone radicals and other reactive oxygen species (Kahl *et al*., 1989). Lungs are very rich in oxygen and in such environment BHA could be metabolized into quinone radicals and other reactive oxygen species at high amounts, which could lead to oxidative stress in the lung environment. Oxidative stress and free radicals could cause damage to lung membranes resulting in increased MDA levels.

In the kidneys, only DTD activity in mice treated with 1000 mg/kg of *Cosmos caudatus* was found to be significantly higher compared to normal controls. There was no significant finding for other enzymes in other *Cosmos caudatus* treated groups. This might be due to the type of *Cosmos caudatus* metabolites that are present in the kidneys, or it could be that the expression of catalase, SOD and GST in the kidneys of our mice are low and much higher concentrations of *Cosmos caudatus* is needed to induce those enzymes in the kidneys. The level of MDA in mice treated with the positive control BHA was significantly reduced compared to normal controls.

BHA also significantly increased catalase and DTD activities, but did not have any significant effects on SOD and GST activities in the kidneys, further suggesting that the expression of SOD and GST in the kidneys of our mice is low.

In the stomach, catalase activities in mice treated with 1000 mg/kg *Cosmos caudatus* and BHA were significantly higher than normal control. There were no significant findings for other enzymes, indicating that the expression of these enzymes in the stomach of our mice is indeed very low.

To date, no data are available on the effects of *Cosmos caudatus* on detoxifying enzmes in extrahepatic tissues. However, studies on other Asian herbs such as *Aegle marmelos* and *Adhatoda vesica* showed that the herbs were able to significantly increase detoxifying enzymes activities in extrahepatic organs at lower doses (50 mg/kg and 100 mg/kg) and shorter duration of treatment (14 days) (Singh *et. al*. 2000a, 2000b). This might partly be due to the fact that the total phenolic content in those herbs are higher than *Cosmos caudatus*, and it might also be that the herbal

extracts used are organic solvent-based rather than aqueous based (Kruawan *et. al*, 2006; Maurya *et. al*, 2010; Rafat *et.al*, 2010).

CONCLUSION

In conclusion, this study showed that *Cosmos caudatus* is able to significantly induce the expression of detoxifying enzymes and reduce the lipid peroxidation level especially in the lungs. However, at higher doses (1000 mg/kg), *Cosmos caudatus* was seen to exert pro-oxidant effects. Therefore, oral supplementation of *Cosmos caudatus* at lower doses might be able to protect the lungs from oxidative stress and chemical insults. Further studies on the active compounds of *Cosmos caudatus* is recommended to determine the substance responsible for those effects. It could also be concluded that at low doses, no beneficial effect was seen in kidneys and stomach. Further studies could be done to evaluate the effect of *Cosmos caudatus* on the level of gene expression of detoxifying enzymes in extrahepatic organs in order to see whether it correlates with detoxifying enzymes activities. Studies using other types of extracts using organic solvents (e.g hydro-alcoholic, ethanolic or methanolic extracts) rather than aqueous extract of *Cosmos caudatus* is also warranted.

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