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Response of Hepatic Metabolizing Enzymes and Oxidative Stress in Orally Administrated Zerumbone Against MIA-Induced Osteoarthritis in Rats

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Abstract: The main objective of this study was to elucidate the extent of hepatic oxidative stress following oral administration of zerumbone against monosodium iodoacetate induced Osteoarthritis (OA) in rats by monitoring microsomal cytochrome P450 and glutathione S-transferase enzymes as well as determination of oxidative stress biomarkers i.e., glutathione and malondialdehyde. Forty rats were randomly assigned into five groups. Rats in the first and second groups were treated with two different doses of zerumbone. Rats in the third group (positive control) were given celecoxib whereas the fourth group (negative control) was given corn oil. Rats of the fifth group were untreated not induced with OA and were used as a basal group. Results showed significant induction of cytochrome P450 and glutathione S-transferase and insignificant changes in both glutathione and lipid peroxidation levels in zerumbone treated groups compared to corn oil and basal groups. Levels of ALT and AST in zerumbone treated groups were comparable to the level in the basal group indicating absence of liver damage. Prostaglandin E_2 level significantly reduced following zerumbone administration. Safety profile of zerumbone in this study, attract new investigation to explore its advantageous effect on using higher dosage regimen and/or longer duration against OA or other disease.

Key words: Zerumbone, oxidative stress, cytochrome P450, glutathione, glutathione S-transferase, malondialdehyde

INTRODUCTION

The use of herbal natural products has gained interest among the world population so that many herbs have been developed into supplement which are claimed to reduce the disease symptoms and improve quality of life. Unfortunately, few adverse affect may be rise beside its pharmacological and therapeutic efficacy.

Apart from that zerumbone which is a crystalline sesquiterpene found in the rhizomes of the edible ginger known as *Zingiber zerumbet* Smith (Szabolcs *et al*., 2007). The underground rhizomes are the medicinally useful part (Ahmed *et al*., 2008) so that extensive studies were carried out through the last decade on their pungent constituents and among the pharmacological effects established were anti-inflammatory (Nharet-Somchit and Nur-Shukriah, 2003), anti-oxidants (Ruslay *et al*., 2007) anti-cancer (Yee *et al*., 2006) and anti-arthritic properties (Al-Saffar *et al*., 2010). Xenobiotics oxidative stress can influence the metabolism of cells in vital organs such as heart, nervous tissue and liver which is awfully hazardous as it does not exhibit any symptom and its identification is great difficult by means of laboratory methods (Kataria *et al*., 2010).

Xenobiotics such as drugs, chemical carcinogens, pesticides, environmental pollutants, ingested natural products and endogenous compounds are undergoing a number of hepatic and extra-hepatic enzymatic reactions referred to as biotransformation which may be categorized into phase I and phase II reactions (Hayes, 2008). Microsomal cytochrome P450s (CYP450) are an important phase I enzymes responsible for the metabolism of numerous xenobiotics.

Liver is the primary site of these enzymes. They render foreign compounds more water-soluble and thus facilitate their excretion (Nelson *et al*., 1996). Paradoxically, these enzymes may activate inert compounds as for example they can activates pro-

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arcinogens to their ultimate carcinogenic form which is rendered suitable for interaction with nucleophilic sites in DNA (Koul *et al*., 2009). One of the most essential enzymes of phase II reactions are Glutathione S-Transferases (GSTs) which play an important role in both intracellular transport of hydrophobic molecules and the metabolism of toxic compounds (Zhu *et al*., 1998). The cytosolic GSTs which comprising over 95% of total cellular GSTs are the major isoforms involved in xenobiotics metabolism.

The cytosolic GST enzymes serve as ideal biomarkers and early indicators of organ damage which is applicable for both human and animals (Kilty *et al*., 1998). In essence, they catalyze the nucleophilic attack of the sulphur atom of GSH on the electrophilic group thus greatly decrease the reactivity of the compounds and make them more water soluble to favor their elimination (Sau *et al*., 2010).

Glutathione (GSH), a key master hepatic antioxidant biomarker is a superoxide radical scavenger where it protects thiol group required for maintaining the cell integrity against oxidation (Altinkaynak *et al*., 2003). It is an endogenous, non enzymatic antioxidant component present at highest concentration in the liver plays an important role in maintaining the intracellular redox equilibrium. It protects tissues from oxidative stress through glutathione peroxidase, glutathione reductase and the conjugation of intermediary metabolites through glutathione transferase (Jusman and Halim, 2009).

Glutathione conjugates have higher molecular weights and are more water soluble and more likely to be excreted in urine and bile than the parent compounds (Pompella *et al*., 2003). Malondialdehyde (MDA) is presumptive biomarker for lipid peroxidation which is enhanced in the presence of oxidative stress in the living organisms. Its elevation in various diseases thought to be related to the free radical damage so that its determination has been widely applied as the most common approach for the assessment of lipoperoxidation (Mateos *et al*., 2005).

There is paucity of research to detect oxidative stress associated with OA and its regulation with zerumbone. The study was designed to explore the effect of oral application of zerumbone on hepatic oxidative stress in the rat by monitoring important hepatic oxidative enzymes such as microsomal CYP 450, cytosolic GST and non enzymatic oxidative stress biomarkers i.e., GSH and MDA.

MATERIALS AND METHODS

Animals and induction of osteoarthritis: The study was performed on forty adult male Sprague Dawley rats each weighing approximately 275-400 g randomly assigned to five groups each of eight. The animals were obtained from the animal house facility and housed in well air-conditioned animal room at 22°C (one rat per cage). The rats were given commercial pellet and tap water *ad libitum* and were left for 2 weeks for acclimation before used.

The study was carried out according to the guideline for animal handling and care regulations and was approved by Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, University Putra Malaysia (Reference: UPM FPV/PS/3.2.1.551/AUP-R44). To induce OA, rats were anesthetized and injected intraarticularly with 50 μL of MIA diluted with saline at a concentration of 60 mg mL^{-1} (Sigma, USA) in their right knee joints at day 0 (Bove *et al*., 2003).

Preparation of zerumbone and protocol of treatments: Zerumbone was prepared according to the method of Murakami *et al*. (1999). The preparation and qualification has been implemented at Analytical Laboratory and Quality Assurance Programmed Technical Services Centre, MARDI, P.O. Box 12301. General post office: 50774, Kuala Lumpur, Malaysia. Oral treatment was started at day 16 after OA induction and lasted for 4 weeks. Rats in the first (ZI) and second (ZII) groups were administered with 2 mL kg^{-1} body weight of 0.2% and 0.4 w/v zerumbone diluted in corn oil, respectively. Third group (CEL) were administered celecoxib (Celebrex®) at a dosage of 30 mg kg^{-1} body weight. Diluted in 5% carboxyl methyl cellulose and served as positive control and rats in the fourth group were received Corn Oil (CO) at a dosage of 2 mL kg^{-1} body weight and served as negative control whereas rats of the fifth group were left untreated and without OA induction as a Basal (B) group.

Preparation of liver microsomal and cytosolic fractions: Rats were euthanized with intraperitoneal injection of 500 mg kg^{-1} sodium phenobarbital in line with current animal care regulations. Liver were excised and washed in cold 1.15% KCl then mixed in 10 times to their weight with 1.15% KCl, minced well, homogenized thoroughly and centrifuged at 10^4 g for 20 min.

A volume of buffer A [0.10 M Tris-acetate buffer (pH 7.4) containing 0.10-M KCl, 1.0 mM Ethylenediamine Tetraacetic Acid (EDTA) and 20 mM Butylated Hydroxytoluene (BHT)] equal to the discarded sediment was added to the supernatant and centrifuged for 60 min at 10^5 g (Beckman, XL-80 ultracentrifuge, 70.1 Ti rotor) to yield supernatant (contain the cytosolic fraction which was saved for subsequent uses) and a microsomal pellet. A volume of buffer B (0.10 M potassium pyrophosphate

buffer (pH 7.4) containing 1.0 mM EDTA and 20 mM BHT) equal to that saved supernatant was added to the pellet and suspended, homogenized and recentrifuged at 10^5 g for 60 min. The resulted pellet was homogenized in 2 mL of buffer C (10 mM Tris-acetate buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (w/v)) and stored at -80°C (Hayes, 2008).

Determination of protein in liver: Unknown protein concentration in liver microsomal pellet, cytosolic fraction and homogenate was assayed using BCA™ protein assay kit (Thermo Scientific, USA) which was performed according to the manufacturer's instructions.

Determination of CYP P450: Aliquot from microsomal pellet was diluted with 0.05 M PBS (pH 7.4) to obtain sample contain protein concentration approximately 2 mg mL^{-1}. Aliquot of 2 mL from this sample was transferred for each reference and sample cuvettes. Their baseline densities were determined using a spectrophotometer by scanning them from 420-490 nm. Carbon monoxide was bubbled gently into the sample cuvette for 20 sec then a small pinch of powdered sodium dithionite was added. The cuvette covered with a parafilm and inverted many times and then bubbled with carbon monoxide again for 20 sec.

The reference cuvette was treated only with a small pinch of sodium dithionite. The cuvettes were shaken vigorously then their observed densities were determined with the spectrum from 420-490 nm. The quantity of CYP450 was calculated from the differences of optical densities and the molar extinction coefficient of 91/mM/cm (Omura and Sato, 1964).

Determination of cytosolic GST activity: The procedure was conducted by the preparation of two cuvettes (sample and reference) each of 3 mL from the followings: 2.2 mL of 0.1 M potassium phosphate buffer (pH 6.5), 0.1 mL of 30 mM CDNB substrate (1-chloro-2, 4-dinitrobenzen from Aldrich Chemical Co.) and 0.1 mL of 30 mM GSH. Both cuvettes were left for 1 min at room temperature (25°C) and read with spectrophotometer at 340 nm. An aliquot of 0.6 mL of the cytosol was added to the sample cuvette and mixed thoroughly. Reading was read after 1-5 min for both cuvettes at 340 nm with spectrophotometer. The specific activity of GST was expressed as nmol of GSH-CDNB conjugates formed/min/mg cytosolic protein using an extinction coefficient of 9.6/mM/cm (Habig *et al*., 1974).

Determination of MDA: Upon euthanasia, livers were excised, weighed, washed and chilled in ice-cold 1.15% KCl. Then 10% of liver homogenates in 1.15% KCl (w/v) were prepared and homogenized. Aliquot of 0.1 mL from the homogenate mixed with 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution (pH 3.5) and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid. The mixture was finally made up to 4.0 mL with distilled water and heated at 95°C for 60 min.

After cooling with tap water, 1.0 mL of distilled water and 5.0 mL of n-butanol were added and the mixture was shaken vigorously and centrifuged at 4000 rpm for 10 min, the upper butanol layer separated and read with spectrophotometer at 532 nm against pure n-butanol. The concentration of MDA was determined by comparison of absorbance to that of a standard curve with a range from 1-100 μmols of authentic MDA (product of Sigma Co.) and expressed as μmol mg^{-1} of protein (Ohkawa *et al*., 1979).

Determination of GSH: Total GSH concentration was measured in the liver homogenate according to the method of Sedlak and Lindsay described by Swarnakar *et al*. (2005). About 2 mL of 10% trichloacetic acid was added to a 2 mL of homogenate and centrifuged at 600 g for 15 min. Then 2 mL of supernatant was added to 2.0 mL of 0.32 mol L^{-1} Na_2HPO_4. About 5 min prior to the measurement of absorbance, 50 μL aliquot of 0.01 mol L^{-1} Ellman's solution (5, 5-dithiobis-2-nitrobenzoic acid) was added. The absorbance was determined at 412 nm. The concentration of GSH was determined by comparison of absorbance to that of a standard curve with a range from 1-150 μmols of authentic GSH (purchased from sigma chemicals Co.) and expressed as μmol mg^{-1} of protein.

Measuring serum enzymes and prostaglandin E_2: Upon euthanasia 5 mL of blood was collected from all rats via cardiac puncture and left for 1 h.

The blood was then centrifuged at 3000 rpm for 10 min and then sera were collected and kept at -20°C until use. Activity of ALT and AST were determined in units per liter using standard auto-analyzer methods (Automatic analyzer 902, Hitachi, Japan) and the kits (from Cobas Roche/Hitachi) used according to the manufacturer's protocol.

Determination of PGE_2 was carried out using enzyme immunoassay kits (Assay Design purchased from USA, Catalog #900-001) which was performed according to the manufacturer's protocol.

Statistical analysis: Statistical calculations were carried out with the SPSS 15.0 for Windows software package. Data was expressed as mean±SEM and analyzed with one way ANOVA and confirmed with student test (Table 1).

Table 1: Hepatic concentrations of proteins, CYP450, GST, GSH and MDA in ZI (treated with 2 mL kg^{-1} body weight of 0.2% w/v zerumbone diluted in corn oil), ZII (treated with 2 mL kg^{-1} body weight of 0.4% w/v zerumbone diluted in corn oil), CEL (treated with 30 mg kg^{-1} body weight diluted in 5% carboxyl methyl cellulose), CO (treated with 2 mL kg^{-1} body weight corn oil) and B (left untreated) groups

Concentration	ZI	ZII	CEL	CO	B
Microsomal protein (mg g^{-1}) wet tissue	31.50±1.3	31.55±0.90	28.40±1.80	31.52±1.90	31.52±1.1
Cytosolic protein (mg g^{-1}) wet tissue1	164.59±3.5	166.80±6.50	143.67±3.80¶	153.52±4.30	164.68±2.6
Homogenate protein (mg g^{-1}) wet tissue	189.10±4.2	195.11±4.10	159.07±2.40†	185.95±3.60	188.43±3.1
Microsomal CYP450 (nmol mg^{-1}) protein	0.698±0.01*	0.701±0.02*	0.685±0.01*	0.582±0.03	0.596±0.03
Cytosolic GST (nmol/min/mg) protein	1623.7±61**	1650.32±56**	1223.4±73***	1197.35±43	1174.8±29
Homogenate GSH (μmol g^{-1}) wet tissue #	3.7±0.17	3.65±0.17	3.74±0.09	3.56±0.18	3.47±0.15
Homogenate MDA (μmol g^{-1}) wet tissue #	6.72±0.2	6.81±0.4	6.82±0.35	6.86±0.44	6.77±0.3
Serum ALT IU L^{-1}	65.62±1.7¥	62.32±0.9¥	72.95±2.5¥	85.02±4.9	61.8±1.5¥
Serum AST IU L^{-1}	154.1±4.1€	149.5±3.1€	165.2±7.6€	200.6±14	144.8±9.7€
Serum PGE2 concentration (pg mL^{-1})	23317.5±713[a]	19735.1±967[a]	22845.1±750[a]	34139.8±2404[a]	17359.1±2123[a]

Data were analyzed using one-way ANOVA followed by student's t-test; All values were expressed as the mean±SEM (n = 8); ¶Significantly (p<0.05) lower versus ZI, ZII and B; † significantly (p<0.01) lowered versus other groups; *Significantly (p<0.05) higher versus CO and B groups; **Significantly (p<0.01) higher versus CEL, CO and B groups; ***Non significant difference (p>0.05) versus CO and B groups; # No significant differences (p>0.05) between all groups; ¥Significantly (p<0.05) lower versus CO group; €Significantly (p<0.01) lower versus CO group; [a]Significantly (p<0.001) lower versus CO group

RESULTS AND DISCUSSION

All rats gained weight over the study period and there was no adverse effect following the treatments on body weight gain (Fig. 1). Protein assay in different preparations and enzymes content and activities as well as GSH and MDA levels in the liver tissues were well summarized and statistically analyzed as shown in Table 1. Assay of protein reveled non significant differences in microsomal pellet concentration between the groups.

While concentration of protein in both cytosolic fraction as well as liver homogenate revealed significant lowering in those of CEL group versus the others. Protein determined in the liver homogenate was higher than the cytosolic fraction and both were significantly higher than the microsomal pellets content. Microsomal content of CYP450 found significantly ($p<0.05$) induced in ZI, ZII and CEL groups compared with CO and B groups. Activity of cytosolic GST enzyme was induced significantly in Zerumbone treated groups (ZII, ZII). However, the activity of GST in rats treated with celecoxib was not significantly affected compared to CO or B groups. Determination of GSH in the liver homogenates of different groups revealed no significant elevation of its concentrations in ZI, ZII and CEL groups compared with CO and B groups. Whereas there was no significant changes in the level of MDA in ZI, ZII and CEL groups versus the levels found in both CO and B groups. The activity of ALT and AST showed no significant changes when compared to CO and B groups.

Assay of the inflammatory PGE_2 showed a significant reduction of this hormone in the Zerumbone (ZI, ZII) and celecoxib treated groups compared to the negative control group (CO). Interestingly, PGE_2 in ZII showed comparable level to that found in the B group. Recently, zerumbone was deliberated by the current team work for its effect on OA. Oral administration of zerumbone for a period of 4 weeks revealed plausible chondroprotective effects on the knee OA in rats (Al-Saffar *et al*., 2010). The purpose of the current research is to explore the status of hepatic oxidative stress following zerumbone oral application to recommend longer course or higher dosage regimen of this natural remedy against OA to obtain further anti-arthritic efficacy without any possible adverse effect.

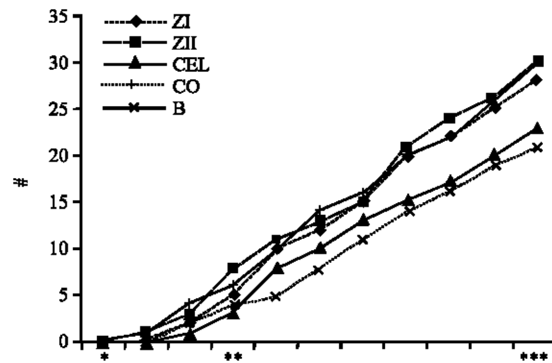

Fig. 1: The figure showed gradual gain (#) in g of the body weight in all groups throughout the experiment. The gain in zerumbone treated groups was comparable to the normal basal group (B). Body weight determined with 4 days interval in the ZI (treated with 2 mL kg^{-1} body weight of 0.2% w/v zerumbone diluted in corn oil), ZII (treated with 2 mL kg^{-1} body weight of 0.4% w/v zerumbone diluted in corn oil), CEL (treated with 30 mg kg^{-1} body weight diluted in 5% carboxyl methyl cellulose), CO (treated with 2 mL kg^{-1} body weight corn oil) and B (left untreated) groups. On day 0 (*), OA was induced and lasted on day 15 (**) then followed with the treatment which was conducted till day 43 (***)

Current data showed significant induction of CYP450 enzyme in zerumbone and celecoxib treated groups. The

inducibility of the CYP450 enzyme system is an important property because it enables the organism to detoxify a wide range of exogenous compounds (Raza *et al*., 1998). Conversely, its reduction following malnutrition, cocaine administration or ether anesthesia will attenuate its metabolizing function (Vitcheva and Mitcheva, 2007). Without doubt, this enzyme is considered the prime metabolic route for the majority of xenobiotics, acting either directly in detoxification or indirectly by priming the xenobiotics for further metabolism (Hayes, 2008). The induction of this enzyme by the given dosage regimen and course of zerumbone in this investigation should be kept in consideration when supplemented with other drugs against OA and other diseases.

The premise is that drug interaction may occur and the inducibility may enhance the metabolism of these drugs or therapies which are substrates to CYP450 or some of its isozymes. Currently, induction of CYP450 by celecoxib also observed which is steady with previous findings (Koul *et al*., 2009).

Zerumbone possess activating properties towards phase II drug metabolizing enzymes (Murakami *et al*., 2004; Abdul *et al*., 2008). In the present research the activity of the hepatic cytosolic GST was induced significantly in Zerumbone treated groups (ZII, ZII) because of its potential inducibility to some of GST isozymes resulting in an induction of the total GST activity. Some of these isozymes being able to utilize major product of lipid peroxidation including fatty acid hydroperoxide and play a role in the protection against oxidative stress (Yang *et al*., 2001).

The enzyme conjugates the xenobiotics with glutathione (glutathionylation) producing metabolites which are more hydrophilic and are readily excreted in bile or urine as inactive conjugates. The induction of GST by zerumbone was parallel to the previous findings (Nakamura *et al*., 2004). The data showed no significant induction of GST activity by celecoxib which was comparable to the previous findings (Bastos-Pereira *et al*., 2010).

This enzyme is not the target of celecoxib activity in the oxidative stress process. Celecoxib failed to induce GST due to failure of further oxidation and -COOH formation (Sandberg *et al*., 2002). Currently, the levels of GSH and MDA biomarkers in the liver homogenates revealed no significant changes of GSH and MDA concentrations in ZI, ZII, CEL groups compared to both CO and B groups. Glutathione is a critical component of the oxidant defense system which helps in scavenging free radicals generated during poisoning. Currently, its level was not markedly different from the basal normal concentration indicating absence of free radical accumulation; otherwise its deficiency will signify an excessive production of reactive oxygen species (Sido *et al*., 1998). There is a strong correlation between MDA and GSH concentrations in the liver tissue because the oxidative stress leads to the formation of profuse reactive oxygen species which triggers the redox signaling cascade so that predispose to GSH depletion. As a response to these events, lipid peroxidation will be enhanced which is monitored by induction of MDA levels in the liver tissue (Jusman and Halim, 2009). Present findings revealed no depletion of GSH and no subsequent MDA induction in the rats treated with zerumbone.

It denotes that zerumbone exhibited antioxidant property by restoring the redox equilibrium (lipid peroxidation and GSH levels). Recently, it was found that zerumbone reduced the extent of liver damage caused by toxic agents as it can attenuate and prevent lipid peroxidation and preserved GSH antioxidant (Ibrahim *et al*., 2009).

Celecoxib and other NSAIDs showed no significant changes in the hepatic lipid peroxidation following its application compared to normal control animals (Karatopuk and Gokcimen, 2010; Bastos-Pereira *et al*., 2010). But they are capable to inhibit the elevated hepatic MDA in the oxidative stress in rats suffered hypercholesterolemia (Dabhi *et al*., 2008). El-Medany *et al*. (2005) found no significant change in GSH following oral administration of celecoxib in the normal wister rats but it can significantly decreases its level in the induced acetic acid colitis.

The determination of the activities of pathological enzymes like transaminases (ALT, AST) is a common means of detecting liver damage. Previously, ALT and AST enzymes considered as a marker of drug-associated hepatotoxicity (Brunner *et al*., 1998). However, the current data revealed no significant changes of ALT and AST activities following the treatment with the zerumbone or celecoxib.

Elevation of these enzymes due to the oxidative stress was well stated before (Modi *et al*., 2007; Fakurazi *et al*., 2008). Ibrahim *et al*. (2009) demonstrated an elevation of these enzymes in hepatic injury whereas he found that the pretreatment with zerumbone can prevents such changes. Also, previously found that celecoxib has no oxidative stress and toxic effect on these enzymes (Park *et al*., 2005).

Serum concentrations of PGE_2 were inhibited significantly in zerumbone treated groups compared to CO group. Reduction of this hormone in the zerumbone treated groups was consistent with previous finding (Murakami *et al*., 2004). The data exposed a role of this

hormone in OA events and following the treatment with zerumbone indicate zerumbone's anti-inflammatory property which was dose dependent. Interestingly, higher dose of zerumbone used in ZII group showed comparable level of this hormone to that of the basal group.

CONCLUSION

Current data showed that zerumbone with a given dose and duration course have significant inducible activity toward both CYP450 and Cytosolic GST enzymes, a property which is very important in biotransformation reactions of exogenous compounds.

Besides, zerumbone also exhibited antioxidant property by restoring the redox equilibrium because it restored the levels of hepatic GSH and MDA indicating absence of free radical accumulation and lipid peroxidation. Zerumbone does not have adverse effect on both ALT and AST as markers of damaged liver tissue.

Zerumbone anti-inflammatory activity was well stated by the inhibition of PGE_2 following its application. Safety profile of zerumbone on the basis of this study, attract additional investigation to explore the advantageous effect of this herbal product on using higher dosage regimen and/or longer duration against OA or other disease.

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