

In vitro antioxidant and anti-inflammatory activities of methanol extract of *Vitellaria paradoxa* seed (shea seed)

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Abstract

Vitellaria paradoxa (shea tree) is the source of shea seed from which the well-known shea butter is derived. The methanol extract of shea seed was evaluated for its anti-inflammatory and antioxidant activities using diclofenac sodium and ascorbic acid as standard respectively in *in vitro* methods. The anti-inflammatory activity was determined by inhibition of protein denaturation of bovine serum albumin (BSA) and erythrocyte membrane stabilization of human red blood cell. The antioxidant activity was evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS) and total antioxidant capacity (TAC) assays. The results showed that methanol extract of *V. paradoxa* seed at different concentration protects the heat induced protein denaturation with the maximum percentage inhibition of 27% (IC₅₀=303.0 µg/mL, p<0.05) at 500µg/mL compared to the standard drug which induced maximum inhibition of 45% (IC₅₀=261.4 µg/mL, p<0.05) at 500 µg/mL and the control. The percentage inhibition of the methanol extract and standard drug in erythrocyte stabilization assay increased in a concentration dependent manner with maximum inhibitory activity of 96.9% (IC₅₀=31.47 µg/mL, p<0.05) and 95% (IC₅₀=33.89 µg/mL, p<0.05) at 2000 µg/ml respectively, which indicates that methanol extract stabilized erythrocyte membrane against hypotonic induced hemolysis in a blood sample better than the standard drug. The maximum percentage inhibition of methanol extract and standard drug in DPPH assay were found to be at 97% (IC₅₀=8.95 µg/mL, p<0.05) and 98% (IC₅₀=6.72 µg/mL, p<0.05) respectively at 100 µg/ml. The absorbance of the reductive capacities in FRAP assay indicates that the methanol extract has higher reducing potency in a concentration dependent manner. The methanol extract exhibited total antioxidant capacity of 0.25 ± 0.04 µg/(AAE/g) when compared to the standard drug 0.87 ± 0.03 µg/(AAE/g) at highest concentration of 100 µg/ml. For TBARS assay, low absorbance value indicate a high level of inhibition of lipid peroxidation. The maximum percentage inhibition of methanol extract was 97.5 % (IC₅₀=51.79 µg/mL, p<0.05) and ascorbic acid was 99% (IC₅₀=52.30 µg/mL, p<0.05) at concentration of 20 µg/ml. The assay indicates that the methanol extract has higher inhibiting potency in a reverse concentration dependent manner. In conclusion, *V. paradoxa* seed may possess strong anti-inflammatory and antioxidant activities which could constitute a potential source for development of new therapy.

Keywords: *Vitellaria paradoxa*; Anti-inflammatory; Antioxidant; Inhibition

1. Introduction

Medicinal plants are of great importance to the health of humans. Medicinal plants are plants that are used to attempt to maintain health, to be administered for a specific condition or both, whether in modern or traditional medicine [1]. The parts of the plants used for medicine may be their leaves, flowers, roots, seeds or bark. Treatment with medicinal

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plants is considered very safe as there is no or minimal side effects. The medicinal value of these plants lies in some of their bioactive constituents (like alkaloids, tannins, flavonoids and phenolic compounds) that produce a definite physiological action on the human body [2, 3]. According to World Health Organization (WHO), around 21, 000 plant species have the potential for being used as medicinal plants. WHO estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary health care needs [4].

Vitellaria paradoxa (shea tree) belongs to the Sapotaceae family. It is the source of shea seed from which the well-known shea butter is derived. Shea tree can be found growing in savannah belt region in countries including Senegal, Mali, Côte d'Ivoire, Burkina Faso, Togo, Ghana, Benin, Nigeria, Niger, Cameroon, Uganda, Sudan and Ethiopia [5]. The shea seed is a hard brown shell that contains the fatty kernel. The shea seed is not edible, it is primarily used to extract shea butter and shea nut oil, rich in nourishing fatty acids and healing components [6]. The chemical composition of the kernel of the seed per 100 g dry matter are approximately, 31-62 g fat, 7-9 g protein, 31-38 g carbohydrate, 2.5-12 g unsaponifiable matter. The non-fat part (kernel) is composed of phenols: tocopherols, triterpenes (alpha-amyrin, lupeol, butyrospermol, parkeol), steroids (campesterols, stigmasterol, beta-sitosterol, alpha-spinasterol, delta-7-avenasterol), and hydrocarbons (2-3% karitene). It also contains terpenic alcohols [7].

Inflammation is the organism's protective response to several stimuli, prolonged inflammatory response or occurrence where it is not needed is fatal to the body system. It involves occurrence such as; increase of vascular permeability, increase in protein denaturation and membrane alteration. Researchers recognize inflammation as a key components of many of the major diseases affecting human health. It sometimes contributes to the damage of the very tissue it is evolved to protect. The human body naturally produces free radicals and the antioxidants to counteract their damaging effects. The generation of free radicals in excess amount outnumber that naturally occurring antioxidants and induce oxidative stress which is associated with many degenerative human diseases such as accelerative aging, certain cancers, diabetes, cardiovascular diseases, inflammation and neurodegenerative diseases have been recognized to be associated to free radicals damage [8]. In order to maintain the balance, a continual supply of external sources of antioxidants is necessary to obtain the maximum benefits of antioxidants. Antioxidant agents benefit the body by neutralizing and removing the free radicals. The synthetic anti-oxidative and anti-inflammatory drugs have a number of adverse side effects, such as gastrointestinal discomfort, inhibition of platelet aggregation, and liver and kidney toxicity [9]. Thus, there is considerable research interest in the identification of new anti-inflammatory and anti-oxidative agents from plants used in traditional medicine. Therefore, this study was designed to investigate the anti-inflammatory and antioxidant activities of methanol extract of shea seed (*V. paradoxa*).

2. Material and methods

2.1. Sample collection and processing

Shea seeds were obtained from Oje market at Ibadan, Oyo state. It was dehulled, washed and dried in a hot air oven at 40°C for 14 days. The seed were pulverized using blending machine. The extract was obtained by weighing 100g of ground shea seed and 800 ml of 70% for maceration with intermittent shaking for 72 hours. The extract was filtered using Whatman filter paper and the filtrate was subsequently concentrated using the rotary evaporator at 40°C. The concentrate was oven dried and kept at 4°C for further use.

2.2. *In vitro* Anti-inflammatory Assays

2.2.1. Inhibition of protein denaturation assay

Anti-denaturation of BSA was assayed using a modified method of Ramalingam et al. (2010) [10]. The assay mixture contained test solution of BSA (45 µL, 0.5% w/v) and 5 µL of methanol extract of *V. paradoxa* prepared in various concentrations 20, 40, 60, 80 and 100 µL/mL respectively. The mixture was incubated at 37°C for 20 mins in a water bath (Uniscop SM801A; Surgifield Medicals) and the temperature was increased to keep the samples at 57°C for 3 mins. After cooling, 2.5 ml of phosphate buffer saline (pH 6.3) was added to each mixture in the tube. The absorbance was measured at 416 nm using a spectrophotometer. 45 µL distilled water and 5µL of test solution (BSA and *V. paradoxa* extract) were used as test control. The experiment was carried out in triplicates and percentage inhibition for protein denaturation was calculated. The IC₅₀ was estimated using linear regression equation.

2.2.2. Human red blood cell (HRBC) membrane stabilization assay

The effect of methanol extract of *V. paradoxa* seed on hypotonicity-induced hemolysis of human red blood cell membrane (HRBC) was carried out in accordance to the protocol described by Oyedapo (2010) [11]. Fresh human whole blood (5ml) was obtained using a 5ml syringe and transferred into an ethylenediamine tetraacetic acid (EDTA) bottle.

The EDTA bottle containing the whole blood was centrifuged at 3000rpm for 10 mins, and the supernatant (plasma and leucocytes) were carefully discarded while the packed red blood cells were washed in fresh normal saline (0.9% w/v NaCl). The process of washing and centrifuging was repeated until the supernatant was clear. 10% HRBC membrane was then resuspended in 0.9% w/v NaCl as stock. The assay mixture contained 1 ml of sodium phosphate buffer (0.15M, 7.4 pH), 2 ml hyposaline (0.36% w/v NaCl), 0.5 ml HRBC suspension (10% w/v) with 0.5 ml diclofenac sodium as standard drug or methanol extract preparations of varying concentrations in test tubes. For the control, distilled water replaced NaCl (0.36%, w/v) to induce 100% hemolysis. The hemolysis produced in the presence of distilled water was taken as 100%. The different test tubes were incubated at 56°C in a water bath (Uniscope SM801A; Surgifield Medicals) for 30 min and then centrifuged at 5000 rpm. The hemoglobin content in each tube was estimated spectrophotometrically at 560 nm. The experiment was performed in triplicates for all the test samples. The percentage of HRBC membrane stabilization was calculated using the formula;

$$\text{Percentage Stabilization} = 100 - \frac{\text{Optical density of extract}}{\text{Optical density of control}} \times 100$$

2.3. *In vitro* Antioxidant Assays

2.3.1. 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging activity was determined as described by Molyneux (2004) [12], with slight modifications. 1 ml of 0.2 mM DPPH prepared with methanol was added to 2.5 ml solution of varying concentrations of methanol extract of *V. paradoxa* seed and ascorbic acid as standard at different concentrations of 20, 40, 60, 80 and 100 µg/mL and allowed to react at room temperature in darkness for 30 min. 1 ml of 0.2 mM DPPH and 2.5 ml methanol served as a control. This assay was carried out in triplicates for each concentration. Absorbance of the resultant mixture was measured using the double beam UV-visible spectrophotometer (Model T80; PG Instruments, Lutterworth, England, UK) at 517 nm. Percentage inhibitions of the methanol extract and the standard were calculated using the formula below:

$$\% \text{ inhibition} = \frac{\text{Optical Density control} - \text{Optical Density sample}}{\text{Optical Density control}} \times 100$$

Percentage inhibition indicates the capacity of fractions to inhibit reactive oxygen species, and the concentration of sample required for 50% inhibition was determined and expressed as an IC₅₀ value.

2.3.2. Ferric reducing antioxidant potential assay

Ferric reducing assay was done using the method described by Benzie and Strain (1999) [13]. A solution of 2.5 mL 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1% w/v) was added into test tubes containing 1 mL methanol extract of *V. paradoxa* at different concentrations. The reaction mixtures were incubated at 50°C for 30 min, followed by the addition of trichloroacetic acid (2.5 mL 10% w/v). The reaction mixtures were centrifuged at 3000 rpm for 10 min, and the upper layer of the solution was collected. A volume of 2.5 ml supernatant solution was mixed with 2.5 mL distilled water and 0.5 mL FeCl₃ (0.1% w/v). The absorbance was then measured at 700 nm against blank sample using the double beam UV-visible spectrophotometer (Model T80; PG Instruments, Lutterworth, England, UK). Ascorbic acid was used as the standard.

2.3.3. Thiobarbituric acid reactive species assay

Thiobarbituric acid reactive substances (TBARS) assay was carried out using the method described by Hodges et al. (1999) [14]. Varying concentrations of ascorbic acid were prepared and made up to 1.0 ml in test tubes using distilled water. Similarly, varying concentrations of methanol extract of *V. paradoxa* seed was prepared and made up to 1.0 ml using distilled water. 2 ml of 20% (w/v) trichloroacetic acid and 2 ml of 0.67% (w/v) of thiobarbituric acid solutions were added to these test solutions. Blank was also prepared in another set of tubes containing the above reagents without any sample. The test tubes were placed in a boiling water bath for 10 min, cooled and centrifuged at 3000 rpm for 20 min. Absorbance of supernatants was measured at 552 nm using the double beam UV-visible spectrophotometer (Model T80; PG Instruments, Lutterworth, England, UK). This assay was carried out in triplicate and percentage inhibition of lipid peroxidation (%) was calculated according to the formula below:

$$\% \text{ inhibition} = \frac{\text{Optical Density control} - \text{Optical Density sample}}{\text{Optical Density control}} \times 100$$

Optical Density control = The absorbance without sample,

Optical Density sample = The absorbance of methanol extract or standard.

2.3.4. Total antioxidant capacity (TAC)

TAC assay was determined using modified phosphomolybdate method as described by Prieto et al. [15]. 0.1 ml of methanol extract of *V. paradoxa* seed in different concentration ranging from 20 to 100 μ l were added to each test tube, 1 ml of Molybdate reagent (0.6 M sulfuric acid, 28 mM sodium dihydrogen phosphate, and 4 mM ammonium molybdate) was also added. The standard consisted of 0.1 ml of varying concentrations of 1 mg/ml ascorbic acid in distilled water with 1 ml working reagent. Solutions were incubated in a water bath (Uniscope SM801A; Surgifield Medicals, Okehampton, England, UK) for 90 mins at 95°C and then allowed to cool. At room temperature, the absorbance of the sample mixture was measured at 695 nm against a blank (1 ml of Molybdate reagent and 0.1 ml of distilled water) using a double beam ultraviolet (UV)-visible spectrophotometer (Model T80; PG Instruments, Lutterworth, England, UK) against a blank solution containing 0.1 ml distilled water and 1 ml working reagent incubated under the same condition. Mean values from three independent samples were calculated for the extract. TAC was expressed as equivalents of ascorbic acid per ml sample.

2.4. Statistical analysis

Statistical analysis and graphical presentations were performed using GraphPad Prism® 6.0 software. It was used for computing the IC_{50} values for antioxidant and anti-inflammatory assays. Values were reported as a mean \pm standard error of the mean of triplicate readings.

3. Results and discussion

Data from figure 1 showed that Diclofenac sodium and methanol extract of *V. paradoxa* seed (VPSME) inhibited heat-induced protein denaturation in a concentration-dependent manner. Maximum inhibition was shown at 500 μ g/mL for both Diclofenac sodium and the methanol extract. Furthermore, Diclofenac sodium ($IC_{50} = 261.4 \mu$ g/mL, $P < 0.05$) had higher anti-denaturation potential than methanol extract of *V. paradoxa* seed ($IC_{50} = 303.0 \mu$ g/mL).

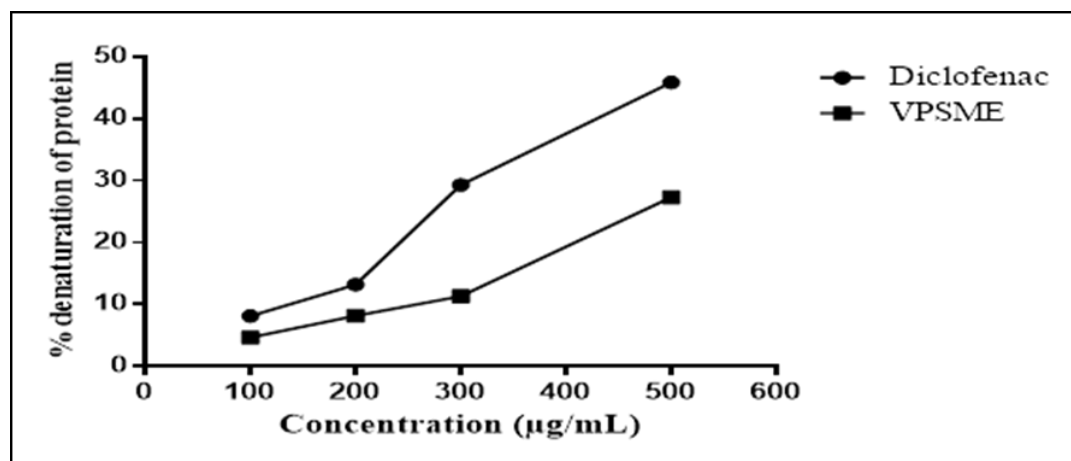


Figure 1 Percentage inhibition of thermal-induced protein denaturation by Diclofenac sodium and methanol extract of *V. paradoxa* seed

Data from Figure 2 exhibited that diclofenac sodium (2-[(2,6-dichlorophenyl) amino] benzene acetic acid sodium salt) and the methanol extract stabilized HRBC membrane against hypotonicity-induced hemolysis in a concentration-dependent manner. The data in figure 2 shows that the methanol extract stabilized HRBC membrane against hypotonic induced haemolysis in a blood sample than Diclofenac sodium. In addition, methanol extract of *V. paradoxa* seed ($IC_{50} = 31.47 \mu$ g/mL, $P < 0.05$) had higher membrane stabilization capacity than Diclofenac sodium ($IC_{50} = 33.89 \mu$ g/mL).

Data from figure 3 showed that the methanol extract (Mean \pm SEM) had a significantly ($P < 0.05$) high radical scavenging activity. The methanol extract exhibited a significant concentration dependent inhibition of DPPH activity. The maximum percentage inhibition of methanol extract and ascorbic acid were found to be at 97% and 98% respectively at 100 μ g/mL. Furthermore, ascorbic acid ($IC_{50} = 6.72 \mu$ g/mL) exhibited higher inhibition of DPPH than methanol extract of *V. paradoxa* seed ($IC_{50} = 8.95 \mu$ g/mL).

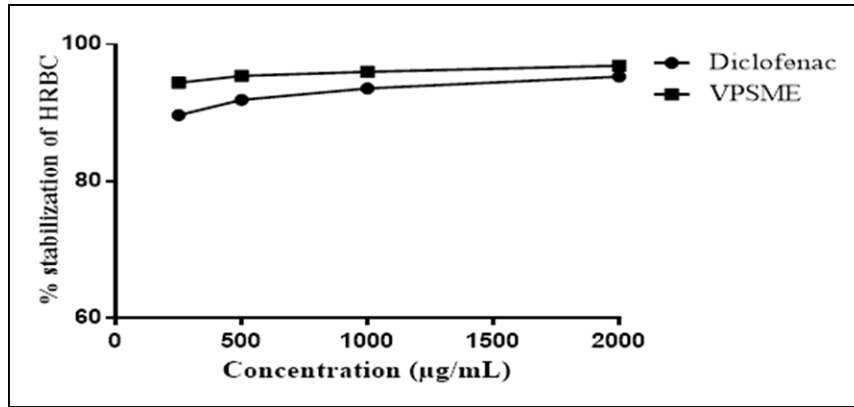


Figure 2 Percentage stabilization of hypotonicity-induced hemolysis of human red blood cell membrane by Diclofenac sodium and methanol extract of *V. paradoxa* seed

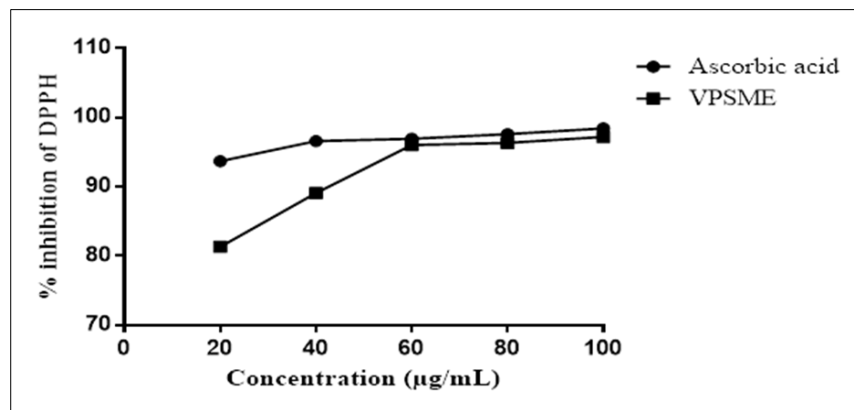


Figure 3 Percentage inhibition of 1,1'-diphenyl-2-picrylhydrazyl radical by methanol extract of *V. paradoxa* seed and Ascorbic acid

Data from figure 4 showed the ferric reducing capacities of 20 – 100 µg/mL ascorbic acid and methanol extract of *V. paradoxa* seed. The absorbances (Mean ± SEM) exhibited by the methanol extract and ascorbic acid range between (0.134 ± 0.006 - 0.837 ± 0.032) and (0.174 ± 0.043 - 0.761 ± 0.016) respectively at 552nm

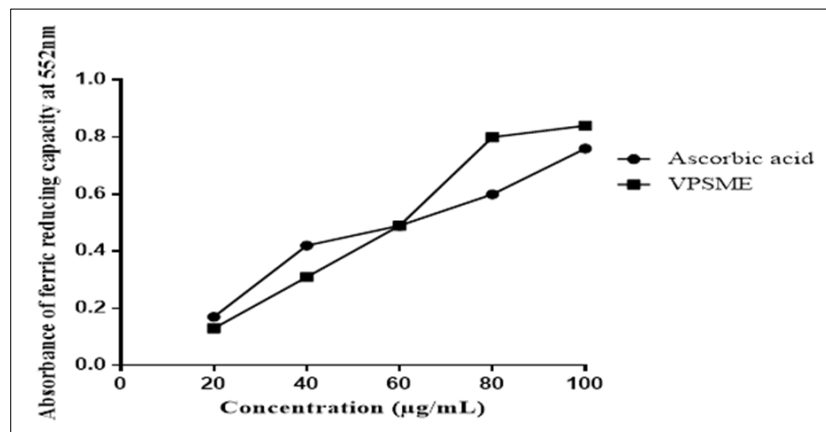


Figure 4 Ferric reducing antioxidant potentials of methanol extract of *V. paradoxa* seed and Ascorbic acid

Data from figure 5 showed that the methanol extract of *V. paradoxa* seed (Mean ± SEM) had a significantly ($P < 0.05$) high inhibition of lipid peroxidation. The maximum percentage inhibition of methanol extract and ascorbic acid were

97.5 % and 99 % respectively at concentration of 20 µg/ml. Also, methanol extract of *V. paradoxa* seed ($IC_{50} = 51.79$ µg/mL) exhibited higher antioxidant potential than ascorbic acid ($IC_{50} = 52.30$ µg/mL). Low IC_{50} value indicate a high level of antioxidant activity.

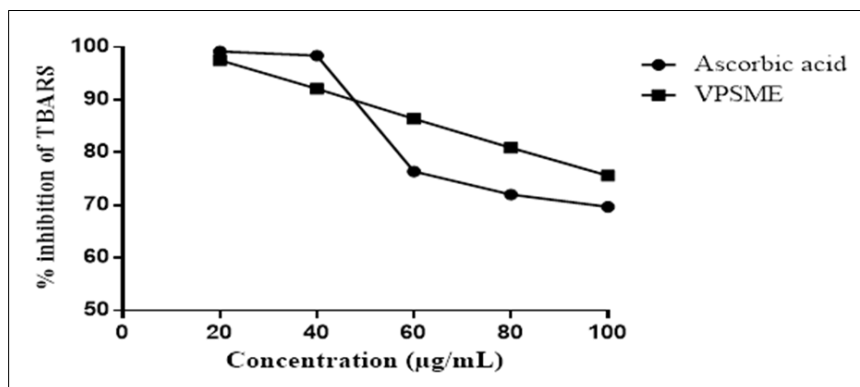


Figure 5 Percentage inhibition of Thiobarbituric acid by Ascorbic acid and methanol extract of *V. paradoxa* seed

Data from figure 6 showed that the methanol extract of *V. paradoxa* seed had a significantly low (0.249 ± 0.04 mg ascorbic acid equivalent [AAE]/g, $P < 0.05$) total antioxidant capacity than the standard (0.838 ± 0.03 mgAAE/g) at the highest concentration of 100 µg/mL. Although the TAC of both ascorbic acid and VPSME was exhibited in a concentration dependent manner, 20 µg/mL – 100 µg/mL.

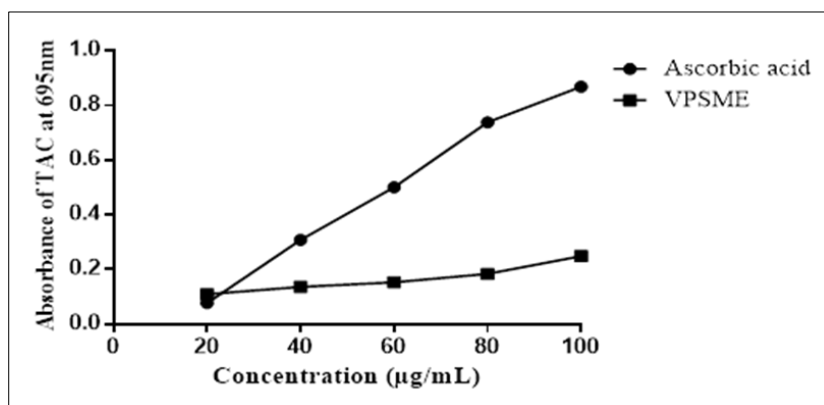


Figure 6 Total antioxidant capacities of methanol extract of *Vitellaria paradoxa* seed and ascorbic acid

Data from Table 1 showed the IC_{50} values of methanol extract of *Vitellaria paradoxa* seed and ascorbic acid on inhibition of TBARS, DPPH, anti-denaturation of BSA and HRBC stabilization at $P < 0.05$.

Table 1 IC_{50} of methanol extract of *Vitellaria paradoxa* seed and ascorbic acid on inhibition of TBARS, DPPH, anti-denaturation of BSA and HRBC stabilization

Test samples	IC_{50}			
	DPPH assay (20-100 µg/mL)	TBARS assay (20-100 µg/mL)	Anti-denaturation of BSA assay (100-500 µg/mL)	Stabilization of HRBC assay (500-2000µg/mL)
Ascorbic acid	6.723	52.30		
VPSME	8.95	51.79	303.0	31.47
Diclofenac sodium			261.4	33.89

4. Discussion

The antioxidant and anti-inflammatory activities are very important for humans because the oxidative stress and the inflammatory process were generated during different infectious diseases [16]. The release and proliferation of oxidants as free radicals has in part revealed the mechanism of tissue inflammation injury. These chemical radicals are key mediators in provoking or sustaining inflammatory processes, and consequently, their neutralization by phyto-antioxidants may attenuate inflammatory response [17]. The result of this study revealed the methanol extract of *Vitellaria paradoxa* seed may possess profound anti-inflammatory and antioxidant activities [18]. As part of the investigation on the anti-inflammatory activity of methanol extract of *V. paradoxa* seed, the inhibition of protein denaturation and stabilization of human erythrocyte were studied. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation. High temperature causes denaturation of BSA (bovine serum albumin) and this denatured protein exhibits the antigens similar to the type III hyper-sensitive reactions [19]. Heat denatured proteins causes delayed hypersensitivity which is associated to diseases like glomerulonephritis, serum disease and rheumatoid arthritis [20]. This study have revealed that the methanol extract of *V. paradoxa* had significant protein denaturation activity compared to the standard Diclofenac sodium. The human red blood cell membrane stabilization assay is studied because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the methanol extract may as well stabilize the lysosomal membrane. Stabilization of lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, which causes further tissue inflammation and damage upon cellular release [18]. The methanol extract had significant membrane stabilizing activity which was compared to the standard, Diclofenac sodium. The result showed that the methanol extract had higher stabilization activity than the standard drug which prove that the *V. paradoxa* seed may possess high anti-inflammatory activities.

Generation of free radicals or reactive oxygen species (ROS) during metabolism beyond the antioxidant capacity of a biological system results in oxidative stress [16]. Antioxidants either scavenge the reactive oxygen species or reduce the free radicals. Low levels of antioxidant in the living system assists the development of ageing related diseases such as atherosclerosis, cancers, heart diseases, diabetic neuropathy, aging and Alzheimer's disease. An anticipated solution to such problem is the enhancement of antioxidant that is present in plants [20]. There is a complex mechanism of scavenging free radicals in the human body and consists of a group of enzymes. Consequently, four different assays have been used in this study to reveal the *in vitro* antioxidant scavenging activities of methanol seed extract of *V. paradoxa*. Bleaching of DPPH solution is extensively used to estimate the electron donating ability of natural plants [21]. The electron donating capacity and concentration of the active constituent in the natural plants contributes towards the free radical scavenging activity. In this study, methanol seed extract of *V. paradoxa* showed significant scavenging ability and was compared with Ascorbic acid which suggested the presence of hydrogen donating constituents. Other plant extracts have been shown to reduce ferric ion and donate H⁺ to DPPH as a mechanism of scavenging these unstable chemicals [22].

FRAP assay measures the reducing capacity by increased sample absorbance based on the formed ferrous ions. The change in absorbance at 593 nm is attributed to the formation of blue coloured Fe²⁺-TPTZ complex from the colourless oxidized Fe³⁺ form by the action of electron donating antioxidants [23]. Higher degree of colour formation indicates the more reducing power of that antioxidant. The result of this study showed that methanol extract of *V. paradoxa* seed had a significantly high ferric-reducing antioxidant power compared to the Ascorbic acid standard. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present, thus it can be reported that methanol extract of *V. paradoxa* seed may act as free radical scavenger, capable of transforming reactive free radicals into stable non radical products [24].

Phosphomolybdenum assay is an additionally important *in vitro* antioxidant assay to evaluate the total antioxidant capacity of the methanol seed extract. The assay is based on the reduction of molybdenum (VI) phosphate to molybdenum (V) phosphate by the methanol extract and subsequent formation of a bluish green coloured molybdenum (V) phosphate complex at acid pH [15]. It involves in thermally generating auto-oxidation during prolonged incubation period are higher temperature [25]. It gives direct estimation of the reducing capacity of antioxidant. According to this study, methanol extract of *V. paradoxa* seed exhibit significant total antioxidant capacity compared to Ascorbic acid standard. This event could be attributed to the readiness of antioxidant phytochemicals present in the test extract to donate a hydrogen ion to the reactive molybdenum (VI) [15].

The TBARS assay has been used to measure the degree of lipid peroxidation. Polyunsaturated lipid contents of the biological membrane are susceptible to oxidative reaction of free radicals, which leads to lipid peroxidation [26]. Products of lipid peroxidation such as malondialdehyde (MDA), 4-hydroxyl 2-ninenal, and some other alkanes react with cell macromolecules to form adducts with significant irreversible effects on cellular functions [27, 28]. Consequently,

the formation of adduct leads to membrane permeability, oxidative nucleic acid damage and eventually to mutation and cancer [29]. Inhibition of lipid peroxidation by methanol extract of *V. paradoxa* seed was very significant compared to Ascorbic acid standard. For TBARS assay, low or reducing absorbance value indicate a high level of antioxidant activity [30]. It will however be necessary to study the structural properties of the active compounds responsible for these activities and investigate their mechanism of actions.

5. Conclusion

The results of our investigations reveal that the methanol extract of *V. paradoxa* may contain vital bioactive metabolites including phenolic compounds with significant antioxidant and anti-inflammatory activities which could be helpful in the management of oxidative and inflammatory disorders and constitutes a potential source for development of new treatment agents.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest amongst the authors as all the authors contributed in one way or the other in conducting the research and in writing the manuscript which was eventually articulated and submitted for publication by the corresponding author.

Statement of ethical approval

This research is in full compliance with ethical standards and was approved by Babcock University Health Research Ethics Committee.

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