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Phytochemical Analysis, Larvicidal Activity and Cytotoxic Properties of Malvarosa (*pelargonium graveolens*) Leaf Extract

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Plants have been utilized as medicines for thousands of years. The pharmacological properties are naturally derived compounds, the isolation and characterization of pharmacologically active compounds from medicinal plants continues today.
Phytochemical analysis, larvicidal activity and cytotoxic properties of malvarosa (*Pelargonium graveolens*) leaf extract was done to determine the secondary metabolites present in *P. graveoleons*. The preliminary phytochemical screening of *P. graveolens* crude extracts showed the presence of bioactive components like Alkaloids, Essential Oils, Flavonoids, Higher Alcohols, Phenols, Steroids, and Tannins. The study identified the larvicidal activity of *Pelagonium graveoleus* ethanolic extract against *Aedes aegypti* mosquito. The Linear Regression Probit Analysis was used to identify the concentration (ppm) that will kill certain percentage of mosquito larvae. The lethal concentrations for the *P. graveolens extract* to achieve 50% and 90% mortality or LC50 and LC90 were estimated at 1,355 and 5,443 ppm respectively with linear regression equation of $Y = 2.12x – 1.64$.
The biological activity of different concentrations of *P. graveolens* leaf ethanolic extract was tested for their ability to inhibit the proliferation of human lung cancer cell lines (A549), at 50 µg/mL concentration inhibited the proliferation of 55.51 % cells. The concentration needed to inhibit the proliferation of 50% (IC_{50}) of the A549 cells is 46.72 µg/mL. Different concentration of *P. graveolens* extracts exhibit growth inhibitory effect and larvicidal activities.

Key Words: *P. graveolens,* phytochemical, larvicidal, cytotoxic properties, cell line (A549), proliferation

Introduction

Several illnesses have emerged throughout time and corresponding cures have been identified by researchers, pharmaceutical companies and chemical laboratories. Some of these cures are derived synthetically while others are from plant-based compounds.

In majority of the studies though, naturally derived compounds were shown to outweigh those that were synthetically- based for a variety of reasons.

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Despite the recent interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, natural products, and particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (Newman *et al*., 2000, Butler, 2004). Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques (Balunas and Kinghorn, 2005).

The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents (Nostro *et al*., 2000). Phytochemicals are primary or secondary compounds that naturally occur in the medicinal plants, leaves, vegetables and roots that have defense mechanism against various diseases (Wadood, 2014).

Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Newman *et al*., 2000; Butler, 2004; Samuelsson, 2004). Isolation and characterization of pharmacologically active compounds from medicinal plants continue today.

Materials and Methods

Plant Materials

The malvarosa (*Pelargonium graveolens*) plants were obtained from Benguet State University Orchidarium in La Trinidad, Benguet.

Plant Preparation and Extraction

Fresh leaves of *P. graveolens* were collected and washed thoroughly. They were then air-dried under shade at room temperature for one week. Once dried, the leaves were finely powdered using an electrical blender (Camel CBL-1020G 1.5L).

Twenty grams of powdered leaves were extracted with 200mL of ethanol to make a 1:10 concentration. The extract was separated from the residue by filtration using a filter paper (Whatman no. 1). The residual solvent of ethanol extracts was removed under pressure at 40 ℃ using rotary evaporator.

Phytochemical Screening

The crude extracts were assessed through TLC using 80% DCM and 20% EtOH (4:1) as solvent system for the roots, while the flowers and leaves made use of 1:1. Afterwards, the TLC plates were visualized under UV365.

Different spray reagents were used to test the class of compounds present in the extracts. Vanillin–H2SO4 was used to test for the presence of Essential Oils, Higher Alcohols, Phenols, and Steroids.

KOH–MeOH H2SO4 was used to identify the occurrence of Anthraquinones, Anthrones, and Coumarins while Magnesium Acetate was used to spot Anthraquinones only.

Tannins and Flavonoids were verified using K3 (FeCN)6– FeCl3 and SbCl3. Also, Dragendorff's reagent was used for the confirmation of Alkaloids.

Larvicidal Activity

Homogeneous batches of 3rd instars to early 4th instars larvae reared in the Insectary of Standards and Testing Division, Industrial Technology Development Institute, Department of Science and Technology were used in the test.

Batches of 20 third instar and early fourth instar larvae were submerged to 250 mL cups containing the appropriate volume of solution concentrations under test (Table 1). Three (3) replicates were set up for each concentration.

Table 1. Final test concentrations of *P. graveolens* ethanolic extract

Final Test Concentrations		**Amount of10% plant extract solution added to water**	**Amount of water needed to make a 50mL.solution(mL)**
Parts per million (ppm)	**Percentage**		
500	0.05%	0.25 mL	49.75 mL
1,000	0.10%	0.50 mL	49.50 mL
2,000	0.20%	1.00 mL	49.00 mL
4,000	0.40%	2.00 mL	48.00 mL
7,000	0.70%	3.50 mL	46.50 mL

Equal numbers of controls were also set up simultaneously. For negative control, dechlorinated tap water with 1% ethanol was used. Abate 1SG mosquito larvicides was used for positive control. The test containers were held at 25–28 ^{o}C temperature and 80 ± 10% relative humidity.

Larval mortality was recorded after 24 hours by counting the number of moribund and dead larvae. These data were used to compute for percentage mortality. Dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region. Moribund larvae are those incapable of rising to the surface or not showing the characteristics diving reaction when the water is disturbed. The results were recorded.

Cytotoxic Property

The cytotoxic property of *P. graveolens* leaf extract was determine through MTT assay using the human lung adenocarcinoma A549 cells cultured at the Mammalian Cell Culture Laboratory, Institute of Biology, College of Science, University of the Philippines- Diliman.

The assay detects the reduction of MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide] by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and hence the measurement of cytotoxicity cell and viability. A549 cells were seeded in sterile 96- well plates until 4 x 104 cells/ mL density was attained. The plates were then incubated overnight at 37℃ and 5% CO2.

Four concentrations of the extract were prepared as treatment: 50µg/mL, 25µg/mL, 12.5µg/mL and 6.25µg/mL. The positive control was Doxorubicin while dimethyl sulfoxide (DMSO) served as negative control. Following incubation, the cells were treated with 10µL per extract dilution. The treated cells were again incubated for 72 hours at 37℃ and 5% CO2.

After incubation, the media was removed and 20µL 3-(4,5-dimethylethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) at 5mg/mL PBS was added. The cells were again incubated at 37℃ and 5% CO2 for four (4) hours. 150µL DMSO was then added to the wells to solubilize the MTT crystals. Absorbance was read at 570nm. Trials were performed in triplicates.

Data interpretation

To determine the larvicidal activity of the extract, the lethal concentrations to kill 50% and 90 % of larval population or LC_{50} and LC_{90} were calculated using Linear regression probit analysis. Percentage cytotoxicity was calculated using the background corrected absorbance as follows (Carmichael, 1987; Manosroi *et al.*, 2006): % Cytotoxicity Index= [1- (OD570-630 treated/ OD570-630 negative control)] x 100. SPSS v.22 was used for statistical computations.

Results

Phytochemical Analysis

Phytochemical screening was done to determine the secondary metabolites present in *P. graveoleons*. Table 2 shows the detected compounds present the extract.

Table 2. Pelargonium graveolens Leaf Extract Phytochemical Result

REAGENTS	COMPOUNDS TESTED	INDICATION OF POSITIVE RESULTS	RESULTS	
			Observations	Positive/ Negative
Preliminary Test (H_2SO_4-H_2O)	Essential Oils	Violet		+
Vanillin- Sulphuric Acid	Higher alcohols Steroids Essential oils Phenols	Triterpenes and sterols appear as blue-violet spots; essential oils form zones with a range of colors; phenols appear as brown spot and fatty acids as yellow spot	Blue-violet spots	+++
Alpha-Naphthol-Sulphuric acid	Sugars	Blue spots	Blue spot	+
KOH-MeOH (Methanolic Potassium Hydroxide)	Anthraquinones Cuomarins Anthrones	Anthraquinones give orange coloration; cuomarins react to form blue (UV 365nm) colored zones; anthrones give yellow (UV 365nm) colored zones	Orange and yellow coloration (UV 365nm)	++
Potassium Ferricyanide- Ferric Chloride	Tannins Flavonoids Phenols	Blue spots	Blue spots	+++
Dragendorf's Reagent	Alkaloids	Brown-orange visible spots immediately on spraying , colors are not stable	Brown-orange	+

The preliminary phytochemical screening of *Pelargonium graveolens* crude extracts showed the presence of bioactive components like Alkaloids, Essential Oils, Flavonoids, Higher Alcohols, Phenols, Steroids, and tannins (Table). The presence of phenolic compounds such as Flavonoids and Tannins indicates the plant's potent to many bioactivities.

It was once a common notion that secondary metabolites are mostly laboratory artifacts but today, there is no doubt that secondary metabolites are natural products (Gloer, 1993).

Secondary metabolites of mixed biosynthesis like alkaloids, flavonoids, oligosaccharides and others with biological activities such as bacteriacidal, fungicidal, antiviral, cytotoxic, analgesic, anticancer, spermicidal, cardiovascular, antiallergic capabilities and many others. It has been also reported that tannins, flavonoids and phenols have antimicrobial activities, antioxidative, anticarcinogenic, and antimutagenic potentials. Flavonoids also important function like antimicrobial activities (Lamb, 2005).

Larvicidal Activity

After 24 hours of exposure, the numbers of dead larva in each concentration of the different treatments were counted. The percentage of larval death for *P. graveolens* extract and the negative control (Dechlorinated tap water with 1% ethanol) were computed and presented in Table 3.

Table 3. Toxicity of P. graveoleus ethanolic extract on Aedes aegypti mosquito larvae

Replicate	Percentage Dead Larvae after 24 hours exposure to different concentrations					
	500 ppm (0.5mg/mL)	**1000 ppm (1.0mg/mL**	**2000 ppm (2.0 mg/mL)**	**4000 ppm (4.0 mg/mL)**	**7000 ppm (7.0mg/mL**	**Control (-)**
1	25	35	60	90	95	0
2	20	35	45	75	95	0
3	20	45	60	90	95	0
Mean	**21.67**	**38.33**	**55.00**	**85.00**	**95.00**	**0**
SD	**2.89**	**5.77**	**8.66**	**8.66**	**0.00**	**0.00**

% dead larvae = no. Dead/total larvae x 100%

The table shows that the mean percentages of dead larva for 500 ppm, 1000 ppm, 2000 ppm, 4000 ppm and 7000 ppm concentrations were 21.67 (±

2.89 SD), 38.33 (± 5.77 SD), 55.00 (± 8.66 SD), 85.00 (± 8.66 SD) and 95.00 (± 0.00 SD) respectively.

Notably, the percentage of dead larvae after 24 hours increases as the concentration increases. This implies that the percentage of mortality is directly proportional to the concentration of the plant extract. Table further shows that as expected, the negative control containing Dechlorinated tap water with 1% Ethanol had 0 mean percentage dead larvae (± 0.00 SD).

The percentage of dead larvae in the different concentrations of the positive control (Abate ISG mosquito larvicide) was computed and presented in Table 4.

Table 4. Toxicity of Abate 1SG mosquito larvicides (Positive Control)

Replicate	Percentage Dead Larvae after 24 hours exposure to different concentrations				
	0.2ppm ($2x10^{-4}$mg/mL)	**0.4ppm ($4x10^{-4}$mg/mL)**	**0.6ppm ($6x10^{-4}$mg/mL)**	**0.8ppm ($8x10^{-4}$mg/mL)**	**1.0 Ppm ($1x10^{-3}$mg/mL)**
1	15	35	55	75	100
2	10	35	50	80	90
3	10	25	50	75	90
Mean	**11.67**	**31.67**	**51.67**	**76.67**	**93.33**
SD	**2.89**	**5.77**	**8.66**	**8.66**	**0.00**

% dead larvae = no. Dead/total larvae x 100%

The table shows that for the positive control, the mean percentages of dead larvae for 0.2 ppm, 0.4 ppm, 0.6 ppm, 0.8 ppm and 1.0 ppm concentrations were 11.67 (± 2.89 SD), 31,67 (± 5.77 SD), 51.67 (± 8.66 SD), 76.67 (±8.66 SD) and 93.33 (± 0.00 SD) respectively.

Similarly the percentage mortality of larvae is directly proportional to the concentration of the positive control. As seen in Table 3, the number of dead larvae increases with an increase in the concentration of the treatment.

The Linear Regression Probit Analysis was used to identify the linear regression equation. This equation is used to calculate for the concentration (ppm) that will kill certain percentage of mosquito larvae.

The linear regression equation and computed concentrations of the different treatments that can kill 50 % (LC_{50}) and 90% (LC_{90}) of the mosquito larvae are presented in Table 5.

Table 5. Lethal concentration of treatments that can kill 50% and 90% of the larvae

Treatments	Linear Regression Equation	LC_{50}	LC_{90}
P. graveolens ethanolic extract	Y= 2.12x - 1.64	1, 355 ppm (1.36 mg/mL)	5,443 ppm (5.44 mg/mL)
Positive control (Abate ISG mosquito larvicide)	Y= 3.69x + 6.16	0.48 ($4.8x10^{-4}$ mg/mL)	1.07 ($1.07x10^{-3}$mg/mL
Negative control (Dechlorinated water with 1% Ethanol	-	-	-

Note: Y= log of percentage mortality; x= concentration in ppm

The above table shows that the needed concentration for *P. graveolens* extract to kill 50% (LC_{50}) of the larvae is 1,355 (1.36 mg/mL) ppm while a concentration of 5,443 ppm (5.44 mg/mL) is needed to kill 90% (LC_{90}) of the larvae.

Cytotoxic Property

Different concentrations of *P. graveolens* leaf ethanolic extract were tested for their ability to inhibit the proliferation of human lung cancer cell lines (A549). Dimethyl sulfoxide was used as a negative control and different concentrations of a chemotherapeutic drug, Doxorubicin were used as the positive control.

The absorbance values were then read at 570nm. There were three replicates for each trial. Results are presented in Figure 1.

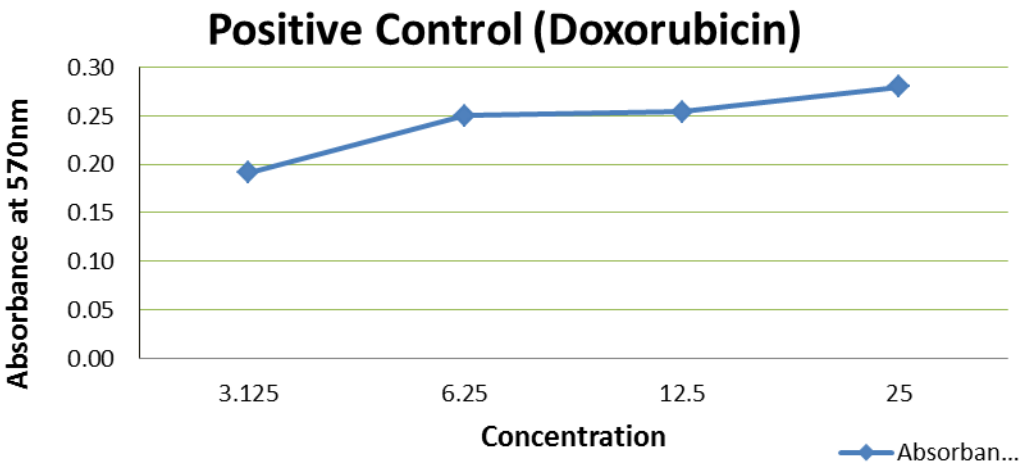

Figure 1. Absorbance rate at 570nm Positive control

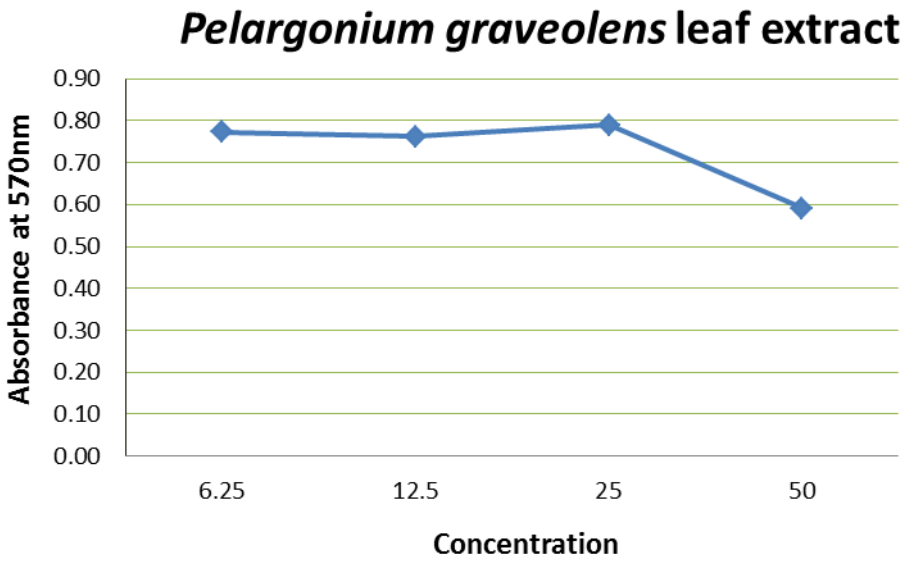

Figure 2. Absorbance rate at 57nm of P. graveolence leaf extract

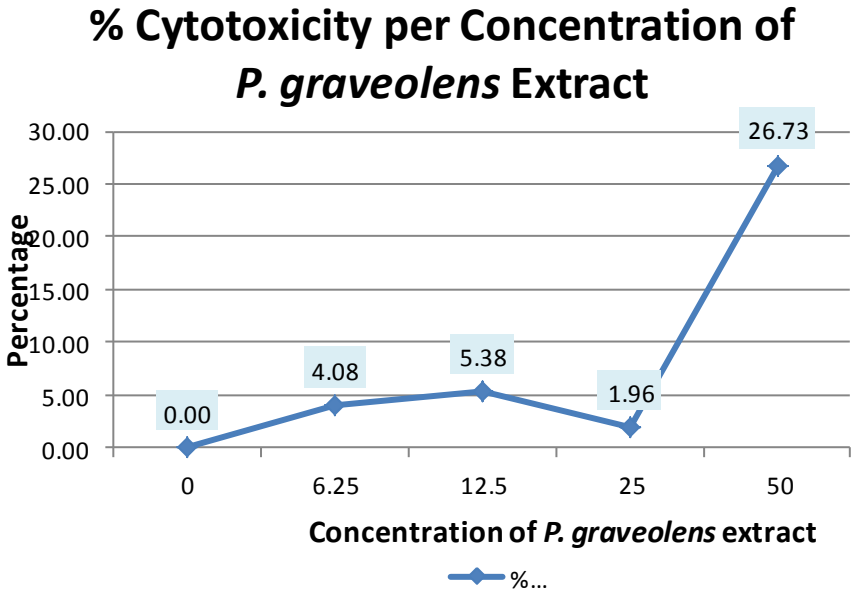

Figure 3. Percentage cytotoxic per concentration of P. graveolence leaf extract

Following treatment and incubation, the cells were viewed and photomicrographs were capture using Carl Zeiss Axio Vert Microscope (Magnification: 100x). Changes in A549 cell morphology after exposure to MTT are shown.

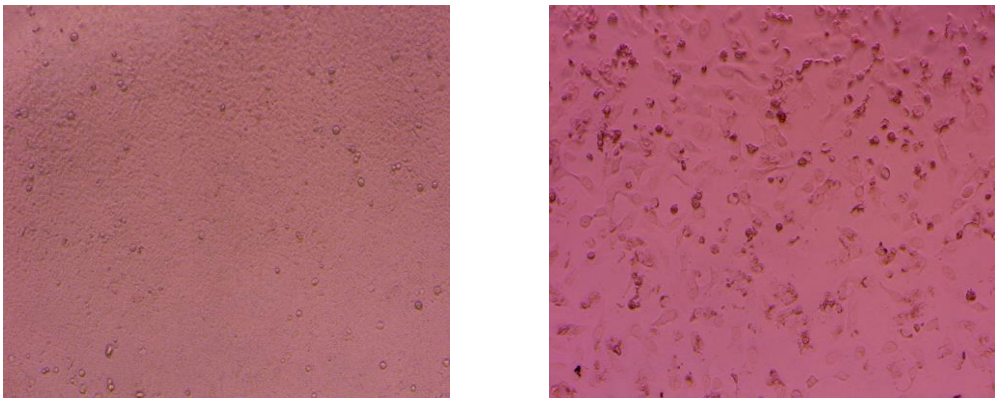

Figure 4. Lung Cancer Cell Lines (A549) with DMSO (left) Lung Cancer Cell Lines (A549) with Doxorubicin (right)

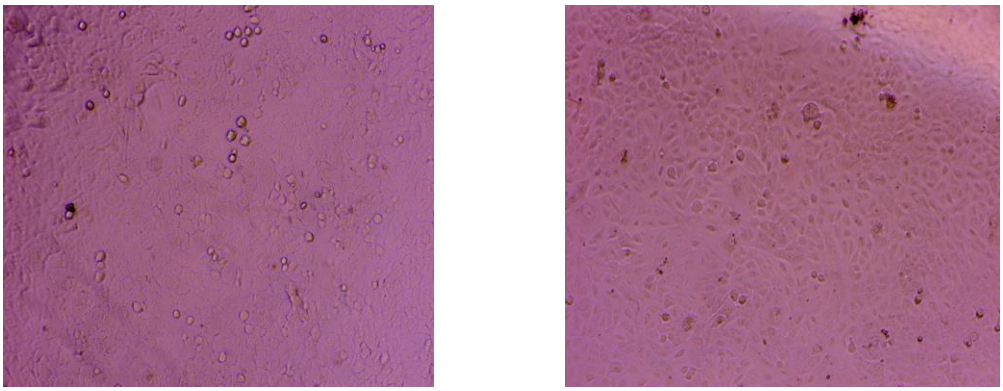

Figure 5. Lung Cancer Cell Lines (A549) with 6.25 µg/ml P. graveolens extract (left,) Lung Cancer Cell Lines (A549) with 12.5 µg/ml P. graveolens extract (right)

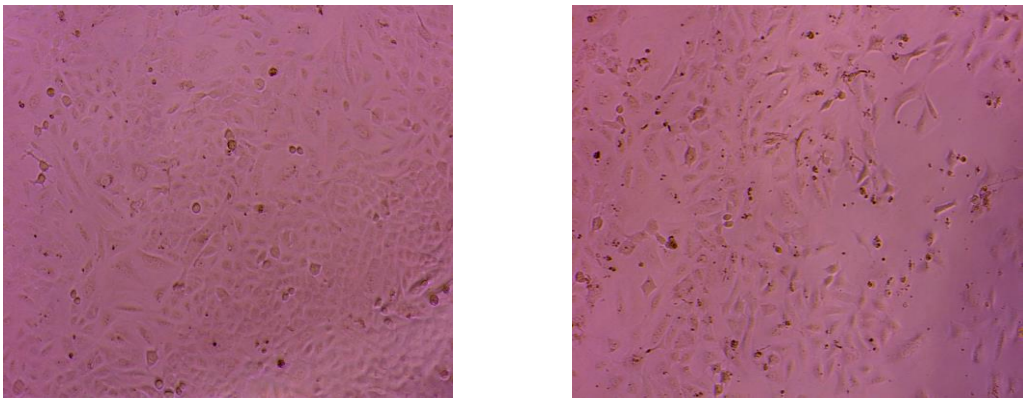

Figure 6. Lung Cancer Cell Lines (A549) with 25 µg/ml P. graveolens extract (left), lung Cancer Cell Lines (A549) with 50 µg/ml P. graveolens extract (right)

Dicussion

Phytochemical screening was done to determine the secondary metabolites present in *P. graveoleons*. The preliminary phytochemical screening of *Pelargonium graveolens* crude extracts showed the presence of bioactive components like alkaloids, essential oils, flavonoids, higher alcohols, sugars, phenols, steroids, anthraquinone, cuomarins and tannins

The presence of higher alcohol content in plants correlated with a wide spectrum of medical, phsyiological, and behavioral, High alcohol causes damage to and functional impairment of several organs affecting protein, carbohydrate, and fat metabolism and demonstrates the direct participation of

mitochondria as potential target of compounds (Avalos *et al*., 2013). Presence of sterols and steroid hormones, are compounds that exert a wide range of biological activities. They are essential for plant growth, reproduction, and responses to various abiotic and biotic stresses (Clouse, 2011). Pharmacological effect and toxicity of alkaloids from plants conducted by (Rujjanawate *et al.*, 2000), Tests in animals showed that the alkaloids exerted analgesic and anti-inflammatory effects. At a lethal dose, the alkaloids produced violent clonic convulsions that led to respiratory failure. While, essential oil benefits come from their antioxidant, antimicrobial and anti-inflammatory properties (Alexander, 2010). Coumarins are of great interest due to their pharmacological properties. In particular, their physiological, bacteriostatic and anti-tumor activity makes these compounds attractive backbone derivatisation and screening as novel therapeutic agents (Jain *et al*., 2012).

Flavonoids are highly diversified plant pigments that are present in a wide range of fruits, vegetables, nuts, and beverages. They are regularly consumed in the human diet and have various biological activities including anti-inflammatory, anti-cancer, and anti-viral properties (Lee *et al*., 1999), decreased maternal weight gain and increased maternal mortality were also observed (Calebresa *et al*., 1991).

The ethanolic extract of *P. graveolens* was tested for their larvicidal activity against *A. aegypti* mosquito during their third instar stage. The regression analysis shows a statistically significant relationship between the mortality rates of the third- instar *A. aegypti* larvae and the concentrations of the ethanolic extract.

The *P. graveolens* plant extract exhibited a concentration-dependent activity against mosquito larvae since the percentage mortality were observed to increase with increasing concentrations Rana *et al.* (2002) identified that citronellol, geraniol, linalool, citronellyl formate and p-menthone are among the chemical constituents of essential oil derived from *P. graveolens* leaves. The presence of phytochemicals in the plant extracts, which have pesticidal activities, causes increase in percentage mortality of the treated mosquito larvae (Gutierrez *et al*, 2014).

The biological activity of different concentrations of *P. graveolens* leaf ethanolic extract was tested for their ability to inhibit the proliferation of human lung cancer cell lines (A549), at 50 µg/mL concentration inhibited the proliferation of 55.51 % cells. The concentration needed to inhibit the proliferation of 50% (IC_{50}) of the A549 cells is 46.72 µg/mL. Different concentration of *P. graveolens* extracts exhibit growth inhibitory effect and larvicidal activities.

Conclusion

Phytochemical screening is conducted to determine the secondary metabolites present in *P. graveoleons*. The preliminary phytochemical screening of *P. graveolens* crude extracts showed the presence of bioactive components like Alkaloids, Essential Oils, Flavonoids, Higher Alcohols, Phenols, Steroids, and Tannins (Table1). The presence of phenolic compounds such as Flavonoids and Tannins indicates the plant's potent to many bioactivities.

The study identified the larvicidal activity of *P. graveoleus* ethanolic extract against *A. aegypti* mosquito. Homogenous batches of 20 third instar larvae are exposed to different concentrations of the *P. graveolens* extract, positive control and negative control. After 24 hours of exposure, the numbers of dead larva in each concentration of the different treatments are counted.

For the *P. graveolens* ethanolic extract, concentration ranged from 500 ppm to 7,000 ppm. The mean percentages of dead larva for 500 ppm, 1000 ppm, 2000 ppm, 4000 ppm and 7000 ppm concentrations are 21.67 (± 2.89 SD), 38.33 (± 5.77 SD), 55.00 (± 8.66 SD), 85.00 (± 8.66 SD) and 95.00 (± 0.00 SD) respectively.

The Linear Regression Probit Analysis is used to identify the concentration (ppm) that will kill certain percentage of mosquito larvae. The lethal concentrations for the *P. graveolens* extract to achieve 50% and 90% mortality or LC50 and LC90 are estimated at 1,355 and 5,443 ppm respectively with linear regression equation of $Y = 2.12x - 1.64$.

The LC50 and LC90 for the positive control are 0.48 and 1.07 ppm respectively with linear regression equation of $Y = 3.69x + 6.16$.

Different concentrations of *P. graveolens* leaf ethanolic extract are tested for their ability to inhibit the proliferation of human lung cancer cell lines (A549). Dimethyl sulfoxide is used as a negative control and different concentrations of a chemotherapeutic drug; Doxorubicin is used as the positive control.

The biological activity of A549 cell line are affected by different concentrations of *P. graveolens* extract. 6.25 µg/mL inhibited the proliferation of 32.24% cells, 12.5 µg/mL concentration inhibited the proliferation of 33.80 % cells, 25 µg/mL concentration inhibited the proliferation of 30.57% % cells, 50 µg/mL concentration inhibited the proliferation of 55.51 % cells. The concentration needed to inhibit the proliferation of 50% (IC_{50}) of the A549 cells is 46.72 µg/mL.

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