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Original Article

Phenolic-antioxidant capacity of mango seed kernels: therapeutic effect against viper venoms

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ABSTRACT

In this study, mango seed kernels extract contained a considerable amount of phenolics and flavonoids (17,400 and 3325 mg/100 g seed, respectively). The HPLC profiling revealed that hesperidin was the major phenolic compound of the mango seed kernels extract. This is the first report find hesperidin in mango extracts. The phenolic compounds of mango seed kernels extract were effective in scavenging free radicals of DPPH and ABTS with IC_{50} values of 47.3 and 7.9 μg/ml, respectively. The total antioxidant activity of mango seed kernels extract based on the reduction of molybdenum was also measured. The phenolic compounds of mango seed kernels extract potentially inhibited the protease, fibrinogenase, phospholipase A_2, L-amino acid oxidase, hyaluronidase, and hemolytic activities of the most dangerous *Cerastes cerastes* and *Echis coloratus* viper venoms. The phenolic compounds of mango seed kernels extract could completely neutralize the hemorrhage and lethality of both venoms in experimental animals. It could be concluded that the mango seed kernels extract phenolic compounds with potential antioxidant activity are considered as a new avenue in the viper bite treatment.

Introduction

Mango (*Mangifera indica* L., Anacardiaceae) is one of the most important tropical fruits in the world. Mango fruit occupies the second position as a tropical crop with a global production exceeding 35 million tons (Jahurul et al., 2015). In Egypt, over 4 million tons of mango fruits are produced (Abdalla et al., 2007). Mango fruits are processed in various products as puree, canned slices, syrup, nectar, leather, pickles, chutney and jam, resulting in a significant amount of wastes. Mango seed kernel represents about 20% of the whole mango fruit and includes rich levels of health-enhancing compounds and natural antioxidants (Sogi et al., 2013; Jahurul et al., 2015). Further, kernels have anti-tyrosinase, anti-inflammatory, anti-obesity, and hepato-protective effects (Kobayashi et al., 2013).

Vipers are the main cause (over 80%) of snake bite worldwide. Venoms of the vipers are plentiful sources of active molecules that affect a large number of physiological functions. These molecules include diverse hydrolytic enzymes that causing local harmful effects like hemorrhage, necrosis and edema end with tissue loss. They also cause systemic effects resulting in coagulopathy, cardiopathy, and neuropathy (Oussedik-Oumehdi and Laraba-Djebari, 2008). Furthermore, viper venom causes various complications as thrombocytopenia, renal abnormalities, hypopituitarism and permanent tissue damage. Oxidative stress plays a main role in viper bite pathophysiology and the constancy of the viper bite complications (Zengin et al., 2012; Sunitha et al., 2015). Insufficiency of anti-venom to reverse these complications leads to viper bite treatment still a challenge till now (Girish and Kemparaju, 2011). *Cerastes cerastes* (Linnaeus, 1758) (horn viper) and *Echis coloratus* Günther, 1878 (red carpet viper) are the most dangerous and medically important vipers in Egypt and are responsible for the greatest incidence of envenomation (Wahby et al., 2012).

The recovery and utilization of valuable compounds from mango by-products are an important challenge, hence, this study aims to determine the total phenolic, flavonoid contents and antioxidant capacity of Egyptian mango seed kernel extract (MSKE) and to assess its anti-venom properties against Egyptian *Cerastes cerastes* and *Echis coloratus* viper venoms.

Materials and methods

Plant

Egyptian mango Hindi cultivar (*Mangifera indica* L., Anacardiaceae) was obtained from Horticulture Institute Research, Agriculture Research Centre, Cairo, Egypt.

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Animal

Cerastes cerastes (Linnaeus, 1758) and *Echis coloratus* Günther, 1878 adult vipers were obtained from laboratory animal unit of Helwan Farm-VACSERA, Egypt. The *C. cerastes* and *E. coloratus* venoms were milked, lyophilized and stored at −20 °C.

Male Swiss-albino mice weighing (20 ± 2.4 g) were used for this study. All animals were housed at the animal house of the National Research Centre (NRC), under standardized conditions and diet. All the experimental protocols described in this study were performed in accordance with the recommendations of the ethical committee, NRC, Egypt (Protocol permit #15-097).

Plant extraction

Mango seed kernel Hindi cultivar (8 g) were chopped, grinded, soaked in 60 ml of 80% methanol and shaken overnight at 120 rpm and room temperature. The extract was centrifuged at 7200 × *g* for 10 min. The supernatant was evaporated under vacuum and dissolved in least volume of 0.1% DMSO and designated as a mango seed kernel extract (MSKE).

Total phenols measurement

The total phenolic content of MSKE was measured according to Velioglu et al. (1998). The reaction mixture includes: 0.1 ml methanol extract, 0.1 ml Folin-Ciocalteu reagent and 0.8 ml distilled water were incubated for 5 min at room temperature. Then 0.5 ml sodium carbonate (20%) was added and incubated at room temperature for 30 min. The absorbance was measured at 750 nm. The results were expressed as mg gallic acid equivalent (GAE)/100 g seed.

Total flavonoids measurement

The total flavonoid content of MSKE was measured according to Zhishen et al. (1999). Incubation of 0.25 ml methanol extract, 1.25 ml distilled water and 0.075 ml of 5% $NaNO_2$ for 6 min, then add 0.15 ml of 10% $AlCl_3$. After 5 min, 0.5 ml of 1.0 M NaOH and 0.275 ml distilled water were added. The absorbance was measured at 510 nm. The results were expressed as mg catechin equivalent (CE)/100 g seed.

HPLC analysis of phenolic compounds

The high performance liquid chromatography (HPLC) analysis was carried out for MSKE according to Kim et al. (2006). The separation and determination were performed on XDB-C18 column (150 × 4.6 μm). The column was eluted by acetonitrile (solvent A) and 2% acetic acid (solvent B) at a flow rate of 1 ml/min. The obtained peaks were monitored simultaneously at 280, 320 and 360 nm. Commercial phenolic compounds were used as standards.

Antioxidant assays

DPPH assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) method was used for determination of the antioxidant activity of MSKE (Ao et al., 2008). The reaction mixture includes: 0.1 ml methanol extract and 0.9 ml of 0.1 mM DPPH dissolved in methanol were incubated for 30 min at room temperature. The absorbance was measured at 517 nm. DPPH scavenging percent = [(O.D. control − O.D. sample)/O.D. control] × 100.

ABTS assay

ABTS (2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) reagent was prepared and used for determination of the antioxidant activity of MSKE (Re et al., 1999). The reaction mixture includes: 1 ml of ABTS reagent and 0.1 ml of the extract were incubated for 1 min at room temperature and the reduction of absorbance was measured at 734 nm. ABTS scavenging percent = [(O.D. control − O.D. sample)/O.D. control] × 100.

Phosphomolybdenum complex assay

The antioxidant activity of MSKE was also evaluated by formation of a phosphomolybdenum complex according to Prieto et al. (1999). The reaction mixture includes: 4 mM ammonium molybdate, 28 mM sodium phosphate, 600 mM sulfuric acid, and 50 μl of the extract were incubated at 95 °C for 90 min. After cooling, the absorbance was read at 695 nm. EC_{50} is defined as a concentration of the MSKE gives absorbance of 0.5.

Protease inhibition

Azocasein assay

The protease activity of crude venom was measured according to Lemos et al. (1991) using azocasein as a substrate. One ml reaction mixture includes: 0.2% azocasein, 10 μg of crude venom and 0.02 M Tris–HCl buffer, pH 7.0 were incubated at 37 °C. The reaction was stopped after 1 h by adding 100 μl of 20% TCA and precipitation removed by centrifugation at 5000 × *g* for 5 min. The change of absorbance (0.01 O.D.) was measured at 366 nm which considered as a one unit/h. Inhibition studies, the crude venom (10 μg) was pre-incubated with various concentrations of MSKE for 15 min at 37 °C and measurement of residual activity.

Gelatin zymography

Gelatin zymography of crude venom (30 μg) was performed in 12% native polyacrylamide gel co-polymerized with 0.2% gelatin according to Bee et al. (2001). After electrophoresis, the gel was incubated overnight in 0.02 M Tris–HCl buffer, pH 7.0 at 37 °C. After gel staining by Coomassie Brilliant Blue R-250, clear zones were appeared. Inhibition experiments, 30 μg of crude venom was pre-incubated with different concentrations of MSKE for 15 min at 37 °C and performed according to the method previously described.

Fibrinogen degradation inhibition

Fibrinogenase activity of crude venom was determined according to Ouyang and Teng (1976). One ml reaction mixture includes: Human plasma fibrinogen (2 mg), venom sample (2 μg) and 5 mM Tris–HCl buffer, pH 7.5 containing 5 mM $CaCl_2$ were incubated at 37 °C. The reaction was stopped after 2 h by adding 100 μl of stopping solution (4% SDS, 4% 2-mercaptoethanol and 10 M urea). The samples were electrophoresis on 10% SDS-PAGE according to Laemmli (1970). Inhibition experiments were performed by pre-incubated venom sample with different concentrations of MSKE for 15 min at 37 °C and performed according to the method previously described.

Phospholipase A_2 (PLA_2) inhibition

PLA_2 activity of crude venom was determined using egg yolk as a substrate according to Marinetti (1965). One ml of egg yolk solution (1:5 w/v saline), crude venom alone (200 μg) or pre-incubated with different phenolic concentrations of MSKE at 37 °C for 15 min were mixed and reached to a final volume of 5 ml with saline. The absorbance was recorded each 5 min for 15 min at 900 nm. PLA_2 activity was also determined according to Gutierrez et al. (1988).

Table 1
Total phenolic, flavonoid contents and antioxidant capacity of the mango seed kernels extract.

Sample	Total phenolic	Total flavonoid	Antioxidant activity		
	mg GAE/100 g seed	mg CE/100 g seed	DPPH (IC_{50}) μg GAE/ml	ABTS (IC_{50}) μg GAE/ml	Total antioxidant capacity (EC_{50}) μg GAE/ml
MSKE	17,400 ± 348	3325 ± 120	47.3 ± 0.85	7.9 ± 0.14	4.0 ± 0.11

GAE, gallic acid equivalent; CE, catchin equivalent; values are presented as means ± S.E. ($n = 3$).

Crude venom (200 μg) alone or preincubated with various concentrations of MSKE for 15 min at 37 °C was loaded into wells (3 mm diameter) of 1% agarose plates containing 4% washed human erythrocytes, 4% egg yolk suspension and 10 mM $CaCl_2$. The fortified plate was incubated for 20 h at 37 °C. The decreasing of the clear zone diameters around the wells was measured and equivalent to the PLA_2 inhibition. The venom PLA_2 activity in the absence of MSKE was taken as a 100%.

L-Amino acid oxidase inhibition

L-Amino acid oxidase (LAAO) activity of crude venom was measured according to Kishimoto and Takahashi (2001). The reaction mixture includes: 0.005 M L-leucine, as a substrate, horseradish peroxidase (5 U/ml) and 0.002 M *O*-phenylenediamine, as a substrate for peroxidase, 2 μg of venom sample and 0.05 M of Tris–HCl buffer, pH 8.0 were incubated at 37 °C. The reaction was stopped after 1 h by adding 50 μl of 2 M H_2SO_4. The absorbance was measured at 490 nm using ELISA reader. One unit of LAAO enzyme is the amount of enzyme which produces 1 μmol of H_2O_2. Inhibition experiments were performed by pre-incubated venom sample with different concentrations of MSKE at 37 °C for 15 min and measurement of remaining activity. Crude venom alone (a positive control) was taken as a 100%.

Hyaluronidase inhibition

Hyaluronidase activity of crude venom was measured according to Pukrittayakamee et al. (1988). The reaction mixture includes: 50 μg hyaluronic acid, 10 μg of crude venom (in a final volume of 500 μl) and 200 mM sodium acetate buffer, pH 5.5, including 0.15 M NaCl were incubated at 37 °C. The reaction was stopped after 15 min by adding 100 μl of (2.5% acetyltrimethylammonium bromide in 2% NaOH). The absorbance was recorded at 400 nm. One unit is defined as a concentration of enzyme that causes 50% turbidity reduction. Inhibition experiments, 10 μg of crude venom was pre-incubated with different concentrations of MSKE at 37 °C for 15 min and measurement of the residual activity. Crude venom alone (a positive control) was taken as a 100%.

Hemolytic inhibition

The hemolytic activity of crude venom was determined according to Al-Abdulla et al. (1991). The reaction mixture includes: 100 μl of egg yolk solution (diluted 1:5 in saline), 1 ml of 2.5% washed human erythrocytes (v/v), 3.8 ml of 0.001 M Tris–HCl buffer, pH 7.5 containing 0.1 M NaCl, 0.1 M KCl and 0.01 M $CaCl_2$ and 10 μg of crude venom alone or pre-incubated with various concentrations of MSKE at 37 °C for 15 min. The reaction mixture was centrifuged at 2500 rpm for 10 min and the concentration of hemoglobin that released was estimated at 540 nm.

Hemorrhagic inhibition

Hemorrhagic activity of crude venom was evaluated by Kondo et al. (1960). Mice groups ($n = 5$) were injected with venom samples dorsally and intradermally in 0.1 ml of saline. Groups (a1) and (a2) were injected with two minimum hemorrhagic doses (2MHD) of *C. cerastes* and *E. coloratus* venoms, respectively. Groups (b1–f1) and (b2–d2) were injected with 2MHD of *C. cerastes* and *E. coloratus* venoms pre-incubated for 15 min at 37 °C with various concentrations of the MSKE, respectively. Groups (g2) and (K2) were injected with 0.1 ml of MSKE and saline, as controls, respectively. After 2 h, the mice skins were removed and the diameters of the hemorrhagic spots were recorded.

Neutralization of lethal potency of crude venoms

The median lethal dose (LD_{50}) of *C. cerastes* and *E. coloratus* venoms was measured according to Meier and Theakston (1986). Mice groups ($n = 5$) were injected intraperitonially with different concentrations of venom (2–16 μg protein/mouse) in 0.5 ml of saline. In neutralization studies, groups 1 and 5 were injected with $2LD_{50}$ of *C. cerastes* and *E. coloratus* venoms, respectively. Groups 2–4 and groups 6–7 were injected with $2LD_{50}$ of *C. cerastes* and *E. coloratus* venoms, respectively, and after 10 min following venom injection different phenolic concentrations of the MSKE were injected individually. Groups 8 and 9 were received saline and 500 μg phenolic of the MSKE, respectively. The survival time of mice in each group was recorded during 24 h.

Statistical analysis

The data were statistically analyzed by a one-way ANOVA, followed by Dennett's post hoc test. Differences were significant at $p < 0.01$. The results were expressed as mean ± S.E.

Results and discussion

The total phenolic content of MSKE was found to be 17,400 ± 348 mg GAE/100 g seed (Table 1). Different of total phenolic contents ranged from 112 to 44,760 mg/100 g seed were reported in other mango cultivars (Abdalla et al., 2007; Ribeiro et al., 2008; Sogi et al., 2013; Dorta et al., 2014). These differences in phenolic contents might be due to mango cultivars, geographical location, extraction conditions and used different standard equivalents.

The total flavonoid content of MSKE was found to be 3325 ± 120 mg CE/100 g seed (Table 1). The obtained results were much higher than that reported for peel and kernel of other mango cultivars (10–1170 mg/100 g) (Ribeiro et al., 2008; Dorta et al., 2012; Ajila and Rao, 2013; Dorta et al., 2014).

Different phenolic compounds were identified and quantified by HPLC technique (Table 2). Hesperidin was the major compound in MSKE which represented 3000 ± 112 mg/100 g seed and 55.6% of total polyphenols, followed by cinnamic and tannic acids which represented 1200 ± 52 and 987 ± 44 mg/100 g seed with 22.2 and 18.3% of total polyphenols, respectively. Whereas MSKE contained minor amount of gallic, protocatechuic, *p*-coumaric, catachine, and cholorgenic. This is the first report find hesperidin in mango extracts. However, hesperidin was extracted in large concentrations from the discarded rinds of the ordinary orange (Kanes et al., 1993).

Table 2
HPLC analysis of the mango seed kernels extract phenolic compounds.

Compounds	mg/100 g	% of total compounds
Hesperidin	3000 ± 112	55.6
Cinnamic acid	1200 ± 52	22.2
Tannic acid	987 ± 44	18.3
Gallic acid	156 ± 7.8	2.9
Protocatechuic acid	21 ± 2.0	0.39
Catachine	15 ± 1.5	0.3
p-Coumaric acid	12.5 ± 1.0	0.23
Chlorogenic acid	8.5 ± 0.82	0.16

Values are presented as means ± S.E. ($n = 3$).

The MSKE showed a concentration dependent scavenging of DPPH and ABTS radicals with IC_{50} values 47.3 ± 0.85 and 7.9 ± 0.14 μg/ml, respectively (Table 1). The scavenging activity of MSKE was attributed to its phenolic compounds, like hesperdin, cinnamic, and tannin (Kim et al., 2004; Abdalla et al., 2007). Total antioxidant capacity of the MSKE based on the reduction of molybdenum (VI) to molybdenum (V) was also measured with EC_{50} value of 4.0 ± 0.11 μg/ml (Table 1). Many studies reported that mango seed kernel is a rich source of natural antioxidants (Abdalla et al., 2007; Jahurul et al., 2015).

Viper venom contains a set of multifunctional proteases like snake venom metalloproteases (SVMP), serine and thrombin like proteases. These proteases cause numerous pathologies including hemorrhage, necrosis, hemostatic disturbances, inflammation and alter the prey redox state (Markland and Swenson, 2013; Sunitha et al., 2015). The MSKE exhibited 50% inhibition (IC_{50}) of the *C. cerastes* and *E. coloratus* protease activity at 18 and 9 μg phenolics, respectively and full inhibition for both venoms was given at 30 μg phenolics (Fig. 1A). However, full inhibition for *C. cerastes* and *E. coloratus* protease zones was achieved at 80 and 40 μg phenolic of the MSKE in gelatin zymography (Fig. 1B and 1C), respectively.

For studying the mode of inhibition by MSKE, the kinetic of the CcHTI and EcoHTI metalloproteases, previously purified from Egyptian *C. cerastes* and *E. coloratus* venoms (Wahby et al., 2012), was evaluated in presence of the MSKE. The k_m values of the CcHTI and EcoHTI enzymes increased from 0.9 and 0.55 to 2.0, 1.25, and 4.1, 3.3 mg at 15 and 30 μg phenolic of the MSKE, respectively. The V_{max} values of the CcHTI and EcoHTI were unaffected (Fig. 2A and B). This increase in the K_m values reflects the decline of the affinity of both enzymes toward the azocasein, as a substrate, and at the same time reflects the competitive inhibitory effect of the phenolic compounds of the MSKE on the active sites of both enzymes.

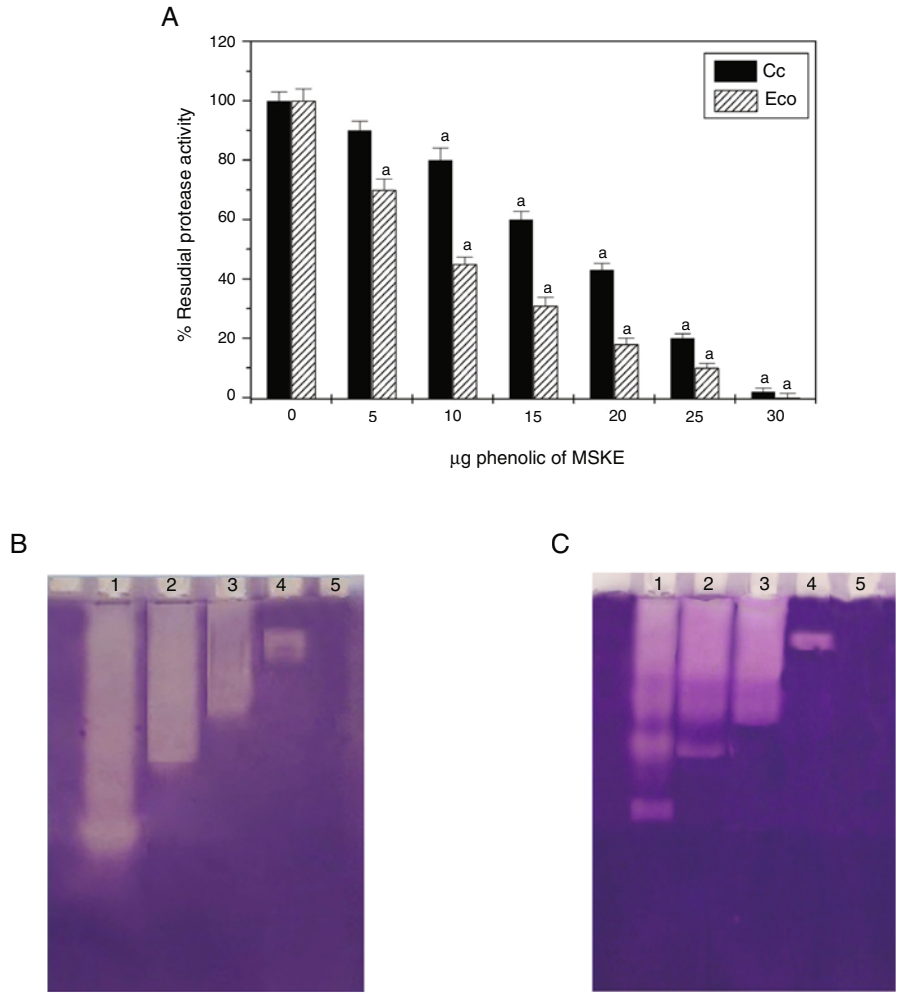

Fig. 1. Inhibition of *Cerastes cerastes* (Cc) and *Echis coloratus* (Eco) protease activity by the mango seed kernels extract (MSKE). (A) Ten μg of crude venoms were pre-incubated individually with different phenolic concentrations of the MSKE for 15 min at 37 °C and the residual activity % was measured using the standard assay. The values represent mean ± S.E. ($n = 3$) and the results $p > 0.01$ ([a]) were considered as significant. (B) Gelatin zymography of *C. cerastes* venom protease inhibition by the MSKE. Lane 1, 30 μg of *C. cerastes* venom sample alone, and lanes (2–5) 30 μg of *C. cerastes* venom pre-incubated with 10, 20, 40 and 80 μg phenolic of the MSKE, respectively. (C) Gelatin zymography of *E. coloratus* venom protease inhibition by the MSKE. Lane 1, 30 μg of *E. coloratus* venom alone, and lanes (2–5) 30 μg of *E. coloratus* venom pre-incubated with 10, 20, 30 and 40 μg phenolic of the MSKE, respectively.

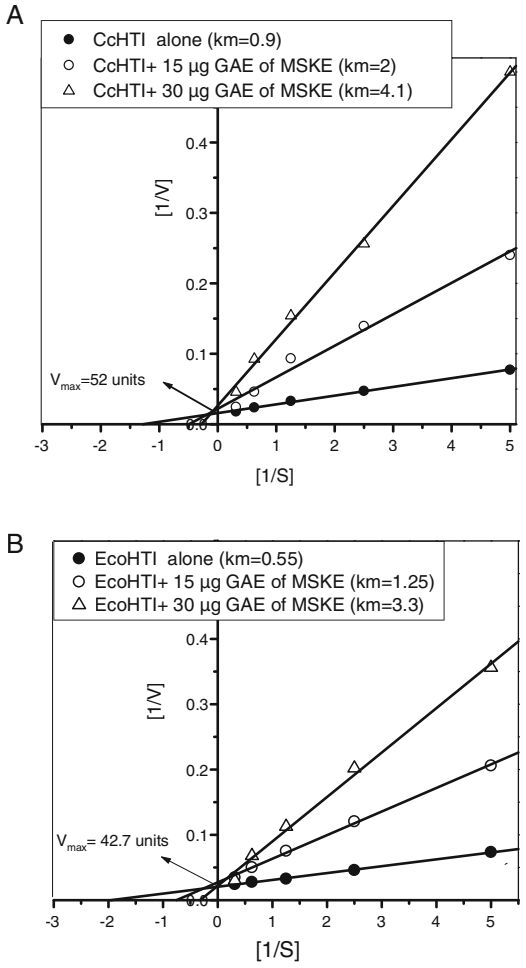

Fig. 2. Lineweaver–Burk plot for inhibition of CcHTI (A) and EcoHTI (B) metlalloproteases toward azocasein as a substrate by 15 and 30 µg phenolic of the mango seed kernels extract (MSKE).

The action of venom proteases on fibrinogen lead to unstable fibrin, causing change in blood coagulation and platelet function (Mc-Cleary and Kini, 2013). When the *C. cerastes* and *E. coloratus* venoms (2 µg) were individually incubated with fibrinogen (2 mg/ml) for 2 h, α and β chains of the fibrinogen were completely digested. Whereas the ϑ-chain of the fibrinogen partially digested, demonstrating that both venoms have a potential fibrinogenase activity. At 1–4 µg of the MSKE, the degradation inhibition of β and ϑ chains was preferred over α chain in both venoms. At 6 and 10 µg of the MSKE, full inhibition of α, β and ϑ chains degradation was achieved in *E. coloratus* and *C. cerastes* venoms, respectively (Fig. 3A and B) suggesting that the MSKE inhibits proteases induced hemostatic disturbances. Thai mango ethanolic extract inhibited the fibriongenase activity of the Malayan and Thai cobra venoms (Pithayanukul et al., 2009).

PLA_2 plays a vital role in systemic oxidative stress and inflammation. It implicated in many necrotic actions on red blood cells (RBC) and skeletal muscle cells resulting in production of inflammatory mediators like prostaglandins and leukotrienes. These mediators are responsible for tissue damage (Barone et al., 2014; Sunitha et al., 2015). For determination of PLA_2 activity, 200 µg of both viper venoms was used because they showed a low PLA_2 activity. This weak activity is probably related to a sub-group (2b) of $sPLA_2$ which contains a Lys at position 49 instead of Asp. These $sPLA_2$ have a low activity but retain high levels of toxicity and are found exclusively in viper venoms (Harris and Scott-Davey, 2013). The MSKE efficiently inhibited the PLA_2 activity of the *C. cerastes* and *E. coloratus* venoms with IC_{50} values of 30 and 35 µg phenolics, respectively. Full inhibition was achieved at 50 µg phenolic of the MSKE for both venoms (Fig. 4A). The PLA_2 inhibition was also confirmed by reduction the activity zone diameters on gel plate (Fig. 4B and C). At 80 µg of MSKE, 88 and 100% inhibition of PLA_2 activity were observed in *C. cerastes* and *E. coloratus* venoms, respectively. Similarly, the hesperidin is a potent anti-inflammatory agent and inhibited snake PLA_2 (Garg et al., 2001; Soares et al., 2005). Further, tannic acid had anti-snake effect which could be precipitated proteins, formed insoluble complexes with many metal ions and inhibited snake PLA_2 (Haslam, 1996). Moreover, cinnamic acid was also tested as an anti-snake agent (Mors et al., 2000).

LAAO are flavoenzymes which catalyze the oxidative deamination of L-amino acids to α-keto acids and liberate ammonia and H_2O_2. The excess production of H_2O_2 affect victim's endogenous antioxidant defense followed by apoptotic, cytotoxic, platelet aggregation, edema and hemorrhage (Sunitha et al., 2015). The MSKE inhibited the LAAO activity of *C. cerastes* and *E. coloratus* venoms with IC_{50} values of 16 and 18 µg phenolics, respectively. Full inhibition of both venoms LAAO activity was achieved at 40 µg phenolic of the MSKE (Fig. 5A). This inhibition may be due to the antioxidant potency of MSKE components. Kalpana et al. (2009) found that the hesperidin prevented H_2O_2-induced oxidative damage on the RBC.

Snake venom hyaluronidases are known as spreading factors. They facilitate inflow of the toxins from the bite site to the circulation (Kemparaju and Girish, 2006). The IC_{50} and full inhibition of *C. cerastes* and *E. coloratus* hyaluronidase activity were recorded at 9, 30 and 14, 40 µg of MSKE, respectively (Fig. 5B). The hesperidin is considered as a potent hyaluronidase inhibitor (Garg et al., 2001).

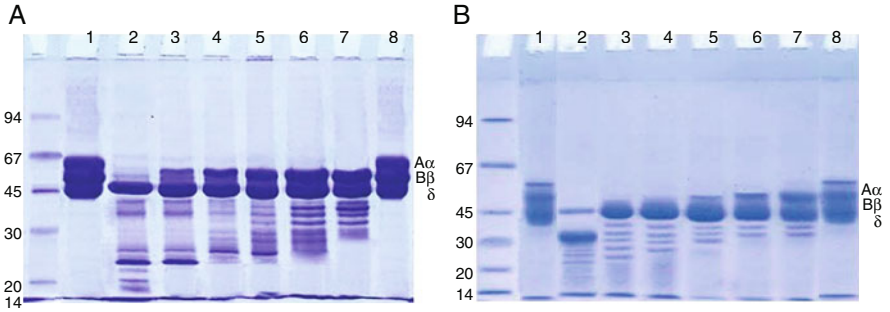

Fig. 3. Inhibition of *Cerastes cerastes* and *Echis coloratus* fibrinogenase activity by the mango seed kernels extract (MSKE). (A) Lane (1) 2 mg fibrinogen/ml alone, lane (2) fibrinogen + 2 µg of *C. cerastes* venom, and lanes (3–8) fibrinogen + 2 µg of *C. cerastes* venom pre-incubated with 1, 2, 3, 4, 8 and 10 µg phenolic of the MSKE, respectively. (B) Lane (1) 2 mg fibrinogen/ml alone, lane (2) fibrinogen + 2 µg of *E. coloratus* venom, and lanes (3–8) fibrinogen + 2 µg of *E. coloratus* venom pre-incubated with 1, 2, 3, 4, 5 and 6 µg phenolic of the MSKE, respectively.

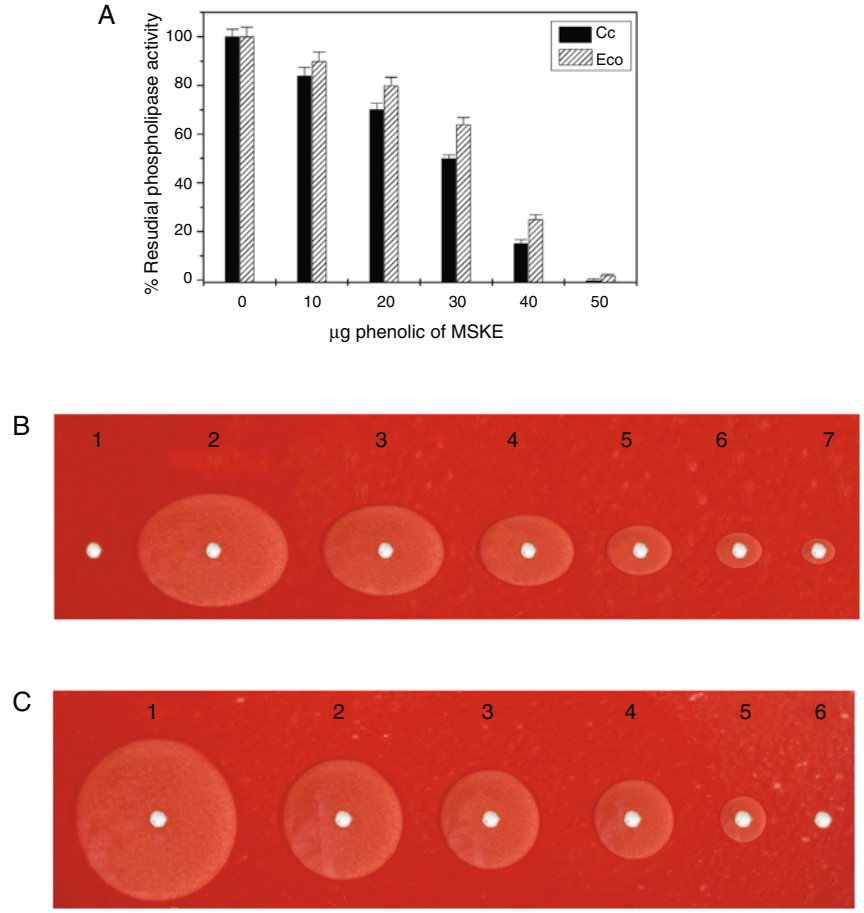

Fig. 4. Inhibition of *Cerastes cerastes* (Cc) and *Echis coloratus* (Eco) PLA_2 activity by the mango seed kernels extract (MSKE). (A) Two hundred µg of each venom sample was pre-incubated with different phenolic concentrations of the MSKE for 15 min at 37 °C and the residual activity % was measured using the standard assay. The values represent mean ± S.E. ($n = 3$) and the results $p > 0.01$ ([a]) were considered as significant. (B) Inhibition of *C. cerastes* PLA_2 activity in fortified agarose gel (1). Eighty µg phenolic of MSKE alone, (2) *C. cerastes* venom alone and (3–7) *C. cerastes* venom pre-incubated with 5, 10, 20, 40 and 80 µg phenolic of the MSKE, respectively. C. Inhibition of *E. coloratus* PLA_2 activity in fortified agarose gel. (1) *E. coloratus* venom alone and (2–6) *E. coloratus* venom pre-incubated with 5, 10, 20, 40 and 80 µg phenolic of the MSKE, respectively.

Table 3
Neutralization of *Cerastes cerastes* and *Echis coloratus* lethality by the mango seed kernels extract (MSKE) administered intraperitoneal. The values represent mean ± S.E. ($n = 5$). Values with superscript (a) were remarkable significant ($p < 0.01$).

Groups (G)	Number of dead mice	Survival time (h)
G 1 $2LD_{50}$ of *C. cerastes* venom ($2LD_{50}$ = 16 µg/mice)	5/5	0.43 ± 0.05
G2 $2LD_{50}$ of *C. cerastes* + 20 µg phenolic of MSKE	3/5	16 ± 0.80[a]
G3 $2LD_{50}$ of *C. cerastes* + 40 µg phenolic of MSKE	1/5	22 ± 1.3[a]
G4 $2LD_{50}$ of *C. cerastes* + 80 µg phenolic of MSKE	0/5	≥24[a]
G5 $2LD_{50}$ of *E. coloratus* venom ($2LD_{50}$ = 8 µg/mice)	5/5	0.49 ± 0.06
G6 $2LD_{50}$ of *E. coloratus* + 20 µg phenolic of MSKE	2/5	19 ± 0.95[a]
G7 $2LD_{50}$ of *E. coloratus* + 40 µg phenolic of MSKE	0/5	≥24[a]
G8 Saline	0/5	≥24
G9 500 µg phenolic of MSKE	0/5	≥24

During viper bite, hemolysis was occurred by the action of both SVMP and $PLAs_2$ on the RBC causing accumulation of Hb and heme and mediated pro-oxidant and pro-inflammatory effects (Sunitha et al., 2015). The IC_{50} and 100% inhibition of *C. cerastes* and *E. coloratus* hemolytic activity were observed at 21, 19 and 40 µg of MSKE (Fig. 5C), respectively. The MSKE alone didn't induce any RBC hemolysis. This proves that MSKE is non-toxic. Hemolytic inhibition could be explained by the ability of pheno-

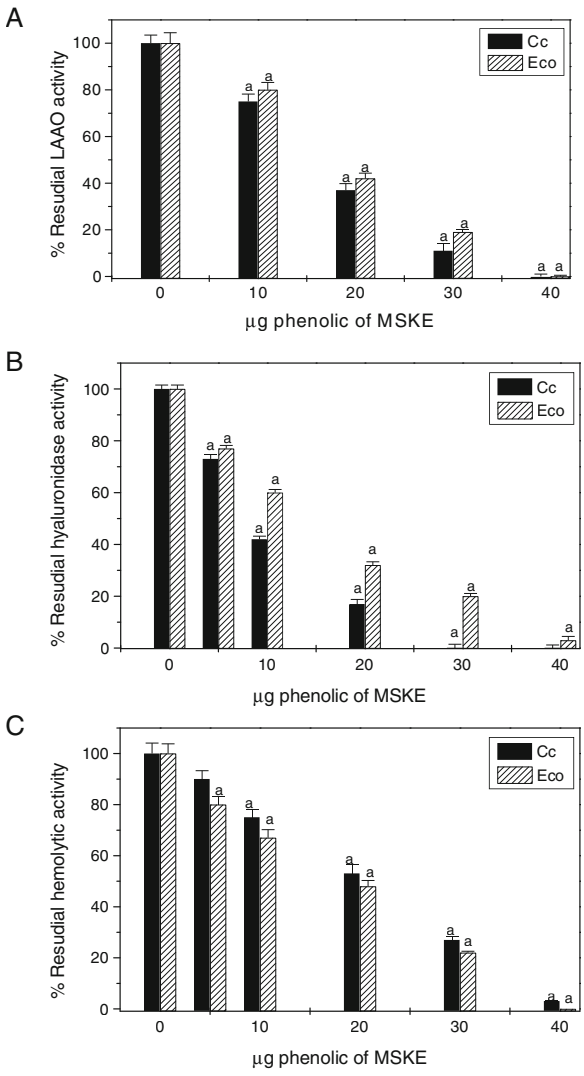

Fig. 5. Inhibition of L-amino acid oxidase activity (A) hyaluronidase activity (B) and hemolytic activity (C) of *Cerastes cerastes* (Cc) and *Echis coloratus* (Eco) venoms by the mango seed kernels extract (MSKE). Venom samples were pre-incubated individually with different phenolic concentrations of the MSKE for 15 min at 37 °C and the residual activities % were measured using the standard assays. The values represent mean ± S.E. ($n = 3$) and the results $p > 0.01$ ([a]) were considered as significant.

lic compounds of MSKE to inhibit the proteases and PLA_2s in both venoms.

Hemorrhagic SVMP are the major components of viper venoms and considered as the basic causative of viper lethality (Sells et al., 1997). Inhibition of viper hemorrhagic activity is considered as a core test to evaluate the extract anti-snake property (Shirwaikar et al., 2004). The results showed that the hemorrhagic spots in mice skin injected with 1 and 3 µg of *C. cerastes* and *E. coloratus* venoms in absence of MSKE were about 20 ± 0.2 and 21 ± 0.3 mm, respectively. The MSKE was impressively able to relieve the hemorrhagic effect of both venoms in mice dose dependently. The hemorrhagic zones of *C. cerastes* and *E. colora*tus venoms were totally disappeared at 25 and 15 µg phenolic of the MSKE, respectively (Fig. 6A and B). Also, the MSKE alone didn't induce any hemorrhage.

The LD_{50} of *C. cerastes* and *E. coloratus* venoms were established at 8 and 4 µg/20 g mouse, respectively (data not shown). Mice injected with $2LD_{50}$ of *C. cerastes* and *E. coloratus* survived for 0.43 and 0.49 h, respectively. The mice could survive up to 16 and 19 h when MSKE administrated by 20 µg phenolics after 10 min of 2 LD_{50} *C. cerastes* and *E. coloratus* venoms injection, respectively, and about fifty percent survival in mice groups. Further, the $2LD_{50}$ of *C. cerastes* and *E. coloratus* venoms totally neutralized at 80 and 40 µg of MSKE, respectively and all mice still alive even after 24 h of injection. In addition, intraperitonial administration of the MSKE did not induce any toxic effect (Table 3). The full inhibition of both venoms lethality may be due to interact of phenolic compounds of the extract with venom harmful enzymatic components (Houghton, 1998). Many recent studies reported that oxidative stress and the complications associated with viper bite could be improved by the antioxidants treatment strategy (Zengin et al., 2012; Katkar et al., 2014). Therefore, the venom neutralization action of the MSKE may be attributed to its phenolic compounds and their antioxidant activity.

Conclusion

In the present study, the MSKE contained a considerable amount of phenolic and flavonoid contents and antioxidant activity. Hesperidin was the major phenolic compounds of MSKE. This is the first report find hesperidin in mango extracts. The phenolic compounds of the MSKE potentially inhibited the protease, fibrinogenase, PLA_2, LAAO, hyaluronidase, hemolytic, hemorrhagic activities and lethality of *Cerastes cerastes* and *Eicus coloratus* venoms. Therefore, the cost effective phenolic compounds from mango waste products could be used as a therapeutic way for viper bites management.

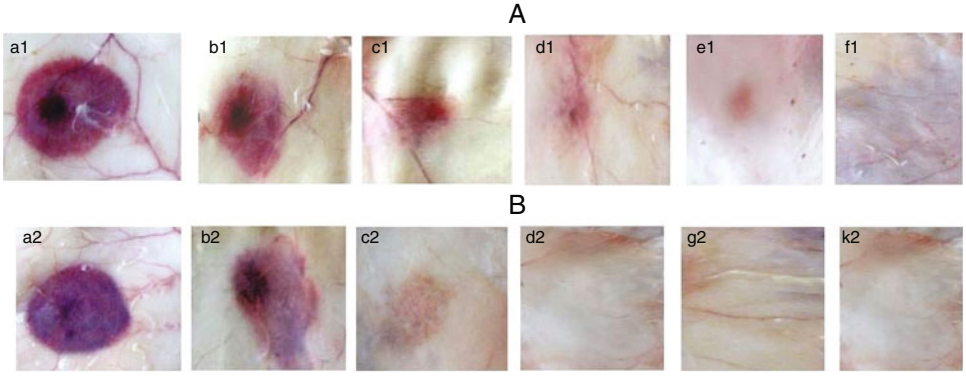

Fig. 6. Inhibition of *Cerastes cerastes* and *Echis coloratus* hemorrhagic activity by the mango seed kernels extract (MSKE). (A) a_1 contains 2 MHD of *C. cerastes* alone, b1, c1, d1, e1 and f1 contain 2 MHD of *C. cerastes* venom pre-incubated with 5, 10, 15, 20 and 25 µg phenolic of the MSKE, respectively. (B) a_2 contains 2MHD of *E. coloratus* alone, b2, c2, and d2 contain 2 MHD of *E. coloratus* venom pre-incubated with 5, 10, and 15 µg phenolic of the MSKE, respectively, (g2) 100 µl of MSKE alone, and (k2) 100 µl of saline as controls. The values represent mean ± S.E. ($n = 5$).

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

AMA, WHS, MBH and ASF and SAM had the original idea for the study and carried out the design. AMA and WHS were responsible for data analysis and data cleaning. Drafted the manuscript was revised by all authors. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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