

In-vitro* and *in-vivo* studies on the cell sap extract of some plant waste and using a natural biocidal to control the red mites *Tetranychus urticae

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Abstract

In the present work, three different samples of plant waste were studied; these are leaves of moringa, olive, and pomegranate trees. All three samples contain phenolic acids, phenols, and flavonoids. The total phenolic compounds (TPCs) in the samples were in moringa, olive, and pomegranate (780, 698, and 650 $\mu\text{g/g}$), respectively. HPLC-fractionation of phenolic compounds revealed the existence of 8 phenolic acids and 4 phenols. The most abundant acid is *p*-hydroxybenzoic acid and the levels are 490.2, 310.5, 300 $\mu\text{g/g}$, and the least abundant acid is ellagic acid (1.0, 20.01, and 5.60 $\mu\text{g/g}$). The highest concentrations of hydroxytyrosol are 190.04, 310.04, and 180.75 $\mu\text{g/g}$, and the levels of vanillin are 50.01, 24.20, and 30.19 $\mu\text{g/g}$ sequentially samples and found 14 compounds is a flavonoid. And when those extracts were tested at four concentrations (2.5, 5, 7.5, and 10 ml/L). The results obtained from the use of natural pesticides under study at the previously mentioned concentrations were as follows (35, 57, 73, and 91 %) moringa extract, (19, 30, 43, and 59%) olive extract, and (33, 45, 65, and 72 %) pomegranate extract with the use of a standard chemical pesticide (Vertimec 1.8%) against mites, the result obtained from it was 33% of insects. The results of the *in-vitro* experiment were confirmed by an *in-vivo* applied field experiment.

Keywords: bioactive compounds; cell sap extract; natural biocides; plant wastes.

1. Introduction

Plant wastes are biomass or plant-matter such as (wood, branches and tree stumps and leaves) (Ghanema *et al.*, 2019). These biomass or plant leaves contains many bioactive compounds within these plants are responsible for their medicinal value such as alkaloids, tannin, flavonoid, phenolic compounds (Shihabudeen *et al.*, 2010).

Olive tree (*Olea europaea* L.) is one of the most important fruit trees in Mediterranean countries (Guinda *et al.*, 2004). Olive leaves were benefits to be derived from utilization of any of its byproducts and contains important for their secondary metabolites such as the secoiridoid compounds oleacein and oleuropein, the former responsible for hypotensive activity and phenolic compounds and flavonoids these are consider defensive compounds (Hansen *et al.*, 2007).


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Drumstick tree (*Moringa oleifera*) has various industrial applications. The leaves have a high protein content 27% and are rich in vitamins A, C and many of elements such as calcium, iron and phosphorus. It has been found useful in nutrition, agriculture, soil control, water purification, industrial applications, cattle feed. and for treating various types of illnesses in humans and livestock (Jed and Fahey, 2005).

The increasing world population necessitates the agricultural sector to develop sustainable systems that can produce enough food for the growing numbers of people. This entails both producing more food and protecting what is currently being produced. To date, efforts to boost food production have led to heavy reliance on mineral fertilizers and synthetic chemicals for improving yields and protecting crops from pathogens and pests to boost food production and reduce crop loss (Carvalho, 2017). Herbivorous arthropods, such as insects and mites, are key pests in several crops, causing considerable yield losses worldwide (Savary *et al.*, 2019).

Phenolic compounds are arguably the most abundant secondary metabolites in plants and

are often found in the cell walls and vacuoles of epidermal and subepidermal cells. These phenolics can be constitutive or induced in plants. Constitutive phenolics are pre-formed in plants, usually being synthesized during normal growth and development, whereas induced phenolics are synthesized in response to biotic and abiotic stresses. Induced phenolics can also be constitutive, but their synthesis and accumulation are increased by exposure of the plant to biotic and abiotic stresses (Lattanzio *et al.*, 2006).

According to previous *in vitro* and *in vivo* studies pomegranate (*Punica granatum* L.) leaves have revealed its beneficial physiological activities, especially its antioxidants, antimicrobial, anti-inflammatory properties and bioactive compounds (secondary metabolites) such as phenolic compounds and flavonoids (Pagliarulo *et al.*, 2016).

Green pesticides provide materials for natural and beneficial pest control and increased production per unit area (Habib *et al.*, 2015). Broad spectrum synthetic insecticides have consequences which badly affect human health, environmental cause pollution, destroy the food chain and kill beneficial insects (Isman and Machial, 2006). The present work was performed to study the insecticidal activities (against of red mites) of moringa, olive and pomegranate cell sap leaves of the cucumber plant infected with the red mites *Tetranychus urticae*.

2. Materials and Methods

2.1. Plant Materials

Samples of leaves of Moringa were collected in November 2020 from the farm of faculty of agriculture, Beni-Suef University. and olive kalamata and Manfaloty pomegranate leaves were collected in November 2020 from the farm of Agricultural Research Station in Sids, Beni-Suef - Egypt. All solvents used throughout the whole work were analytical grade and distilled before use. Caffeic acid (98%) and folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Gerbsaure Chemical Co. Ltd., Germany, respectively.

2.1.1. Preparation of plant extract

Leave of moringa, olive and pomegranate were manually separated, cleaned from dust followed by seed removal then mechanically pressed by a Carver hydraulic laboratory press according to (Radwan and Sekina, 2016; Abdou *et al.*, 2020; Hassan, H.M. *et al.*, 2021).

2.2. Phytochemical analysis

2.2.1. Total phenolic compounds (TPCs)

Following the Folin's reagent, spectrophotometric ally method, TPCs of samples were measured as described by (Siriwoharn *et al.*, 2014) with minor modification for 96-well micro-plates. Briefly, 15 μ l of diluted samples were placed into wells of 96-well micro- plates (GS, USA). Consequently, 240 μ l of Folin was added and left for half an hour in darkness at ambient temperature. Then, 15 μ l of Na₂CO₃ 20% (wt/wt) were added to each well, adjust the micro-plate reader at shaken mode before start reading the TPC concentrations. The absorbance was measured at $\lambda=755$ nm with the micro-plate reader ACCURIS Smart Reader (Edison, NJ, USA). TPC was calculated using a standard curve set of serial dilutions of gallic acid (GAE). TPC values were performed in triplicate and expressed as [mg GAE/g(FM)] (Hassan, A.A. *et al.*, 2021).

2.2.2. Estimation of total flavonoid content (TFC)

Following previously described method (Chang *et al.*, 2002). To determine the content of total flavonoid with minor modifications. 25 μ l of samples were added to 75 μ l of MeOH 96% (v/v). Then, 5 μ l of 10% Aluminum chloride (AlCl₃) and 5 μ l of potassium acetate, then 140 μ l with distilled water. Kept for half an hour in darkness at 25°C, the readings was measured at $\lambda=415$ nm. TFC content was calculated using a standard curve prepared using gradient dilutions of quercetin. The TFC was presented as mg QE/g (FM) (Ahmed *et al.*, 2020).

2.3. High performance liquid chromatography (HPLC) analysis

Identification of phenolic acids in the plant cell sap was performed on a Varian HPLC instrument central laboratory - institute of food technology - Agricultural Research Center -

Giza – Egypt using an ODS2 C18 reversed phase column according to (Chapuis-Lardy *et al.*, 2002).

2.4. Insecticidal activity

2.4.1. *In vitro* study

The insecticidal studies were performed at the chemistry laboratory of faculty of Agriculture, Beni-Suef University, Egypt. The leaves of the cucumber plant infected with the red mites *Tetranychus urticae* were obtained and the activity of the insect was confirmed on the surface of the leaves by examining them by microscope and then placing them in Petri dishes and dividing them into an untreated group (control) - and a group treated with the cell sap of the leaves under study (at a concentration of 2.5, 5, 7.5, 10 ml /liter) and a group treated with a standard chemical pesticide (Vertimec 1.8% 40ml/100l) used against the insect under study (Ascher and Mirian, 1981).

The percentage of dead insects is calculated from the following equation:

$$D. I. \% = \frac{A-B}{A} \times 100$$

*Where: A= Total number of insects and B= Number of live insects

2.4.2. *In vivo* study

This experiment was carried out in a greenhouse planted with cucumber and 100 plants infected with red mite were selected for each treatment. The percentage of sterility was calculated (Radwan and Sekina, 2016).

$$\text{Sterility, \%} = 100 - [a \times b / A \times B] \times 100$$

*Where: a = No. of eggs laid / female in treatment, b = % of hatch in treatment, A = No. of eggs laid / female in control and B = % of hatch in control.

2.5. Statistical analysis

The statistical analysis was performed using SPSS statistical software. Differences in mortality percentages of 4th instar larvae of cotton leaf worm were compared using one-way analysis of variance (ANOVA). Data were expressed as mean \pm standard error. The statistical significance of differences between individual means was determined by student "t"

test for paired observations (IBM® SPSS®, 2011).

3. Results and discussion

3.1. Phytochemical content

3.1.1. Total phenolic compounds (TPCs) and total flavonoids (TFs)

The amount of total phenolic compounds and total flavonoids differed significantly in extracts sample (Table 1). The values of phenolics in the samples were moringa, olive and pomegranate, respectively (780, 698 and 650 $\mu\text{g/g}$) measured by Folin's reagent method.

Table 1. Levels of total phenolic compounds (TPCs).

Samples	TPCs ($\mu\text{g/gm}$)
Moringa leaves	780 \pm 7.7
Olive leaves	698 \pm 6.7
Pomegranate leaves	650 \pm 6.3

Value are mean \pm S.D. (N=3)

3.2. High performance liquid chromatography (HPLC) analysis to fractionation of phenolics and flavonoids

When making the fractionation of simple phenolic compounds by HPLC analysis, found: (8 phenolic acids compounds and 4 phenol) (Table 2) (catechin, p-Hydroxy benzoic acid, Vanilin, p-Coumaric acid, Gallic acid, Caffeic acid, Tyrosol, Ferulic acid, Hydroxy tyrosol, Tannic acid, Ellagic acid and Syringic acid).

The highest value was P- hydroxy benzoic acid (490.2, 310.5 and 300 $\mu\text{g/g}$) and the lowest value is Ellagic acid (1.0, 20.01 and 5.60 $\mu\text{g/g}$) in the phenols was the highest value is Hydroxy tyrosol (190.04, 310.04 and 180.75 $\mu\text{g/g}$) and the lowest value is Vanilin (50.01, 24.20 and 30.19 $\mu\text{g/g}$) sequentially samples.

Table 3 showed that the fractionation of flavonoids by HPLC analysis and found 14 compounds (APig.6-arabinose 8-glucos, APig.6-rhamnose 8-glucose, Rutin, Narengin, Luteo-7-glucose, Hesperidine, Rosmarinin, Quercetrin, Apigenin-7-glucose, Quercetin, Naringenin, Kamp-3, (2-p-comaroyl) glucose, Kampferol and Apigenin) was the highest value is Hesperidine (100, 90.87 and 70.23 $\mu\text{g/g}$) and the lowest value is Luteo-7-glucose (0.90, 0.08 and 0.01 $\mu\text{g/g}$).

Table 2. HPLC-fractionation of phenolic compounds ($\mu\text{g/g}$) in moringa, olive and pomegranate leave cell sap.

Phenolic substance	Moringa leaves	Olive leaves	pomegranate leaves
Catechin	110.00	90.00	67.00
Vanilin	50.01	24.2	30.19
Tyrosol	150.30	212.50	122.00
Hydroxy tyrosol	190.50	310.04	180.75
Gallic acid	40.00.20	19.04	11.00
Caffeic acid	68.30	44.30	50.00
p-Coumaric acid	166.00.20	140.04	150.80
Ferulic acid	78.50	31.02	20.91
p-Hydroxybenzoic acid	490.02	310.50	300.00
Tannic acid	100.00	78.00	11011
Ellagic acid	1.00	20.01	5.60
Syringic acid	40.00	11.10	10.00

Table 3. The concentration ($\mu\text{g/g}$) of flavonoids by HPLC in moringa, olive and pomegranate leave cell sap

Flavonoids	Moringa leaves	Olive leaves	pomegranate leaves
Rutin	7.01	2.32	1.19
Narengin	1.02	4.30	0.08
Hesperidine	100.00	90.87	70.23
Rosmarinine	2.00	5.01	1.15
Quercetrin	1.00	3.90	6.90
Quercetin	70.00	89.10	99.00
Narengenin	10.04	3.72	1.00
Kamferol	60.11	24.07	18.00
Apigenin	1.11	0.18	0.04
Luteo-7-glucose	0.90	0.08	0.01
Apigenin-7-glucose	10.11	9.40	7.45
Kamp-3, (2-p-comaroyl) glucose	3.00	100.00	45.13
APig.6-arabinose 8-glucose	60.11	76.45	54.00
APig.6-rhamnose 8-glucose	5.00	1.00	8.23

The flavonoids listed in Table 3 show existence of 5 different glycosides and 9 components as aglycone form. All assayed flavonoids reported here are ideal antioxidants with high potential. The APig.6-arabinose 8-glucose, APig.6-rhamnose 8-glucose, Luteo-7-glucose, Apigenin-7-glucose and Kamp-3, (2-p-comaroyl) glucose are sugar sources and functional phytochemical. Many flavonoids are shown to have antioxidative activity, free radical scavenging capacity, coronary heart disease prevention, hepatoprotective, anti-inflammatory, and anticancer activities, while some flavonoids exhibit potential antiviral activities. In plant systems, flavonoids help in combating oxidative stress and act as growth regulators. For pharmaceutical purposes cost-effective bulk production of different types of flavonoids has been made possible with the help of microbial biotechnology (Kumar and Pandey, 2013).

3.3. Anti-insect activity of moringa, olive and pomegranate leaves cell sap

3.3.1. In vitro study

In the laboratory study, the leaves infected with the red spider were treated by the cell sap of the leaves of the aforementioned samples at the mentioned concentrations, and the leaves infected with the whole insect of the red spider were examined by a microscope before and after the treatment. Fig. (1) and (2) showed that all concentrations had a significant effect on killing insects in different percentages, where the effect of concentrations were 2.5 ml/l (33, 19, 35%), 5 ml/l (45, 30, 57%), 7.5 ml/l (65, 43, 73%) and 10 ml/l (72, 59, 91%) and the effect of the standard insecticide was 33%. Therefore, the cell saps of the leaves under study are considered anti-growth, development and also lethal to the whole insect of the red mite, The results obtained explain that the leaf cell saps of the samples under study contain phenolic compounds (tyrosol and catechin) phenolic acids (*p*-hydroxybenzoic acid and tannic acid) and

flavonoids (quercetin and kampferol), which are considered defensive compounds that kill insects

such as the red mite. Those results are consistent with (Radwan and Sekina, 2016).

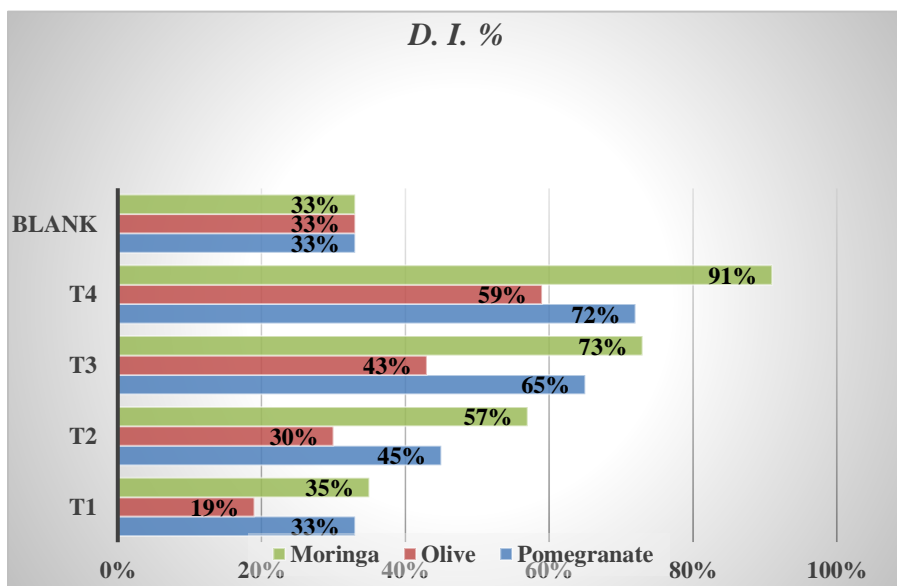


Figure 1. Effect of the leaves cell sap on D. I. % "In vitro study" *D. I. %: Dead Insect %

3.3.1. In vivo study

The results of the *in vivo* study confirmed the results of *in vitro* study, where the treatments in all their concentrations showed a significant effect of the plants infected with the red mite compared to the standard chemical pesticide (Vertimec 1.8%) used to combat that insect in the field, with the

plants recovering from the infection morphologically and physiologically, and a good health condition appeared on the treated plants, as shown in Fig. (2, 3) and Table (4). The results of the experiment were in agreement with the results by (Farag *et. al.* 2014).



Figure 2. Design of laboratory experiment and examination of insect vitality under the microscope

Table 4. Effect of the leaves cell sap on sterility % "In vivo study"

Treatments	Moringa	Olive	Pomegranate
T1 (2.5 ml/l)	26 %	11%	20 %
T2 (5 ml/l)	43 %	14 %	30 %
T3 (7.5 ml/l)	60 %	36 %	52 %
T4 (10 ml/l)	80 %	51 %	61 %
Blank	24 %	24 %	24 %

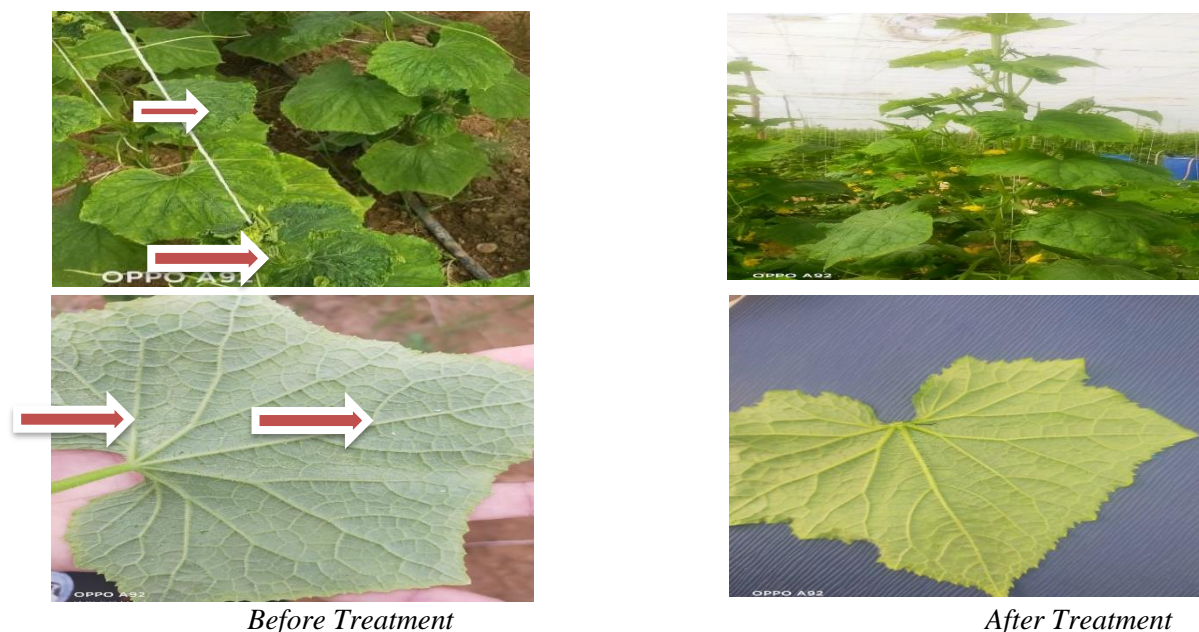


Figure 3. The effect of treatments for the same plant before and after treatment

4. Conclusion

At the end of our research, we recommend the interested and workers in the agricultural field to use the cell sap of moringa, olive and pomegranate leaves to control the red mite and insects that are similar to it in its life cycle and the naturalness of its growth and the plant families that grow on it as natural, vital and safe pesticides in everything in terms of extraction. It contains any chemical solvents. At the same time, those plant leaves are considered polluting wastes of the environment. Here comes the role of getting rid of these wastes in a way that benefits the environment and at the same time is very economical.

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