Whey Protein Concentrate and Ginseng Extract Exhibit Antioxidant Properties *in vitro* and Reduce Hepatotoxicity and Oxidative Stress of Aflatoxin *in vivo*

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Abstract: Aflatoxins the major toxic metabolites of fungi which are able to induce chronic liver damages. The antioxidant and hepatoprotective effects of whey protein concentrate (WPC) and/or Korean ginseng extract (KGE) were evaluated *in vitro* in *in vivo*. In the *in vitro* study, five concentrations (e.g. 20, 40, 60, 80 and 100 mg/100 ml) of WPC and/or KGE were tested for their antioxidant activity using ABTS and thiobarbituric acid reactive substances (TBARS) assays. In the *in vivo* study, male Sprague-Dawley rats were divided into eight groups including the control group, the group fed AFs-contaminated diet (2.5 mg/kg diet) and the groups treated with WPC (500 mg/kg b.w) and/or KGE (20 mg/kg b.w) with or without aflatoxins for 30 days. The results indicated that both WPC and KGE exhibit antioxidant activity in vitro and the combined treatment showed the potential effect. Both agents showed a potential hepatoprotective effects against aflatoxins-induced liver damage and oxidative stress. They succeeded to restore the biochemical parameters and improve the histological and histochemical picture of the liver. This improvement was pronounced in the group received the combined treatment of WPC and KGE. It could be concluded that WPC should be incorporated with KGE when used as functional foods for people suffering from liver diseases. [New York Science Journal 2010;3(11):37-51]. (ISSN: 1554-0200).

Key words: whey protein concentrate; ginseng, aflatoxin; mycotoxins; liver; oxidative stress; antioxidants.

1. Introduction

Aflatoxins (AFs) are a group of fungal toxins, produced mainly by *Aspergillus flavus* and *A. parasiticus*, which occur naturally in several important feedstuffs. Major crops in which AFs are produced are peanuts, corn, and cottonseed. AFs has been extensively linked to human primary liver cancer in which it acts synergistically with HBV infection and AFB_1 was classified by the International Agency for Research on Cancer (IARC) as a human carcinogen (group 1 carcinogen) (IARC 1993a). This combination represents a heavy cancer burden in developing countries. A recent comparison of the estimated population risk between Kenya and France highlighted the greater burden that can be placed on developing countries (Shephard 2006). Based on respective estimates for aflatoxin exposure of 133 and 0.12 ng/kg body weight/ day and respective *hepatitis B* virus (HBV) prevalence of 25 and 1%, the liver cancer risk would be 11 vs. 0.0015 cancers per year per 100 000 population, respectively. Given recently published liver cancer incidence rates in the European Union of 10.0 per 100 000 for males and 3.3 per 100 000 for females (Bray et al. 2002). It is clear that aflatoxin plays a significant role in liver cancer in developing countries, but not in the developed world where other risk factors such as cirrhosis are more important.

Whey proteins (WP), by-products of the manufacture of cheese and curd, were shown to be tumor protective in animal models (Eason et al., 2004), and in limited clinical trials of cancer patients (Kennedy et al., 1995; Marshall 2004). In addition, whey has the ability to act as an antioxidant (Bounous, 2000), antihypertensive, antitumor (Yoo et al., 1998), hypolipidemic, antiviral (Low et al. 2003), antibacterial (Ajello et al. 2002), and chelating agent (Weinberg, 1996). The use of whey protein concentrates in formulating products is increasing due to the nutritional and health benefits attributed to these proteins (Onwulata, et al. 2004). These proteins exhibit potent antioxidant activities by inducing cellular biosynthesis of glutathione (GSH), which can boost the immune system and detoxify potential carcinogens (Bayram et al., 2008). In an earlier work, Eason et al. (2004) showed that WP reduced mammary tumor incidence and increased mammary tumor latency in the lifetime of 7,12-dimethylbenz[a]anthracene (DMBA)-treated female rats.

Panax ginseng C.A. Meyer is an herbal root that has been used for more than 2000 years

throughout Far Eastern countries including China, Japan, and Korea. Its beneficial effects have been analyzed by extensive preclinical and epidemiological studies (Yun, 2003). Recently, 20-O-(h-D-glucopyranosyl)-20(S)-protopanaxadiol (IH-901), a novel ginseng saponin metabolite, formed from ginsenosides Rb1, Rb2, and Rc was isolated and purified after giving ginseng extract p.o. to humans and rats (Hasegawa et al., 1996). IH-901 has been shown to enhance the efficacy of anticancer drugs in cancer cell lines previously resistant to several anticancer drugs (Lee et al. 1999), to exhibit antigenotoxic and anticlastogenic activity in rats concurrently treated with benzo(a)pyrene (Lee et al., 1998), and to induce apoptosis (Choi et al., 2003). These studies found that the antitumor activity of IH-901 is attributable to the induction of apoptosis. The aims of the current study were to evaluate the antioxidant and radical scavenger properties of whey protein which use in formulating products and ginseng extract alone or in combination *in vitro* and to evaluate their protective effects against aflatoxin-induced oxidative stress and in rats.

2. Materials and Methods

2.1. Chemicals

Free stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH·), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), $FeSO_4.7H_2O$ and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Kits

Transaminase (ALT and AST), alkaline phosphatase (ALP), Total protein (TP) and albumin kits were purchased from Randex Laboratories (San Francisco, CA, USA). Total and direct bilirubin (TB, DB) kits were purchased from Biomerieux, Laboratory of Reagents and Products (Marcy Létoile, France). Cholesterol kits was purchased form Biodiagnostics Company (Cairo, Egypt). Malondialdehyde (MDA) was obtained from Eagle Diagnostics (Dallas, TX, USA). Total antioxidant capacity (TAC) kit was purchased from Biodiagnostic Co. (Cairo, Egypt). Other chemicals were of the highest purity commercially available.

2.3. Materials

2.3.1. Whey protein concentrate (WPC)

Concentrated whey powder contains 80% proteins was purchased from Davisco Foods International, Inc. (Eden Prairie, Minnesota, USA).
Ginseng: Korean Red ginseng was provided by the Korean Society of ginseng, Seoul, Korea.

2.3.2. Preparation of WPC and KGE

WPC was mixed with water; the mixture was stirring with a magnetic stirrer and left in refrigerator over night to fully hydrate (Kennedy et al., 1995). However, Korean ginseng was extracted with 10 volumes of distilled water at 85 ^{O}C for 8 hours five times. The aqueous extracts were combined and concentrated under reduced pressure to give a darkish brown syrup (KGE). The moisture content of KGE was 37.21%.

2.3.3. Determination of WPC and KGE content

To verify the manufacturers claim of WPC, we determined the Thiol (SH) as major components which are responsible for the antioxidant activity. Thiol (SH) content was determined using a modification Ellman's assay (Anema and Lioyd, 1999) and was expressed as cysteine equivalents µM. Whereas, the ginsenoside content in the KGE was determined as follows. Briefly, an aliquot of KGE dissolved in distilled water was passed through Sep-Pak C_{18} cartridge, and the cartridge was washed with distilled water. Subsequently, ginsenosides were eluted with 90% methanol and then analyzed by high performance liquid chromatography (Ko et al., 1989).

2.4. In vitro study

2.4.1. WPC Solubility

Protein Solubility (PS) was determined in duplicate by the method described by Bera and Mukherjee (1989). Two hundred milligrams of proteins were dispersed in 10 ml of deionized water. The pH of suspensions was adjusted to different levels (2.0 to 8.0) by using 1 mol/L HCl or 1 mol/L NaOH. The suspensions were stirred at room temperature for 30 min then centrifuged at 10000 X g for 30 min (Kika Ultra Turrax T18 basic, Germany). Protein contents in supernates were determined by Kjeldahl method (Ceirwyn, 1995). The percentage of protein solubility in each suspension was calculated by the ratio of protein in the supernatant to protein in 200 mg sample.

2.4.2. Preparation of working solution

Five concentrations (20, 40, 60, 80 and 100 mg/100 ml) of WPC and KGE alone or in combination were tested for their antioxidant activity. The antioxidant potential of these ingredients as a free radical scavenging activity was determined by ABTS and TBAR assays.

2.4.3. Antioxidant activity test:

2.45 mM. The reaction mixture was left to stand at room temperature for 12 h in the dark before

usage. The resultant intensely-colored $ABTS^{\bullet+}$ radical cation was diluted with 0.01 M phosphate buffered saline (PBS), pH 7.4, to give an absorbance value of ~0.70 at 734 nm. The tested materials were diluted 100x with the $ABTS^{\bullet+}$ solution to a total volume of 1 ml and the absorbance was measured spectrophotometrically. The assay was performed in triplicates. To allow for any absorbance of the tested materials themselves, 990 µl of PBS was added to the controls instead of $ABTS^{\bullet+}$. Trolox, the water-soluble α-tocopherol (vitamin E) analogue was used as a standard. The results of the assay were expressed relative to Trolox in terms of TEAC (Trolox equivalent antioxidant capacity).

2.4.4. Radical Scavenging activity

Lipid peroxidation measured on the basis of MDA levels was carried out using the method described by Huerta et al. (2007). The concentration of the tested materials that showed the best antioxidant activity in ABTS assay was used in this test. Briefly, 1 ml reaction mixture was incubated at 95 °C for 1 h with 250 ml of TBA (0.67%) and 100 ml of H_3PO_4 (0.44 M) then 150 ml of TCA (20%) were added. The mixture was centrifuged and the lipid peroxidation products in the tested samples (WPC and/or KGE) were estimated by the formation of thiobarbaturic acid reactive substances (TBARS) and quantified in term of malonyldialdehyde (MDA) as described by Haraguchi et al. (1997). Inhibition of TBARS formation by the KGE and/or WPC was calculated compared to control, which did not contain the extracts and reference antioxidant substance (0.01% BHA). TBARS were assayed as previously described (Hseu et al., 2002) and were expressed in terms of MDA equivalents in µmol/L.

2.5. In vivo study

2.5.1. Aflatoxin Production

The aflatoxin was produced via fermentation of rice by *Aspergillus parasiticus* NRRL 2999 as described by Demet et al. (1995). The fermented rice was autoclaved, dried and ground to a powder, and the aflatoxin content was measured by the use of HPLC (Hustchins and Hagler, 1983). The aflatoxins within the rice powder consisted of 83.1% B_1, 12.9% B_2, 2.8% G_1 and 1.2% G_2 based on the total aflatoxins in the rice powder. The rice powder was incorporated into the basal diet to provide the desired level of 2.5 mg/kg diet. The diet containing the aflatoxins was analyzed and the presence of parent aflatoxins was confirmed and determined as mentioned above.

2.5.2. Experimental animals

Three-months old Sprague-Dawley male rats (100-120 g, purchased from animal house colony, Giza, Egypt) were maintained on standard lab diet (protein: 160.4; fat: 36.3; fiber: 41 g/kg and metabolizable energy 12.08 MJ) purchased from Meladco Feed Co. (Aubor City, Cairo, Egypt). Animals were housed in a room free from any source of chemical contamination, artificially illuminated and thermally controlled, at the Animal House Lab., National Research Centre, Dokki, Cairo, Egypt. After an acclimatization period of one week, the animals were divided into eight groups (10 rats/group) and housed in filter-top polycarbonate cages. All animals were received humane care in compliance with the guidelines of the Animal Care and Use Committee of the National Research Center, Dokki, Cairo, Egypt.

2.5.3. Experimental design

Animals within different treatment groups received their respective treatment by oral intubations for 30 days as shown in Table (1). The animals were observed daily for signs of toxicity. At the end of experimentation period (i.e. day 31), blood samples were collected from all animals from retro-orbital venous plexus for biochemical analysis. The following biochemical methods were performed according to the kits instructions ALT and AST, ALP, TP, albumin, cholesterol, TB and DB.

Table (1) Experimental groups and corresponding treatments

Treatment / Groups	Basal Diet	AF-contaminated diet (2.5 mg/ kg)	WPC (500 mg/kg b.w)	KGE (20 mg/kg b.w)	WPC + KGE
Group1	■				
Group2		■			
Group3			■		
Group4				■	
Group5					■
Group6		■	■		
Group7		■		■	
Group8		■			■

2.5.4. Determination of lipid peroxidation and total antioxidant capacity in liver tissues

Samples from liver tissues (approximately 0.05-0.1 g) were homogenized in phosphate buffer (pH 7.4) to give 20% w/v homogenate (Lin *et al*., 1998). This homogenate was centrifuged at 1700 rpm and 4 °C for 10 min; the supernatant was stored at -70 °C until analysis. This supernatant (20%) was used for the determination of hepatic lipid peroxidation according to the method described by Ruiz-Larrea *et al.* (1994). In brief, 4.5 ml working reagent [1 volume of (0.8 g thiobarbituric acid dissolved in 100 ml of 10% perchloric acid) and 3 volume of (20% trichloroacetic acid) were added to 0.5 ml sample and incubated for 20 min in boiling water bath then left to cool at room temperature before centrifuge at 3000 rpm for 5 min at 0 ^{O}C. the pink color was measured at wavelength 532 nm, against blank solution which was prepared by the addition of 0.5 ml of distilled water to 4.5 ml working reagent, using V-530 UV/Vis spectrophotometer. The level of lipid was expressed as n mol malondialdehyde (MDA)/g liver tissue. The homogenate was further diluted to give 5% homogenate (w/v), centrifuged at 3000 rpm for 5 min at 0 ^{O}C and used for the determination of total antioxidant capacity (TAC) according to the method described by Koracevic *et al.* (2001).

Samples of the liver from all animals within different treatment groups were excised and fixed in 10% formal saline followed by dehydration in ascending grades of alcohol, clearing in xylene and embedding in paraffin wax. Liver sections (5 µm thickness) were stained with hematoxylin and eosin (H&E) for the histological examination (Drury and Wallington, 1980). Another liver section from all groups was stained with Bromophenol blue technique to demonstrate total protein contents (Mazia et al., 1953). The optical density (O.D.) of total protein were measured using computerized image analyzer.

2.6. Statistical analysis:

All data were statistically analyzed using the General Linear Model Procedure of the Statistical Analysis System (SAS, 1982). The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio (Waller and Duncan, 1969). All statements of significance were based on probability of $P \leq 0.05$.

3. Results:

The determination of the major components of the tested materials indicated that WPC contained 523 µM cysteine equivalents. Where as, the HPLC analyses of the GE are presented in Table (2). These results showed that the sum of ginsenosides contents was 19.3 mg/g (1.93%).

Table (2). Concentrations of ginsenosides in KGE as determined by HPLC

Ginsenosides	Concentration (mg/g)
Rg1	0.54
Re	0.95
Rf	1.02
Rh1	0.88
Rg2	3.16
Rb1	3.72
Rc	1.89
Rb2	1.71
Rd	1.32
Rg3	4.04
Rh2	0.11
Total	19.3 (1.93%)

3.1. In vitro results

3.1.1 WPC solubility

The results of WPC solubility measured in different pH ranged from 2-8 showed that the minimum solubility was found at pH 4.0-5.0. However, the solubility in the isoelectric point (pI) range was 75.0 and the highest solubility values were found at the both acidity pH range between 2.0 -3.0 and alkaline pH range between 7.0 -8.0. WPC have high solubility over a wide range of pH, (lower the pH, higher the solubility).

3.1.2. Antioxidant activity

3.1.2.1. ABTS (2,2'-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid]) assay

The results of in vitro test for the antioxidant activity of different concentrates (20-100 mg/100 ml) of WPC and/or KGE revealed that they have potential antioxidant activity singly or in combination in a dose dependent manner. Both agents were able to scavenge the $ABTS^{\bullet+}$ radical cation (Fig. 1). It is clear that the combination of WPC plus KGE gave the highest ABTS radical scavenging activity followed by KGE then WPC which showed the least scavenging activity. On the other hand, the highest scavenging activity of WPC plus KGE was observed at the highest concentration (100 mg/100ml).

3.1.2.2. Thiobarbituric acid reactive substances (TBARS) assay

TBARS assay was used for the determination of lipid peroxide formation expressed as malondialdehyde (MDA). The results of lipid peroxide formation showed that there is no significant difference between the antioxidant activity for both WPC and KGE at the concentration of 100 mg/100 ml (Fig. 2). However, both had more

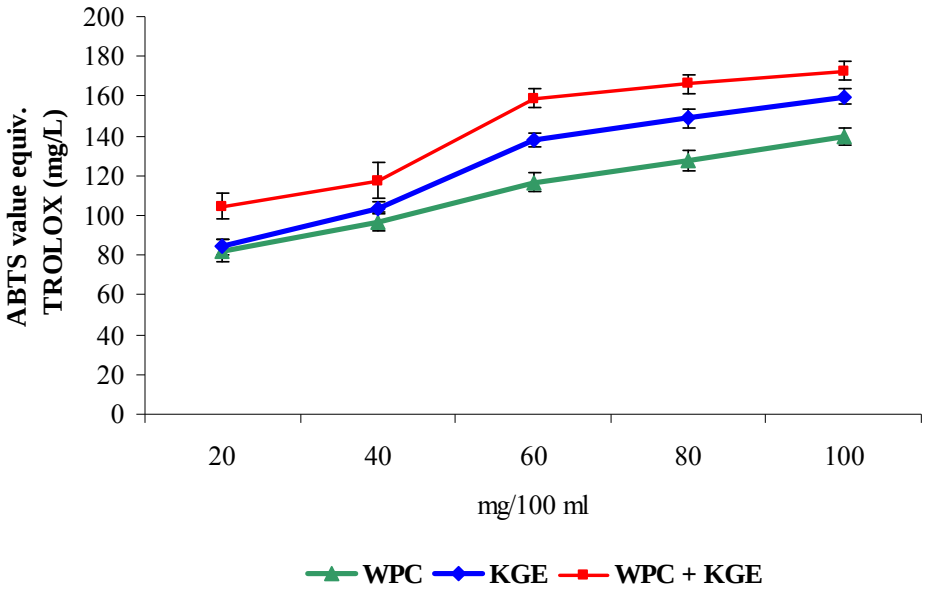

Fig.(1). Antioxidant activity using ABTS method in different concentration of ingredients additives. Data are expressed as Trolox equivalent mg/L.

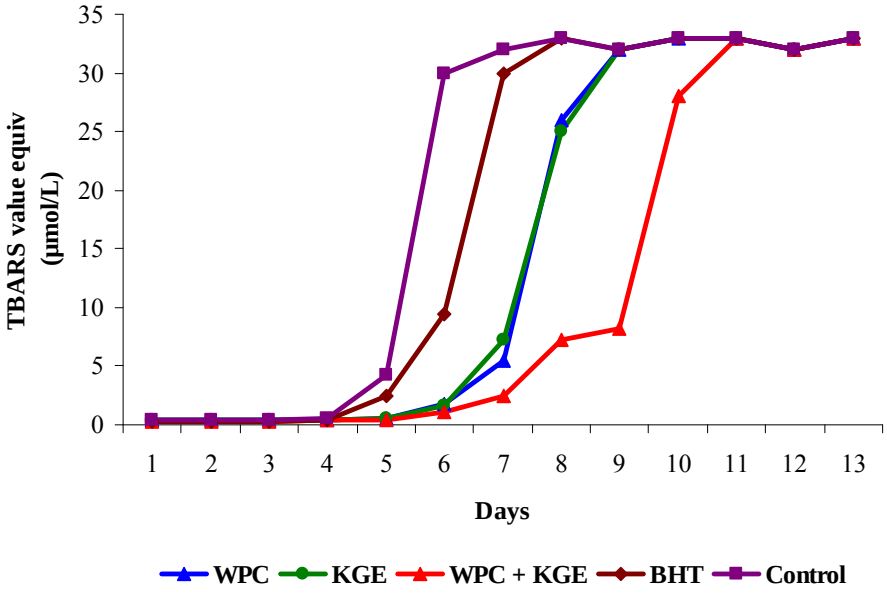

Fig.(2). Antioxidant activity using TBARS method in different concentration in ingredients additives. Data are expressed as µmol MDA/L.

significant activity than BHA which was obvious after 6 days as TBARS formed. The level of lipid peroxidation in the presence of BHA, WPC and KGE were 9.4, 1.75 and 1.6 µmol/L respectively. On the other hand, lipid peroxidation in the presence of WPC plus KGE recorded 1.1 µmol/L TBARS. Furthermore, by the seventh day, TBARS formed recorded 30, 5.5, 7.2 and 2.5µmol/L in the presence of BHA, WPC, KGE and WPC plus KGE respectively. Interestingly, the mixture of WPC plus KGE succeeded to inhibit the lipid oxidation significantly until day nine since it recorded 8.2 µmol/L TBARS, whereas it recorded 32 µmol/L for the other tested samples.

3.2 In vivo results

The results of the biochemical parameters (Table 3) revealed that rats fed aflatoxin-contaminated diet showed a significant increase in ALT, AST, ALP, TB, DB, cholesterol and lipid peroxidation accompanied with a significant decrease in TP, albumin and TAC. Animals treated with KGE alone showed a significant increase in ALT and DB accompanied with a significant decrease in AST, ALP and cholesterol whereas; TP and albumin were comparable to the controls. Animals fed AFs-contaminated diet and treated with KGE showed a significant improvement in all biochemical parameters towards the control values although it did not normalize them but it succeeded to normalize TB and cholesterol. Animals treated with WPC alone showed a significant increase in ALP and DB accompanied with a significant decrease in cholesterol whoever the other tested parameters were not significantly affected. The combined treatment with WPC and KGE increased ALT and decreased AST, cholesterol and lipid peroxidation but did not significantly affect the other parameters. Animals fed AFs-contaminated diet and treated with WPC and/or KGE showed a significant improvement in all biochemical parameters towards the control values although these treatments failed to normalize these levels. Moreover, the recorded improvement was more pronounced in the group fed AFs-contaminated diet and received the combined treatment.

Table (3). Effect of WPC and KGE alone or in combination on different biochemical parameters in rats fed AFs-contaminated diet

Groups / Parameters	Control	AFs	WPC	KGE	WPC + KGE	WPC + AFs	KGE + AFs	WPC + KGE + AFs
ALT (U/L)	24.43 ± 0.8^{a}	105.57 ± 4.0^{b}	22.43 ± 2.6^{a}	23.29 ± 2.7^{a}	24.14 ± 3.3^{a}	44.86 ± 3.2^{c}	40.57 ± 1.6^{c}	35 ± 0.8^{d}
AST (U/L)	59.29 ± 4.7^{a}	124 ± 4.1^{b}	57.49 ± 3.5^{a}	52.71 ± 3.7^{c}	50.14 ± 3.7^{c}	68.43 ± 1.1^{d}	69.43 ± 1.6^{d}	57.14 ± 1.7^{a}
ALP (U/L)	53.88 ± 4.3^{a}	99.04 ± 3.0^{b}	57.56 ± 3.8^{a}	50.96 ± 6.3^{a}	51.83 ± 5.19^{a}	74.71 ± 3.7^{c}	76.91 ± 1.7^{c}	73.64 ± 1.6^{c}
TP (mg/dl)	7.01 ± 0.3^{a}	4.09 ± 0.2^{b}	7.03 ± 0.1^{a}	6.98 ± 0.1^{a}	6.96 ± 0.1^{a}	5.56 ± 0.2^{c}	5.64 ± 0.1^{c}	6.45 ± 0.3^{a}
Alb (mg/dl)	3.01 ± 0.2^{a}	1.64 ± 0.2^{b}	2.95 ± 0.2^{a}	3.10 ± 0.2^{a}	2.95 ± 0.2^{a}	2.54 ± 0.2^{a}	2.84 ± 0.2^{a}	2.74 ± 0.2^{a}
TB (mg/dl)	3.35 ± 0.1^{a}	7.57 ± 0.2^{b}	3.16 ± 0.2^{a}	3.8 ± 0.4^{a}	2.98 ± 0.2^{a}	4.33 ± 0.2^{c}	3.34 ± 0.3^{a}	3.95 ± 0.4^{a}
DB (mg/dl)	1.76 ± 0.1^{a}	5.24 ± 0.4^{b}	2.00 ± 0.3^{c}	2.06 ± 0.3^{c}	1.70 ± 0.2^{a}	3.45 ± 0.1^{d}	3.26 ± 0.4^{d}	2.17 ± 0.2^{c}
Cho (mg/dl)	82.8 ± 4.9^{a}	208.27 ± 20.2^{b}	59.3 ± 7.8^{c}	59.61 ± 3.3^{c}	56.86 ± 6.1^{c}	75.74 ± 6.1^{a}	85.37 ± 7.2^{a}	41.43 ± 2.9^{d}
MDA (ng/g liver tissue)	90.16 ± 0.8^{a}	203.28 ± 15.1^{b}	89.35 ± 0.9^{a}	90.18 ± 1.3^{a}	83.63 ± 1.4^{c}	111.44 ± 4.1^{d}	118.3 ± 4.9^{d}	96.98 ± 6.7^{a}
TAC (µmol/g liver tissue)	47.42 ± 47.4^{a}	27.5 ± 1.9^{b}	47.01 ± 0.1^{a}	47.27 ± 0.2^{a}	47.01 ± 0.1^{a}	42.24 ± 1.7^{a}	44.25 ± 1.4^{a}	45.88 ± 0.7^{a}

Within each row, means superscript with different letters are significantly different ($P \leq 0.05$)

The histological examination of the liver sections of the control animals revealed normal hepatocytes and central vein (Fig 3a). Microscopic examination of the liver sections of animals fed AFs-contaminated diet showed the hepatocytes with fatty degeneration and necrosis scattered in all liver tissue

(Fig. 3b). The liver sections of rats treated with WPC alone showed normal hepatocytes architecture, central veins, portal tracts and hepatic cords separated with blood sinusoids (Fig. 3c). Livers of rats treated with KGE alone showed nearly normal hepatocytes structure (Fig. 3d). Animals treated with WPC plus KGE raveled more or less normal hepatocytes structure (Fig. 4a). Animals fed AFs-contaminated diet and treated with WPC showed decrease in different featuers of degenerative changes and improvement in hepatocytes in central zone and around blood vessels (Fig. 4b) however; those fed AFs-contaminated diet and treated with KGE alone or plus WPC showed prominent improvement in all hepatocytes, blood vessels and connective tissue and the liver sections become nearly normal (Fig. 4c, 4d).

The histochemical inspection of total protein content in the liver tissue as determined by the optical density (Fig. 5) revealed that strong protein materials in the cytoplasm and nuclear membrane of the control rats. Rats fed AFs-contaminated diet showed moderate decrease in the distribution of protein materials in the cytoplasm and nuclear membrane. Rats treated with WPC alone showed a marked increased in protein materials in the hepatocytes. Whereas, those treated with KGE alone showed a marked increase in protein content of the majority of hepatocytes. Rats treated with WPC plus KGE showed a remarkable restoration in the stain of protein particles in the majority of hepatocytes. Rats fed AFs-contaminated diet and treated with WPC showed a moderate restoration in the stain of protein particles in the majority of hepatocytes. Rats fed AFs-contaminated diet and treated with KGE showed a decrease in protein particles in the majority of hepatocytes which still abnormal whereas, those fed AFs-contaminated diet and treated with WPC plus KGE showing mild improvement in protein contents.

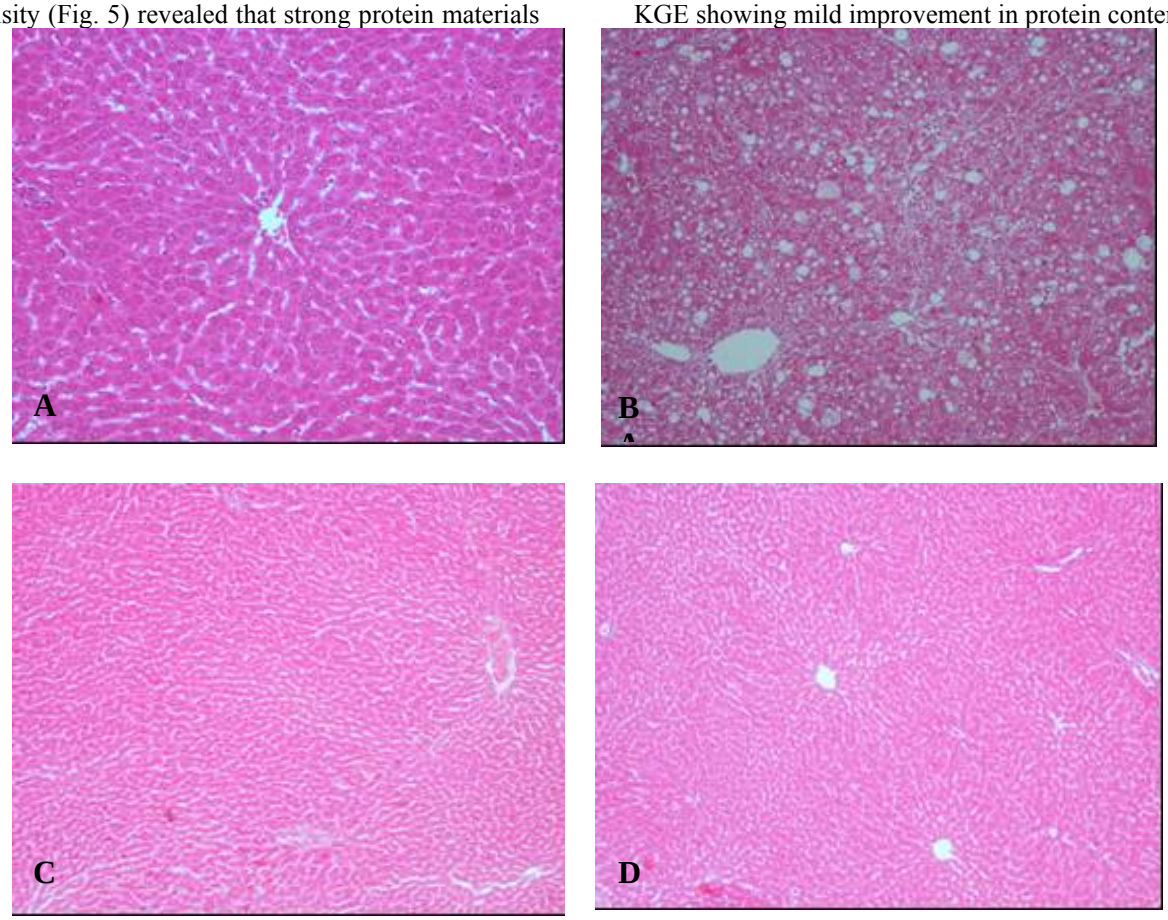

Fig. (3). A photomicrograph in a liver section of: **(A)** Control rat showing normal hepatocytes and central vein, **(B)** Rats treated with AFs alone showing hepatocytes with fatty degeneration and necrosis scattered in all liver tissue, **(C)** Rats treated with WPC alone showing normal hepatocytes architecture, central veins, portal tracts and hepatic cords separated with blood sinusoids and **(D)** Rats treated with KGE alone showing marked improvement in hepatocytes structure.
(H&E X150).

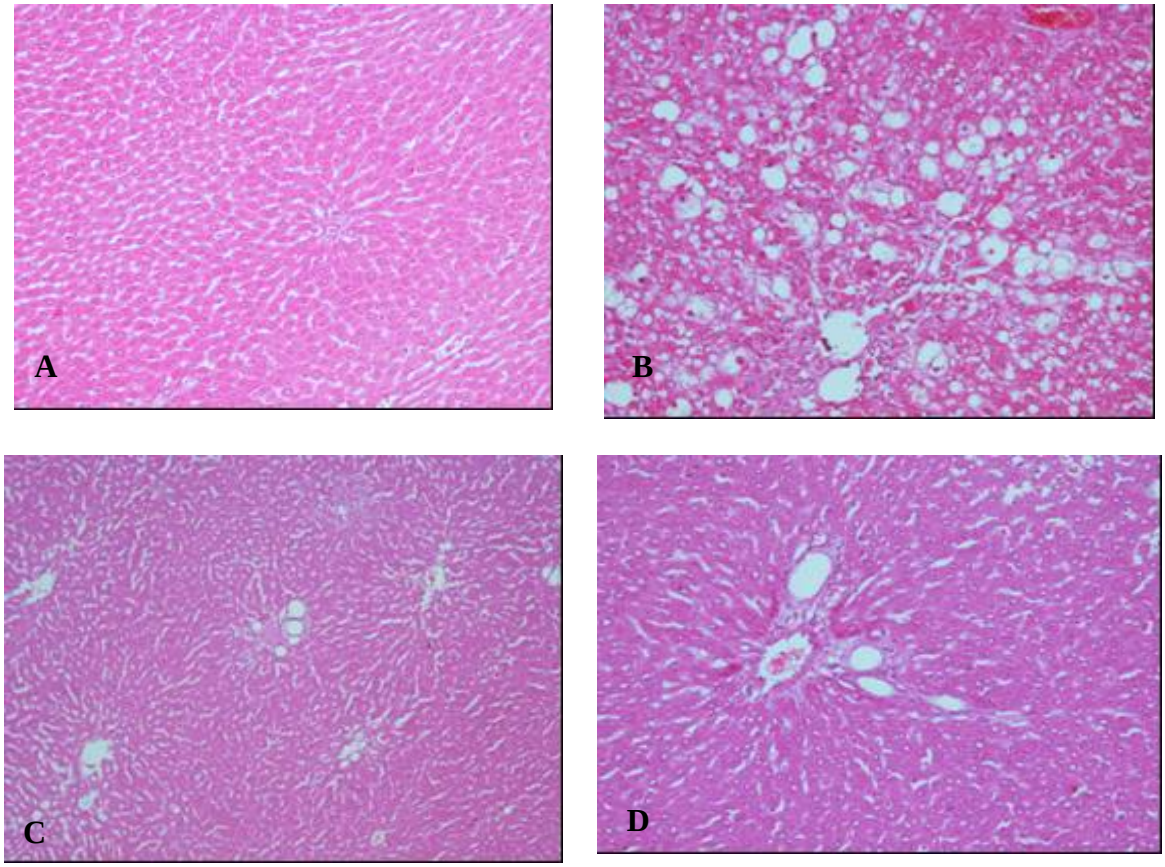

Fig. (4). A photomicrograph in a liver section of: **(A)** Rats treated with WPC plus KGE alone showing normal hepatocytes structure, **(B)** Rats treated with AFs plus WPC showing decrease in different features of degenerative changes and improvement in hepatocytes in central zone, **(C)** Rats treated with AFs plus KGE showing prominent improvement in all hepatocytes, no inflammation or connective tissue around blood vessels and it is nearly normal . **(D)** Rats treated with AFs plus WPC and KGE showing prominent improvement in all hepatocytes, blood vessels and connective tissue; the section become nearly normal (H&E X150)

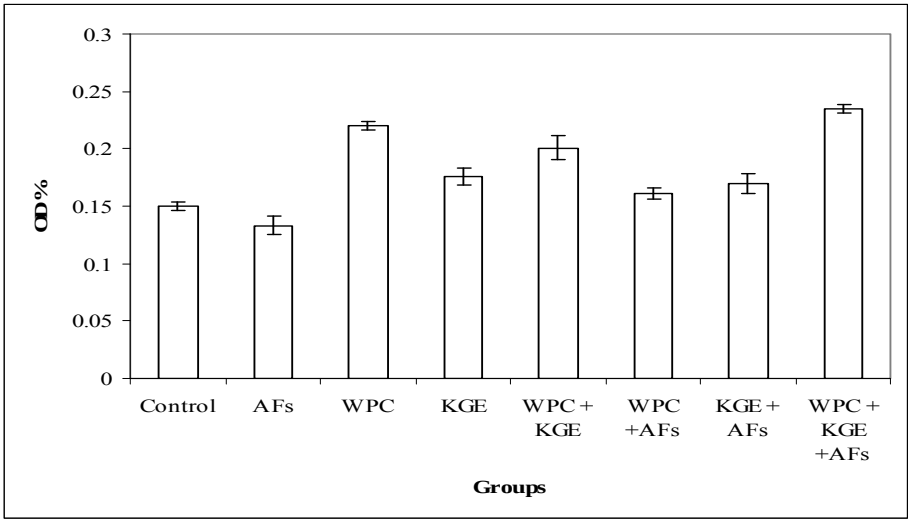

Fig. (5) Optical density (O.D) of total protein content of liver in rats fed AFs-contaminated diet and treated with WPC and/or KGE

4. Discussions:

The high incidence of primary liver cancer in human in aflatoxin infected area in the Africa and Asia have been documented previously (Peers et al., 1976). Domngang et al. (1984) reported that AF content in some African and Asian foods is >10-fold the recommended maximum level. This metabolite can induce several toxic effects in the human body (Krishnamachari et al., 1975) consequently; it is important to find a method for protection since the consumption of contaminated food in some areas is expected. Food supplements are most likely to be effective in this respect. In the current study, we evaluated the antioxidant and radical scavenging properties of WPC and/or KGE in vitro and their protective role in vivo during aflatoxin exposure.

Despite the fact that the compositions of WPC are already known, we further verified the manufactures claim by the analysis of the major components responsible for the antioxidant activity. The results indicated that the major components including Thiol (SH) groups in WPC and confirmed the manufactures claim. According to Onwulata et al. (2004) the solubility of commercial WPC has been shown to be highly variable. The level of insoluble denatured proteins in WPC products affects its solubility which in turn determines functionality (Puyol et al., 1999). The current results showed that pH affected WPC solubility which showed minimum value at the pH 4.5 and indicated the isoelectric point of WPC (Pelegrine and Gasparetto, 2005). Although there are many variables affecting the chemical properties of WPC, the major process variable that affects functionality is related to the extent of protein denaturation during processing (Onwulata, et al., 2004). The extremely high solubility index reported in the current study revealed that the proteins present are in the tested WPC is in undenatured form which consequently, when ingested, exhibit higher level of tissue glutathione the major intracellular antioxidant (Lands et al. 1999).

The current in vitro antioxidant results showed that WPC exhibit a weak scavenger to acid radical ($ABTS^{\cdot+}$) compared to KGE which indicate that the ability of WPC to donate hydrogen to reduce free radicals was low (Colbert and Decker, 1991). The higher radical scavenging activity for KGE reported in the current study may be due to not only the ginenosides but also to the phenolic substances (maltol, salicylic acid, vanillic acid) which have the ability to scavenge free radicals (Zhang et al. 1996; Kim et al. 2002). The potential of radical scavenging activity of WPC plus KGE at all tested concentrations demonstrated a possible synergistic action.

Lipid peroxidation results when free radicals attack membranes and seems to be closely related to toxicity, disease and aging. In the present study, TBARS was used as markers of lipid peroxidation which is a useful assay for the measurement of antioxidant activity of several compounds (Villa-Caballero et. al. 2000) to protect unsaturated fatty acids from oxidation. Using this model system to measure the antioxidant activity, WPC is considered to be a mild antioxidant. ß-Lactoglobulin (ß-LG), a major protein that accounts for approximately 10 to 15% of total milk proteins has a mild antioxidant role however, Cys-121 plays an essential role in the antioxidant nature of ß-LG (Liu et al. 2007). A recent evaluation of the activity of Cys, Trp, and Met of ß-LG using oil-in-water emulsions suggests that free Cys and Trp are involved, but not Met (Elias et al., 2005). The results also showed that the combination of WPC and KGE had more ability to restrict the unsaturated fatty acids from oxidation than BHA. WPC which are rich in sulfur amino acids, cysteine and methionine that can inhibit lipid oxidation had the same ability of ginsenosides in KGE which were shown previously to protect against lipid peroxidation and LDL oxidation *in vitro* (Zhang et al. 1996). These authors also suggested that hydroxyl radical formed by the Fenton reaction were completely inhibited by ginseng extract. Generally, the combination of WPC and KGE was the most effective to protect unsaturated fatty acids from oxidation which may contribute to stabilizing the structure of the lipid membrane perturbed by free radical attack.

In the current study we further evaluated the antioxidant and radical scavenging properties of WPC and/or KGE *in vivo* in rats fed AFs-contaminated diet. The selected doses of AFs and KGE were based on our previous work (Abdel-Wahhab and Aly, 2005; Abdel-Wahhab and Ahmed, 2004) whereas, the selected dose of WPC was based on the results of the current *in vitro* study. These results indicated that the increase in ALT, AST, ALP, cholesterol, TB and DB in animals fed AF-contaminated revealed degenerative changes and hypofunction of liver (Abdel-Wahhab et al., 2005, 2006). However, the decrease in serum levels of TP and albumin may indicate protein catabolism and/or kidney dysfunction (Abdel-Wahhab et al., 2007). These results clearly showed that aflatoxin has a harmful and stressful influence on the hepatic tissue consistent with those reported in the literature of aflatoxicosis (Miller and Willson, 1994).

The decrease in TAC with the increase in LP reported herein in the animals fed AFs-contaminated diet revealed that aflatoxins induced oxidative stress in liver tissue. Aflatoxins especially AFB_1 is metabolized by the cellular cytochrome P450 enzyme system to form the reactive intermediate AFB_1-8,9-epoxide as well as reactive oxygen species (ROS)

like superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) during the metabolic processing of AFB_1 (Abdel-Wahhab et al., 2005; Preetha et al., 2006), which in turn reacts with macromolecules such as lipids and DNA, leading to lipid peroxidation and cellular injury (Abdel-Wahhab et al., 2010). The aflatoxin-induced alterations in the hepatic antioxidant status may therefore be considered as manifestation of increased oxidative stress caused by aflatoxin and its metabolites. It is well known that TAC includes enzymes such as SOD, catalase and GPX. TAC may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids. Both GPX and SOD are considered enzymatic free-radical scavengers in cells. The decrease in TAC and the increase in LP reported in the current study in AFs-treated leading to an indirect increase in oxidative DNA damage. These results were in agreement with those reported by Meki *et al.* (2004) and Abdel-Wahhab et al. (2010). Moreover, Azeredo-Martins et al. (2003) stated that SOD plays a role in the suppression of oxygen free-radical formation and the decrease of nitric oxide generation. The current results also indicated that LP is one of the most prominent factors in aflatoxin toxicity and carcinogenicity (Rastogi *et al.*, 2001; Abdel-Wahhab and Aly 2005; Abdel-Wahhab et al., 2005, 2006). In the same concern, the mechanisms of aflatoxins induced liver injury have been demonstrated that glutathione plays the major important role in the detoxification of the reactive and toxic metabolites of aflatoxins, and the liver necrosis begins when the glutathione stores are almost exhausted (Abdel-Wahhab and Aly, 2003, 2005).

The histological and histochemical results reported in the current study confirmed the biochemical results and indicated that aflatoxin induced severe histological and histochemical changes in the hepatic tissues. The histological changes observed in the liver induced by aflatoxin have been documented previously (Mayura, et al., 1998; Abdel-Wahhab et al., 1998, 2007, 2010).

In the current study, animals fed AFs-contaminated diet and treated with WPC and/or KGE showed a significant improvement the biochemical parameters, the histological and the histochemical picture of the liver. Moreover, this improvement was more pronounced in the group received the combined treatment of WPC and KGE. This group showed a significant decrease in LP compared to the control group. Previous reports indicated that WPC has a potential antioxidant activity due to its ability to increase glutathione levels (Peng et al., 2009; Bayram et al., 2008). WPC is well known to be rich in cysteine, α-lactalbumin, β-lactoglobulin, and bovine serum albumin (Morr and Ha, 1993). Cysteine is an amino acid regulates the *in vivo* concentrations of GSH and supplementation of the diet with whey protein high cysteine may promote GSH biosynthesis (Kent et al., 2003). GSH was reported to be an antioxidant and anticarcinogenic tripeptide, and thereby improving protection against oxidant-induced cell damage (Peng et al., 2009). The increased TAC production reported herein in rats treated with WPC accompanied with the decrease level of LP supported the earlier findings of Watanabe et al. (2000). The results of the current study and others suggested that cysteine content is responsible, in part, for the observed increase TAC via the increase in GSH. According to Bounous (2000), the suggested mechanism by which WPC induced its protection has been attributed to the increase in blood and tissue GSH concentration, which in turn increased the scavenger of the free radicals produced by AFs.

In previous reports, we showed that KGE protects against aflatoxin and fumonisins-induced liver cancer in rats (Abdel-Wahhab et al., 2010), the toxic effects of chromium VI (Abdel-Wahhab and Ahmed 2004), acrylamide (Mannaa et al., 2006), PCBs (El-Kady et al., 2006) and EDTA (Khalil et al., 2008). In the present study, administration of KGE to rats received AFs-contaminated diet resulted in a significant improvement in all biochemical parameters, histological and histochemical pictures of the liver. Jeong et al. (1997) suggested that KGE displays a pronounced hepatoprotective effect, assessed through the transaminases (ALT, AST) activities following hepatotoxicity in rats treated with carbon tetrachloride. Moreover, administration of KGE significantly reduced the activities of ALT and AST induced by dexamethasone (DEN) administration in rats (Lin et al., 1995). Ginseng also was found to protect against liver toxicity induced by CCL_4 through the inhibition of cytochrome P450-associated monoxygenase activities (Kim et al., 1997). Therefore, the protective effect of KGE in the current study is attributable to its free radical scavenging activity (Abdel-Wahhab and Ahmed, 2004; Mannaa et al., 2006). Similar to the current observations, Yun et al. (1987) reported that prolonged administration of Korean red ginseng extract resulted in substantial suppression of pulmonary tumorigenesis induced by AFB_1. Non-saponin components in red ginseng suppressed the harmful effects of free oxygen radicals (O_2, H_2O_2, OH_2), which exercise an important role in tissue degeneration (Kim et al., 1997). Furthermore, the hydroxyl radical formed by the Fenton reaction were

completely inhibited by ginseng extract (Zhang et al., 1996). These antioxidant effects of ginseng may be responsible for its wide pharmacological actions in clinical practice by a free radical reaction-inhibition mechanism consequently decreased risk for most cancers including carcinomas of the esophagus, stomach, colon, pancreas, lung and liver (Jeong et al., 1997; Abdel-Wahhab et al., 2010).

Li et al. (2008) postulated that Ginsenoside Rg1, cinnamic acid, and tanshinone IIA isolated from ginseng could serve as protective agents in cancer prevention and treatment. A 20-O-(beta-D-glucopyranosyl)-20(S)-protopanaxadiol (IH901), an intestinal bacterial metabolite of ginseng saponin formed from ginsenosides Rb1, Rb2, and Rc, is suggested to be a potential chemopreventive agent and induces apoptosis in human hepatoblastoma HepG2 cells (Yim et al., 2005). In the same concern, Konoshima et al. (1999) isolated a ocotillol-type saponin, majonoside-R2 (MR2) from the rhizome and root of ginseng and reported that this active constituent exhibited potent anti-tumor-promoting activity on two-stage carcinogenesis test of mouse hepatic tumor. Moreover, Kim et al. (1999) suggested that G-Rs4 induces apoptosis through the down regulation of both cyclins E- and A-dependent kinase activity as a consequence of selectively elevating protein levels of p53 and p21WAF1 in SK-HEP-1 cells.

Ginsenoside Rh2 (GS-Rh2) is one of the ginsenosides presented in KGE plays an essential role in the prevention and treatment of liver cancer by mechanisms postulated in a serious of reports. Zeng and Tu (2004a) stated that GS-Rh2 may effectively reduce telomerase activity and arresting cell cycle progression. The down-regulation of telomerase activity in SMMC-7721 cells may be closely related to GS-Rh2-induced differentiations which were found to be tended to normal (Zeng and Tu, 2004b). Moreover, Oh et al. (1999) suggested that GS-Rh2 inhibited the growth of MCF-7 cells, by inducing protein expression of p21 and reducing the protein levels of cyclin D which resulted in the down-regulation of cyclin/Cdk complex kinase activity, decreasing phosphorylation of pRb, and inhibiting E_2F release. GS-Rh2 was also found to arrest Eca-109 cells at G0/G1 phase and induce cell differentiation to be normal (Li et al., 2005). The panaxadiol fraction and its ginsenosides could induce the antioxidant enzymes which are important for maintaining cell viability by lowering the level of oxygen radical generated from intracellular metabolism (Chang et al., 1999). Taken together, these results indicated that KGE have protective effects against liver injury induced by aflatoxins and it plays a role in increasing the antioxidant status as well as lowering the oxidative damage of nucleic acids in the body (Abdel-Wahhab and Ahmed, 2004; Mannaa et al., 2006).

5. Conclusion:

The current study revealed that WPC and KGE exhibited a potential antioxidant and radical scavenging properties in vitro and in vivo. They were able to protect against the oxidative stress of aflatoxins singly or in combination. KGE was effective than WPC however, the combined treatment was found to be more effective than the single treatment. It could be concluded that incorporation of WPC and KGE may be useful for people suffering from liver diseases.

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