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Evaluation of cytotoxicity and oxidative stress induced by alcoholic extract and oil of Lepidium Sativum seeds in human liver cell line HepG2

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Since, the primary site of drug metabolism is the liver, that plays a major role in metabolism, digestion, detoxification, and elimination of substances from the body, the present studies were designed to investigate the possible adverse effect of alcoholic extract of seeds of Lepidium sativum (LSA) and Lepidium sativum seed oil (LSO) on HepG2 cells, a human liver cell line. LSA and LSO induced cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and neutral red uptake (NRU) assays. Morphological changes, lipid peroxidation, glutathione, catalase, and superoxide dismutase activities in HepG2 cells were studied. Cells were exposed to 25 to 1000 µg/ml of LSA and LSO for 24 h. The results show that LSA and LSO reduced cell viability, and altered the cellular morphology in dose dependent manner. Concentrations (100 to 1000 µg/ml) of LSA and LSO were found to be cytotoxic, whereas 50 µg/ml and lower concentrations did not cause any significant adverse effect in cell viability of HepG2 cells. LSA and LSO were also found to induce oxidative stress in dose-dependent manner indicated by decrease in glutathione level, catalase activity, and SOD activity and an increase in lipid peroxidation. The results indicate that LSA and LSO induced oxidative stress mediated cytotoxicity in HepG2 cells.

Key words: Lepidium sativum, HepG2 cells, oxidative stress, cytotoxicity.

INTRODUCTION

Garden cress (Lepidium sativum) has been widely used to treat a number of ailments in traditional system of medicine. Lepidium sativum (L. sativum) is a fast-growing edible herb that belongs to the family Cruciferae (Brassicaceae; mustard family), and is being cultivated as culinary vegetable in North America, Europe, and all over Asia including India (Nadkarni, 1976). Also, this is a popular herbal plant grown in many regions of Saudi Arabia, such as Hijaz, AL-Qaseem, and the Eastern Province and is called “Habel Rashaad” or “Thufa” (Ageel et al., 1987; Rahman et al., 2004). In Europe and America, the leaves are used in salad. In various countries of Africa, Lepidium sativum seeds are thought to be an effective medicinal remedy to cure respiratory disorders, like bronchitis and asthma (Kloos, 1976; Nadkarni, 1976). The edible whole seed is known to have health promoting properties hence; it was assumed that these seeds can serve as raw material for functional foods; its peppery tangy flavor and aroma (Snehal et al., 2012; Rehman et al., 2012). L. sativum plant is known to contain ascorbic

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Abbreviations: ALP, SGOT etc
acid, β-carotenes, linoleic acid, palmitic acid, stearic acid (Duke et al., 1992), sinapic acid and sinapin (Schultz and Gmelin, 1952), and imidazole, lepidine, semilepidinoside A and B (Maier et al., 1998). L. sativum seeds are known to be useful as poultices for sprains, and in leprosy, skin diseases, dysentery, diarrhoea, splenomegaly and asthma (Kirtikar and Basu, 2006). Seeds of L. sativum contain carbohydrate, protein, fatty acid, riboflavin, niacin, flavor- noids, isothiocynates glycoside, essential aromatic oils, and fatty oils (Nadakarn, 1995). Previous studies showed that L. sativum have antiasthmatic (Paranjape and Mehta, 2006), bronchodilatory (Mali et al., 2008), anti- inflammatory, analgesic, anticoagulant (Al-Yahya et al., 1994), antirheumatic (Ahsan et al., 1989), hypoglycemic (Patele, 1998), laxative, prokinetic (Rehman et al., 2011), antihypertensive (Maghrani et al., 2005) and diuretic (Patel et al., 2009) activities. The aqueous seeds extract of L. sativum showed hypoglycemic activity, and were used in the treatment of bronchial asthma (Archana and Mehta, 2006).

L. sativum have been widely used to treat a number of ailments in traditional system of medicine throughout India. Preliminary phytochemical study of L. sativum showed presence of flavonoids, coumarins, sulphur glycosides, triterpenes, sterols and various imidazole alkaloids (Patel et al., 2009). The major secondary compounds of this plant are glucosinolates (Gill and Macleod, 1980). The alkaloids of L. sativum are member of the rare imidazole alkaloids that is known as lepidine (Maier et al., 1998). Despite the widespread traditional/edible uses of L. sativum, there are very few reports in the literature available regarding the toxicity of L. sativum. In one of the study, investigators have shown that seeds of L. sativum in Wistar albino rats treated for 6 weeks did not cause any toxicity at 2%, but caused toxicity at 10% (w/w) (Adam, 1999). In another study, Datta et al. (2011) found that clinical enzymes viz., lactate dehydrogenase (LDH), serum glutamic pyruvate transaminase (SGPT) were within normal levels, however, the serum ALP and SGOT were significantly increased in male rats receiving 5 and 10% of L. sativum seeds (Datta et al., 2011). Hepatotoxicity in rats fed with suspension of L. sativum seed extract at 2, 4 and 8 g/100 ml in albino rats fed with high doses of seeds have also been reported (Bafeel and Ali, 2009).

Thus, the present investigations were carried to study the cytotoxicity and oxidative stress induced by L. sativum seeds in human hepatocarcinoma cell line HepG2.

MATERIALS AND METHODS

Chemicals and consumables

DMEM culture medium, antibiotics-atimycotic solution, fetal bovine serum (FBS) and trypsin were purchased from Invitogen, Life Sciences, USA. Consumables and culture wares used in the study were procured from Nunc, Denmark. Ethanol and all other specified reagents and solvents were purchased from Sigma Chemical Company Pvt. Ltd. St. Louis, MO, USA.

Plant material and extractions

The L. sativum seeds used in this work were obtained from the local market of Riyadh, Saudi Arabia. The seeds were screened manually to remove bad ones. They were then dried to constant weight in an oven at 70°C, ground using mechanical grinder, put in air-tight containers and stored in a desiccator.

The oil from L. sativum seeds was extracted by continuous extraction in Soxhlet apparatus for 12 h using petroleum ether (60 to 80°C boiling range) as a solvent according to the method described by AOCS (Horwitz, 1980). At the end of the extraction, the solvent was evaporated. The oil thus obtained was dried over anhydrous sodium sulphate and stored at -4°C for further analysis.

For the preparation of alcoholic extract, the seeds were mace- rated in alcohol and then filtered. The procedure was repeated several times. The solvent was then evaporated using a rotary evaporator and the residue so obtained was called as the alcoholic extract.

Cell culture

Human hepatocellular carcinoma cell line (HepG2) was cultured in DMEM, supplemented with 10% FBS, 0.2% sodium bicarbonate and antibiotic/antimycotic solution (100x, 1 ml/100 ml of medium). Cells were grown in 5% CO₂ at 37°C in high humid atmosphere. Before the experiments, viability of cells was assessed following the protocol of Siddiqui et al. (2008). HepG2 cells showing more than 95% cell viability and passage number between 12 and 14 were used in the present study.

Experimental design

HepG2 cells were exposed to various concentrations of alcoholic extract (LSA) and oil (LSO) of L. sativum seeds (10 to 1000 μg/ml) for a period of 24 h. Following the exposures of LSA and LSO, cells were subjected to assess the cytotoxic responses using MTT, and NRU assays. Further, cellular morphological alterations, oxidative stress parameters such as glutathione, lipid peroxidation, cata- lase and superoxide dismutase induced by LSA and LSO were studied.

Drug solutions

The extracts of L. sativum were not completely soluble in aqueous medium solution; therefore the stock solutions of all the extracts were prepared in dimethylsulphoxide (DMSO) and diluted in culture medium to reach the desired concentrations. The concentration of DMSO in culture medium was not more that 0.1% and this medium was used as control.

MTT assay

Percent cell viability was assessed using the 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described (Siddiqui et al., 2008). Briefly, cells (1x10⁴) were allowed to adhere for 24 h CO₂ incubator at 37°C in 96 well culture plates. After the respective exposure, MTT (5 mg/ml of stock in PBS) was added (10 μl/well in 100 μl of cell suspension), and plates were incubated for 4 h. Then, supernatants were discarded and 200 μl of DMSO were added to each well and mixed gently. The developed color was read at 550 nm using Multiwell Microplate Reader (Thermo Scientific, USA). Untreated sets were also run under identi- cal conditions and served as control.

Neutral red uptake (NRU) assay

Neutral red uptake (NRU) assay was carried out following the pro-
protocol described by Siddiqui et al. (2010). Briefly, after the respective exposure, the medium was aspirated and cells were washed twice with PBS, and incubated for 3 h in a medium supplemented with neutral red (50 µg/ml). Medium was washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. Cells were subjected to further incubation of 20 min at 37°C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. The plates were read at 540 nm using Multifor Microplate Reader (Thermo Scientific, USA). The values were compared with the control sets run under identical conditions.

**Morphological analysis**

Morphological changes in HepG2 cells exposed to increasing concentrations (10 to 1000 µg/ml) of LSA and LSO were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20X magnification.

**Lipid peroxidation (LPO)**

Lipid peroxidation was performed using thiobarbituric acid-reactive substances (TBARS) protocol (Buege and Aust, 1978). Briefly, after the exposure, HepG2 cells were collected by centrifugation and sonicated in ice cold potassium chloride (1.15%) and centrifuged for 10 min at 3000 x g. Resulting supernatant (1 ml) was added to 2 ml of thiobarbituric acid (TBA) reagent (15% TCA, 0.7% TBA and 0.25N HCl) and heated at 100°C for 15 min in a boiling bath. Then sample was placed in cold and centrifuged at 1000 x g for 10 min. Absorbance of the supernatant was measured at 535 nm.

**Glutathione (GSH) content**

Intracellular GSH content was estimated as described by Chandra et al. (2002) with specific modifications. Briefly, after respective exposure, cells were collected by centrifugation and cellular protein were precipitated by incubating 1 ml sonicated cell suspension with 1 ml TCA (10%) and placed on ice for 1 h followed by a 10 min centrifugation at 3000 rpm. The supernatant was added to 2 ml of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA followed by an addition of 0.01 M 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to a final volume of 3 ml. The tubes were incubated for 10 min at 37°C in water bath with shaking. The absorbance of yellow colour developed was read at 412 nm using multiplate reader (Thermo Scientific, USA).

**Catalase activity**

The activity of catalase in cells was assayed following the protocol of Sinha (1972) using H2O2 as substrate. Reaction mixture in a final volume of 1 ml consisted of phosphate buffer (pH 7.0), 0.08 µmol of H2O2 and enzyme protein. The enzyme activity was measured following disappearance of H2O2 at 570 nm using Spectrophotometer.

**Superoxide dismutase (SOD) activity**

The assay of SOD activity was carried out using protocol described by Kakkar et al. (1984). The final volume of 3 ml contained 0.052 M sodium pyrophosphate buffer (pH 8.3), 186 µM phenoxazine methosulphate (PMS), 300 µM nitroblue tetrazolium (NBT), 780 µM NADH, sonicated enzyme preparation and water. The reaction was started by addition of NADH followed by incubation at 37°C for 90 s. After the incubation, the reaction was stopped by the addition of 1.0 ml of glacial acetic acid and the content was rigorously shaken with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 min, cen-trifuged, and butanol layer was separated. The colour intensity of chromogen in butanol was measured at 560 nm against butanol using a spectrophotometer. A mixture devoid of enzyme containing cell suspension served as control.

**Protein estimation**

Protein estimation of each sample was done following the method of Lowry et al. (1951) using bovine serum albumin as a reference standard.

**Statistical analysis**

The results are expressed as mean and standard error of means (SEM). One way ANOVA was employed to detect differences between the groups of treated and control. The values showing p<0.05 were considered as statistically significant.

**RESULTS**

**MTT and NRU assays**

The percent cell viability in HepG2 cells observed by MTT and NRU assays are presented in Figures 1 and 2. Cytotoxicity of alcoholic extract (LSA) and oil (LSO) of Lepidium sativum seeds were assessed using MTT and NRU assays, after exposing the HepG2 cells to 10 to 1000 µg/ml. Figures 1A and 1B shows that LSA induced statistically significant (p<0.01) decrease in cell viability of HepG2 cells in a concentration dependent manner. HepG2 cells exposed to 100 µg/ml concentration and above concentrations of LSA for 24 h were found to be cytotoxic, and cell viability was found to be 8% by MTT and 81% by NRU assays at 100 µg/ml, respectively, whereas, maximum reduction in cell viability at 1000 µg/ml was found to be 68% by MTT (Figure 1A) and 43% by NRU (Figure 1B) assays. Similar kind of reduction in cell viability of HepG2 cells was also observed in the case of LSO. Figures 2A and 2B shows that LSO induced statistically significant (p<0.01) decrease in cell viability of HepG2 cells in a concentration dependent manner. HepG2 cells exposed to 100 µg/ml and above concentrations of LSO for 24 h were found to be cytotoxic. Cell viability at 100 µg/ml, was found to be 86% by MTT (Figure 2A) and 89% by NRU (Figure 2B) assays, whereas, maximum reduction in cell viability at 1000 µg/ml was found to be 49% by MTT (Figure 2A) and 46% by NRU assays (Figure 2B), respectively. Concentrations of LSA and LSO at 50 µg/ml and lower did not cause any adverse effect on the viability of HepG2 cells by both the parameters that is, MTT and NRU assays (Figures 1 and 2). Although both LSA and LSO were found to reduce significantly the cell viability in a concentration manner, but the magnitude of reduction was high in case of LSO.

**Morphological changes**

The morphological changes observed in HepG2 cells are...
Figure 1. Cytotoxicity assessments by (A) MTT and (B) NRU assays in HepG2 cells following the exposure of various concentrations of alcoholic extract of *Lepidium sativum* seeds (LSA) for 24 h. Values are mean±SE of three independent experiments (*p<0.01, **p<0.001 Vs Control).

Changes in morphology were found in concentration dependent manner. Cells exposed to 250 µg/ml and above concentrations of LSA for 24 h reduced the normal morphology of HepG2 cells, and cell adhesion capacity compared to the control (Figure 3). But in case of LSO, the morphology of HepG2 cells at 100 µg/ml reduced the normal morphology and cell adhesion capacity compared to the control (Figure 4). Most of the cells exposed to 500 and 1000 µg/ml of LSA and LSO lost their typical morphology and appeared smaller in size, but the lower concentrations did not cause any effect on cellular morphology of HepG2 cells (Figures 3 and 4).

**Glutathione level**

Glutathione level in the cultured HepG2 cells exposed to 10 to 1000 µg/ml concentration of LSA and LSO for 24 h is summarized in Figure 5. The result indicates that LSA and LSO decreased the GSH levels in a dose dependent manner. Figure 5 shows a significant decrease in the GSH activity at higher concentrations as compared to the control. A concentration dependent significant decrease of 18, 30 and 49% (p<0.01) in GSH activity at 250, 500 and 1000 µg/ml of LSA, whereas, 25, 38, and 54% were observed at 250, 500, and 1000 µg/ml of LSO, respectively compared to the untreated control in HepG2.
Figure 2. Cytotoxicity assessments by (A) MTT and (B) NRU assays in HepG2 cells following the exposure of various concentrations of *Lepidium Sativum* seed oil (LSO) for 24 h. Values are mean±SE of three independent experiments. (*p<0.01, **p<0.001 Vs Control)

Lipid peroxidation

The LSA and LSO induced lipid peroxidation in HepG2 cells are summarized in Figure 6. A concentration dependent increase in lipid peroxidation was also observed. An increase of 18, 35, 53, 81, 95% at 50, 100, 250, 500, 1000 µg/ml of LSA and 20, 45, 67, 92, 112% at 50, 100, 250, 500, 1000 µg/ml of LSO, respectively were found in HepG2 cells exposed for 24 h (Figure 6).

Catalase activity

Results of the catalase activity are summarized in Figure 7. A clear-cut significant decrease of 20% and 25% was observed at 250 µg/ml of LSA and LSO, respectively compared to the control (Figure 7). However, the effect on catalase level was decreased with the increase in concentrations that is 500 µg/ml (35 and 40%) and 1000 µg/ml (50 and 55%) of LSA and LSO, respectively (Figure 7).

Superoxide dismutase activity

Figure 8 shows the pattern of SOD levels in HepG2 cells exposed with (10 to1000 µg/ml) to LSA and LSO for 24 h. A concentration dependent decrease in the level of SOD was observed. The decrease in SOD started at 100 µg/ml LSA and LSO; the results show a decrease in SOD activity at 100 µg/ml (15, 20% of control), 250 µg/ml (27,
Figure 3. Morphological changes in HepG2 cells exposed to various concentrations of alcoholic extract of *Lepidium sativum* seeds (LSA) for 24 h. Images were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20 X magnification.

Figure 4. Morphological changes in HepG2 cells exposed to various concentrations of *Lepidium sativum* seed oil (LSO) for 24 h. Images were taken using an inverted phase contrast microscope (OLYMPUS CKX 41) at 20 X magnification.

35% of control), 500 µg/ml (39, 45% of control), 1000 µg/ml (45, 50% of control) of LSA and LSO, respectively (Figure 8).

**DISCUSSION**

Leaves, roots and seeds of the plant are attributed with a variety of medicinal properties and are used in traditional medicine in various parts of India (Nadkarni, 1976), and the other parts of the world including Saudi Arabia (Ageel et al., 1987; Rahman et al., 2004). *L. sativum* have widely been used to treat a variety of diseases in traditional system of medicine. Phytochemical studies of *L. sativum* showed the presence of flavonoids, coumarins, sulphur glycosides, triterpenes, sterols and various imidazole alkaloids (Patel et al., 2009), and glucosinolates (Gill and Macleod, 1980). The seeds are traditionally used in the diet of lactating woman to induce milk secretion (Sahsrabudde and De, 1943) and in the treatment of some inflammatory conditions like asthma, skin disease and diabetes (Gill and Macleod, 1980; Maier et al., 1998). Despite the widespread traditional/edible uses of *L. sativum*, there is no report available in the literature on the toxicity of alcoholic extracts and seed oil of *L.*
Figure 5. Glutathione depletion in HepG-2 cells following the exposure of various concentrations of *Lepidium sativum* seed oil (LSO) and alcoholic extract of *Lepidium sativum* seeds (LSA) for 24 h. All values represent the mean ± SE (*p<0.01, **p<0.001 Vs Control).

Figure 6. Lipid peroxidation in HepG-2 cells following the exposure of various concentrations of *Lepidium sativum* seed oil (LSO) and alcoholic extract of *Lepidium sativum* seeds (LSA) for 24 h. All values represent the mean ±SE (*p<0.01, **p<0.001 Vs Control).
**Figure 7.** Catalase activity in HepG-2 cells following the exposure of various concentrations of *Lepidium sativum* seed oil (LSO) and alcoholic extract of *Lepidium sativum* seeds (LSA) for 24 h. All values represent the mean ±SE (*p<0.01, **p<0.001 Vs Control).

**Figure 8.** Superoxide dismutase (SOD) activity in HepG-2 cells following the exposure of various concentrations of *Lepidium sativum* seed oil (LSO) and alcoholic extract of *Lepidium sativum* seeds (LSA) for 24 h. All values represent the mean ±SE (*p<0.01, **p<0.001 Vs Control).
Therefore, the present investigations were initiated to screen the cytotoxicity and oxidative stress induced by *L. sativum* in human hepatocarcinoma cell line HepG2. Our results show that alcoholic extract and oil of *L. sativum* seeds induced cytoxicity and oxidative stress in HepG2 cells. In this study, we found that alcoholic extract and oil of *L. sativum* seeds significantly reduced cell viability in HepG2 cells in a concentration dependent manner. The concentration at 100 µg/ml and above of *L. sativum* were found to be cytotoxic, whereas the concentration at 50 µg/ml and below did not show any significant cell death in HepG2 cells. The *in vitro* concentrations used in this study were selected from the previous studies showing the toxicity in this range. The ethanolic and seed extracts of *Moringa stenopetala* plant extracts have been shown to significantly decrease the cell viability of HepG2 exposed to 500 µg/ml concentrations (Mekonnen et al., 2005). The exposures of various natural products have also been investigated in this concentration range by various investigators in different *in vitro* models (Maria et al., 1997; Nguta et al., 2012; Solanki et al., 2013). There are very few reports in the literature regarding the toxicity of *L. sativum*. In one of the studies, investigators have shown that seeds of *L. sativum* in Wistar albino rats treated for 6 weeks did not cause any toxicity at 2%, but toxicity at 10% (w/w) (Adam, 1999). In another study, Datta et al. (2011) found that clinical enzymes viz., LDH, SGPT were within normal levels, however, the serum ALP and SGOT were significantly increased in male rats receiving 5 and 10% of *L. sativum* seeds (Datta et al., 2011). Liver toxicity in rats fed with suspension of *L. sativum* seed extract at 2, 4 and 8 g/100/ml in albino rats fed with high doses of seeds have also been reported (Bafeel and Ali, 2009). Our results also well agree with these findings, where higher concentrations of *L. sativum* induced toxicity in HepG2 cells. Reactive oxygen species (ROS) are one of the important factors not only in the apoptosis process but also oxidative stress induced damages, DNA damage, and many other cellular processes. In this study, we found an alteration in the oxidative stress markers that has remarkable increase in the level of LPO and decreases in antioxidant enzymes, superoxide dismutase (SOD), glutathione (GSH) content, and catalase activities in HepG2 cells following the exposure of alcoholic extract and oil of *L. sativum* seeds which gradually decrease due to the continuously increasing concentrations. We believe that, the exposure of alcoholic extract and oil of *L. sativum* seeds give rise to the formation of 8-hydroxy guanosine and revealed the DNA damage occurring during the time of cells exposure to toxicant (Dusinska and Collins, 1996; Singh et al., 1988), and limited the burden of oxidative stress (Collins et al., 1993). Therefore, in our experiments, when cells were exposed to alcoholic extract and oil of *L. sativum* seeds, the level of antioxidant enzymes, superoxide dismutase (SOD), glutathione (GSH) content, and catalase activities showed alterations towards the oxidative damage. This might be due to the ROS generation, which leads to oxidative stress to a certain extent. The varied biological responses of alcoholic extracts and oil of *L. sativum* seeds in the studies could be due to the difference in the extract, and high concentrations of *L. sativum* extracts (Adam, 1999; Bafeel and Ali, 2009).

**Conclusion**

This study for the first time demonstrate the concentration dependent cytoxicity of alcoholic extract and oil of *L. sativum* seeds in human liver cells (HepG2). A significant reduction in antioxidant enzymes, and increase in lipid peroxidation suggested their role in inducing oxidative stress in HepG2 cells exposed to *L. sativum*. This significant variation in biological end-points in our experiments also revealed the utility of HepG2 cells as *in vitro* model system for the screening of various extracts of plant origin for their hepatotoxicity.

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