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Original Research Article

Evaluation of Antibacterial, Antioxidant and Anti-inflammatory Properties of Methanol Extract of *Varthemia iphionoides*

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ABSTRACT

Medicinal plants represent an essential source of active biological compounds. The current study examined the methanol extract of *V. iphionoides* for its antibacterial, antioxidant, and anti-inflammatory activities. The antibacterial activity was evaluated against six bacterial strains (*Staphylococcus xyloses*, *Escherichia coli*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Achromabacter xylosoxidans*, and *Pseudomonas aerugenosa*). *V. iphionoides* extract showed good antibacterial activity against *Staphylococcus xylosus* followed by, *pseudomonas aeruginosa* and *Klebsiella oxytoca*, and the minimum inhibitory concentrations (MICs) were 33, 33, and 11 µg/ml, respectively. However, the extract did not show any antibacterial activity against the other bacterial strains. The antioxidant activity of the plant extract was evaluated using ABTS and DPPH radical scavenging assays, and the Trolox equivalents were 93±0.31 and 84.8±0.28 mg Trolox/g plant extract, respectively. Anti-inflammatory activity in LPS-induced mice was investigated by measuring the concentration of pro-inflammatory cytokines TNF-alpha, IL 1beta, and antioxidant enzyme catalase and glutathione peroxidase. The results demonstrated that the highest concentration of *V. iphionoides* extract was the most efficient. Anti-inflammatory activity was exhibited by reducing the concentrations of pro-inflammatory TNF-α and IL 1 beta and reducing the concentrations of antioxidant enzyme catalase and glutathione peroxidase compared to the negative control group.

Keywords: Antibacterial, Antioxidant, Anti-inflammatory, DPPH, ABTS, Interleukin.

Introduction

Medicinal plants continue to be an important source to treat many diseases. World Health Organization (WHO) estimated that 80% of the population still depends on alternative medicines for the treatment of diseases.[1] Medicinal plants represent an essential source of active biological compounds, therefore they have played an important role in the health of human beings and their value is appreciated to date.[2] The possible hazards of antibiotic in public health, and the incorrect use of antibiotics has contributed to the increase in bacterial resistance.[3,4] This has led to the failure of the treatment of many microbial diseases,[5] therefore, scientists are challenged to come up with new ideas of alternative and novel drugs to overcome microbial resistance.[6] Inflammation is defined as a normal body response to foreign agents, such as microbial pathogens, and tissue damage.[7]

V. phionoides is one of the most important medicinal plants found in the east of the Mediterranean region. This plant is a member of the Asteraceae family. It is a 20 to 50 cm long (in some regions, it can reach 80 cm) green dwarf shrub with hairy leaves and aromatic glands. It has tubular flowers, and the blooming season extends from September to December. It has been used in traditional medicine to treat pains, wounds, complaints, and urine retention.[8–10] Many studies have shown that *V. iphionoides* contains many chemical constituents that have different effects such as antimicrobial,[11] anti-thrombosis.[12] cytotoxic, and antioxidant activities.[13,14] Moreover, it has been reported that *V. iphionoides* has antidiabetic and anticancer properties.[15,16] There are few studies on its potential as anti-nflammatory agent.[17] *V. iphionoides* is utilized in the treatment of various conditions: stomach ailments, diabetes, male and female infertility, eye disease, kidney stones, and as an anti-inflammatory. The areal parts of *V. iphionoides* have been utilized for the treatment of some infections in sheep and goats such as colic, fever, and scabies.[18] This study aimed to investigate the effects of *V. iphionoides* as an antibacterial and antioxidant agent as well as evaluating the anti- inflammatory effects and its ability to decrease the concentration of pro-inflammatory cytokine in mice.[19]

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Materials

Plant material

The fresh plant aerial parts were collected from different regions of Karak city during April and May 2021, from Al-Karak Governorate/ South Jordan. The chosen parts were washed thoroughly with tap water to remove dirt, then dried in shade at room temperature for 14 days. A voucher specimen with the number (MU 2021-33) has been deposited in the Department of Biology at Mutah University.

Sample preparation

Dried leaves were crushed by blender into uniform powder to increase the surface area for extraction. Two hundred grams of the powder were placed into a sterile conical flask containing 1000 ml of methanol

(99%). The Conical flask was tightly covered, shaken vigorously, and soaked for 3 days to enhance the proper dissolution of the bioactive compounds in the sample. The sample solution was filtered using a Buchner funnel with 0.45 µm filter paper (Whatman Int. Ltd., Maidstone, U.K) at room temperature. The filtrate was then centrifuged at 3000 rpm for 10 minutes and concentrated using a rotary evaporator (BUCHI Rotavapor BL-710D, Switzerland) at 45°C until the extract become completely dry. The extract was stored in a plastic container at 4°C in a refrigerator until used.[11]

Determination of extraction yield
The percentage of yield (w/w) from all the dried extracts was calculated as follows:
Yield (%) = (W1 * 100%)/W2
Where W1 is the weight of the extract, and W2 is the weight of the plant powder [11].

Antibacterial assay
Preparation of bacterial suspension
The culture medium was prepared according to the Laboratory Standards Institute (CLSI M7-A7, 2012) standards. One bacterial colony was selected and cultured in sterile 5 ml nutrient broth (NB) overnight at 37 °C. Bacterial growth was set at a value of 0.5 McFarland Standard using sterile NB, with the final absorbance being 0.1 at 620 nm. Six human pathogenic bacterial strains (*Staphylococcus xylosus*, *Escherichia coli*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Achromabacter xylosoxidans*, and *pseudomonas aerugenosa*) were obtained from Dr. Yaseen Al Qaisi (Microbiology Research Lab., Department of Biology, Mu'tah University).

Antibacterial activity of the extract
The antibacterial activity of the extract was investigated using the disc diffusion method.[20] The crude plant extract was dissolved in 10% DMSO in addition to MeOH. Four to five well-isolated colonies of the same morphological type were selected and inoculated into tubes containing 5 ml Muller-Hinton broth and incubated at 37°C with shaking at 150 rpm until the turbidity of the bacterial growth was achieved. The antibacterial action was examined at two different concentrations: 300 and 450 µg/disc. The growth medium was prepared, autoclaved, and allowed to cool to 48–50ºC and a standard inoculum (2×10^8 CFU/mL) was then added under aseptic conditions to the molten agar and poured into sterile Petri dishes to give a solid plate. Then, sterile antimicrobial susceptibility discs (6 mm diameter) were loaded with 10 µL of the extract and placed on the inoculated plates. For each bacterial strain, negative control (10% DMSO in methanol) was included. Teicoplanin (TEI) and Olfaxcin (OFX) were used as positive controls. The cultures were incubated at 37 ºC for 24 h. The antibacterial activity was determined according to the size of the inhibition zone around each disc. Each test was done in triplicate.[11]

Determination of minimum inhibitory concentration (MIC)
The MIC values were determined using the microdilution broth susceptibility assay, following the National Committee for Clinical Laboratory Standardization's standards. All trials began with a typical initial inoculum ($2x10^6$ cell/mL) which was inoculated into microwells (96-wells microplate) containing a serial dilution of plant extract ranging from 0 to 2000 µg /mL. Each experiment comprised negative controls (media, cell suspension, and the solvent, which was always maintained at 1%, without extract) and blanks (medium containing extract but without cell suspension). The microplates were incubated at 37°C for 24 hrs. After 24 hours of incubation, the MIC was determined as the lowest extract concentration that inhibits the visible growth of the bacteria when compared to a negative control [21–23].

Evaluation of the antioxidant activity of the extract
DPPH radical scavenging assay
The hydrogen atom or electron-donating capacity of the corresponding extract was measured by bleaching a purple-colored methanol solution of DPPH[24]. Aliquots (50 µL) of different concentrations of the plant extract in the range of 0.1–2 mg/mL were mixed with 5 mL of 0.004% 2,2-Diphenyl-1-picrylhydrazyl (DPPH) in methanol and incubated in dark at room temperature. After 30 minutes of incubation, the absorbance was measured against methanol at 517 nm. The following equation was used to calculate the inhibition of DPPH radical by the extract:

I % = [(A blank – A sample) / A blank] x 100%

Where A blank is the absorbance of the control reaction (containing all reagents except the test compound), and A Sample is the absorbance of the test compound. For each plant extract, the DPPH inhibition curve was constructed, and the concentration of extract providing 50% inhibition of DPPH (IC50) was calculated. Trolox was used for the construction of a standard curve to calculate the Trolox equivalent antioxidant capacity of the plant extract [25].

ABTS radical scavenging assay
The ABTS radical scavenging activity was evaluated using a modified version of the method described by Yu et al [11]. Mixing an equal volume of a 7 mM ABTS stock solution and a 2.45 mM potassium persulfate solution produced the ABTS solution. The mixture was then kept at room temperature in the dark for 12 to 16 hours. The ABTS solution was diluted to an absorbance of 0.70±0.02 at 734 nm by adding 10 mM of phosphate-buffered saline (PBS, pH 7.4) Then, 3 mL of the diluted ABTS solution was combined with 50 lL of the sample solution. After a 6-minute dark, room-temperature incubation, the absorbance of the mixture was immediately measured at 734 nm. The control was made by substituting PBS for the ABTS solution, and the blank was made by substituting distilled water for the sample. The ABTS radical scavenging activity (%) was calculated using the formula:

ABTS radical scavenging activity (%) = [1 - (As - Ac)/Ab],

where As, Ac, and Ab represent the absorbance values of the sample, control, and blank, respectively.

Evaluation of Anti-inflammatory effects
Animals
Female BALB/c mice weighing 25 g were purchased from the Applied Science University animal house. All BALB/c mice received water and a standard rodent diet.

Acute toxicity study and dose selection
The dose of the *V. iphionoides* methanolic extract was selected based on the lethal dose (LD50). LD50 experiment was conducted on 30 BALB/c mice to determine the dose that is considered safe to be administered. The experiment was conducted by selecting five different doses (100, 200, 400, 500, 1000, and 2000 mg/kg), which were administered orally to each group of six mice. After oral administration, clinical observations were performed at 4, 8, 12, and 16 hours after dose administration. After 24 hours, the number of survived mice was counted in each group, and the survival rate was calculated based on Reed–Muench method [26] as (number of live mice/ total number of mice tested) ×100%.

Study design
In this study, a total of 30 BALB/c mice were used. The mice were divided into 5 groups (6 for each group): Group 1: 6 mice were given water (i.p); Group 2: 6 mice were given only LPS as a negative control (at a dose of 2.5 mg/kg i.p); Group 3: 6 mice were given LPS (2.5 mg/kg i.p) and after 24 h given ascorbic acid as a positive control (500 mg/kg i.p); Group 4: 6 mice were given LPS (2.5 mg/kg i.p) and after 24 h given *V. iphionoides* extract (100 mg/kg i.p); Group 5: 6 mice were given LPS (2.5 mg/kg i.p) and after 24 h given *V. iphionoides* methanolic extract (200 mg/kg i.p).

Biochemical analysis
Blood samples (1 mL) were collected in sterile tubes and allowed to clot at room temperature for 10 minutes. Then, the blood samples were centrifuged at 2500 rpm for 10 min and the sera were stored at -21°C until used. Measurements of serum concentrations of TNF- alpha, IL-

1β, CAT, and GP-X were performed according to manufacturers' instructions.[27]

Statistical analysis

One-way analysis of variance (ANOVA) was used followed by Dunnett's post hoc test. The data was analyzed by SPSS© 22 (SPSS, Inc., USA). The rest of the results were presented as means± standard deviation (SD) of 3-4 independent experiments. Statistical differences between control and different treatment groups were determined using Graph Pad Prism ANOVA followed by Dunnett's post hoc test.

Results and Discussion

Dry weight and yield percentage of plant extract

The weight of yielded plant extract and the yield percentage are shown in Table 1.

Antibacterial activity of plant extracts

The results of the antibacterial activity of the plant extract are shown in Table 2. The inhibition zones of tested bacterial strains were measured and recorded in mm. Different responses to the plant extract have been seen in the different bacterial species, and the response was dose-dependent in the same species. The highest antibacterial effect was seen against *Staphylococcus xylosus* followed by, *pseudomonas aeruginosa* and *Klebsiella oxytoca*. However, no effect was seen on the other bacterial species as demonstrated in Table 2. The negative control discs in all investigations did not show any inhibition against bacteria growth. Positive controls showed different activities against the bacterial strains and their inhibition zone was used as a reference to determine the efficiency of chosen plants.

MIC of plant extracts against bacterial growth

To determine the MIC values of the plant extract against the tested bacterial species, the microdilution method was employed. According to the results (Table 3), the tested plant extract possessed antibacterial activities in various degrees. The MIC values were between 11-110 µg/mL.

The inappropriate uses of antibiotics are often associated with resistance in bacterial strains. This has resulted in many health problems during the treatment of microbial disease [28]. Most of these compounds should be tested in *in vivo* studies to investigate their effectiveness in whole-organism systems, including toxicity studies as well as an tested of their effects on the beneficial bacteria in the body such as microflora in the digestive, respiratory and others system.[29,30] It was observed that the activity of medicinal plant extracts varies from one plant to another and from different country of the world in different research's, and this may be resulted due to many factors such as different of climate, soil composition, age and vegetation cycle stage on the quality, quantity and composition of extracted product.[31] In the current study, the antimicrobial, antioxidants and anti-inflammatory effects of *V. iphionoides* was studied. The results of the current study demonstrated that the *V. iphionoides* methanolic extract have different levels of antimicrobial activities comparing to standard antibiotics (Teicoplanin (TEI) and Ofloxacin (OFX)). The results of inhibition zone were in accordance with the values of MICs for *V. iphionoides* methonolic extract. The methanol extraction of *V. iphionoides* was effective against the following bacteria *S. xylosus*, *K. oxytoca* and *p. aerugenosa* at different level but no any antimicrobial activities against *E. aerogenas*, *A. xylosoxidans* and *E. coli* in both concentration Table (3). The gram-positive *S. xylosus* showed the maximum activity against *V. iphionoides* methanolic extract with mean of inhibitory zone 19.6 mm. This finding indicates that some compounds present in *V. iphionoides* methanolic extract have antibacterial activity against Gram-positive and Gram-negative bacteria. These results agreement with Masadeh et al., 2013, Afifi et al., 1991.[32,33] who showed the *V. iphionoides* methanolic extract have antibacterial agents against *Staphylococcus* species, but there is no antibacterial activity against *E. coli*. The variations in antibacterial activity response to *V. iphionoides* methanolic extract may refer to the differences in cell wall structure between gram positive and gram-negative bacteria or the mode of action of the *V. iphionoides* methanolic extract against tested bacterial species. *V. iphionoides* is most commonly used in traditional medicine for the treatment of gastrointestinal infection in Jordan.[33]

Antioxidant activity

Antioxidant activities of *V. iphionoides* methanolic extract was assessed by detecting their ability to scavenge free radicals by using DPPH, and ABTS radical scavenging activity methods.

ABTS and DPPH

The antioxidant activity of *V. iphionoides* methanolic extract was measured by ABTS scavenging assay. which is one of the main method which used in determining the free radical-scavenging activity of various natural products. *V. iphionoides* methanolic extract showed high antioxidant activity in $TEAC_{DPPH}$ and $TEAC_{ABTS}$ results, *V. iphionoides* contained high amount of phenols as shown in the Table 4.

Table 1: Weight and yield percentage of crude plant extract.

Plant	Dry weight of plant (g)	Weight of plant extract (g)	Yield of plant extract (%)
V. iphionoides	200	22	11%

Table 2: Antibacterial activity of methanol extract of *V. iphionoides* at different concentrations on the bacterial growth. Data are shown as Mean ±SD, n=3.

Bacterial strain	Zone of inhibition (mm)	
	300 µg/disc	450 µg/disc
S. xylosus	16 ± 1	19.6 ± 1.08
K. oxytoca	9.6 ± 0.59	13.3 ± 1.47
E. aerogenes	-ve*	-ve*
P. aerugenosa	12.6 ± 1.47	15.33 ± 0.58
A. xylosoxidans	-ve	-ve
E. coli	-ve	-ve

*-ve: No inhibition zone was detected

Table 3: The MIC values of *V. iphionoides* extract on the different bacterial strains in (µg/mL)

Bacterial strain	MIC (µg/mL)
S. xylosus	22
K. oxytoca	33
P. aerugenosa	33

Table 4: Effect of plant extract as antioxidant

Test	Antioxidant activity
$TEAC_{DPPH}$	84.8 ± 0.28 mg Trolox/g
$TEAC_{ABTS}$	93 ± 0.31 mg Trolox/g

Acute toxicity study and selection of the experimental dose

The LD_{50} value of the *V. iphionoides* methanolic extract was 1000 mg/kg. In *in-vivo* study 100 mg/kg (1/10 LD_{50}) and 200 mg/kg (1/5 LD_{50}) was used.

The results support the use of *V. iphionoides* plant in folk medicine in treatment of microbial infection. Phenolic compounds have been previously identified as one of the main phytochemical in *V. iphionoides,* and the antimicrobial activities of *V. iphionoides* methanolic extract are associated in plant with high content of phenolic compounds.[34] Phenolic compounds have been suggested as antimicrobial agents by lysis of the plasma membrane, and disturbing the transport, electron flow, and microbial enzymes [35]. In addition, flavonoids and essential oils are also considered as one of the main important *V. iphionoides* plant phytochemical, which may be attributed to the antimicrobial activity of this plant[36,37] showed flavonoids and essential oils isolated from *V. iphionoides* revealed antimicrobial agents through inhibition of genetic material DNA synthesis, disturbing plasma membrane function and energy metabolism process. Essential oils especially, were reported to disrupt mitochondria in the cell through disintegrating double membrane permeability [38]. The total antioxidant activities of *V. iphionoides* methanolic extracts was measured by using two commonly accepted assays, DPPH and ABTS, were employed to evaluate the total antioxidant activity of *V. iphionoides* methanolic extract, and TEAC-DPPH and TEAC-ABTS were indicated. The antioxidant activity of *V. iphionoides* methanolic extract was measured by DPPH scavenging assay, which is one of the main method which used in determining the free radical-scavenging activity of various natural products.[39] The absorbance of the DPPH-solution decreases as the plant extract is added because of a color change from purple to yellow showing that the radical is scavenged by antioxidants through donation of hydrogen radicals (H·) to form the stable DPPH molecule.[40] The phenolic compounds are one of the major plant content and found in many plants in different concentration and show different antioxidant activity.[41] Phenolic compounds have the ability to scavenge free radicals such as the Reactive Oxygen Species (ROS) which are determined by their reactivity as hydrogen-or electron donating agents.[42] In current study *V. iphionoides* methanolic extract show high antioxidant activity in $TEAC_{DPPH}$ and $TEAC_{ABTS}$ results, *V. iphionoides* contained high amount of phenolic $TEAC_{DPPH}$ (84.8±0.28 Mg trolox/g) and $TEAC_{ABTS}$ (93±0.31 Mg trolox/g). These results are in agreement with Mallinckrodt *et al*., (2007)[43] results, who reported that the extracts of plant with high amounts of phenolic compounds, which have highly potential as protecting agents against the lethal effects of oxidative stress and protection of DNA from damage. Also, these observations agreed with several previous findings [44,45]. The antioxidant activities of *V. iphionoides* methanolic extract in the present study, are in accordance with the previous findings of. [46,47] who reported moderate antioxidant activity for *V. iphionoides*. The present study showed a strong correlation between the mean values of $TEAC_{DPPH}$ and $TEAC_{ABTS}$ which indicated that compounds present in the *V. iphionoides* methanolic extract capable of reducing DPPH radicals were also able to reduce ABTS. Pro-inflammatory cytokines production in high concentration accelerate the chronic inflammations, which leads to more activations of immune system, and continuous activation leads to tissue destruction and cancer development.[48]

Effect of V. iphionoides extract on the TNF alpha concentration

Figure 1 shows the TNF alpha concentration in different groups. LPS which was used as negative control significantly induce the cell to produce TNF comparing with normal non treated group and ascorbic acid as positive control significant reduce the TNF production compared with negative group. *V. iphionoides* methanolic extract at the dose of 100 mg/kg and 200 mg/kg significant decrease the production of TNF respectively as concentration increase compared with negative groups and have similar effect as positive control.

Effect of V. iphionoides methanolic extract on the expression on IL1 beta concentration.

Figure 2 shows the **IL1 beta** concentration in different groups. LPS significantly induce the cell to produce **IL1 beta** compared with normal non treated group and ascorbic acid as positive control highly significant reduce the production comparing with negative group. *V. iphionoides* methanolic extract at concentration 100 mg/k.g and 200 mg/k.g highly significant decrease the production of **IL1 beta** respectively as concentration increase compared with negative groups and have similar effect as positive control.

Effect of V. iphionoides methanolic extract on the expression on catalase concentration.

Figure 3 shows the catalase enzyme concentration in different groups. LPS which was used as negative control significantly induce the cell to produce catalase enzyme comparing with normal non treated group and ascorbic acid as positive control significantly reduce the production compared with negative group. *V. phionoides* methanolic extract at concentration 100 mg/k.g and 200 mg/k.g significantly decrease the production of catalase enzyme respectively as concentration increase compared with negative groups and have similar effect as positive control.

Effect of V. iphionoides methonlic extract on the expression on Glutathione peroxidase concentration.

Figure 4 shows the Glutathione peroxidase enzyme concentration in different groups. LPS which was used as negative control significantly induce the cell to produce Glutathione peroxidase enzyme compared with normal non treated group and ascorbic acid as positive control highly significant reduce the production comparing with negative group. *V. iphionoides* methanolic extract at concentration 100 mg/k.g and 200 mg/k.g significantly decrease the production of Glutathione peroxidase enzyme respectively as concentration increase compared with negative groups and have similar effect as positive control.

The current study aims to investigate the effect of *V. iphionoides* methanolic extract as ant-inflammatory in LPS induced mice by reducing the concentration of pro-inflammatory cytokines TNF-alpha and IL 1beta and reducing the oxidative stress *in vivo* by decrease the concentrations of antioxidant enzyme such as catalase and glutathione peroxidase.

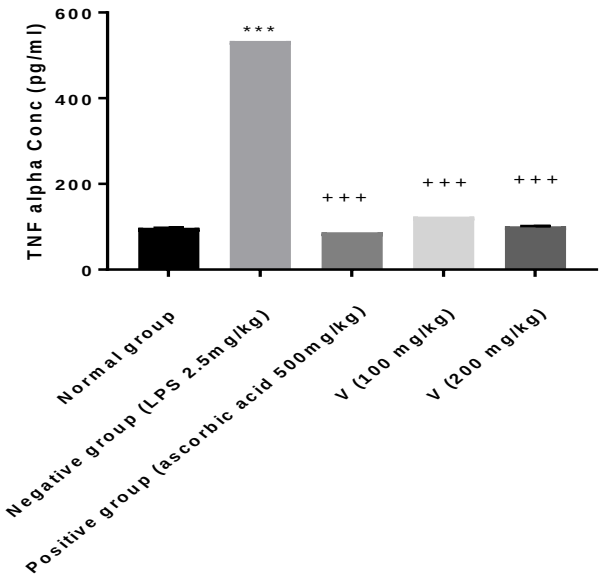

Figure 1: Effect of *V. iphionoides* methanolic extract on the expression on TNF alpha concentration. The result represents the concentration of TNF after 24h exposure to LPS in negative group then treated with ascorbic acid in positive control group and treated with 100, 200 mg/k.g of *V. iphionoides* methanolic extract respectively. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ LPS group compared to normal group, +: $p<0.05$, ++ $p<0.01$, +++: $p<0.001$ compared to LPS group.

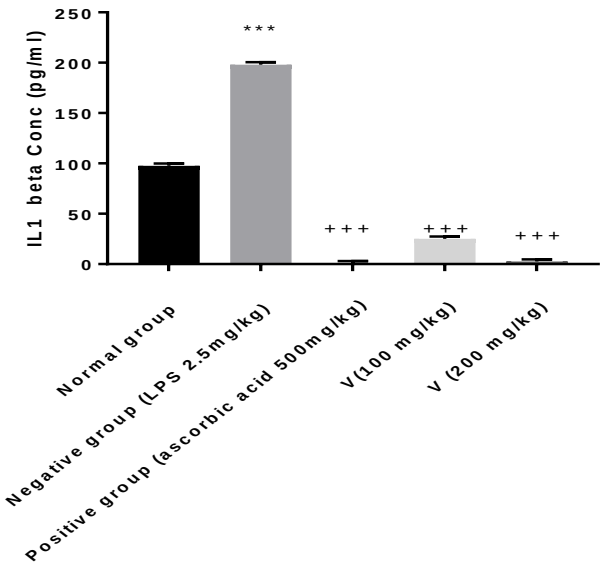

Figure 2: Effect of *V.a iphionoides* methanolic extract on the expression on IL1 beta concentration. The result represents the concentration of IL1 beta after 24h exposure to LPS in negative group then treated with ascorbic acid in positive control group and treated with 100, 200 mg/k.g of *V. iphionoides* methanolic extract respectively. *: p<0.05, ** p<0.01, ***: p<0.001 LPS group compared to normal group, +: p<0.05, ++ p<0.01, +++: p<0.001 compared to LPS group.

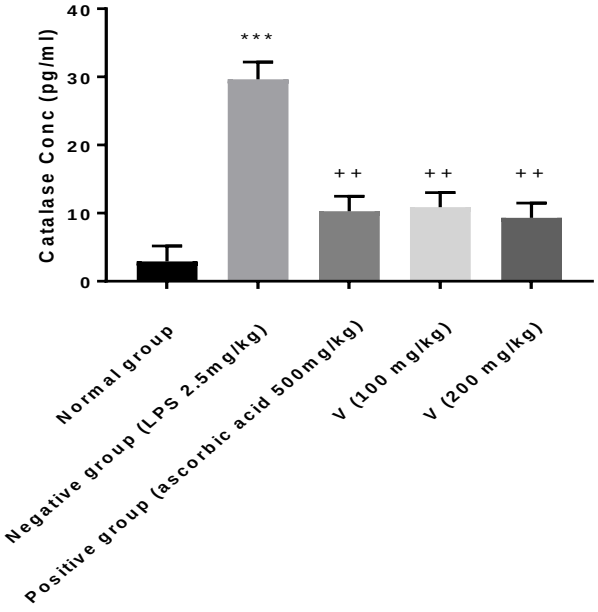

Figure 3: Effect of *V.iphionoides* methanolic extract on the expression on catalase enzyme concentration. The result represents the concentration of catalase enzyme after 24h exposure to LPS in negative group then treated with ascorbic acid in positive control group and treated with 100, 200 mg/k.g of *V. iphionoides* methanolic extract respectively. *: p<0.05, ** p<0.01, ***: p<0.001 LPS group compared to normal group, +: p<0.05, ++ p<0.01, +++ : p<0.001 compared to LPS group.

The results of the current study demonstrated that the *V. iphionoides* methanolic extract acts as anti-inflammatory by reducing the concentrations of pro-inflammatory TNF and IL 1 beta and reducing the concentrations of antioxidant enzyme catalase and glutathione peroxidase comparing with negative group which treated by LPS and similar to positive control group which treated with ascorbic acids and the higher concentration (1/5 LD50) of *V. iphionoides* methanolic extract is more effective as anti-inflammatory in reducing pro-inflammatory mediators level and antioxidant enzyme level than 1/10 of the LD50. LPS, a bacterial endotoxin, was able to activate immune cells and cause them to release pro-inflammatory cytokines.[49] As a result, we used LPS in this study and did not measure the level of pro-inflammatory mediators and antioxidant enzymes in response to *V. iphionoides* methanolic extract only without LPS because we wanted to see if *V. iphionoides* methanolic extract could have an anti-inflammatory effect by reducing pro-inflammatory mediators and oxidative stress caused by LPS. The LD50 of the *V. iphionoides* plant clearly indicated that the *V. iphionoides* methanolic extract did not causes any death or toxicity during experiment at any concentration used in the experiment and we used safe concentration under the LD50 (1/10 and 1/5 of LD50). Therefore, we can hypothesis that the anti-inflammatory effect of *V. iphionoides* methanolic extract against the significant increase of pro-inflammatory levels induced by LPS could be resulted by modulation of inflammatory pathways. TNF- and IL-1 are commonly pro-inflammatory mediators produced by various immune cells and act in the promotion and progression of the inflammatory process, but when expressed and released in high levels, these mediators have a direct effect on the progression and formation of multiple inflammatory and immunological disorders.[50] Pro-inflammatory inhibitors are chemically synthesized medications that are often used to treat inflammatory disease. These treatments have some negative effects, including an increased risk of infection, heart failure and heart difficulties, neuropathy disease, and autoimmunity-related issues. [51] However, researchers are making major efforts to produce and develop novel anti-pro-inflammatory medications from medicinal plant components that interfere with pro-inflammatory production that are safer, less toxic, and more useful. Novel inhibitors or blockers may prove to be an alternative for treating inflammatory and immunological disorders. in current study *V. iphionoides* methanolic extract acts as pro inflammatory inhibitors by decreasing the level of TNF-α and IL-1 comparing with LPS treated groups. The results of the current study are in accordance with the results by Justino.[52] who provided that *V. iphionoides* methanolic extract acts as anti-inflammatory by reducing the concentrations of pro-inflammatory IL-6 comparing with LPS treated group. We believe that the inhibition activity of *V. iphionoides* methanolic extract and its ability to lower the levels of pro-inflammatory mediators TNF-α and IL 1 can be attributed to one of the following mechanisms: inhibiting TNF-α binding to specific receptors, lowering the activation of transcriptional factors that reduce pro-inflammatory mediator production at the gene level, lowering the activation signaling cascade that is responsible for pro-inflammatory mediator production, or a combination of these mechanisms.

Because ascorbic acid is one of the key supplements that promote the activity of the immune system by enhancing T-lymphocyte proliferation in response to inflammation and increasing immunoglobulin production from B-lymphocytes, it was employed as an anti-inflammatory positive control in this investigation. Many previous studies found that in many illnesses linked with chronic inflammation and oxidative stress, ascorbic acid levels are depleted when compared to healthy controls.[53] Increased intracellular oxidative stress mechanisms and antioxidant enzyme levels in tissues, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), are caused by the inflammatory process, which leads to the development and causes of many inflammatory diseases and cancers.[54] In this study, *V. iphionoides* methanolic extract behaves as an anti-inflammatory by reducing oxidative stress in mice serum by acting as a scavenger and reducing the level of antioxidant enzyme catalase (CAT) and glutathione peroxidase (GPx). These results agreement with Yoshinari *et al*., (2013).[55] reported that the natural products suppress the oxidative stress and inflammatory process. Phenolic compounds, which have been discovered as one of the primary phytochemicals in *V. iphionoides*, have

been shown to minimize oxidative stress by acting as one of the main antioxidants, stabilizing cell membranes and reducing lipid peroxidation. They can either directly trap and remove free radicals or scavenge them through a sequence of interactions.[56] Phenolic acids and flavonoids are also among the most physiologically active antioxidant phytochemicals found in *V. iphionoides*, owing to their important functions in the protection of illnesses caused by increased oxidative stress.[57] Flavonoid molecules serve as free radical scavengers through donation of a hydrogen atom to radicals, the radical-scavenging activity is dependent on the chemical structure and/or the substitution pattern of hydroxyl groups in the free radicals[58] Ascorbic acid was utilized as a positive control in this investigation because it protects cells from oxidative stress caused by reactive oxygen substances (ROS) and is a strong free radical scavenger. Ascorbic acid's antioxidant properties originate from its ability to mimic potentially harmful reactive oxygen species (ROS) and produce comparatively stable ascorbate free radicals instead.[59]

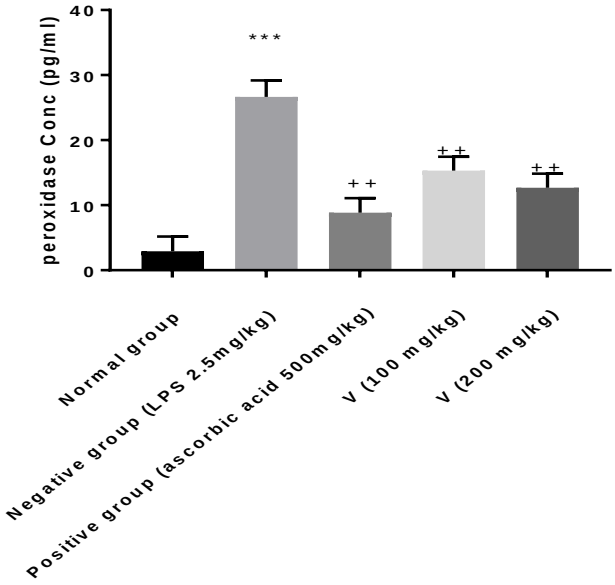

Figure 4: Effect of *V. iphionoides* methanolic extract on the expression on Glutathione peroxidase enzyme concentration. The result represents the concentration iphionoides methanolic extract respectively. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ LPS group compared to normal group, +: $p<0.05$, ++: $p<0.01$, +++: $p<0.001$ compared to LPS group. Of peroxidase enzyme after 24h exposure to LPS in negative group then treated with ascorbic acid in positive control group and treated with 100, 200 mg/k.g of *V. iphionoides*

Conclusion

V. iphionoides have antibacterial activity against several bacterial strains, also it showed an antioxidant and anti-inflammatory activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Ethical Clearance

All experimental protocols were approved under the Department of biology, Mutah University, Jordan, and all experiments were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

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