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INVESTIGATION OF ANTIHEMOLYTIC, XANTHINE OXIDASE INHIBITION, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *SALVIA VERBENACA L.* AERIAL PART EXTRACTS.

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Abstract

Background: In this study, *Salvia verbenaca L*. aerial part extracts (SVEs): were screened for their antihemolytic, xanthine oxidase (XO) inhibition, antioxidant and antimicrobial activities.
Material and methods: To investigate SVEs antihemolytic activity, the 2,2,-azobis (2-amidinopropane) dihydrochloride (AAPH) was used to induce erythrocyte oxidative hemolysis. In XO inhibition test, xanthine was used as substrate and cytochrome c for generating superoxide anions. The antioxidant activity of SVEs was examined by means of reducing power, DPPH free radical scavenging and iron chelating assays. In addition, SVEs were tested for their antimicrobial effects by evaluating antibacterial and antifungal activities.
Results: Ethyl acetate extract (EAE) contains the highest amount of total polyphenols and flavonoids (661.78 ± 4.00 mg GAE / g E) and (28.81 ± 0.38 mg QE / g E) respectively. In antihemolytic test EAE was the most active extract with an HT_{50} value of 165 min. SVEs gave significant inhibitory effects on XO, especially the chloroform extract (ChE) with IC_{50} value of 0.0088 ± 0.000 mg/ml. EAE was the most active extract in reducing power essay (EC_{50}: 0.0047 ± 0.000 mg/ml) and in DPPH radical scavenging essay (IC_{50}: 0.0086 ± 0.000 mg/ml). Finally, the EAE has inhibited the growth of nine bacterial strains with inhibition zone diameters of (12 to 16 mm), but no activities have found against fungal strains.
Conclusion: *S. verbenaca* could be considered as a potential source of natural antihemolytic, enzyme modulator, antioxidant and antibacterial agents.

Key words: *Salvia verbenaca L.*, Erythrocyte Oxidative Hemolysis, Xanthine Oxidase Inhibition, Antioxidant Activity, Antibacterial Activity and Antifungal Activity.

Abbreviations: SVEs: *Salvia Verbenaca L*. aerial part Extracts; CrE: Crud Extract; ChE: Chloroform Extract ; EAE: Ethyl Acetate Extract; AqE : Aqueous Extract ; ROS: Reactive Oxygen Spices; AAPH : 2,2, -Azobis (2-AmidinoPropane) Dihydrochloride ; DPPH: DiPhenyl-Picryl-Hydrazyl; XO: Xanthine Oxidase; Gen: Gentamicin antibiotic; MeOH: Methanol; VitC: Vitamin C; PBS: Phosphate Buffer Saline; Allop: Allopurinol.

Introduction

Free radicals are atoms or groups of atoms with an unpaired number of electrons, which are highly reactive substances that can start a chain reaction (Halliwell, 2007). The reactive oxygen spices (ROS) including hydrogen peroxide (H_2O_2), superoxide radical ($O2^{\bullet-}$), and hydroxyl radical ($OH^{\bullet}$) represent the most important class of free radicals generated in living systems. These substances are highly toxic in nature and if allowed to accumulate, they can destroy all the macromolecules of the cells like lipids, proteins and DNA (Wu et al 2013). Under normal conditions, (ROS) produced are tightly regulated by balancing systems consisting of antioxidants, antioxidant enzymes and proteins. The disequilibrium between the level of free radicals and the capacity of the body to neutralize them, leads to oxidative stress. Panat et al., 2016 have reported that oxidative stress plays a key role in the development of several human pathologies involving age-related degenerative diseases, cancers, cardiovascular diseases, neurological disorders, diabetes, ischemia/reperfusion, trauma, stroke, asthma, hyperoxia, arthritis, age pigments, dermatitis, retinal damage, liver injury and periodontis. However, exogenous antioxidants, usually found in foods, can delay or inhibit the initiation or propagation of oxidative chain reactions (Guo et al., 2011). Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they may possess some side effects and toxic properties to human health (Senguttuvan et al., 2014). Therefore, many researchers are interested in medicinal plants for evaluation of antioxidant phytochemicals such as phenols, flavonoids and tannins which have received more attention for their potential role in the prevention of human diseases (Senguttuvan et al., 2014).

In the Algerian traditional medicine, *Salvia verbenaca L.* is one of the most popular plant remedies. The areal part of this plant is particularly used in the healing of wounds. It was also reported to be used for this purpose in Morocco (Khlifi et al., 2010). Salvia species are rich in phenolic compounds which in most cases are responsible for the pharmacological properties of these plants. Some bio-active compounds have been isolated and various phenolic acids and flavonoids have been identified in many Salvia species including caffeic acid, carnosic acid, kaempferol, oleanolic acid, rosmarinic acid and ursolic acid (Kamatou et al. 2008). The aim of our study was to evaluate the potential of *S. verbenaca* areal part extracts to act as an antioxidant, antihemolytic and antimicrobial agent and as enzyme modulators.

Materials and Methods

Preparation of *S. verbenaca* extracts (SVEs)

The areal part of *Salvia verbenaca L*. was collected from Bordj-Bou-Arerridj region, East of Algeria during the spring (April-May) at the flowering stage. The extraction of phenolic compounds from the aerial part of *S. verbenaca* is carried out according to the method of Markham (1982) using increasing polarity of organic solvents for the fractionation of the crude extract. Briefly, 100 g of crushed dry material

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from plant were placed in an extraction with a methanol / water solution (1000 ml: 85% methanol and 15% distilled water v/v). The mixture underwent stirring overnight at 4°C then allowed some time to settle, we obtain a pellet and a supernatant, it is filtered through a Buchner funnel and concentrated under reduced pressure on a rotary evaporator to give crud extract (CrE). 50 ml of the crud extract undergoes lyophilization and stored at -20 ° C, the rest is delipidated with hexane until the total absence of color. An organic phase is recovered (not useful) and another aqueous, the latter was extracted with chloroform to give an organic fraction (chloroform extract ChE) and an aqueous fraction. The aqueous fraction undergoes a last extraction with ethyl acetate to obtain an organic phase which represents the ethyl acetate extract (EAE) and the final aqueous fraction represents the aqueous extract (AqE).

Total polyphenolics content
Phenolics

The content of phenolic compounds of the various extracts is estimated according to the Folin-Ciocalteau method (Li et al., 2007). This method is based on the reduction in alkaline media of the phosphotungstic (WO42-) phosphomolybdic (MoO42-) mixture of the Folin-Ciocalteau reagent by the oxidizablegroupements of phenolic compounds, leading to the formation of blue reduction products. The latter have a maximum absorption at 765 nm, whose intensity is proportional to the amount of polyphenols present in the sample. Indeed, 1 ml of Folin-Ciocalteau reagent is added to 200 µl of extract or standard (prepared in methanol or distilled water) with suitable dilutions. After 4 min, 800 µl of a sodium carbonate solution (75 mg / ml) are added to the reaction medium. After 2 h incubation at room temperature, the absorbance is measured at 765 nm. The total polyphenol content is estimated from the regression equation of the calibration line established with gallic acid (0-160 mg / ml) and is expressed in mg of gallic acid equivalents per milligram of extract (mg GAE / mg extract).

Flavonoids

The method of aluminum trichloride (AlCl3) (Bahorun et al., 1996) is used to quantify the flavonoids in extracts. The method consisted of adding 1 ml sample or standard to 1 ml of the solution of AlCl3 (2% in methanol). After 10 minutes of reaction, the absorbance is read at 430 nm. Flavonoid content is calculated from a calibration line prepared with quercetin or rutin(0-40 µg / ml) and is expressed in microgram equivalents quercetin or rutinper milligram extract (EQ µg / mg of extract).

Inhibition of erythrocyte oxidative hemolysis induced by AAPH

In order to induce free radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of 2,2,-azobis (2-amidinopropane) dihydrochloride (AAPH) (dissolved in PBS, final concentration 300 mM). To study the protective effects of SVEs against AAPH-induced hemolysis, an erythrocyte suspension at 2% hematocrit was prepared. Blood was collected from male Wistar albino mice in EDTA tubes and centrifuged at 6000 rpm for 10 min. The lysed erythrocytes were discarded by repeated PBS wash and 4 % v/v erythrocyte suspension was prepared in PBS (pH=7.4). According to the procedure established by (Girard et al., 2006) with slight modifications, an erythrocyte suspension at 2% hematocrit was preincubated with samples, followed by incubation with and without AAPH (300 mM). Briefly, 80 µl of erythrocyte suspension in PBS were preincubated in micro plaques with 20 µl of samples (0.1 mg/ml) at 37 °C for 15 min. After, 136 µl AAPH (300 mM) were added and reaction mixtures while being incubate at 37 °C for 4 to 5 h. The extent of hemolysis was determined spectrophotometrically at 620 nm where the optical density was read every 15 min, with the aim of measuring most correctly possible time of half hemolysis. In all experiments, a negative control (erythrocytes in PBS with AAPH), as well as extract controls (erythrocytes in PBS with each extract) were used. The results were expressed as percentage inhibition of erythrocyte hemolysis. The half-time of hemolysis corresponds in necessary time so that the initial optical density decreases in 50%. Vitamin C (0.1 mg/mL) was used as a reference antihemolytic agent.

Effects on XO Activities
Inhibition of xanthine oxidation

The activity of xanthine oxidase (XO) is determined by colorimetric method by following the production of uric acid at 295 nm (ε acide uric = 9600 M-1cm-1, Bray, 1975) in the presence of 100 mM of xanthine in phosphate buffer (Baghiani et al., 2003). Assays were performed at room temperature, in air saturated sodium phosphate buffer (50 mM, pH 7.4) with various amounts of SVEs dissolved in methanol. The results were expressed as percentage inhibition of XO.

Inhibition of superoxide anions generation by XO

The XO superoxide anions generation can be measured by following the cytochrome c reduction at 550 nm (εCytc = 21100 M-1cm-1) (Robak and Gryglewski, 1988). The reaction mixture containing: 50 mM phosphate buffer (pH 7.4), 0.1 mM EDTA, 100 µM xanthine, 25 µM cytochrome c and various concentrations of plant extracts. Reaction was started by the addition of XO. The results were expressed as percentage inhibition of cytochrome c reduction.

Antioxidant activities of SVEs
Reducing power assay

Reducing power was determined according to the method of Oyaizu (1986). Briefly, 200 µl of SVEs in different concentrations diluted in distilled water were mixed with phosphate buffer (200 µl, 0.2 M, pH 6.6) and potassium ferricyanide (200 µl, 1 %). The mixture was incubated at 50 ºC for 20 min. Trichloroacetic acid (TCA) (200 µl, 10 %) was added to the mixture and centrifuged at 3000 x g for 10 min. The resulting supernatant (400 µl) was mixed with distilled water (400 µl) and $FeCl_3$ (80 µl, 0.1 %) and the absorbance of the sample and ascorbic acid (standard) were measured at 700 nm after 10 min.

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DPPH free radical scavenging assay

This spectrophotometric assay uses stable radical diphenyl-picrylhydrazyl (DPPH) as a reagent. As described by (Burits and Bucar, 2000) with slight modifications. 50 µL of various concentrations of the extracts in methanol was added to 1.25 ml of a 0.004% methanol solution of DPPH. All determinations were performed in triplicate. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm.

Ferrous iron chelating assay

Metal chelating activity was performed as described by Decker and Welch1990 with slight modifications (Le et al., 2007). Appropriately diluted extracts (250 µl) were mixed with 50 µl of 0.6 mM $FeCl_2$ and 450 µl methanol. The reaction was initiated after 5 min by the addition of 50 µl of 5 mM ferrozine and incubated for 10 min at room temperature. The absorbance of the Fe^{2+}-ferrozine complex was measured at 562 nm. The chelating effect was calculated as a percentage and EC_{50} was defined as the effective concentration of test material which produces 50 % of maximal chelating effect. EDTA was used as a standard.

Antimicrobial activities of SVEs
Antibacterial assay

SVEs were individually tested against a panel of bacteria; *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 10876, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhi* ATCC 13311, *Enterococcus faecalis* ATCC 49452, *Citrobacter freundii* ATCC 8090, *Acenetobacter baumanii* ATCC 19306 and *Lysteria monocytogenes* ATCC 15313. Bacterial strains were cultured overnight at 37 °C in Mueller Hinton agar (MHA). Disc-diffusion method was employed for the determination of antibacterial activities of SVEs (Sokmen et al., 2004) with slight modifications. Briefly, suspensions of the bacterial strains tested were prepared in sterile saline (equivalent to 0.5 MacFarland or have D.O of 0.08 to 0.10). From these suspensions, strains were spread on the solid media plates (MHA). Filter paper discs (7 mm in diameter) were placed on the inoculated plates and impregnated with 30 µL of SVEs (100 and 200 mg/ml in DMSO). These plates were incubated at 37 °C for 24 h. The diameters of the inhibition zones were measured in millimeters. Results were compared with that of Gentamicin (6 mm, 10 µg /disc). All the tests were performed in duplicate.

Antifungal assay

SVEs were tested against four fungal strains: *Aspergillus flavus* NRRL 391, *Aspergillus niger* 2CA 936, *Penicillum sp* and *Fusarium sp*. Fungal strains were activated on PDA plats (200g potatoes, 10g dextrose and 15g Agar in 1l distilled water). Antifungal activity was assessed from spores obtained after seven days at 28°C. SVEs were dissolved in DMSO in two concentrations (100 and 200 mg/ml). Briefly, suspensions of the tested fungal strains were prepared in sterile saline. The resulting suspensions were vortexed and adjust the turbidity to yield $1x10^6 – 5 x 10^6$ spores / ml. From these suspensions, strains were spread on the solid media plates (PDA). Filter paper discs (7 mm in diameter) were placed on the inoculated plates and impregnated with 30 µl of SVEs (3 and 6 mg / disc) or with an antifungal agent as Fluconazole or Griseofulvine (5, 10 and 30 µg/disc). These plates were incubated at 28°C for up to 72 hours. The diameters of the inhibition zones were measured in millimeters every 48 hours of incubation. Results were compared with the antifungal agents. All the tests were performed in duplicate.

Statistical analysis

Results obtained were reported as mean ± SD of triplicate measurements. Significance differences for multiple comparisons were determined by one way analysis of variance (ANOVA) followed by Duncan's test with $p<0.05$.

Results and Discussion
Total polyphenolics and flavonoid contents

The quantitative determination of total polyphenols and flavonoids in SVEs are shown in Table 01. There was a wide range of phenol concentrations in SVEs. The value varied from 123.18±4.20 to 661.78±4.00 mg EGA/g E and from 06.74±0.14 to 28.81±0.38 mg EQ/g E for polyphenols and flavonoids, respectively. The highest phenol and flavonoid content was found in EAE.

Table 1: Total polyphenolics and flavonoid contents of SVEs.

SVEs	Polyphenols	Flavonoids
	mg EGA / g E	mg EQ / g E
CrE	177.56 ± 2.51	08.40 ± 0.32
ChE	156.81 ± 1.57	14.87 ± 0.81
EAE	661.78 ± 4.00	28.81± 0.38
AqE	123.18 ± 4.20	06.74± 0.14

* mg EGA / g E: mg equivalent gallic acid / g extrait. * mg EQ / g E: mg equivalent quercetin / g extrait.
Each value represents the mean ± SD (n = 3)

Antihemolytic activity

Recent research has demonstrated an increasing interest in the protective biochemical function of naturally occurring antioxidants in biological systems and on their mechanism of action. The human erythrocyte has been proposed as a valuable *in vitro* model to study the

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oxidant/antioxidant interaction, since its membrane is rich in polyunsaturated fatty acids, which are extremely susceptible to free radical-mediated peroxidation, and it is considered to be representative of the plasma membrane in general (Oliveira et al., 2012; Abirami et al., 2014). Erythrocyte lipid peroxidation may be involved in normal cell aging and has been associated with a variety of pathological events (Oliveira et al., 2012). SVEs were evaluated for their protection capacity on red blood cells (RBCs) against oxidative damage by AAPH, a peroxyl radical generator that attacks the erythrocytes to induce the chain oxidation of lipids and proteins, disturbs the membrane organization and eventually leads to hemolysis (Abirami et al., 2014; Kumar et al., 2015). The results are shown in Figure 1.

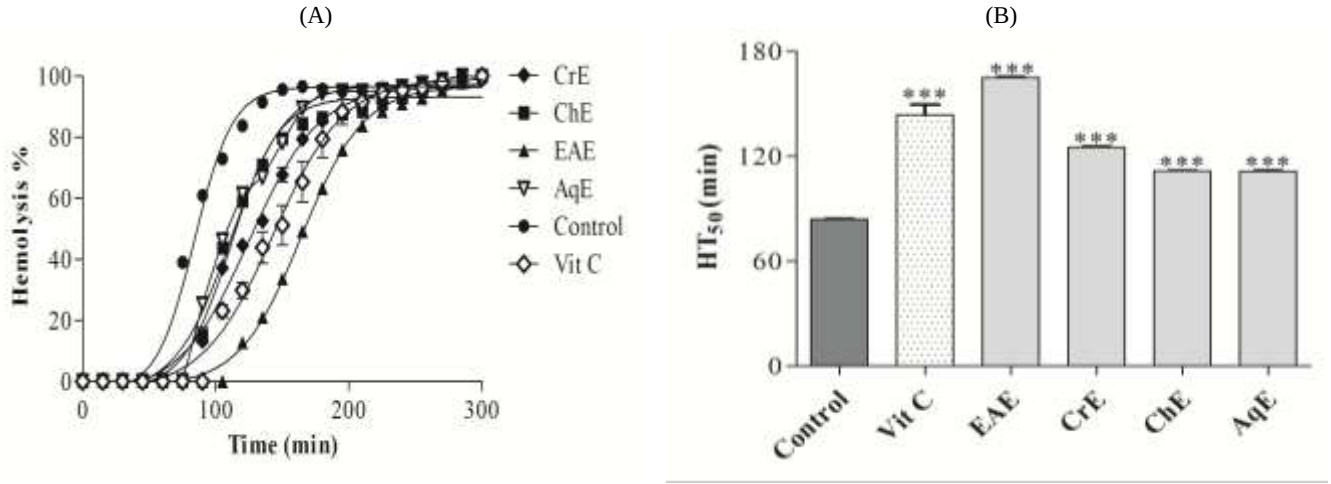

Figure 1: Inhibitory effects of SVEs on AAPH-induced erythrocyte hemolysis. (**A**): Hemolysis percentage variation in the time. (**B**): (HT_{50}) necessary time for 50% of initial erythrocyte hemolysis. **Vit C**: standard antioxidant, **Control**: negative control (erythrocytes in PBS + AAPH). Values are expressed as the mean ± SD (n=3). (*** $p \leq 0.001$).

SVEs and VitC significantly protected the erythrocyte membrane from hemolysis in a time-dependent manner. A significant deviation to the right was signed in al sigmoidal curbs obtained by adding of SVEs and VitC (Fig 1-A). This deviation explains the increase in the total time of hemolysis so the protective effects of SVEs and VitC on erythrocytes against AAPH. It is note-worthy that the positive standard VitC showed a higher activity than CrE, ChE and AqE of *S. verbenaca* but a lower activity than EAE. These results were confirmed by calculation of HT_{50} (half-time of hemolysis in min) corresponding to 50 % of hemolysis of the initial RBCs. According to HT_{50} values (Fig 1-B, $p \leq 0.001$), among the different SVEs, EAE (165 min) exhibited the highest hemolytic inhibition activity better than that of VitC, followed by CrE (125.17 min) then ChE and AqE with the same HT_{50} (111.50 min).

Using this test there was a high correlation between hemolytic inhibition activities caused by SVEs and their flavonoid and polyphenol contents (Table 1). This activity could be related to the presence of polyphenols especially flavonoids in SVEs. Our results are in agreement with other studies showing that phenolics are able to protect erythrocytes from oxidative stress or increase their resistance to damage caused by oxidants (Costa et al., 2009; Magalhães et al., 2009; Carvalho et al., 2010; Nithiyanantham et al., 2013). The strong antioxidant effects of polyphenols have been highlighted by several studies, with underlying mechanisms involving both free radical scavenging (Bors et al., 1990) and redox-active metal chelation (van Acker et al., 1998). In our model, these phytochemicals present in the incubation medium can protect against lipid peroxidation by trapping the peroxyl radicals in the aqueous phase before these radicals attack the lipid molecules of the erythrocyte membrane. This breaks the free radical chain reaction and inhibits subsequent oxidative hemolysis (Sowndhararajan et al. 2011). Dai et al. (2006) reported that flavonols and their glycosides are effective antioxidants which can protect human RBCs from free radical induced oxidative hemolysis. Similarly, Chaudhuri et al. (2007) noted that binding of flavonoids to the RBCs membranes significantly inhibits lipid peroxidation and at the same time enhances their integrity against lysis.

Inhibition of XO Activities

SVEs were evaluated for their XO inhibitory activity by measuring uric acid production at 295 nm. As shown in Figure 2 (A), all extracts have inhibited the XO activity in a dose-dependent manner. SVEs showed significant inhibition with ChE exhibiting the highest activity (IC_{50} = 0.009 mg/ml) followed by EAE, then CrE and finally AqE with IC_{50} (mg/ml) values of 0.017, 0.052 and 0.98 respectively (Table 2).

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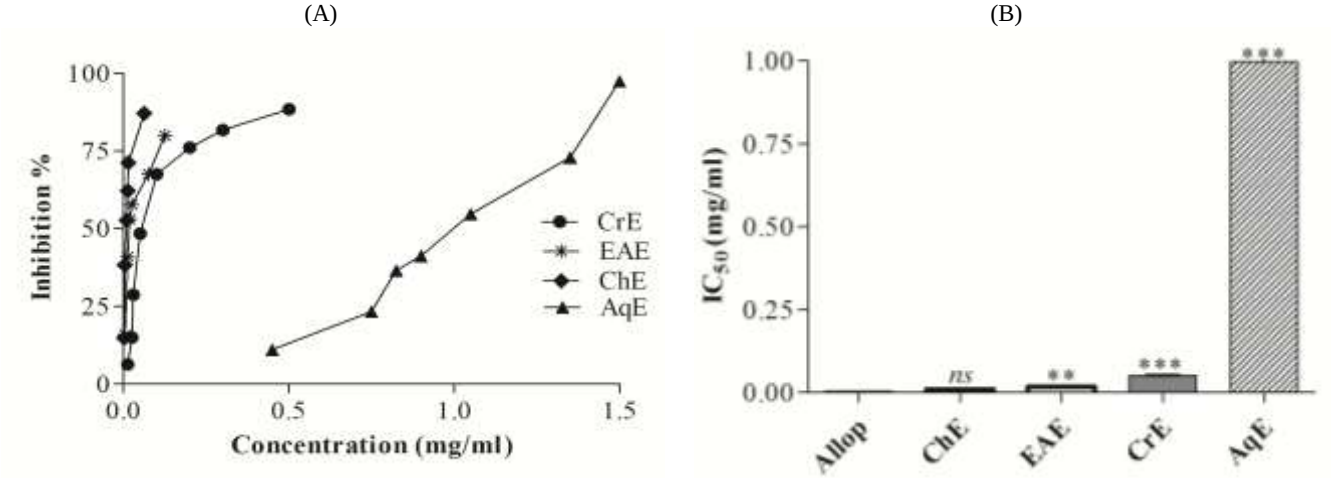

Figure 2: Inhibitory actions of SVEs on XO activity. (**A**): Inhibition percentage of XO in the presence of SVEs. (**B**): IC_{50} (Inhibition concentration of 50% XO). Each value is represented as mean ± S.D (n = 3).

The effects of SVEs on XO activity were compared with the Allopurinol effect, presented an IC_{50} of 0.0041±0.0004 mg/ml. Allopurinol is rapidly oxidized by XO *in vivo* to its active metabolite oxypurinol, which also inhibits XO. However, it induces serious side effects such as renal failure, impaired liver function and allergic reactions (Argulla and Chichioco-Hernandez, 2014). Hence, there is a need to look for new XO inhibitors. Previous research had shown that the XO inhibition may be related to the presence of phenolic compounds (Costantino et al., 1992), of flavonoids (Lin et al., 2002) and tannins (Schmeda-Hirschmann et al., 1996) in plant extracts. Flavonoids were found to inhibit XO activity (Sowndhararajan et al., 2012). XO converts xanthine to uric by transferring an oxygen atom to xanthine from the enzyme's molybdenum center. Flavonoids work by competitively binding at the binding site of xanthine (Masuoka et al., 2012). SVEs are rich in flavonoids especially EAE, ChE and CrE (Table 01).

The ChE have a potent scavenging activity on superoxide anion generation higher than the other extracts, with an IC_{50} (mg/ml) value of (0.016 ± 0.0004), followed by EAE (0.060 ± 0.0005) then the CrE (0.211 ± 0.014) and finally AqE (2.860 ± 0.014) (Table 02). SVEs have an inhibitory effect on XO activity, indicating that their scavenging effects on superoxide anion is due to dual effect of the extracts on XO activity and superoxide anion scavenging. This activity could be related to the presence of flavonoids in SVEs and their structural differences. The insaturation in the C ring and the free hydroxyl group at C-7 enhanced the activity (Baghiani et al., 2012).

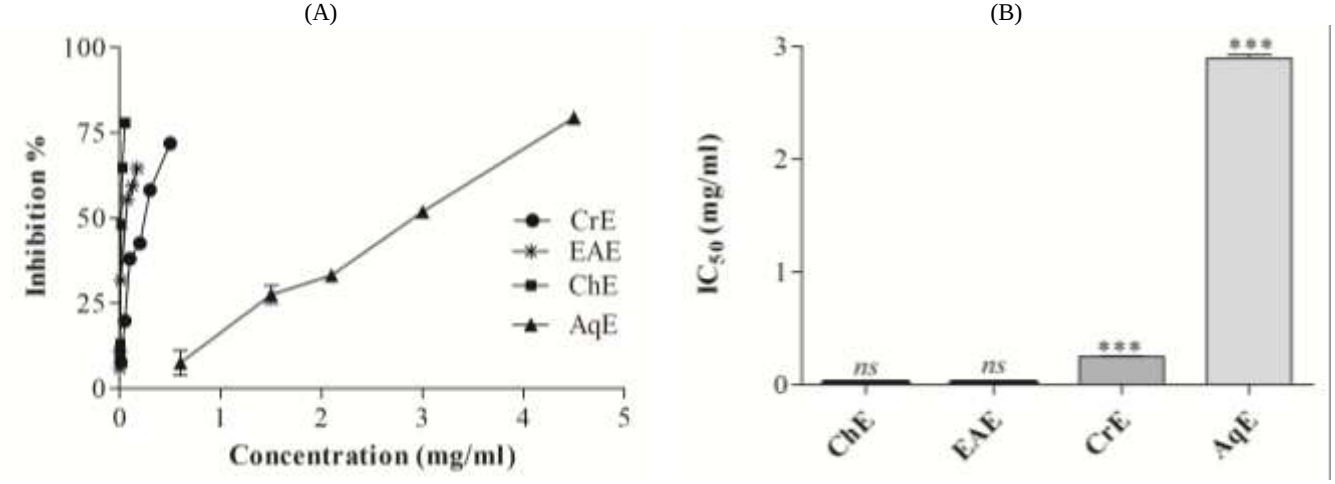

Figure 3: Inhibition of superoxide anion generating from Xanthine/XO system by SVEs. (**A**): Inhibition percentage, (**B**): IC_{50} (Inhibition concentration of 50% superoxide anion generation). Each value is represented as mean ± S.D (n = 2), (***p ≤0.001; *ns:* not significant).

Table 2: IC_{50} values (mg / ml) of SVEs in inhibition of xanthine oxidation and superoxide anion generation.

SVEs	Xanthine oxidation inhibition	Superoxide anion scavenging
CrE	0.0520 ± 0.0030	0.211 ± 0.014
ChE	0.0088 ± 0.0004	0.016 ± 0.0004
EAE	0.0165 ± 0.0010	0.060 ± 0.0005
AqE	0.9800 ± 0.0040	2.860 ± 0.014
Allopurinol	0.0041± 0.0004	NT

NT: not tested. Each value is represented as mean ± SD (n = 3).

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Antioxidant activities

In this work, the antioxidant properties of SVEs were evaluated by different *in vitro* antioxidant assays such as reducing power; iron chelating and DPPH radical scavenging activities. All this experiments are chemical-based assays. As expected, owing to its antioxidant composition, all assayed models demonstrated antioxidant and scavenging efficiency for SVEs.

Reducing power

In the reducing power assay, both SVEs displayed a concentration-dependent antioxidant potential (Fig. 4-A).

In this assay, the presence of reducing agents in the extracts causes the conversion of the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form. Fe^{2+} is monitorized by measuring the formation of Perl's Prussian blue at 700 nm, with rising absorbances indicating an increase in reducing power. The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity (Meir et al., 1995). Statistically significant differences ($p<0.05$) were observed in the EC_{50} values calculated for SVEs and Ascorbic A. (Fig. 4-B). EAE exhibited the strongest capacity (EC_{50} value of 0.0047±0.000 mg/ml), followed by CrE (0.0178±0.000 mg/ml), while ChE and AqE were less active (EC_{50} values of 0.0453±0.0006 and 0.0455±0.000 mg/ml) respectively. But all SVEs have signed a reducing power better than Ascorbic A. (EC_{50} value of 0.0489±0.0014 mg/ml).

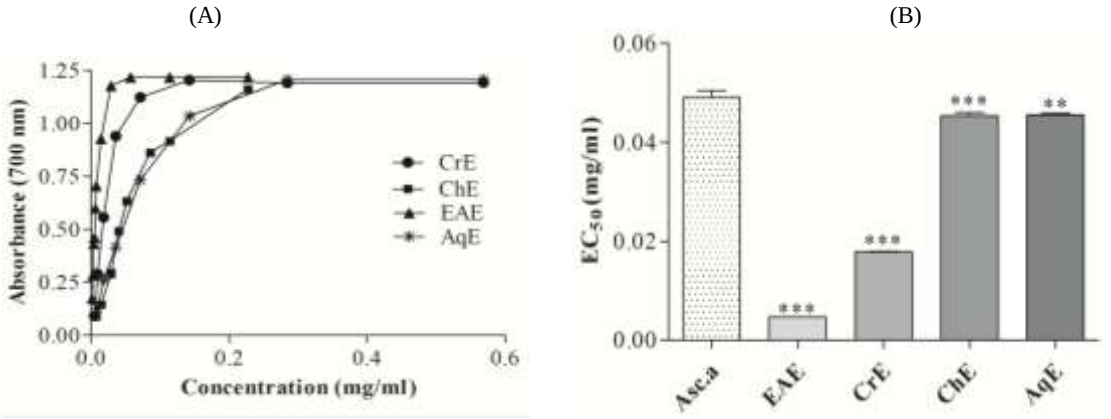

Figure 4: Reducing power of SVEs. (**A**): Absorbance at 700 nm in function of concentration increase. (**B**): EC_{50} (effect concentration of 50% of reduction). Each value is expressed as mean ± SD (n=3), (*** $p<0.001$, ** $p<0.01$). **Asc.a**: Ascorbic acid (positive control).

DPPH free radical scavenging activity

This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the radical-scavenging activity of specific compounds or extracts (Barros et al. 2007, Amarowicz et al., 2004). Results showed that scavenging activity of both SVEs on DPPH was increased as the concentration of extract increased until a plateau where activity stabilizes (Fig 5-A). IC_{50} value was determined from the plotted graph of scavenging activity against the concentration of SVEs. From values of IC_{50} (Fig 5-B) the radical-scavenging effects were excellent for the EAE with an IC_{50} (0.0086 mg/ml), good for the CrE (0.0336 mg/ml) and AqE (0.0389 mg/ml) and moderate for ChE (0.0725 mg/ml). The scavenger can be explained by the presence of polyphenols and flavonoids in the extract. Marsden Blois (1958) has recorded that flavonoids reduce and decolourise DPPH by their hydrogen donating ability (Yokozawa et al., 1998).

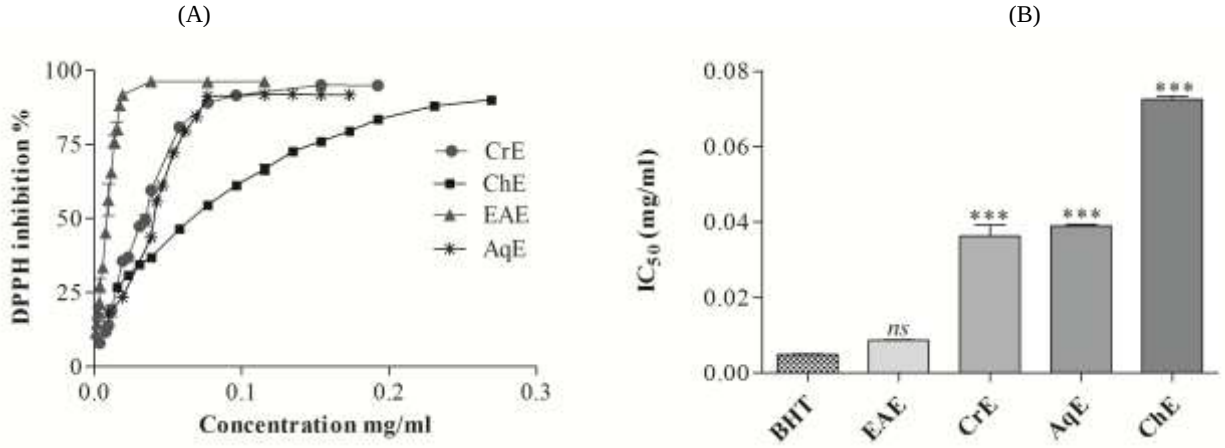

Figure 5: DPPH free radical scavenging activity of SVEs; (**A**): Inhibition percentage of DPPH radical by SVEs, (**B**): IC_{50} (Inhibition concentration of 50% DPPH). (*** $p<0.001$, ** $p<0.01$, *ns*: not significant)

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Metal chelating activity

An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which catalyze hydro peroxide decomposition and Fenton-type reactions. Therefore, it was considered of importance to screen the iron(II) chelating ability of extracts (Manian et al., 2008). The Fe^{2+} chelating capacity of various samples was determined by measuring the iron-ferrozine complex and results are summarized in Fig. 6. Among the treatments, the highest activity was noted for the polar extracts; AqE and CrE. While non-polar extracts; ChE and EAE were less active especially EAE with a chelation percentage does not exceed 20%. Iron can stimulate the lipid peroxidation by Haber–Weiss and Fenton reactions, resulting in the generation of hydroxyl radicals. Ferrous ions are also commonly found in food systems and considered as prooxidants. Ferrozine form complexes with the ferrous ion, generating a violet color (Guo et al., 2011). The formation of this color can be interrupted by metal chelating agents. No correlation was found between iron-chelating capacity and phenolic content for SVEs. This may indicate the presence of other antioxidants responsible for metal chelation. Non-phenolic metal chelators include phosphoric acid, citric acid, ascorbic acid, carnosine, some amino acids, peptides and proteins such as transferrin and ovotransferrin (Lee et al., 2004).

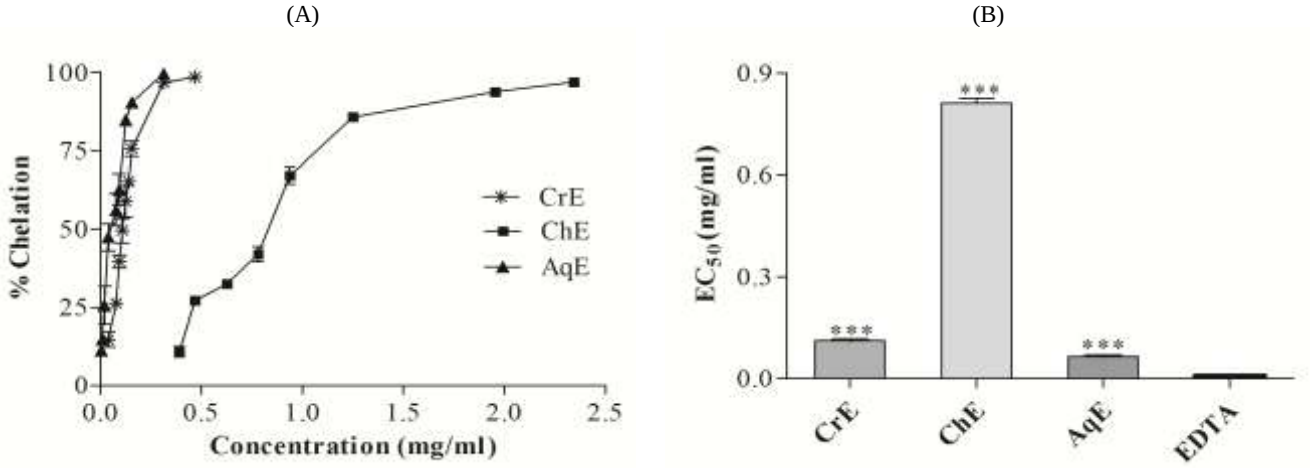

Figure 6: Iron chelating activity of SVEs; (**A**): Iron Chelating percentage by SVEs, (**B**): EC_{50} (Chelation concentration of 50% iron), (*** $p<0.001$, ns: not significant).

Antimicrobial activities

The application of SVEs on the ten bacterial strains tested, showed that EAE was the most active extract, that can inhibited the growth of nine bacterial strains; *E. coli*, *P. aeruginosa*, *S. aureus*, *B. cereus*, *K. pneumoniae*, *E.faecalis*, *C.freundii*, *A. baumanii* and *L. monocytogenes*, with inhibition zones diameters form 09 to 12 mm with a concentration of 100 mg /ml and from 12 to 16 mm with 200 mg /ml (Table 2).

Table 2: Antibacterial activities of SVEs, Zone of Inhibition (diameter in mm, n=2).

Bacterial strains	**ChE**		**EAE**		**CrE**		**AqE**		**Gen** (10µg /disc)
	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	
E. coli ATCC 25922	09	12	11	14	-	-	-		19
P. aeruginosa ATCC 27853	-	13	12	15	-	09	-		27
S. aureus ATCC2592 3	11	10	12	16	11	11	-		-
B. cereus ATCC 10876	11	15	13	15	-	12	-		-
S. typhi ATCC 13311	-	-	-	-	-	-	-		14
E. faecalis ATCC 49452	09	12	12	14	-	11	-		14
C. freundii ATCC 8090	-	14	12	14	11	-	-		34
A. baumanii ATCC 19306	-	14	10	15	-	10	-		27
L. monocytogenes ATCC 15313	08	-	10	14	-	12	-		13
P. mirabilis ATCC 35659	-	13	13	13	-	-	-		26

Gen: Gentamicin antibiotic, $\mathbf{C_1}$: 100 mg/ml (3mg/disc), $\mathbf{C_2}$: 200 mg/ml (6mg/disc), -: no inhibition.

ChE has inhibited the growth of eight bacterial strains by the concentration of 200 mg/ml (6 mg/disc), except*S. typhi*and *L. monocytogenes*with inhibition diameters of 09 to 14 mm. while, CrE showed a lower activity against the panel of bacteria, it has inhibited the growth of six bacterial strains but with low diameters (08 to 11 mm) by 200 mg/ml. Phytochemical analysis of plant extracts indicates that the presence of one or more groups of phytoconstituents like flavonoids, tannins, glycoside, phenols, saponin, etc. is responsible for antibacterial activity alone or in combinations (Ahmed and Aqil, 2007).

The antifungal activities of the SVEs were tested against four fungal strains. No activities have signed after 48-72 hours of incubation against all fungal strains tested.

Conclusion

The present study is a report demonstrating that the extract from *S. verbenaca* areal part and its four different solvent sub-fractions have antihemolytic, antioxidant and antibacterial activities, as seen in the inhibition of AAPH inducing erythrocyte hemolysis, inhibition of XO activity and inhibition of superoxide anion generating by the xanthine/XO system. Also in chemicals based assays; reducing power assay, DPPH free radical assay and ferrous ion-chelating activity assay. The present study reveals that the SVEs possess marked antihemolytic and antioxidant activities but lower antibacterial and antifungal activities. Of all the four SVEs, EAE showed the most potent antioxidant properties. It may be due to the presence of respective secondary metabolites such as phenolics, flavonoids, tannins etc. in these extracts. The strong correlation between the contents of total phenolics and flavonoids and antihemolytic, antioxidant and antibacterial activities indicates that these phytochemical constituents are major contributors of these activities. Further work is needed to isolate and identify the specific compounds in EAE that are responsible for the antioxidant capability.

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