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Rhynchophorus ferrugineus midgut cell line to evaluate insecticidal potency of different plant essential oils

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Abstract Cell cultures can be a potent and strong tool to evaluate the insecticidal efficiency of natural products. Plant essential oils have long been used as the fragrance or curative products around the world which means that they are safer to be used in close proximity of humans and mammals. In this study, a midgut cell line, developed from *Rhynchophorus ferrugineus* (RPW-1), was used for screening essential oils from nine different plants. Assays revealed that higher cell mortality was observed at 500 ppm which reached to 86, 65, 60, 59, 56, 54, 54, 53, and 53%, whereas lowest cell mortality at 1 ppm remained at 41, 23, 20, 17, 16, 15, 14, 13, and 10%, for *Azadirachta indica*, *Piper nigrum*, *Mentha spicata*, *Cammiphora myrrha*, *Elettaria cardamomum*, *Zingiber officinale*, *Curcuma longa*, *Schinus molle*, and *Rosmarinus officinalis*, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay revealed the percentage of cell growth inhibition was highest at 500 ppm and remained at 48, 45, 42, 37, 34, 29, 24, 22, and 18% against *A. indica*, *P. nigrum*, *M. spicata*, *C. myrrha*, *E. cardamomum*, *Z. officinale*, *C. longa*, *S. molle*, and *R. officinalis*, respectively. Lowest LC_{50} value (7.98 ppm) was found for *A. indica*, whereas the highest LC_{50} (483.11 ppm) was against *R. officinalis*. Thus, in this study, essential oils of *A. indica* exhibited the highest levels of toxicity, whereas those from *R. officinalis* exhibited the lowest levels of toxicity toward RPW-1 cells.

Keywords Midgut cell line RPW-1 · Plant essential oils · Cell mortality · Cell proliferation

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Introduction

The development of rapid assessment tools is required to increase the availability of new control methods for insect pests. Traditional studies involving the entire pest insect life cycle slow the pace of discovery because they often need to be conducted under laboratory as well as field conditions. In this regard, cell cultures from notorious pests can be helpful by providing a quick analysis of the problem. Smagghe *et al.* (2009) reported that cell lines are perfect tools not only for the screening of insecticides but also for unraveling their mechanism(s) of action. Midgut tissue in insects is important for the production and secretion of luminal enzymes and final digestion by microvillar enzymes. Midgut epithelium consists of columnar absorptive cells with apical microvilli, pear-shaped goblet cells that transport ions, and small round stem cells located at the base of the epithelium (Hakim *et al.* 2001).

Plant oils are generally considered broad spectrum and safe for the environment as the compounds they contain degrade under field conditions (Yuan *et al.* 2010). Essential oils are defined as oils with aromatic components which give distinctive odor, flavor, or scent to a plant. These are by-products of plant metabolism and are referred to as volatile plant secondary metabolites. Many plant essential oils show a broad spectrum of activity against pest insects and plant pathogenic fungi ranging from insecticidal, antifeedant, repellent, oviposition deterrent, and growth regulatory activities. Essential oils when mixed with emulsifying agent such as dimethyl sulfoxide become more effective against insects as they can more easily penetrate the waxy insect cuticle (Sampson *et al.* 2005). Development of insect resistance is an alarming issue for many synthetic pesticides, but it is assumed that resistance will develop more slowly to essential oil-based pesticides because of the complex mixtures of constituents in essential oils. Essential oils are presumed to have greater impact in future integrated pest management because of safety to

nontarget organisms and the environment. As their target site is not shared with mammals, most essential oils are relatively nontoxic to mammals and fish and are more compatible with the environment than synthetic pesticides (Isman and Machial 2006).

Rhynchophorus ferrugineus was initially reported on coconut *Cocos nucifera* in South Asia, but it became an invasive pest of date palm in several Middle Eastern countries (Faleiro 2006). *R. ferrugineus* is a concealed tissue borer and has been reported to attack 17 palm species worldwide and has especially become a major source of economic loss in date production in the Middle East (Abid *et al.* 2013). Although integrated control methods have been used to control this pest, the main method is the use of synthetic insecticides (Mahmoud *et al.* 2013). Increased resistance of this pest against synthetic insecticides urges scientists to look for safer alternatives. Environmental problems caused by overuse of pesticides have been a matter of concern in recent years. About 2.5 million tons of synthetic pesticides are used on crops each year, and the worldwide damage caused by pesticides reaches $100 billion annually because of the high toxicity and residues in soil, water resources, and crops (Koul *et al.* 2008).

With these perspective in mind, the current study was carried out, using the RPW-1 midgut cell line established during our early studies (Aljabr *et al.* 2014), to screen potent insecticidal essential oils from different plants (*Piper nigrum*, *Zingiber officinale*, *Mentha spicata*, *E. cardamomum*, *Cammiphora myrrha*, *Schinus molle*, *Curcuma longa*, *Rosmarinus officinalis*, *Azadirachta indica*) as alternative natural pesticides against the red palm weevil.

Material and Methods

Reagents. All the reagents including cell growth media (Grace's insect cell media containing L-glutamine (0.6 g/L) and sodium bicarbonate (0.35 g/L)) required for the cell culturing were purchased from Sigma-Aldrich (St. Louis, MO). Essential oils obtained with steam distillation (*P. nigrum*, *Z. officinale*, *M. spicata*, *E. cardamomum*, *C. myrrha*, *S. molle*, *C. longa*, *R. officinalis*, *A. indica*) were purchased from Eden Botanicals (Petaluma, California) (www.edenbotanicals.com).

Essential oil applications. Five concentrations of each essential oil at 500, 100, 50, 10, and 1 ppm were prepared in 0.01% DMSO. The 1×10^6 cells/mL were seeded in six-well plate containing 3 mL of Grace's insect cell media (added with antibiotic and antimycotic solution (Sigma-Aldrich A5955)) and incubated for 4 h at 27°C. Cell cultures were then treated with 10 μl/mL media of each oil concentration. Controls were treated with equal volumes of 0.01% DMSO.

Cell mortality percentages. To assess percent cell mortality, a trypan blue assay was conducted according to Oh *et al.* (2004) after 24-h exposure to each concentration (500, 100, 50, 10, and 1 ppm) of all the essential oils (*P. nigrum*, *Z. officinale*, *M. spicata*, *E. cardamomum*, *C. myrrha*, *S. molle*, *C. longa*, *R. officinalis*, *A. indica*). After harvesting, 10 μl of cells were mixed with 10 μl of 0.4% trypan blue solution (Sigma-Aldrich) and incubated for 3 min. The numbers of blue (dead) cells were counted under the microscope using neubauer hemocytometer, and percentage of dead and viable cells were calculated.

Formulae:

%age of cell mortality=total dead cells (stained)/total cells (viable+dead)×100

Viable cells/mL=average viable cell count per square× dilution factor×10^4

LC_{50} values of essential oils. Five concentrations of each essential oil were used to assess their efficacy as RPW-1 cell mortality percentages, and based on the data retrieved, probit regression analysis was carried out to determine the LC_{50} values of the five essential oil concentrations against RPW-1 (Grimm *et al.* 2001).

MTT cell proliferation assay. Vybrant® MTT Cell Proliferation Assay Kit was used to perform 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. RPW-1 cells at 1×10^6 cells/mL were seeded in 96-well plates. After 24 h of incubation, 20 μl of each of the five concentrations (500, 100, 50, 10, and 1 ppm) from essential oils (*P. nigrum*, *Z. officinale*, *M. spicata*, *E. cardamomum*, *C. myrrha*, *S. molle*, *C. longa*, *R. officinalis*, *A. indica*) were added and 0.01% DMSO was used as the control. After 24 h of treatment, 10 μL of the 12 mM MTT stock solution was added to each well and incubated for 4 h at 37°C. Then, 100 μL of the SDS-HCl solution was added to each well and mix thoroughly using a pipette. Plate was again incubated at 37°C for 4 h to dissolve the formazan crystals. Later on each sample was mixed properly with a pipette and absorbance was measured at 570 nm by microplate reader. Cytotoxic effect was expressed as a relative percentage of inhibition as follows:

$$\text{Cell growth inhibition (\%)} = ((\text{Control} - \text{Insecticide Treatment})/\text{Control}) \times 100$$

Statistical analysis. Data analysis was carried out using analysis of variance (ANOVA) and statistical significance was established by using SAS software (SAS 2000). Differences

between the treatments were determined by Tukey's multiple range tests at $P<0.05$. All experiments were performed in triplicate with three replications.

Results

Cell mortality percentages. Cell mortality percentage varied with different essential oils ranging from highest (*A. indica*) to the lowest (*R. officinalis*). Higher cell mortality was observed at 500 ppm which gradually decreased with concentrations to the lowest at 1 ppm as shown in Table 1. Cell mortality was noted after 24 h which revealed that *A. indica* caused the highest 86% cell mortality at 500 ppm. At 500 ppm, the cell mortality percentage varied at 65, 60, 59, 56, 54, 54, 53, and 53% against *P. nigrum*, *M. spicata*, *C. myrrha*, *E. cardamomum*, *Z. officinale*, *C. longa*, *S. molle*, and *R. officinalis*, respectively, as shown in Table 1. The lowest cell mortality was observed at 1 ppm where percent cell mortality was decreased to 41, 23, 20, 17, 16, 15, 14, 13, and 10% for *A. indica*, *P. nigrum*, *M. spicata*, *C. myrrha*, *E. cardamomum*, *Z. officinale*, *C. longa*, *S. molle*, and *R. officinalis*, respectively, as shown in Table 2. *R. officinalis* showed a 10% cell mortality at 1 ppm, the lowest of all the treatments. Figure 1 shows the cell viability of RPW-1 increased from higher to lower concentrations of essential oils.

LC_{50} values of different essential oils. Probit regression analysis was carried out to determine the LC_{50} values of essential oils against RPW-1 cells. The LC_{50} of *A. indica* was noted to be the lowest and remained as 7.98 ppm, whereas the highest LC_{50} was found for *R. officinalis* reaching to 483.11 ppm, as shown in Table 2.

Table 2. LC_{50} values of different essential oils against *Rhynchophorus ferrugineus*, RPW-1 cells

Insecticides	LC_{50}	Slope	χ^2
A. indica	7.98±0.33	0.41±0.03	0.29
P. nigrum	92.4±1.79	1.88±0.28	2.91
M. spicata	142.64±3.19	2.35±0.37	3.14
C. myrrha	236.23±3.57	2.61±0.36	3.55
E. cardamomum	317.43±3.81	3.04±0.59	3.90
Z. officinale	406.79±3.99	3.46±0.78	4.15
C. longa	458.92±4.50	3.88±0.88	4.42
S. molle	471.7±4.57	4.40±0.80	5.17
R. officinalis	483.11±4.84	4.68±0.85	5.51

Cell proliferation by MTT bioassay. Table 3 reveals the inhibitory effect on RPW-1 cell growth by the nine essential oils (*A. indica*, *P. nigrum*, *M. spicata*, *C. myrrha*, *E. cardamomum*, *Z. officinale*, *C. longa*, *S. molle*, *R. officinalis*) based on the MTT assay. These botanical essential oils acted on percent cell growth in a dose-dependent manner. The level of inhibition of RPW-1 cell proliferation after a 24-h exposure to the plant oils ranged from a high of 32% for *A. indica* to a low of 6% for *R. officinalis* at 1 ppm after 24-h exposure. Inhibition of cell proliferation increased with concentrations at 10, 50, and 100 ppm and further showed a maximum inhibition from 48 to 18% at 500 ppm after 24-h exposure (Table 3).

Discussion

Insecticidal bioassays have been useful for identifying insecticidal efficacies; yet, these studies with insect specimens are very laborious and time-consuming. Insect cell cultures from a

Table 1. RPW-1 cell mortality percentage against different plant essential oils after 24 h of treatment

Concentrations (ppm)					
	500	100	50	10	1
A. indica	86±3.56 a	72±2.87 a	60±2.39 a	49±2.12 a	41±2.70 a
P. nigrum	65±2.48 b	55±1.84 b	46±1.92 b	33±1.51 b	23±1.88 b
M. spicata	60±2.31 c	53±2.01 b	42±2.26 b	32±2.16 b	20±1.82 bc
C. myrrha	59±2.33 c	48±1.73 c	36±1.53 c	25±1.77 c	17±1.56 bcd
E. cardamomum	56±3.01 cd	47±1.33 cd	33±1.45 cd	24±1.65 c	16±1.63 cde
Z. officinale	54±2.27 d	44±2.87 d	31±2.48 d	23±1.88 cd	15±1.26 cde
C. longa	54±2.19 d	40±1.58 e	30±2.27 de	21±1.41 cd	14±0.70 cde
S. molle	53±1.85 d	38±2.87 ef	29±1.61 de	20±1.11 cd	13±0.80 de
R. officinalis	53±2.00 d	36±1.68 f	26±2.39 e	17±1.66 d	10±0.73 ef
Control	4±0.78 e	4±0.78 g	4±0.78 f	4±0.78 e	4±0.78 f

Within *columns*, means followed by the same *letter* are not significantly different at $P<0.05$ level of confidence according to Tukey's test. Values in the table indicate means±standard error. The experiment was repeated three times with three replicates of each treatment

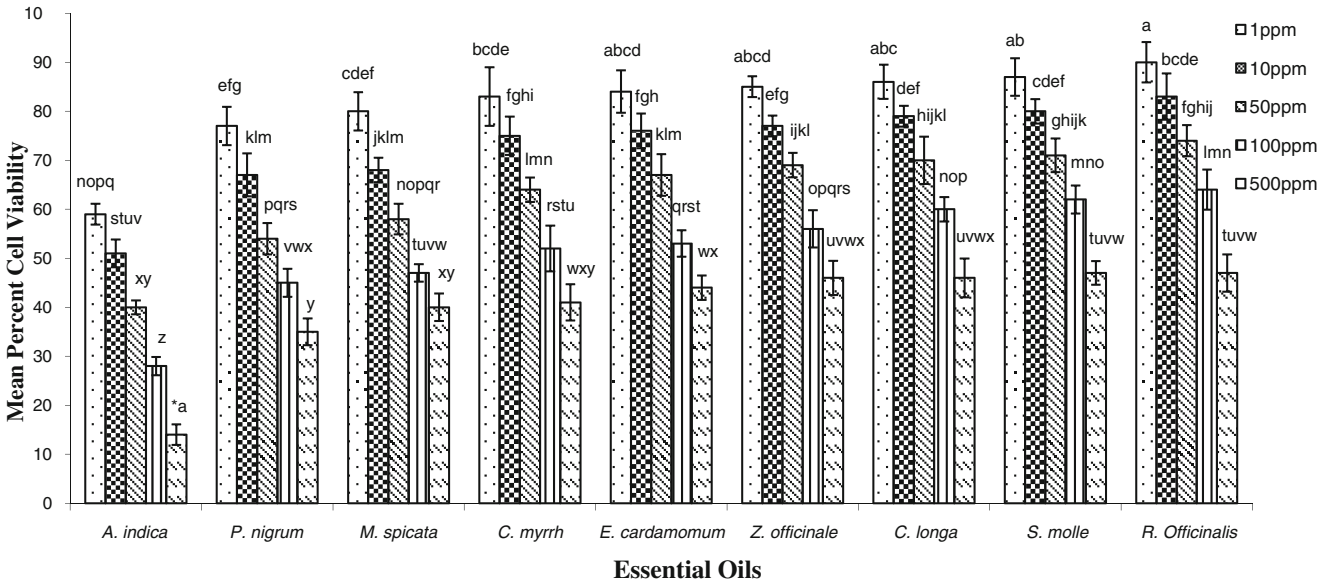

Figure 1. Cell viability trends against different essential oils. *Bars* with the same *letters* are not significantly different from each other at $P<0.05$ according to Tukey's test.

target species can be a useful tool to screen potential insecticides like essential oils and can be used for biochemical and molecular studies (Grasela *et al.* 2012). Cytotoxicity has long been studied for DNA damage, morphological changes, and apoptosis in response to the synthetic insecticides (Yoon *et al.* 2001; Nandi *et al.* 2006; Sonoda and Tsumuki 2007; Huang *et al.* 2011). However, little work has been reported relating to the studies of essential oils and insect cells. Previously, midgut epithelial cell culture (RPW-1) from the red palm weevil was developed in our laboratory (Aljabr *et al.* 2014). As midgut cells have been reported to show high sensitivity to the toxicity (Giner *et al.* 2012), RPW-1 cell line was used as a tool to screen essential oils derived from different plants as potential insecticidal agents.

The quest to find an alternative for synthetic insecticides against red palm weevil led us to the screening of essential oils belonging to different plant species. Research has focused on the natural products to achieve the most bioactive plant essential oils that have shown good insecticidal properties (Isman 2006). The chemical composition and broad spectrum of biological activity for essential oils can vary with plant age, plant tissues, or organs (Choi *et al.* 2003; Sedy and Koschier 2003). The action of essential oils against some pests is indicative of a neurotoxic mode of action, and there is evidence for interference with the neuromodulator octopamine (Kostyukovsky *et al.* 2002) by some oils and with GABA-gated chloride channels by others (Priestley *et al.* 2003).

Table 3. MTT assay-based RPW-1 cell growth inhibition percentage against different 437 essential oils after 24 h of treatment

Concentrations (ppm)					
	500	100	50	10	1
A. indica	48±2.82 a	42±3.07 a	40±3.20 a	37±3.13 a	32±1.79 a
P. nigrum	45±3.53 ab	39±3.70 ab	37±2.88 b	34±1.83 ab	29±1.68 b
M. spicata	42±2.21 b	38±2.28 bc	36±2.34 b	32±1.53 b	26±1.58 c
C. myrrha	37±3.25 c	35±2.14 cd	32±1.66 c	28±1.38 c	21±2.55 d
E. cardamomum	34±1.98 c	32±2.42 d	32±1.55 c	24±1.46 d	16±1.48 e
Z. officinale	29±3.41 d	27±1.47 e	25±2.00 d	21±1.49 de	13±1.65 f
C. longa	24±1.41 e	22±1.99 f	20±1.73 e	18±1.34 e	11±0.77 g
S. molle	22±2.33 ef	18±1.49 g	17±0.74 e	14±0.97 f	8±0.79 h
R. officinalis	18±2.83 f	14±1.43 h	11±0.82 f	10±0.75 g	6±0.74 i

Within *columns*, means followed by the same *letter* are not significantly different at $P<0.05$ level of confidence according to Tukey's test. Values in the table indicate means±standard error. The experiment was repeated three times with three replicates of each treatment

The aromatic characteristics of essential oils provide various functions for plants including attracting or repelling insects and utilizing chemical constituents as a defense mechanism (Koul *et al.* 2008). Biological activity of essential oils is also affected by synergistic and antagonistic interactions among structural components (Chiasson *et al.* 2001) and that might be a reason of better *A. indica* and lower *R. officinalis* efficacy.

Toxicity of some of the essential oils used in this study has also been documented by other researchers against different insect species. Essential oils from *E. cardamomum* have been proven toxic to other coleopteran insects such as *Callosobruchus maculates* and *Tribolium castaneum* and also showed good efficacy as an oviposition deterrent for *C. maculates* (Abbasipour *et al.* 2011). Knaak *et al.* (2012) found that the toxicity of the extracts of *Z. officinale* caused damage such as vacuolization of cytoplasm, disruption of microvilli, peritrophic membrane destruction, and cell changes in the midgut of *Spodoptera frugiperda*. Some essential oils like *P. nigrum* and *S. molle* have been reported to exhibit the bioactivity on *Zabrotes subfasciatus* (Oliveira *et al.* 2005) and *Triatoma infestans* (Laurent *et al.* 1997).

Similarly, mode of action of essential oils is based on disruption of several cell functions (Yang *et al.* 2009; Marchial *et al.* 2010; Perumalsamy *et al.* 2010). Anuradha *et al.* (2007) and Kumar *et al.* (2007) reported that in *Drosophila melanogaster* cells, azadirachtin induced depolymerization of actin, leading to cell cycle arrest and subsequently apoptosis in a caspase-independent manner, thus suggesting that actin might be a target of azadirachtin. Furthermore, Jingfei *et al.* (2011) observed morphological changes in SL-1 cells after treatment with azadirachtin. Essential oils are mainly mixtures of different compounds (Bakkali *et al.* 2008) that show insecticidal characteristics (Leelaja *et al.* 2007; Escriba *et al.* 2009). In general, no adverse effects toward human health or the environment are expected as several essential oils are approved as fragrances and food flavors (Burdock 2010). Cells in the intact insect and cell cultures can behave differently as in intact insect detoxification mechanisms can be more active than in cells cultures. However, this requires more study to fully understand the potential counteractive mechanisms to toxicity in cells and insects.

In conclusion, the data provides a clear picture about the differential insecticidal capabilities of the essential oils against midgut cell line of red palm weevil (RPW-1). Botanical insecticides are good alternatives because they often have lower mammalian toxicity and environmental persistence (Isman 2006). However, further studies with chromatographic analysis are required to fully understand the constituents and potential of the essential oils for controlling red palm weevil.

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