The Effects of *Cosmos caudatus* (Ulam Raja) on the Levels of Expression of Nrf2 Target Genes in Mice Liver

Azman Abdullah[1,*], Tan Chung Phern[1], Nur Fatihah Hanis Mohamad Rahim[1], Nur Izzati Zaharuddin[1], Nurdayana Maulat Salihin[1], Ahmad Hilmi Yusof[1], Kamsiah Jaarin[2], Yusof Kamisah[1], Qodriyah Haji Mohd Saad[1], Nur Azlina Mohd Fahami[1] and Harizz Miszard Radman[3]

[1]*Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia*

[2]*Department of Pharmacology, Faculty of Medicine and Defence Health, National Defence University of Malaysia, Kem Sungai Besi, 57000 Kuala Lumpur, Malaysia*

[3]*Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

Abstract: *Background*: *Cosmos caudatus* (Ulam Raja) is an appetizer (ulam) eaten with rice in Malaysia. Previous studies showed that *Cosmos caudatus* possess high antioxidant content. Nrf2 is a transcription factor which regulates the expression of phase II enzymes and antioxidant proteins. The aim of this study is to investigate the effects of *Cosmos caudatus* aqueous extract (UR) on the expression of Nrf2 target genes in mice liver.

Methods: ICR white mice were treated for 21 days with different doses of UR (100, 500, 1000 mg/kg) through oral gavage. Control mice were only given distilled water. After 21 days, the mice were sacrificed and their livers harvested. Total RNA was extracted, reverse transcribed and subjected to qPCR to detect Nrf2 target genes expression.

Results: Administration of 100 mg/kg UR significantly increased NQO1 expression in mice liver. Administration of 500 mg/kg UR significantly increased HO-1 liver expression. Administration of 100 and 500 mg/kg UR significantly increased GSTA1 liver expression. Administration of 500 and 1000 mg/kg UR significantly increased GSTM3 liver expression, whereas GSTP and GSTM1 liver expression was significantly decreased at similar doses. Administration of all doses of UR significantly decreased the expression of GSTA3, SOD3 and GCLC in mice liver.

Conclusion: UR administration mostly resulted in downregulation of Nrf2 target genes. However, conclusive evidence can only be made through the use of Nrf2 knockout mice or by performing Nrf2 nuclear translocation studies.

Keywords: *Cosmos caudatus*, Nrf2, mice, liver, gene expression.

1. INTRODUCTION

Cosmos caudatus is an indigenous herb of tropical America, but can be found grown in other tropical countries including Malaysia and Thailand [1]. In Malaysia, it is known as “Ulam Raja”. “Ulam” refers to a group of traditional Malay herbs, usually consumed raw. The raw leaves and shoots of *Cosmos caudatus* is eaten by dipping in a condiment made with chili and shrimp paste, accompanied with rice. In Malaysia, *Cosmos caudatus* has been traditionally used to improve blood circulation, as an anti-aging agent, to reduce body heat, to strengthen the bones (because of its high calcium content), to promote fresh breath and to treat infections associated with pathogenic microorganisms [2, 3]. *Cosmos caudatus* has been shown to exhibit potent antioxidant activity [4]. The antioxidant activities of aqueous extracts of several tropical herbs that are widely consumed in the Southeast Asian region had been evaluated, and it was discovered that *Cosmos caudatus* possess considerably high antioxidant activity compared to other Southeast Asian herbs [5]. *Cosmos caudatus* extract was highly effective in the prevention of formation of conjugated diene and in scavenging DPPH radicals [6]. A previous research which evaluated the total phenolic content and antioxidant activity of five local Malaysian herbs using DPPH and FRAP assays discovered that *Cosmos caudatus* and *Oenanthe javanica* aqueous extracts were found to have the highest DPPH and FRAP values, further suggesting that *Cosmos caudatus* is a good source of antioxidant [7]. It is therefore implied that the consumption of *Cosmos caudatus* might prevent the formation of free radicals *in vivo* and consequently reduce the damage caused by these radicals. If left unattended, free radicals are capable of attacking cells which leads to irreparable cellular damage. However, cellular damage

*Address of correspondence to this author at the Department of Pharmacology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia; Tel: 006-03-91459569; Mob: 006-016-3185342;
Fax: 006-03-91459547; E-mail: manlah1969@yahoo.com

ISSN: 2223-3806 / E-ISSN: 1927-5951/17

caused by free radicals can be prevented or reduced by the presence of *in vivo* antioxidant proteins such as superoxide dismutase, glutathione peroxidase and peroxiredoxin, in which their expression are Nrf2-regulated [8]. In this study, we would like to see whether supplementation of *Cosmos caudatus* aqueous extract is capable of enhancing the *in vivo* antioxidant/detoxification response through the Nrf2 pathway. Nrf2 is a member of the Cap'n'Collar (CNC) family of transcription factors, which share a basic region-leucine zipper (bZip) structure. Nrf2 heterodimerizes with other bZip proteins e.g. the small Maf proteins and binds to its cognate DNA sequence, the antioxidant response element (ARE), for which the consensus DNA sequence 5'-TGACNNNGCA-3' has been proposed [8]. It has been shown that Nrf2 is sequestered in the cytoplasm coupled to an inhibitor protein, Keap1, which prevents its access to the nucleus and also directs its degradation by the proteasome [8]. Exposure to chemically reactive species and/or a decrease in reducing conditions within the cell, results in dissociation of Nrf2 from Keap1. The precise mechanism involved in the dissociation process is not clearly understood, but may involve oxidation and/or covalent modification of critical sulfhydryl group within the Keap1 protein. In addition to Keap1, certain protein kinases may be candidate sensor molecules of electrophilic and oxidative stresses [8]. In the nucleus, Nrf2 associates with small Maf protein (or other binding partners), forming a heterodimer that binds to the ARE in order to stimulate the gene expression mechanism. Nrf2-Maf heterodimer target genes include those that encode prototypical phase II detoxification enzymes and antioxidant proteins [8].

The importance of Nrf2 in regulating the cellular antioxidant response has been most convincingly established through studies with transgenic Nrf2 deficient mice. Studies performed on Nrf2 knockout mice indicated that Nrf2 was influential in the expression of ARE-associated genes. Nrf2 knockout mice appeared to be more susceptible to the toxic effects of compounds such as paracetamol and cigarette smoke [8]. Studies performed on Nrf2 knockout mouse indicated that the presence of Nrf2 is required for enhanced expression of various genes related to antioxidant response/chemical defense mechanism, following administration of chemo-protective agents; however, constitutive expression of the same genes is often unaffected by the deletion of Nrf2 gene [8]. Microarray studies carried out on wild-type and Nrf2 knockout mouse suggest that the full range of proteins regulated by Nrf2 might be extensive. Some of the Nrf2 target genes include the glutathione S-transferases (GSTs), NAD(P)H quinoneoxido-reductase 1 (NQO1), superoxide dismutase (SOD), heme oxygenase-1 (HO-1) and glutamate cysteine ligase catalytic subunit (GCLC) [8].

The result of a previous study showed that *Cosmos caudatus* was able to significantly increase the activity of phase II enzymes such as catalase, superoxide dismutase, glutathione S-transferase and DT-diaphorase [NAD(P)H quinoneoxidoreductase 1] and significantly reduce the lipid peroxidation level in mice organs such as the lungs [9]. Another study showed that *Cosmos caudatus* was able to significantly increase the activity of phase II enzymes such as catalase, superoxide dismutase, glutathione S-transferase and DT-diaphorase and significantly reduce the lipid peroxidation level in mice livers [10]. In summary, what was discovered before was the positive effect of *Cosmos caudatus* on the activity of phase II enzymes, which implied that *Cosmos caudatus* could be chemoprotective or hepatopro-tective. However, there is no evidence of studies being done to examine the effects of *Cosmos caudatus* on the level of gene expression of these phase II enzymes (which are Nrf2 target genes) in the liver. Therefore, in this study, we would like to examine the effect of *Cosmos caudatus* aqueous extract (UR) supple-mentation in mice for 21 days on the expression levels of Nrf2 target genes (GSTs, NQO1, SOD, HO-1 and GCLC) in mice liver. Butylated hydroxyanisole (BHA) was used as a positive control since it has been proven to be chemoprotectant in previous animal studies [11].

2. MATERIALS AND METHODS

2.1. Reagents

TRIzol reagent and RNAse free water were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA). iScript cDNA synthesis kit and iQ SYBR Green supermix (2X) kit were obtained from Bio-Rad (Hercules, California, USA). NQO1, GSTA1, GSTA3, GSTM1, GSTM3, GSTP, SOD3, HO-1, GCLC and GAPDH primers for qPCR were synthesized by Vivantis Technologies (Oceanside, CA, USA). BHA and Isopropyl alcohol were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Chloroform was purchased from Merck KGaA (Darmstadt, Germany). Ethanol was purchased from BDH chemicals (Radnor, PA, USA).

2.2. 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.

2.3. 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 an acclimatization period of seven days to room temperature and relative humidity of 28.5°C and 50%, respectively. They were housed in standard mice cages and put under 12 hour 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 (UKMAEC) which had approved the study design of the experiment.

2.4. 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 (standard rat chow) and sham-treated with distilled water (0.1 ml) 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. The chosen dose was based on a previous study [9]. Mice were sacrificed after 21 days of treatment via cervical dislocation. Liver tissues were excised, immediately frozen in liquid nitrogen, and stored at -80 ° C until further analysis.

2.5. RNA Extraction and Quality Test

Total RNA from the liver was isolated using TRIzol reagent, according to the manufacturer's instructions. Isopropyl alcohol (Sigma, USA) was added in each extraction step to precipitate the total RNA. Extracted total RNA pellet was then washed with 75 % ethanol and dried before being dissolved in RNase free water. Total RNA was stored at -80^{0}C immediately after extraction. Concentration and purity of the extracted RNA were determined by NanoDrop spectrophotometer 2000c (Thermo Scientific, USA) at a wavelength of 260 nm (OD260). RNA with RNA integrity number (RIN) ranging from 7 to 10 and absorbance ratio of A260 to A280 ranging from 1.5 to 2.0 was used for cDNA synthesis.

2.6. Reverse Transcription

Generation of cDNA from RNA was done using iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, total RNA (1 µg) from each sample was added to a mixture of 4 µl of 5X iScript reaction mix, 1 µl of iScript reverse transcriptase, and 14 µl of nuclease-free water in a total volume of 20 µl. The final reaction mix was kept at 25°C for 5 min, 42°C for 30 min, and heated to 85°C for 5 min in a thermocycler (TC-412, Techne, Barloworld Scientific, UK). The cDNA was then used as a template for amplification by PCR.

2.7. Quantitative Real-Time PCR

Both forward and reverse primers for the genes of interest in this study were designed according to previous studies [12-16] and synthesized by Vivantis Technologies (Oceanside, CA, USA). The primer sequences for our gene of interest are shown in Table **1**.

Quantitative real-time PCR (qPCR) was performed on the MiniOpticon cycler (Bio-Rad, USA). The total reaction volume used was 20 µl, consisting of 1µl of

Table 1: Primers Sequences Used for Real-Time PCR Reactions

Gene	Primer sequence	References
GAPDH	F: 5' -GTGGAGTCTACTGGTGTCTTCA-3' R: 5' -TTGCTGACAATCTTGAGTGAGT-3'	[12]
NQO1	F: 5' -GCATTGGCCACAATCCACCAG-3' R: 5' -ATGGCCCACAGAGAGGCCAAA-3'	[13]
GSTA1	F: 5' -AGAATGGAGTGCATCAGGTGGTGGCTC-3' R: 5' -GGCAGGCAAGTAACGGTTTTTGGT-3'	[14]
GSTA3	F: 5' -GAGATCGACGGGATGAAACTGGTG-3' R: 5' -GCGCTTTCAGGAGAGGGAAGTTGT-3'	[14]
GSTM1	F: 5' -AGCACCCTGGCCTTCTGCACT-3' R: 5' -TTCGCAGAAACGGGCTGTGAG-3'	[14]
GSTM3	F: 5' -TGATTAGGCCCCTGCCATGCT-3' R: 5' -TTGGGTCTGGGCACCAATGAA-3'	[14]
GSTP	F: 5' -TTTGGGGGCTTTATGGGAAAAACCA-3' R: 5' -ACATAGGCAGAGAGCAGGGGGAAG-3'	[14]
SOD3	F: 5' -CCTTCTTGTTCTACGGCTTGC-3' R: 5' -TCGCCTATCTTCTCAACCAGG-3'	[15]
HO-1	F: 5' -CCTCACTGGCAGGAAATCATC-3' R: 5' -TATGTAAAGCGTCTCCACGAGG-3'	[16]
GCLC	F: 5' -GCACGGCATCCTCCAGTTCCT-3' R: 5' -TCGGATGGTTGGGGTTTGTCC-3'	[13]

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; NQO1: NAD(P)H: quinone oxidoreductase 1; GSTA1: Glutathione S-transferase alpha1; GSTA3: Glutathione S-transferase alpha3; GSTM1: Glutathione S-transferase mu1; GSTM3: Glutathione S-transferase mu3; GSTP: Glutathione S-transferase pi; SOD3: Superoxide dismutase 3; HO-1: Heme oxygenase-1; GCLC: Glutamate cysteine ligase catalytic subunit.

10µM forward primer and 1µl of 10µM reverse primer (500 nM final concentration of each primer), 10.0 µl of iQ™ SYBR®Green Supermix (2X) (Bio-Rad, USA), 7.0 µl of nuclease-free water and 1.0 µl of cDNA. All measurements were performed in triplicate and no-template controls (NTC) were incorporated onto the same set of PCR tubes to test for contamination by any assay reagents. The thermocycling conditions were initiated at 95°C for 30 sec, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing / extension at 60°C for 30 sec. Melting curve was performed by the end of each cycle to confirm the specificity of the primers and the purity of the final PCR product. Threshold cycles were determined for each gene and quantification of templates was performed according to the relative standard curve method. The relative standard curve method, as defined in the Applied Biosystems User Bulletin No. 2 [17] was used to analyse the real-time PCR data. In short, the expression level of each target gene was given as relative amount normalized against GAPDH standard controls. Performing qPCR eliminates the need for post-processing gel electrophoresis [18].

2.8. Statistical Analysis

Data were expressed as mean ± S.E.M. Significant differences between mean values of multiple groups were determined by one-way analysis of variance (ANOVA) with Tukey's HSD post-hoc test. Data not normally distributed was log transformed and analyzed using the Kruskal–Wallis one-way analysis of variance on ranks hypotheses. Statistical analysis was conducted using the SPSS software version 22. Differences were considered significant at $P < 0.05$.

3. RESULTS

3.1. Mortality

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

Table 2: 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 mice treated with *Cosmos caudatus* aqueous extract orally 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 significant difference in body weight was found in control vs. treated groups within day 1 and within day 21 (ANOVA).

3.2. Body Weight

There was an increase in daily body weight from day 0 until day 21 which corresponded to normal growth of mice (Table **2**).

3.3. Liver GSTA1, GSTA3, GSTP, GSTM1 and GSTM3 Gene Expressions

Liver GSTA1 gene expression levels in mice treated with 100 and 500 mg/kg *Cosmos caudatus* aqueous extract were significantly increased compared to the expression levels of GSTA1 gene in control mice livers. However, liver GSTA1 gene expression levels in mice treated with 1000 mg/kg *Cosmos caudatus* aqueous extract and 0.5% BHA were not significantly different from control mice. Liver GSTA3 gene expression levels in mice treated with 100, 500 and 1000 mg/kg *Cosmos caudatus* aqueous extract, as well as in mice treated with 0.5% BHA, were found to be significantly reduced compared to GSTA3 expression levels in the livers of control mice. Liver GSTM1 and GSTP gene expression levels in mice treated with 100 mg/kg *Cosmos caudatus* aqueous extract were not significantly different from GSTM1 and GSTP expression levels in the livers of control mice. However, liver GSTM1 and GSTP gene expression levels in mice treated with 500 and 1000 mg/kg *Cosmos caudatus* aqueous extract, as well as in mice treated with 0.5% BHA, were significantly decreased compared to the expression levels of GSTM1 and GSTP genes in control mice livers. GSTM3 liver gene expression levels were significantly increased in mice treated with intermediate and high doses of *Cosmos caudatus* (500 and 1000 mg/kg) compared to control mice. However, there was significant decrease in GSTM3 gene expression levels in mice treated with 100 mg/kg of *Cosmos caudatus* and also in the 0.5% BHA group, compared to control mice (Figure **1**).

3.4. Liver NQO1, SOD3, HO-1 and GCLC Gene Expressions

NQO1 liver gene expression levels were significantly increased in mice treated with the lowest dose of *Cosmos caudatus* aqueous extract (100 mg/kg), compared to control mice. However, there was significant decrease in liver NQO1 gene expression levels in mice treated with 1000 mg/kg of *Cosmos caudatus* aqueous extract and also in the 0.5% BHA group, compared to control mice. The levels of NQO1 liver gene expression in mice treated with 500 mg/kg aqueous extract was not significantly different to that of control mice. Liver SOD3 gene expression levels were significantly decreased in mice treated with 500 and 1000 mg/kg *Cosmos caudatus* aqueous extract as well as in mice treated with 0.5% BHA, compared to control mice. However, there was no significant difference in liver SOD3 gene expression levels in mice treated with 100 mg/kg of *Cosmos caudatus* aqueous extract compared to control mice. Treatment of mice with 500 mg/kg *Cosmos caudatus* aqueous extract and 0.5% BHA resulted in significant increase in liver HO-1 gene expression levels compared to control mice expression level. However, there was no significant difference in liver HO-1 gene expression levels in mice treated with 100 and 1000 mg/kg *Cosmos caudatus* aqueous extract, compared to control mice. Liver GCLC gene expression levels in mice treated with 100, 500 and 1000 mg/kg *Cosmos caudatus* aqueous extract, as well as in mice treated with 0.5% BHA, were all found to be significantly reduced compared to GCLC expression levels in the livers of control mice (Figure **2**).

4. DISCUSSION

The function of phase I metabolism is to facilitate a chemical compound for phase II metabolism. True detoxification of drugs and xenobiotic occurs in phase II whereby phase I metabolites are converted to water soluble products that can be easily excreted through the body. GST is one of the phase II enzymes involved in the conjugation of cytochrome P450 metabolites with glutathione, thus favoring their elimination from the body [19]. Results from previous studies support the notion that induction of GST conferred protection against cytotoxic, mutagenic and carcinogenic chemicals [20]. The transcription factor Nrf2, which binds to the ARE, seemed to be essential for the

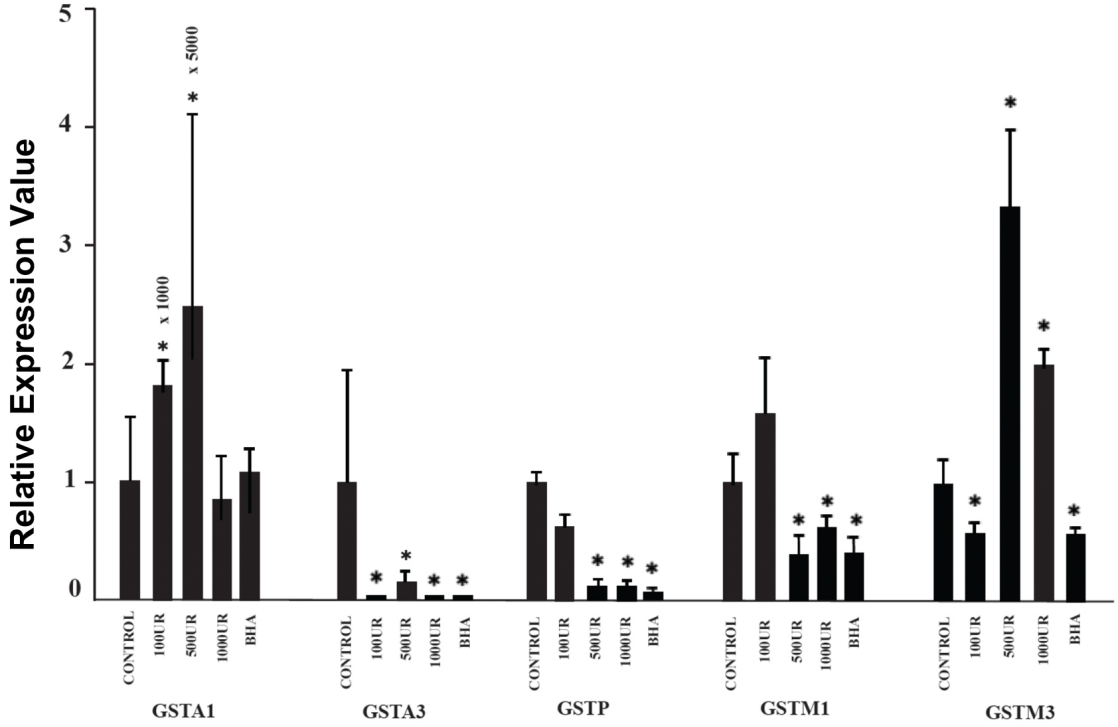

Figure 1: GSTA1, GSTA3, GSTP, GSTM1 and GSTM3 gene expressions in the liver of mice treated with *Cosmos caudatus* aqueous extracts for 21 days. Data is expressed relative to control (mean ± SEM, n=6/group). Asterisks (*) indicate statistically significant difference from the control group ($p < 0.05$). 100UR, 500UR, 1000UR: Groups of mice treated with *Cosmos caudatus* aqueous extract orally at a daily dose of 100, 500 and 1000 mg/kg body weight for 21 days, respectively. CONTROL, control mice; BHA, mice were fed normal diet fortified with 0.5 % BHA (w/w) for 21 days.

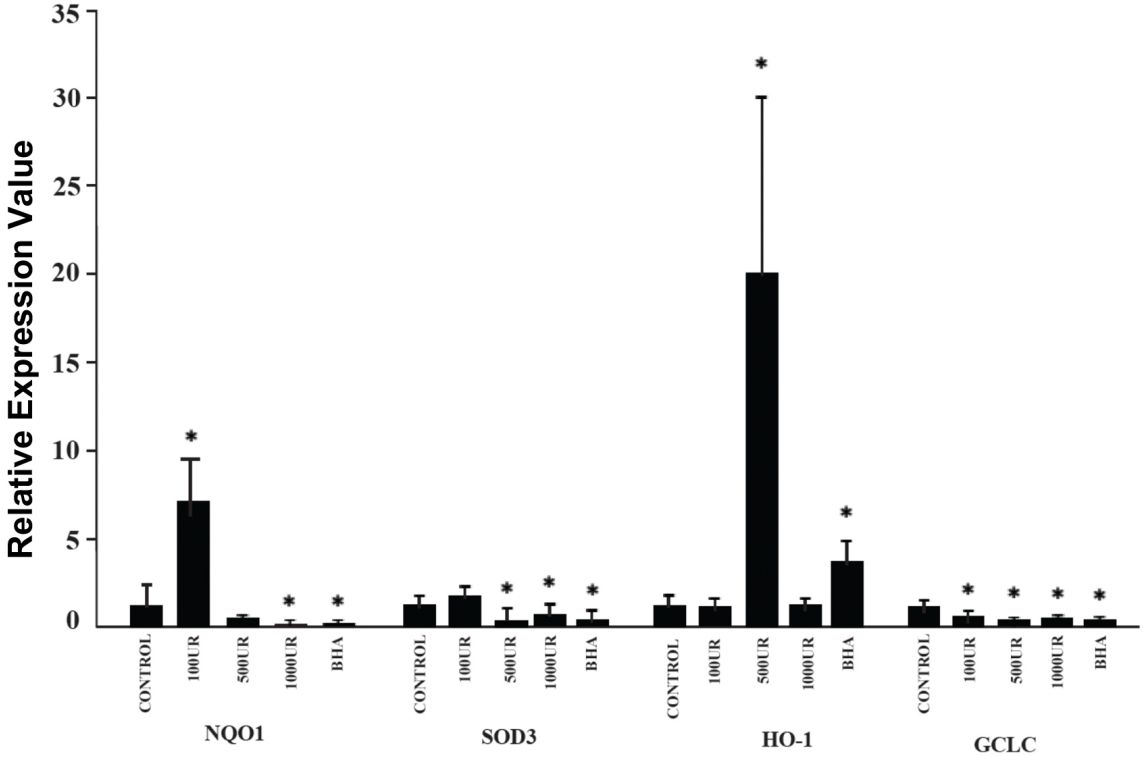

Figure 2: NQO1,SOD3, HO-1 and GCLC gene expressions in the liver of mice treated with *Cosmos caudatus* aqueous extracts for 21 days. Data is expressed relative to control (mean ± SEM, n=6/group). Asterisks (*) indicate statistically significant difference from the control group ($p < 0.05$). 100UR, 500UR, 1000UR: Groups of mice treated with *Cosmos caudatus* aqueous extract orally at a daily dose of 100, 500 and 1000 mg/kg body weight for 21 days, respectively. CONTROL, control mice; BHA, mice were fed normal diet fortified with 0.5 % BHA (w/w) for 21 days.

induction of glutathione *S*-transferases (GSTs) [8]. In this study, the effects of three doses of *Cosmos caudatus* aqueous extract (UR) on the gene expression of levels several GST isoforms [Alpha1 (GSTA1), Alpha3 (GSTA3), Mu1 (GSTM1), Mu3 (GSTM3) and Pi (GSTP)] in mice liver was determined.

Significantly increased expression of GSTA1 gene in mice liver was noted after the mice were treated with 100 and 500 mg/kg *Cosmos caudatus* aqueous extract (UR), compared to control mice liver. It was suggested that at these doses, UR or its metabolites might be able to influence the Nrf2 transcriptional activation pathway, which resulted in the increased expression of GSTA1 gene. However, there was no significant difference in the liver expression of GSTA1 gene after the treatment of mice with 1000 mg/kg UR and 0.5% BHA, compared to controls. These results indicated that treatment with 1000 mg/kg UR and 0.5% BHA were unable to further increase GSTA1 gene expression. It could be suggested that 1000 mg/kg UR and 0.5% BHA or their metabolites might somehow were able to inhibit the Nrf2 transcriptional activation pathway for GSTA1, or directly inhibited the GSTA1 gene expression, through mechanisms which are yet to be ascertained. Further studies are needed to confirm these suggestions.

Liver GSTA3 gene expression was found to be significantly reduced in mice treated with all three doses of UR, compared to controls. In mice treated with 500 and 1000 mg/kg UR, liver GSTP and GSTM1 gene expression was found to be significantly reduced compared to controls. This indicated that at these doses, UR might inhibit the Nrf2 transcription activation pathway related to GSTA3, GSTP and GSTM1, or directly inhibited the GSTA3, GSTP and GSTM1 gene expressions, through mechanisms which are yet to be determined. However, previous studies had shown that *Cosmos caudatus* contained potent antioxidants and it was suggested that UR protects liver cells against liver damage and lipid peroxidation [10, 21, 22]. Therefore, it could be suggested that the livers of mice treated with these doses of UR were not in stress, and in this case there was no urgency for the liver to upregulate GSTA3, GSTP and GSTM1 gene expressions in this situation. Since upregulation of GSTA3, GSTP and GSTM1 enzyme activities was thought to be unnecessary in this case, it was suggested that the liver might downregulate GSTA3, GSTP and GSTM1 gene expressions instead, hence resulting in significant reduction in GSTA3, GSTP and GSTM1 gene expressions found in this study.

GSTM3 liver gene expression was significantly increased in mice treated with high doses of UR (500 and 1000 mg/kg). However, there was significant decrease in GSTM3 gene expression in mice treated with 100 mg/kg UR. The results suggested that normal mice treated with 100 mg/kg UR was not subjected to any stressors, and therefore there was no requirement to increase GSTM3 enzyme activity. This might cause the GSTM3 gene expression to be downregulated compared to mice in control group. However, when treated with higher doses of UR (500 and 1000 mg/kg), the livers of mice might produce phase 1 metabolites of UR which were needed to be metabolized by GSTM3. This could trigger increased gene expression of GSTM3, most probably through activation of the Nrf2 pathway related to GSTM3. Further studies are needed to confirm these possibilities.

We found that treatment with 0.5% BHA significantly reduced the gene expression of GSTA3, GSTP, GSTM1 and GSTM3 in mice liver. The dose of BHA chosen was based on a previous study [9] whereby phase II liver enzymes activities were found to be significantly increased when this dietary dose was fed to mice. In order to induce phase II liver enzymes (such as GSTs) BHA must be metabolized in the liver to form t-BHQ, quinone radicals and/or other reactive oxygen species. The resulting increase in oxidative stress due to the presence of quinone radicals would trigger an increase in phase II enzymes such as GSTs [23]. It could be that in the mice used in our study, not enough oxidative stress was generated in the liver by the dose of BHA given. We therefore postulate that an increase in the dose of BHA in the diet up to 1% might increase the gene expression of phase II enzymes [23]**.** We further postulate that even after feeding the mice in our study with 0.5% BHA diet, their livers were still far from being stressed, which could trigger the downregulation of phase II enzymes gene expression since their activities were not critically required, however, further studies need to be done to confirm this. The result of another previous study that utilized similar dose of BHA as our mice showed that BHA increased the activity of phase II enzymes such as NQO1, SOD and GSTs [24]. However, they used a different strain of mice from our study. The strain of mice used in our study might possibly need a higher concentration of dietary BHA in order to produce the same effect.

NQO gene family belongs to the flavoprotein clan and, in the human genome, consists of two genes (NQO1 and NQO2) [25]. NQO1 is of particular interest,

because in mouse it is highly inducible by electrophiles and has key cytoprotective functions [26]. It is also capable of scavenging superoxide anions generated during oxidative stress and regenerating reduced forms of protective endogenous antioxidant compounds [25]. Nrf2 is the key regulatory pathway for NQO1 expression. The transcription factor Nrf2, which binds to the ARE, appears to be essential for the induction of NQO1 [8].

Our results showed that NQO1 liver gene expression was significantly increased in mice treated with lowest dose of UR (100 mg/kg). There was significant decrease in NQO1 gene expression in mice treated with 500 and 1000 mg/kg of UR and also in the 0.5% BHA group. Previous study showed that there was significant increase in NQO1 enzyme activity after 1000 UR and 0.5% BHA treatment [10]. We postulate that the significant increase in NQO1 enzyme activity in the livers of mice treated with 1000 UR and 0.5 % BHA might cause concomitant reduction in NQO1 gene expression in these mice, due to negative feedback regulation. In the case of mice treated with 100 mg/kg UR, it could be that at this dose, UR or its metabolites were able to activate the Nrf2 transcription activation pathway related to NQO1, thus resulting in significantly increased NQO1 gene expression compared to control mice. However, due to unknown reasons, the increased gene expression did not translate to increased enzyme activity in the 100 UR mice liver [10]. It could be that in mice treated with 100 mg/kg UR, the level of oxidative/chemical stress in the liver was still very minimal, and there was no urgency to translate the increased gene expression to increased detoxification activity related to NQO1 enzyme activity, as indicated by a previous study [10]. Similarly, mice treated with 500 mg/kg UR were also postulated not to encounter any excessive oxidative/chemical insult and stress. Therefore, it was suggested in mice treated at this dose, there was no urgency for the livers of these mice to upregulate the expression of NQO1 since its increased activity was not detected (nor critically required), as indicated by a previous study [10]. However, further studies on NQO1 activity assays and NQO1 expression at post translational level should be carried out to confirm these results.

SOD is the first and most significant line of antioxidant enzyme defence system against several reactive oxygen species (ROS). It catalyses the dismutation of two superoxide radicals to form hydrogen peroxide and oxygen. The product of this gene is thought to defend the lungs and other tissues from oxidative stress. Researchers have identified that the SOD3 is induced by antioxidants and regulated through Nrf2. SOD is therefore an important enzyme in combatting oxidative stress [27]. SOD catalyzes the conversion of superoxide (O2-) 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 [28].

In this study, we found that liver SOD3 gene expression was significantly decreased in mice treated with 500 mg/kg UR, 1000 mg/kg UR and 0.5% BHA. Liver SOD3 gene expression in mice treated with 100 mg/kg UR was not significantly different compared to mice in the control group. Radman *et al*. (2014) showed that liver SOD enzyme activity was significantly increased only in mice treated with 500 mg/kg UR and 0.5% BHA [10]. It is therefore suggested that in mice treated with 500 mg/kg UR and 0.5% BHA, the SOD enzyme activity had already peaked and therefore there is no need to further increase enzyme activity, thus causing the SOD3 gene expression to be downregulated by negative feedback mechanism, which was indicated by our result where there was significant reduction in liver SOD3 gene expression in mice treated with 500 mg/kg UR and 0.5% BHA, compared to control mice. There was no significant increase in liver SOD activity in mice treated with 1000 mg/kg UR [10], suggesting that this particular UR dose might inhibit the liver SOD3 gene expression mechanism, which was indicated in this study, where the liver SOD3 gene expression was significantly decreased compared to control mice. Mice treated with 100 mg/kg UR did not display significantly increased liver SOD enzyme activity compared to control mice [10], suggesting that this particular dose of UR was not able to activate the liver SOD3 gene expression mechanism, as indicated by the result of this study, where liver SOD3 gene expression was not significantly different compared to controls.

HO catalyzes the rate-limiting step in the principal degradative mechanism of heme catabolism. HO-1 expression is upregulated not only by its substrate, heme, but also by various non heme inducers such as heat shock, inflammatory cytokines, endotoxin, and oxidative stress, suggesting that HO-1 may play a vital role in maintaining cellular homeostasis [29]. Increases in HO-1 protein expression and activity have clear anti-

inflammatory and antioxidant effects and can protect tissues, organs, and entire animal models from septic shock, oxidative injury, and hypoxia [30-33]. HO-1 expression is primarily regulated at the transcriptional level, and its induction by various inducers is related to Nrf2 [8]. Heme oxygenase-1 is identified as particularly important in protection against a large number of diseases resulting from increased production or decreased removal of reactive oxygen species. HO-1 also impacts cancer progression through modulating tumor microenvironment [34].

In this study, there was a significant increase of HO-1 gene expression level in mice treated with 500 mg/kg UR, compared to control mice. This suggested that this is the optimum dose for mice liver HO-1 expression. The level of HO-1 gene expression in mice treated with 100 mg/kg UR was not significantly different compared to mice in the control group, suggesting that this particular dose was not enough to induce HO-1 gene expression. The level of HO-1 gene expression in mice treated with 1000 mg/kg UR was also not significantly different compared to mice in the control group, suggesting that this particular dose inhibited HO-1 gene expression, through mechanisms which have yet to be determined. There was a significant increase of HO-1 gene expression level in mice treated with 0.5% BHA, compared to control mice, indicating that BHA was able to positively induce the Nrf2 activation of HO-1 transcription machinery.

Glutamate cysteine ligase catalytic (GCLC) is the rate limiting enzyme in the synthesis of glutathione, an important endogenous antioxidant. Glutathione (GSH) is the main non-protein thiol in mammalian cells that participates in many critical cellular functions, including antioxidant defense and cell growth. GCLC subunits are encoded by different genes and dissociate under reducing conditions [35]. GCLC expression in the liver was found to be regulated by Nrf2 [8]. In this study, we found that the GCLC gene expression was significantly decreased at all UR doses used to treat the mice. This result correlates strongly with the result obtained by Radman *et al*. (2014), which showed that GSH level was significantly reduced at the same UR doses used [10]. GCLC is involved in glutathione (GSH) synthesis, therefore, the result of this study suggested that decreased GCLC gene expression was responsible for the decrease in GSH levels seen in the study by Radman *et al*. 2014 [10].

5. CONCLUSION

Treatment of mice with aqueous extract of *Cosmos caudatus* resulted in significantly increased liver expression of certain Nrf2 target genes at specific doses only – GSTA1 (100 & 500 mg/kg), GSTM3 (500 & 1000 mg/kg), NQO1 (100 mg/kg) and HO-1 (500 mg/kg). For these genes, other doses given to mice did not significantly affect their liver expression, or resulted in significantly reduced liver expression. For GSTA3, GSTP, GSTM1, SOD3 and GCLC, most of the UR doses administered to mice resulted in significantly reduced liver gene expressions compared to control mice. The results of this study indicated that *Cosmos caudatus* aqueous extract might influence the expression of Nrf2 target gene, either negatively (decreased expression) or positively (increased expression). The reasons why such upregulation and downregulation of these genes occurred could partly be explained in conjunction with the results of enzyme activities of these genes products which was detailed in a previous study [10]. However, in order to conclusively prove that *Cosmos caudatus* aqueous extract affect the expression of Nrf2 target genes, similar experiments should be performed on Nrf2 knockout mouse as well, or by conducting Nrf2 nuclear translocation studies in wild-type and Nrf2-depleted mice liver cells using *Cosmos caudatus* aqueous extract. In this study, we had used the aqueous extract of *Cosmos caudatus*. A previous study had suggested that ethanolic extract of *Cosmos caudatus* exhibited stronger antioxidant activity due to the presence of numerous flavonoids and phenolics compounds in the ethanolic extract [36]. Therefore, it is suggested that in future studies, we should use organic solvent-based extract of *Cosmos caudatus* instead of aqueous extract, in order to obtain a more comprehensive result.

ACKNOWLEDGEMENT

This work is funded by the Universiti Kebangsaan Malaysia (UKM) Faculty of Medicine Fundamental Grant FF-2016-155. The authors would like to thank Dr. Dominic Williams from the University of Liverpool, United Kingdom for the English language editing of this manuscript.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES

[1] Samy J, Sugumaran M, Lee KLW. Herbs of Malaysia: An introduction to the medicinal, culinary, aromatic and cosmetic use of herbs. 1st ed. Kuala Lumpur: Times Editions; 2005.

[2] Bodeker G. Health and beauty from the rainforest: Malaysian traditions of ramuan. 1st ed. Kuala Lumpur: Didier Millet; 2009.

[3] Hassan W. Healing herbs of Malaysia. 1st ed. Kuala Lumpur: Federal Land Development Agency; 2006.

[4] Abas F, Lajis NH, Israf D, Khozirah S, Kalsom YU. Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables. Food Chem 2006; 95(4): 566-73. https://doi.org/10.1016/j.foodchem.2005.01.034

[5] Wong SP, Leong LP, Koh JHW. Antioxidant activities of aqueous extracts of selected plants. Food Chem 2006; 99(4): 775-83. https://doi.org/10.1016/j.foodchem.2005.07.058

[6] Mustafa R, Hamid AA, Mohamed S, Bakar FA. Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants. J Food Sci 2010; 75(1): C28-C35. https://doi.org/10.1111/j.1750-3841.2009.01401.x

[7] Reihani SFS, Azhar ME. Antioxidant activity and total phenolic content in aqueous extracts of selected traditional Malay salads (*Ulam*). Int Food Res J 2012; 19: 1439-44.

[8] Ma Q. Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol 2013; 53: 401-26. https://doi.org/10.1146/annurev-pharmtox-011112-140320

[9] Abdullah A, Dhaliwal KK, Roslan NNF, Lee CH, Kalaiselvam M, Radman HM, Saad QHM, Yusof K, Jaarin K. The effects of *Cosmos caudatus* (ulam raja) on detoxifying enzymes in extrahepatic organs in mice. Journal of Applied Pharmaceutical Science 2015; 5(1): 082-088. https://doi.org/10.7324/JAPS.2015.50115

[10] Radman HM, Yusof K, Saad QHM, Wan-Ngah WZ, Abdullah A. The effect of Ulam Raja (*Cosmos caudatus*) on drug metabolizing enzymes, lipid peroxidation and antioxidant status in mice liver. Int J PharmTech Res 2014; 6(4):1213-25.

[11] Dinkova-Kostova AT. Chemoprotection against cancer: an idea whose time has come. Altern Ther Health Med 2007; 13(2): S122-7.

[12] Kong L, Tanito M, Huang Z, Li F, Zhou X, Zaharia A, Yodoi J, Mcginnis JF, Cao W. Delay of photoreceptor degeneration in tubby mouse by sulforaphane. J Neurochem 2007; 101(4): 1041-52. https://doi.org/10.1111/j.1471-4159.2007.04481.x

[13] Leung L, Kwong M, Hou S, Lee C, Chan JY. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. J Biol Chem 2003; 278(48): 48021-29. https://doi.org/10.1074/jbc.M308439200

[14] Xu Z, Chen L, Leung L, Yen TB, Lee C, & Chan JY. Liver-specific inactivation of the Nrf1 Gene in adult mouse leads to nonalcoholic steatohepatitis and hepatic neoplasia. Proc Natl Acad Sci USA 2005; 102(11): 4120-25. https://doi.org/10.1073/pnas.0500660102

[15] Seta F, Rahmani M, Turner PV, Funk CD. Pulmonary oxidative stress is increased in cyclooxygenase-2 knockdown mice with mild pulmonary hypertension induced by monocrotaline. PLoS ONE 2011; 6(8): e23439. https://doi.org/10.1371/journal.pone.0023439

[16] Lu H, Koshkin V, Allister EM, Gyulkhandanyan AV, Wheeler MB. Molecular and metabolic evidence for mitochondrial defects associated with B-Cell dysfunction in a mouse model of type 2 diabetes. Diabetes 2010; 59(2): 448-59. https://doi.org/10.2337/db09-0129

[17] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001; 25(4): 402-8. https://doi.org/10.1006/meth.2001.1262

[18] Ong YL, Irvine A. Quantitative real-time PCR: a critique of method and practical considerations. Hematology 2002; 7(1): 59-67. https://doi.org/10.1080/10245330290015573

[19] Katzung BG, Masters SB, Trevor AJ. Basic & clinical pharmacology. 13th ed. New York: McGraw-Hill Medical; 2014.

[20] Higgins LG, Hayes JD. Mechanisms of induction of cytosolic and microsomal glutathione transferase (GST) genes by xenobiotics and pro-inflammatory agents. Drug Metab Rev 2011; 43(2): 92-137. https://doi.org/10.3109/03602532.2011.567391

[21] Sukrasno S, Fidriany I, Anggadiredja K, Handayani WA, Anam K. Influence of drying method on flavonoid content of *Cosmos caudatus* (Kunth) leaves. Res J Med Plant 2011; 5(2): 189-95. https://doi.org/10.3923/rjmp.2011.189.195

[22] Andarwulan N, Kurniasih D, Apriady RA, Rahmat H, Roto AV, Bolling BW. Polyphenols, carotenoids, and ascorbic acid in underutilized medicinal vegetables. J Funct Foods 2012; 4(1): 339-47. https://doi.org/10.1016/j.jff.2012.01.003

[23] Iverson F. *In vivo* studies on buthylated hydroxyanisole. Food Chem Toxicol 1999; 37: 993-7. https://doi.org/10.1016/S0278-6915(99)00091-5

[24] Singh RP, Padmavathi B, Rao AR. Modulatory influence of Adhatoda vesica (Justicia adhatoda) leaf extract on the enzymes of xenobiotic metabolism, antioxidant status and lipid peroxidation in mice. Mol Cell Biochem 2000; 213(1-2): 99-109.

[25] Aleksunes LM, Goedken M, Manautou JE. Up-Regulation of NAD(P)H quinone oxidoreductase 1 during human liver injury. World J Gastroenterol 2006; 12(12): 1937-40. https://doi.org/10.3748%2Fwjg.v12.i12.1937

[26] Dinkova-Kostova AT, Talalay P. Persuasive evidence that quinone reductase type 1 (DT-diaphorase) protects cells against the toxicity of electrophiles and reactive forms of oxygen. Free Radic Biol Med 2000; 29(3): 231-40. https://doi.org/10.1016/S0891-5849(00)00300-2

[27] Singh B, Bhat HK. Superoxide dismutase 3 is induced by antioxidants, inhibits oxidative DNA damage and is associated with inhibition of estrogen-induced breast cancer. Carcinogenesis 2012; 33(12): 2601-10. https://doi.org/10.1093/carcin/bgs300

[28] Weidinger A, Kozlov AV. Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction. Biomolecules 2015; 5(2): 472-84. https://doi.org/10.3390/biom5020472

[29] Agarwal A, Bolisetty S. Adaptive responses to tissue injury: Role of heme oxygenase-1. Trans Am Clin Climatol Assoc 2013; 124: 111-22.

[30] Chung SW, Hall SR, Perrella MA. Role of haem oxygenase-1 in microbial host defence. Cell Microbiol 2009; 11(2): 199-207. https://doi.org/10.1111%2Fj.1462-5822.2008.01261.x

[31] Farombi EO, Surh Y-J. Heme oxygenase-1 as a potential therapeutic target for hepatoprotection. BMB Reports 2006; 39(5): 479-91. https://doi.org/10.5483/BMBRep.2006.39.5.479

[32] Gozzelino R, Jeney V, Soares MP. Mechanisms of cell protection by heme oxygenase-1. Annu Rev Pharmacol Toxicol 2010; 50: 323-54. https://doi.org/10.1146/annurev.pharmtox.010909.105600

[33] Pae H-O, Chung H-T. Heme oxygenase-1: its therapeutic roles in inflammatory diseases. Immune Netw 2009; 9(1): 12-19. https://doi.org/10.4110/in.2009.9.1.12

[34] Chau L-Y. Heme oxygenase-1: emerging target of cancer therapy. J Biomed Sci 2015; 22: 22 (7 pages). https://doi.org/10.1186/s12929-015-0128-0

[35] Yang H, Magilnick N, Lee C, Kalmaz D, Ou X, Chan JY, Lu SC. Nrf1 and Nrf2 regulate rat glutamate-cysteine ligase catalytic subunit transcription indirectly via NF-kappaB and AP-1. Mol Cell Biol 2005; 25(14): 5933-46. https://doi.org/10.1128/MCB.25.14.5933-5946.2005

[36] Lee TK, Vairappan CS. Antioxidant, antibacterial and cytotoxic activities of essential oils and ethanol extracts of selected South East Asian herbs. J Med Plants Res 2011; 5(21): 5284-90.

Received on 29-05-2017 Accepted on 14-09-2017 Published on 06-11-2017

DOI: https://doi.org/10.6000/1927-5951.2017.07.04.1