Bioactivity of *Viscum album* extracts from Olive and Almond host plants in Palestine

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ABSTRACT

Introduction: *Viscum album* is a semi-parasitic medicinal plant which has been used for many years as a remedy in traditional medicine. The plant is widely used in folk medicine in Palestine; mainly in the treatment of cancer, diabetes and heart disease. Since no previous reports on bioactivity of this plant in association with host plant specificity in Palestine, the current study aimed at evaluating bioactivity of almond and olive variants of this plant. **Method:** Methanolic extract of viscum *album* cultivated from almond and olive host plants were tested for antioxidant, antimicrobial . Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were also used to assess anticoagulant activities of plant extracts. **Result:** The result demonstrated that *Viscum album* have an IC_{50} of $25.34 \pm 3.8\mu$g/ml when hosted by olive while the IC_{50} was $15.37 \pm 2.2\mu$g/ml when hosted by almond. Crude extracts of both *Viscum album* plants showed strong inhibition effects on the growth of the studied *Staphylococcus aureus* strains (ATCC 25923 and MRSA) with a pronounced effect when extracts of almond host was used. However, the effects of both host extracts were very limited or absent when tested against Gram-negative reference and clinical strains. Plant extracts of both host showed prolonged PT and PTT compared to phosphate buffered saline control solution. **Conclusion:** In conclusion, variations in the bioactivity of *Viscum album* is clearly influenced by host type and further studies required to illustrate such variations using other host plants.

Keywords: Viscum album, Antioxidant, Prothrombin time, Antimicrobial.

INTRODUCTION

Complementary and alternative medicine (CAM) has become increasingly popular for various conditions and diseases over the last decades. Most of these complementary treatments are herbal remedies and among these is *Viscum album* (Mistletoe) extracts.[1] A number of biological effects were reported for *Viscum album* including anticancer, apoptosis–inducing, antimycotic, antibacterial, antiviral, antidiabetic, and immunomodulatory activities have been reported.[2]

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Viscum album is a small, dioecious and shrubby semi-parasitic plant that grows wild on trees, bushes and other plants.[3] It has an oblong evergreen leathery leaves, clear dichasial branching and four-part flowers which form white sticky berries with a faint but characteristic odor and a bitter taste.[4] Mistletoe is considered a semiparasitic plant because it synthesizes its own chlorophyll but depends on the host for its supply of water and minerals.[5]

Recent scientific research has confirmed that *Viscum album* extract induced apoptotic killing of cultured human tumor cells and lymphocytes, and stimulated the immune system[6–9] so that, it affects positively on the lifespan of individuals respectively.[10]

The phytochemical profile of mistletoe depends of the host trees of this plant.[11] The main bioactive compounds found in mistletoe are lectins, viscotoxin, flavonoids, as well as acidic arabinogalactan.[12–14] The alkaloid

concentrations also depends on the host tree type.[15] These constituents suggesting that this plant may be an important source of natural products with chemopreventive and chemotherapeutic activities.[16]

The antioxidant molecules found in mistletoe are represented by flavonoids quercetin and quercetin methyl ethers.[14] Quercetin and flavonol, has been demonstrated to display a very strong antioxidant activity, often accompanied by antiviral and antibacterial activity.[17] Sengul et al. showed that *Viscum album* had the highest antioxidant activity (82.23%) among some medicinal plants.[18] Phenolic acids in mistletoe plants, such as digallic acid and *o*-coumaric acid in the free or glycosilated forms are also considered to be compounds with antioxidant activity,[11] they readily forms a resonance stabilized phenoxy radical which accounts for their potent antioxidant potential.[19]

To evaluate the antioxidant capacities of plant extracts, numerous in vitro methods have been developed.[20] In the current study, DPPH method, which relies on the reduction of 2, 2-diphenylpicrylhydrazyl (DPPH) radical was used. This method is simple, fast and inexpensive for measuring the antioxidant capacity. Furthermore, it is not specific to any particular antioxidant component and could be applied to either solid or liquid samples. The DPPH with free radical has a purple color and a strong absorption maximum at 517nm. As shown in Figure 1, when the odd electron of DPPH radical becomes paired with a hydrogen from a free radical scavenging antioxidant it will be reduced to DPPH-H, the color becomes yellow and the molar absorptivity of the DPPH radical at 517nm reduces from 9660 to 1640.[21]

The anti antioxidants activity is usually compared with a reference standard and a common example is Trolox.[21,22] Trolox is a (Hoffman-La Roche) trade name for (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid); a water soluble vitamin E analogue used in this research as an antioxidant standard.[23]

Anticoagulants play a role in the prevention and treatment of thromboembolic disorders.[24,25] Anticoagulant drugs consisting of heparin and its derivatives, and vitamin K antagonists, have been the main anticoagulants in clinical practice. Despite their efficacy, major and life-threatening side effects of these agents have also been reported.[26,27] Plants may serve as an alternative source for the development of new anticoagulants due to their biological activities. There is compelling evidence demonstrating that the consumption of dietary anticoagulants or phytochemicals with anticoagulant properties can ultimately minimize the risks of thromboembolic diseases.[28,29]

In Palestine, mistletoe is widely used in the folk medicine as antimicrobial, antidiabetic and anticancer. The plant mainly cultivated from almond and olive hosts grown in northern Palestine. No previous bioactivity studies have been reported on *Viscum album* in the country. This study aimed to investigate bioactivity of this plant and search for differences in activity that might be influenced by host type.

METHODOLOGY

Materials and reagents

Trolox ((S)-(-)-6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich. Methanol analytical grade was used for extraction purposes. Other chemical reagents were purchased from reliable commercial sources.

Microorganisms used in the current study were reference strains obtained from the American Type Culture

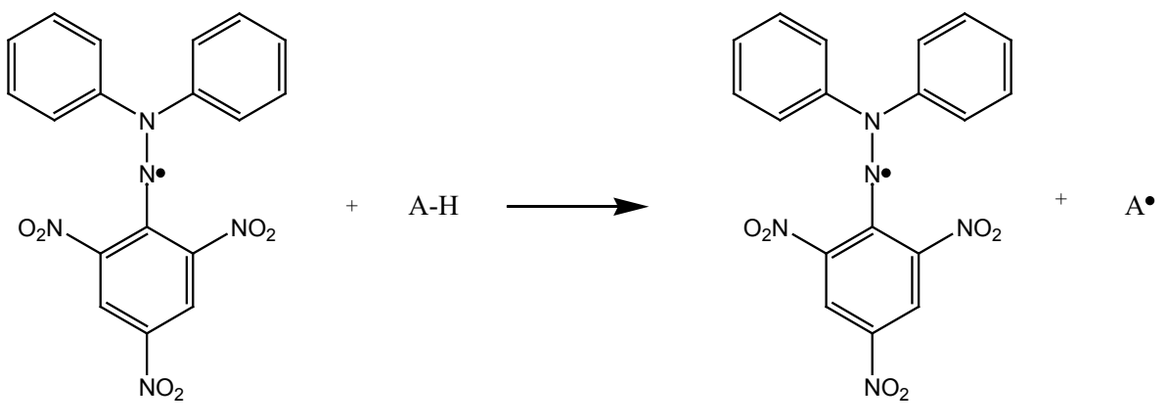

Figure 1. DPPH reduces to DPPH by the antioxidant agent.

Collection (ATCC), including *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (MRSA Positive), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853). In addition, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Proteus mirabilis* clinical isolates were included. Isolates were identified by Gram stain, growth on MacConkey, and API20E (BioMericux, France).

Instrumentation

Shaker device (LabTech Shaking Incubator) was used in extraction of the plants, rotatory evaporator [Heidolph OB2000 (the heater) and Heidolph VV2000 (the rotator)] was used for condensation purpose, Spectrophotometer (Jenway 6505 UV/Vis Spectrophotometer) was used to measure the optical density.

Anticoagulant activity tests were done using HumaClot Duo plus Hemostasis Analyzers, HUMAN, Germany. The samples were collected in sodium citrated blood tubes (Vacutainer, BD) and centrifuged by Hettich Zentrifugen, Germany.

Plant material

Leaves of *Viscum album* were collected from olive and almond trees in spring from places in north Palestine. The leaves of plants were dried in dark and stored in dry place for the research until it was started in summer.

Extract preparation

Leaves of *Viscum album* hosted by olive and almond were powdered separately using a grinder. The extraction was performed at room temperature. About 100g of the grounded leaves were soaked in 1 Liter of methanol (99%) and put in a shaker device at 100 rounds per minute for 72 hours and stored in refrigerator for 4 days. The extracts were then filtered using filter papers. The extract was then concentrated under vacuum on a rotatory evaporator. The crude extract was stored at 4ºC for further use.

Data analysis

The antioxidant activity was reported as percentage of inhibition. The inhibition of the host plants and Trolox standard at different concentration were plotted and tabulated and the IC_{50} for each of them was calculated using the BioDataFit fitting program in which the sigmoidal fitting model was the adapted model.

The antimicrobial activity of the plant was compared to different standards by measuring the inhibition zone, the tests were carried out in duplicates for each concentration and inhibition zones of bacterial growth were measured and reported in mm.

For PT and PTT tests the plant extracts of varying concentrations was carried out in duplicate and the average clotting time in seconds were measured and reported.

Anti oxidant activity

Trolox standard and plant working solutions

A stock solution of a concentration of 1mg/1ml in methanol was firstly prepared for the two plant extracts and trolox. The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100µg/ml) were prepared by suitable dilution with methanol from the stock solution.

Spectrophotometric measurements

DPPH was freshly prepared at a concentration of 0.002% w/v. The DPPH solution was mixed with methanol and the above prepared working concentration in a ration of 1:1:1 respectively. The spectrophotometer was zeroed using methanol as a blank solution. The first solution of the series concentration was DPPH with methanol only. The solutions were incubated in dark for 30 minute at room temperature before the absorbance readings were recorded at 517nm.

Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of the plants and the Trolox standard were calculated using the following formula:

$$\text{Percentage of inhibition of DPPH activity (\%)} = \frac{A - B}{A} \times 100\%$$

where: A = optical density of the blank,

B = optical density of the sample.

The antioxidant half maximal inhibitory concentration (IC_{50}) for the plant samples and the standard were calculated using BioDataFit edition 1.2 (data fit for biologist).[30]

Antimicrobial activity assays

Preparation of inoculum

Stock cultures were maintained at 4ºC on slant of nutrient agar. Active cultures prepared by transferring a loop full of cells from the stock cultures to test tubes of nutrient

broth and incubated at 37°C for 24 hrs. Cultures were then diluted with fresh nutrient broth to achieve optical densities corresponding to 10^8 CFU/ml (turbidity = McFarland barium sulfate standard 0.5) as described by Smânia *et al.*, 1999.

Antimicrobial susceptibility test

Agar diffusion well-variant was performed as described by Smânia *et al.,* 1999.[31] Bacterial inoculums were uniformly spread using sterile cotton swab on a Petri dish of Mueller Hinton Agar (MHA). Six wells (6mm in diameter) were made on each plate. Three concentrations (10, 20, and 30%) of each plant extract were prepared using DMSO and methanol separately. A sample of 50µl of each extract was loaded into each well and plates were then incubated at 37°C for 24 hours under aerobic conditions. Cultures were carried out in duplicates and inhibition zones of bacterial growth were measured in mm.

Agar diffusion disc-variant was performed using selected staphylococcal reference strains and the wells were replaced by 6mm sterile disc loaded with 20µl of sample of plant extract. Reference commercial antibiotic discs were used as positive or negative controls. These included Vancomycin (30µg), Gentamicin (10µg), Oxacillin (5µg), and Cefotaxime (30µg). The inoculation of MHA and measurements of inhibition zones were performed as shown above in the well method. Negative controls were made by replacing plant extracts with DMSO solution.

Anticoagulant activities

Blood sample collection

Blood samples from three healthy volunteers were collected in sodium citrated blood tubes (Vacutainer, BD) and centrifuged for 15 minutes at 1500g to prepare platelet poor citrated plasma.

PT and a PTT tests

Tests were performed using HumaClot Duo plus Hemostasis Analyzers, HUMAN, Germany.

Equal volumes of plant extracts of varying concentrations and citrated platelet poor plasma were incubated for 5 min at 37°C.

For PT test, 100µl of tissue thromboplastin (HUMAN) was added to 50µl to the pre-warmed mixture and clotting time was measured.

For aPTT test, 50µl of rabbit brain extract was added to equal volume of the pre-warmed platelet poor plasma-extract mixture, incubated for 1 minute after which 50µl of 0.025M calcium chloride (HUMAN) was added and clotting time was measured PBS was used as a control.

RESULTS AND DISCUSSION

Antioxidant activity

The free radical scavenging activity of the methanolic extract of *Viscum album* hosted by olive and almond trees has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100µg/ml. The zero inhibition was considered for the solution which contained only DPPH without any plant extract. The results are shown in Table 1. The table readings are presented in Figure 2.

The results showed that the anti-oxidant activity reaches a platuea at a concentration more than 100µg/ml for

Table 1. Absorbance and the calculated percentage inhibition activity for Trolox standard and *Viscum album* hosted on almond and olive.

Concentration µg/ml	%inhibition Trolox	%inhibition Olive	%inhibition Almond
1	37.705	1.098	5.738
2	54.098	1.639	6.557
3	81.148	4.918	11.475
5	86.066	5.639	13.115
7	91.803	9.836	17.213
10	92.614	17.614	31.25
20	93.75	43.182	55.682
30	94.886	59.091	85.227
40	94.886	71.591	89.773
50	95.455	82.955	91.477
80	96.023	89.773	92.614
100	97.159	90.341	92.773

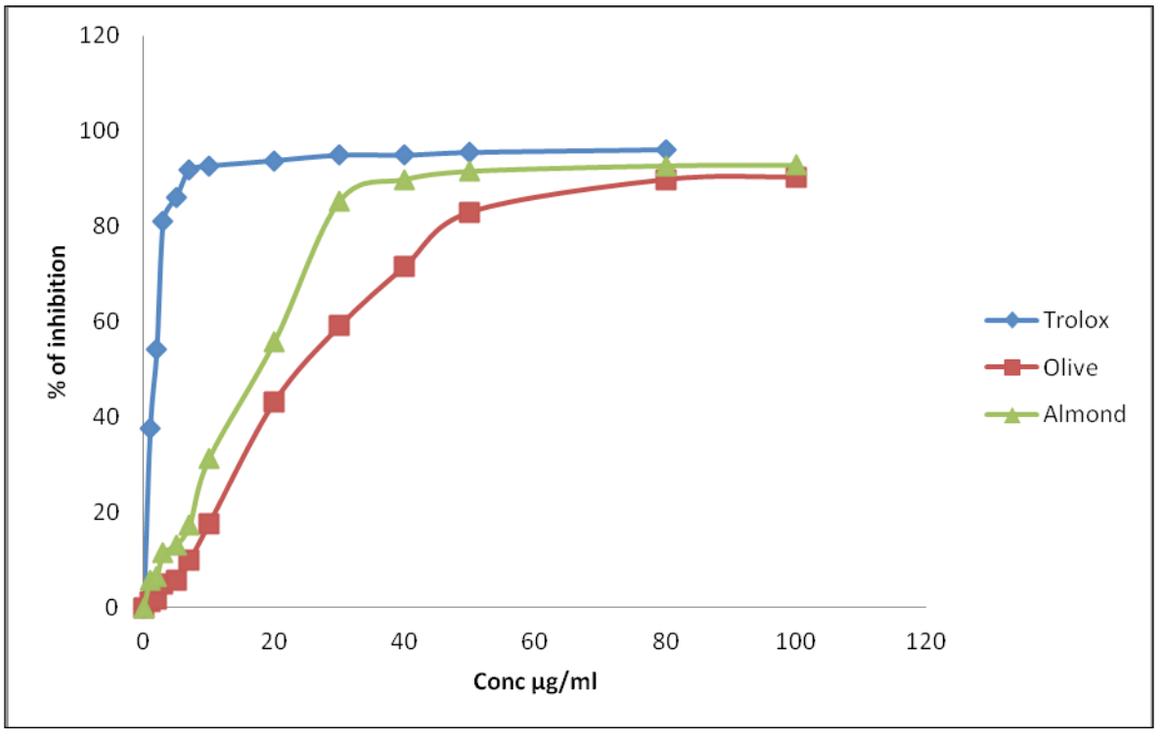

Figure 2. Inhibition activity of Trolox standard and *Viscum album* hosted on almond and olive.

Trolox standard as well as both *Viscum album* hosted by both of the plants. The Graphs show a difference in anti oxidant activity for two host plants. The more potent activity was for *Viscum album* hosted on almond. The Calculated antioxidant IC_{50} for olive *Viscum album* was 25.34±3.8µg/ml and for almond *Viscum album* 15.37± 2.2µg/ml, while it was 1.52±0.05µg/ml for the trolox standard. The results clearly demonstrate a difference in antioxidant activity for *Viscum album* cultivated from different plants. The antioxidant activity was comparatively lower compared to the Trolox reference standard which is known to have a huge antioxidant activity.

Antimicrobial activity

Antimicrobial activity of *Viscum album* methonolic and DMSO crude extracts was investigated against several reference and clinical isolates. The activity was assayed using different extract concentrations on agar plates. Data presented in Table 2 show the results of agar diffusion well-variant using DMSO. Crude DMSO extracts of both variants of *Viscum album* showed strong inhibition effects on the growth of *Staphylococcus aureus* (ATCC 25923) and MRSA. However, the inhibition effect of almond variant was more pronounced compared to that of olive in all examined concentrations. Agar diffusion disc-variant method showed similar effects on bacterial growth. Metabolic extracts of both variants showed similar effect on the growth of Gram positive tested strains (data not shown).

The effect of crude DMSO extracts of both variants showed limited growth inhibition activity in all tested Gram-negative reference and clinical isolates except *Proteus mirabilis*. These extracts failed to show any effect on the growth of *Proteus mirabilis*. No clear differences in growth inhibition zones of both variants on Gram-negative bacteria were observed.

Methanolic extracts of both variants of *Viscum album* showed limited effect on the growth of *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus mirabilis*, however, almond extracts showed some inhibitory effects on the growth of *Escherichia coli* (ATCC 25922) (data not shown). Antibiotic susceptibility testing results of the reference and clinical strains are shown in Table 2.

Anti coagulant activity

The PT and aPTT are indicators of coagulation and are used to determine the clotting of the tissue factor and contact pathways respectively (Table 3).

A significant prolongation of PT was observed with the extract of the *Viscum album* hosted on almond and olive

Table 2. Antimicrobial activity of DMSO extracts of *Viscum album* hosts.

Bacterial Strains	Viscum. album-Olive (%)			Viscum. album-Almond (%)			Antibiotics			
	30	20	10	30	20	10	OX	VA	CN	CTX
Gram-positive strains										
Staphylococcus aureus (ATCC 25923)	32	26	20	35	30	25	20	19	25	30
Staphylococcus aureus (MRSA Positive)	26	24	20	32	30	24	0	25	25	0
Gram-negative strains										
Escherichia coli (ATCC 25922)	12	15	16	16	16	14	–	–	24	35
Pseudomonas aeruginosa (ATCC 27853)	15	14	13	13	14	14	–	–	21	22
Klebsiella pneumoniae	11	12	12	11	11	10	–	–	21	32
Enterobacter cloacae	10	13	14	12	11	10	–	–	21	33
Proteus mirabilis	0	0	0	0	0	0	–	–	0	35

Abbreviations: OX, Oxacillin; VA, Vancomycin; CN, Gentamicin; CTX, Cefotaxime.

Table 3. Viscum album on prothrombin time and Partial Thromboblastin Time.

Effect of organic extract of *Viscum album* on prothrombin time (s)				
	Almond		Olive	
	%	Mean + SD	%	Mean + SD
Control 22.5 + 0.3	10%	*	10%	*
	5%	*	5%	195 + 3.2
	2.50%	31.6 + 0.6	2.50%	26.9 + 1.1
	1.25%	22.9 + 0.5	1.25%	21.6 + 1.2
Effect of organic extract of *Viscum album* on Activated Partial Thromboblastin Time(s)				
	Almond		Olive	
	%	Mean + SD	%	Mean + SD
Control 32.6 + 2.1	10%	*	10%	*
	5%	*	5%	*
	2.50%	55.7 + 3.5	2.50%	67 + 1.5
	1.25%	27.3 + 2.1	1.25%	54.3 + 3.1
	0.75%	25 + 1.7	0.75%	+ 0.6

(*) Clot detection time was greater than 200 seconds.

trees at a concentration of 2.5%. The extract of the *Viscum album* hosted on almond trees prolonged aPTT at a concentration of 2.5% while the extract of the *Viscum album* hosted on olive trees were noticed at a concentration as low as 0.75%.

CONCLUSION AND FUTURE WORK

The observed variations in bioactivity of the tested variants strongly indicate that bioactivity is influenced by host plant. This suggests a careful consideration of the host plant when administering this plant in to folkloric medicine. Further research is required to determine active ingredient involved in each bioactivity.

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