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Original article

Larvicidal activity and Histopathological changes of *Cinnamomum burmannii, Syzygium aromaticum* extracts and their combination on *Culex pipiens*

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ABSTRACT

In order to develop an eco-friendly botanical larvicide alternative to the synthetic larvicides, extracts were prepared from the *Cinnamomum burmannii* (C.B.) and Syzygium aromaticum (S.A.) with hexane using a sonicator. The extracts were evaluated for larvicidal activity individually and in combination against the *Culex pipiens* larvae. The LC_{50} value of C.B. and the S.A. hexane extracts tested individually were 184.2 and 363.7 μg/mL against *Cx. pipiens* respectively. All the combinations of the extract of C.B. and S.A. showed synergistic factors higher than one. Among the different ratios of extracts, the SA25%: CB75% extract was found to be more toxic than the other combinations (LC_{50}:125.7 μg/mL). Midgut cells treated with S.A. 25%: C.B. 75% extract showed severe morphological alterations such as degradation of microvilli; degeneration of epithelial cells, and peritrophic membrane; loss of nuclei, irregular and damage of microvilli. The extract has a promising larvicidal potential against *Cx. pipiens*, However, the extract was toxic against HUVEC cells, as evident from MTT and cell morphology. Further investigation is required to assess the toxicity of the extract on aquatic animals.

1. Introduction

Mosquitoes are a significant vector for many illnesses that affect animals and humans (Kovendan et al., 2012), such as Malaria, Dengue, Chikungunya, Yellow Fever, Filariasis, Schistosomiasis, and Japanese encephalitis (James, 1992). Furthermore, mosquitoes cause allergic reactions, including local (skin allergy) and systematic reactions (angioedema) (Gubler, 1998).

Cx. pipiens (L.) (Diptera: Culicidae) is widely spread in tropical and subtropical nations and bites a wide range of hosts (ECDC, 2021). *Cx. pipiens* are well-known carriers of West Nile Virus (WNV), Usutu virus (USUV), Rift Valley fever virus (RVFV), Japanese encephalitis virus (JEV), Sindbis virus (SINV), Tahyna virus (TAHV), Batai virus, Dirofilarial worms, and Avian malaria (ECDPC, 2021). Managing mosquito vectors relies largely on synthetic insecticide spraying, and due to higher insecticide resistance, controlling mosquitoes is a considerable challenge (Ali et al., 2012; Organization, 2021). *Cx. pipiens* mosquitoes collected and screened for resistance from three different localities in Riyadh city were found resistant to deltamethrin, lambda-cyhalothrin, and beta-cyfluthrin. However, no resistance was detected for fenitrothion (Al-Sarar, 2010).

Larvicidal agents derived from natural sources are alternative tools, especially from bioactive secondary metabolites extracted from the plants because they are cheap, biodegradable, and non-toxic to other non-target organisms (Ghosh et al., 2012). Secondary metabolites from plants such as steroids, alkaloids, phenolics, terpenoids, and essential oils have been documented for their insecticidal, repellent, and adulticidal activities (Senthil-Nathan, 2020). The larvicidal potenial of *Cassia fistula* hexane-methanol soluble fraction showed a promising LC_{50} value. The LC_{50} value was 21.04 μg/ml after 24 h of exposure. The extract of *C. fistula* showed no toxicity to *Danio rerio* embryos, and BEAS-2B at the highest concentration tested. (Abutaha et al., 2020). In addition, previous research of (Al-Solami, 2021) illustrates that the acetone extracts of *Lantana camara*, *Rhazya stricta, Ruta chalepensis*, and *Acalypha fruticose* showed different percentages of mortality rate against the early fourth instar of *Cx. pipiens*. The results showed that *L. camara* extract (LD_{50}: 264 mg/l) was significantly higher as com-

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Table 1
Mortality percent of *Cx. pipiens* larvae treated with the combination of the hexane of *C. burmannii* and S. aromaticum LC_{50} as well as LC_{90} (mg) values 24 h postexposure.

Combinations	Concentration (mg)	% mortality	LC_{50} (mg)	LC_{90} (mg)	df	F
CB 100%	Control	0.00 ± 0.00c				
	62.5	0.00 ± 0.00c				
	125	6.67 ± 3.33c				
	200	50.00 ± 5.77b	184.28	263.92	4	152.00
	250	93.33 ± 3.33 a				
	375	100.00 ± 0.00 a				
	500	100.00 ± 0.00 a				
SA 100%	Control	0.00 ± 0.00				
	62.5	0.00 ± 0.00c				
	125	0.00 ± 0.00c				
	200	0.00 ± 0.00c	363.70	483.50	4	166.80
	250	3.33 ± 3.33c				
	375	60.00 ± 5.77b				
	500	93.33 ± 3.33 a				
SA 75% : CB 25%	Control	0.00 ± 0.00c				
	62.5	6.67 ± 3.33 bc				
	125	10.00 ± 0.00b				
	200	100.00 ± 0.00 a	145.13	202.60	4	1210.00
	250	100.00 ± 0.00 a				
	375	100.00 ± 0.00 a				
	500	100.00 ± 0.00 a				
SA 50% : CB 50%	Control	0.00 ± 0.00c				
	62.5	0.00 ± 0.00c				
	125	30.00 ± 5.77b				
	200	100.00 ± 0.00 a	138.24	192.73	4	387.00
	250	100.00 ± 0.00 a				
	375	100.00 ± 0.00 a				
	500	100.00 ± 0.00 a				
SA 25% : CB 75%	Control	0.00 ± 0.00 d				
	62.5	10.00 ± 0.00c				
	125	46.67 ± 3.33b				
	200	100.00 ± 0.00a	125.78	186.72	4	1024.00
	250	100.00 ± 0.00 a				
	375	100.00 ± 0.00 a				
	500	100.00 ± 0.00 a				

pared to *R. stricta* (293.4 mg/l), *A. fruticose* (435.6 mg/l) and *R. chalepensis* (611.9 mg/l) extract. A previous report by (Maheswaran and Ignacimuthu, 2015) synthesized a new novel plant-based (based on Pongamia glabra and Azadirachtaindica) extract named "PONNEEM" commercially synthesized to control *Cx. quinquefasciatus*, and An. stephensi.

Plant extracts could enable the discovery of new larvicidal agents for effective mosquito management. This research aimed to assess the larvicidal activity of *C. burmannii* (Family: Lauraceae) and *S. aromaticum* (Family: Myrtaceae) extracts and their blend against insectary-reared *Cx. pipiens* larvae.

2. Materials and methods

2.1. Preparation of extract

C. burmannii (C.B.) and *S. aromaticum* (S.A.) were purchased from the herbal shop in Riyadh and pulverized to powder using an electrical blender (SFstardust, Japan). The pulverized powder of the two plants was extracted using hexane as an extraction solvent in a sonicator (WiseClean, China) for 30 mins at 40 °C. Each extract was filtered using Whatman filter paper No. 1 and evaporated under reduced pressure (Heidolph, Germany) at 45 °C. The process was repeated twice for each solvent, and the extracts were combined. The yield was calculated, and stock solution (25 mg/mL) was prepared and kept at −4C until used. Hexane extract from each extract was mixed using the different ratios (S.A. 75%: C.B. 25%, S.A. 50%: C.B. 50%, and S.A. 25%: C.B. 75%) and further tested to evaluate the synergistic potential of the combined extract using the following formula:

$$\text{Synergistic factor (S.F.)} = \text{LC50 of the plant extract alone} / \text{LC50 of the mixture}$$

Value of S.F. < 1 represents the antagonistic action, and S.F. > 1 represents synergistic action (Kalyanasundaram and Das, 1985).

2.2. Mosquito culture

The larvae of *Culex pipiens* were maintained in the Zoology Department insectary, Riyadh, Saudi Arabia, and kept in plastic trays filled with de-chlorinated tap water. Tests were carried out at 30 ± 1 °C and under a light/dark (14:10) phase. Larvae were fed with fish flakes (DAJANA, Czech Public).

2.2.1. Larvicidal bioassay

The larvicidal bioassay was performed based on a previously carried out procedure (Al-Mekhlafi, 2018). The 20 larvae in each replica were placed into disposable plastic six-well plates (NIST, China) containing 8 mL of the test concentrations (500, 375, 250,200, 125, 62.5 μg/mL). Larvae were recorded as dead if no response when the plates were disturbed or touched with a glass rod. The results were reported after 24hrs of exposure. Methanol was used as a negative control. LC_{50} and LC_{90} values were calculated at 24hrs, using SPSS 20.

2.2.2. Histological assay

The treated and control of *Cx. pipiens* third instars were fixed in 10% formalin solutions overnight and processed as reported by (Al-Mekhlafi et al., 2021), followed by dehydration, mounting using paraffin, and. The prepared slides were sectioned using a microtome (Leica, Germany) and stained with eosin and hematoxylin.

Table 2
Synergistic factor values of individual and combined extracts of *C. burmannii* and S. *aromaticum* against laboratory *Culex pipiens* third instar.

Name of extract	Combination of extract	LC_{50} μg/mL	Synergistic factor	effect
C.burmannii	CB 100%	184.2	–	–
S. aromaticum	SA 100%	363.7	–	–
C. burmannii & S. aromaticum	75% : 25%	145.1	2.54	synergism
C. burmannii & S. aromaticum	50% : 50%	138.2	2.63	synergism
C. burmannii & S. aromaticum	25% : 75%	125.7	2.89	synergism

The sections were observed for pathological alternations using a light microscope (Olympus, Japan). Midgut cells of *Cx. pipiens* were photographed, and the alternations in the midgut of treated larvae were observed and compared with control.

2.3. Cell culture

Normal Human Umbilical Endothelial Cells (HUV-EC) (ATCC, USA) were seeded in DMEM medium (UFC, KSA), supplemented with 10% and 1% of fetal bovine serum (Gibco, USA) and Antibiotic-Antimycotic solution (UFC, KSA), respectively. Plates were incubated at 37C with 5% CO2 in a humidified incubator (BINDER, Germany).

2.4. Evaluation of cell viability

Cells (50,000 cells/well) were seeded in 24-well plates (NIST, China) and incubated at 37C for 24 h. Plated cells were incubated with the five different concentrations of the extract (250–12.5 μg/mL), each dissolved in DMSO (Sigma, USA). DMSO (0.01%) was used as a negative control. After treatment (24 h), the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Invitrogen, USA) at 37C for 2 h. The crystals (purple) formed were dissolve in 0.01% HCL-methanol, and the optical density (O.D.) was read (595 nm) using a microplate reader (ChroMate, England). The graph was plotted using OriginPro 8.5.

3. Results

The total yield of hexane extract of S.A. and C.B. was 130 mg and 280 mg, respectively.

3.1. Larvicidal activity

The larvicidal potential of hexane extract of *Cinnamomum burmannii* (C.B.) and *Syzygium aromaticum* (S.A.) against *Cx. pipiens* third instar was recorded after 24 h of treatment. The C.B. extract showed a higher larvicidal effect than the S.A. extract. The 50% (LC_{50}) and 90% (LC_{90}) mortality of larval treated with the plants with hexane extracts individually or in combinations against *Cx. pipiens* larvae are reported in Table 1. C.B. extract caused mosquito larval mortality with LC_{50} and LC_{90} values of 184.28 and 263.92 μg/mL against *Cx. pipiens*. Similarly, the hexane extract of S.A. was effective with respective LC_{50} and LC_{90} values of 363.70 and 483.50 μg/mL against *Cx. pipiens*. The combination of the C.B. and

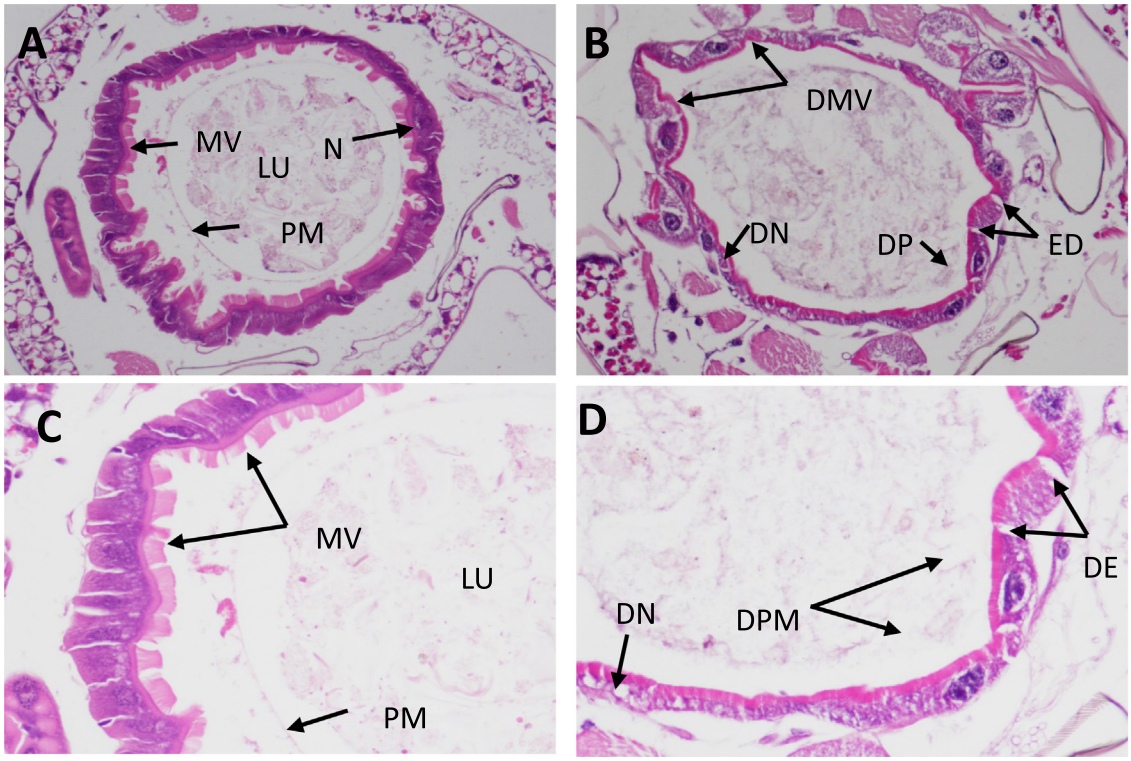

Fig. 1. The midguts of *Cx. pipiens* fourth instars treated with hexane extract of *C. burmannii* (75%) and *S. aromaticum* (25%). A and B are the midgut sections of the control group. C and D are sections of the treated larvae. Degenerating epithelial cells (DE), degraded microvilli (DMV), lumen (lu), degenerating nuclei (D.N.), degenerating peritrophic membrane (DPM).

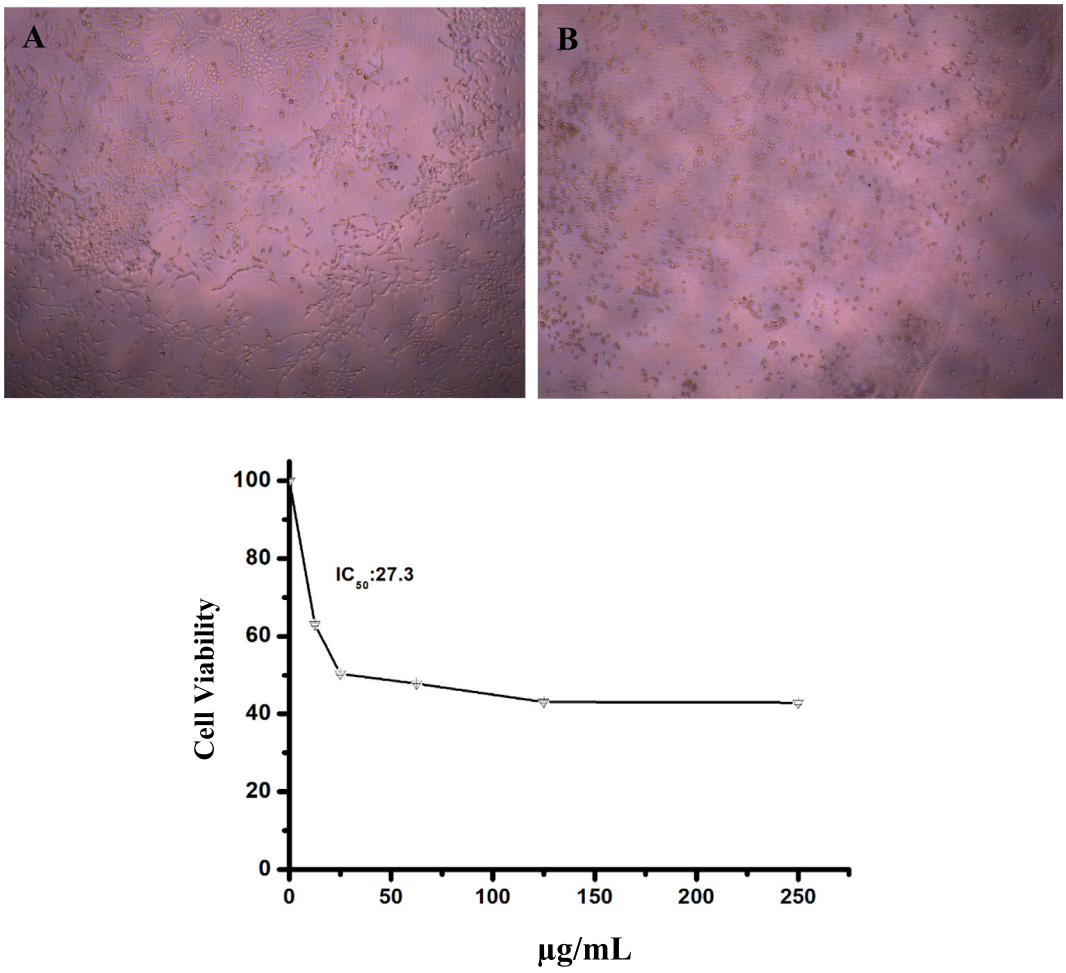

Fig. 2. A: Cell survival curve on HUVEC normal cell lines assessed by MTT test after 24 h of treatment with increasing concentrations (12.6 to 250 µg/mL) of combined hexane extracts of *C. burmannii* and *S. aromaticum* extracts. Data are shown as mean ± S.D. of three independent experiments. B: Morphological changes of cells treated with 2-fold IC50 (34.6 µg/mL) for 24 h. Black arrows indicated changes in cell morphology.

S.A. extracts using the different ratios (1:3, 1:1. 3:1) showed promising LC_{50} and LC_{90} values compared to the individual plant.

Combination of C.B. and S.A. hexane extract enhanced the effectiveness of each extract by decreasing the LC_{50} values 145.13, 138.24 and 125.78 µg/ml for combinations of SA75%: CB25%, S.A. 50%: C.B. 50%, and S.A. 25%: CB75%, respectively compared to LC_{50} of C.B. (184.28) and S.A. (363.70) extracts tested singly. Table 2 illustrated the synergistic activity of the combination of the hexane extracts C.B. and S.A. against the *Cx. pipiens* larvae. All the combinations of the extract of C.B. and S.A. showed synergistic factors higher than one. Among the different ratios of extracts, the SA25%: CB75% extract was found to be more toxic than the other combinations. (Table 2). No larval mortality was detected in the control groups.

3.2. Gut-Histological activity

The midgut cells of *Cx. pipiens* third instar treated with sublethal dosages of S.A. 25%: C.B. 75% extract (125.7 ppm) (Fig. 1 C and D) and the control is shown in Fig. 1 A and B. The control midgut sections appeared normal, with intact microvilli (MV), nuclei (N), and normal peritrophic membrane (Pm). Midgut cells treated with S.A. 25%: C.B. 75% extract showed severe morphological alterations such as degradation of microvilli (DMV); degeneration of epithelial cells (D.E.), and peritrophic membrane (DPM); loss of nuclei, irregular and damage of microvilli.

3.3. Cell viability and cell morphology

The HUV-EC cells morphology was altered by C. cassia and Z. officinale extract, whereas the control (0.01% DMSO) cells retained normal morphology. Loss of cellular integrity, shrinkage of cytoplasmic materials, and cell detachment were noticed in HUV-EC cells by S.A. 25%: C.B. 75% extract (Figure 0000). The extract of S. A. 25%: C.B. 75% showed cytotoxic activity against HUV-EC cells screened, with the IC_{50} value of 32.4 µg/mL. The extract showed dose-dependent cytotoxic activity against the HUV-EC cell line with 47.8%, 50.3%, and 63.0 % cell viability at 62 µg/ ml, 31 µg/ml, and 15 µg/ml concentration, respectively (Fig. 2).

4. Discussion

Botanical-based formulations are an economical approach to combat mosquito-borne diseases. Moreover, mosquito larvae are the ideal stage for insecticides screening using Botanical -based formulations (Benelli et al., 2017). *C. burmannii* and *S. aromaticum* extracts showed larvicidal activities individually and in combination, demonstrating the larvicidal capabilities of these two plants. The result showed that mortalities increased significantly with concentration and time ($P \leq 0.05$). The present investigation is consistent with the reports that showed a positive correlation between concentration, time, and the percentage of larval mortality (Mehra and Hiradhar, 2000; Pelah et al., 2002). However, on an

individual basis, *C. burmannii* exhibited a higher larvicidal effect than *S. aromaticum*, as was observed with their LC_{50}. The LC_{50} values of the two plants show that they can cause 50% larval mortality at 184.28 mg/ml and 363.70 mg/ml, which makes them promising botanical larvicides. Synergistic effects of larvicidal agents have been reported to be advantageous in the control of various pests (Seyoum et al., 2002).

Plant extracts combined formulations improve the larvicidal activity by decreasing the needed dose and lowering the time required to kill the larvae, making them more economical and practical (George and Vincent, 2005; Mohan et al., 2007). Mixtures of insecticide with a different mechanism of action are effective for managing resistant insects (Intirach et al., 2012; Ru et al., 1998). Therefore, they are very beneficial in mosquito control management (Mohan et al., 2010).

In a previous study, assessment of the larvicidal effectiveness of combinations Piper sarmentosum, and *Zanthoxylum piperitum,* Foeniculum vulgare, *Myristica fragrans*, and *Curcuma longa* at different ratios (25%:75%, 50%:50%, and 75%:25%) revealed that at the highest ratio (75%:25%) the extracts displayed synergistic action. All combinations at the lower ratios (50%: 50% and 25%:75%) revealed antagonistic activity. Similarly, The mixture of *Pongamia glabra* and *Annona squamosa* extracts exhibited a synergistic effect against *Culex quinquefasciatus* larvae. Among the combined extracts (25%: 75%, 50%:50%, and 75%:25%) used, the 'A 50%: P 50%' extract was reported to be most effective extract than the other combinations and revealed the maximum synergism (Synergistic Effect:15.1) (George and Vincent, 2005).

Histopathology evaluation of the third instar of *Cx. pipiens* exposed to S.A. 25%: C.B. 75%. The result revleaed that the midgut was affected by the extract. Midgut was severely damaged, especially the basal membrane, epithelium cells, and microvilli. These damages could be attributed to the larvicidal secondary metabolites (phenols, cinnamaldehyde, flavonoids, alkaloids, eugenol, coumarin, tannins, steroids, and saponins) that were reported previously in the plants used (Davis and Stout, 1971). Phytochemicals have the potential as a larvicide and work as an insect growth regulator, a feeding deterrent, and by interrupting nerve impulses, blocking respiration and stomach poison (Al-Mekhlafi, 2018).

The mosquito midgut plays a significant role in the enzymes secretion, absorption of nutrients (Christophers, 1960), ion transport, osmoregulation (Sina and Shukri, 2016), and defense against pathogens (Terra, 2001). In addition, the regenerative cells in the midgut play a vital role in metamorphosis (Procopio et al., 2015). Botanical extracts have been reported to alter the midgut and the survivability of insects. Mosquito larvae treated with *Capparis cartilaginea, Melia azedarach*, *Derris urucu*, and *Averrhoa bilimbi* extracts have been reported to alter the midgut epithelium, microvilli, peritrophic matrix, enlargement of intercellular spaces, and cytoplasmic vacuolization (Abutaha and Al-Mekhlafi, 2014; Al-Mehmadi and Al-Khalaf, 2010; Gusmão et al., 2002). Previously, (Rey et al., 1999) and (Amala et al., 2021) stated that the primary target of any plant metabolite is the midgut epithelium cells and peritrophic membrane, in which the latter is mainly accountable for growth stimulus in the insects. The damage of the midgut region found in the larvae treated with S.A. 25%: C.B. 75% could be related to the damage to digestive absorption processes and the regenerative cells in the larval midgut, disrupting larval mosquito development and compromising survival.

Although the *C. burmannii* and *S. aromaticum* extract have a promising larvicidal potential against *Cx, pipiens* (LC50: 0000 μg/mL), it possesses higher toxicity (IC50: μg/mL) against HUVEC cells. The cell morphology also confirmed the cytotoxicity of the extract against HUVEC cells. The extract was toxic to human HUVEC cell lines, and hence precaution should be considered when using the extract. This finding does not agree with those reported by Silva et al. for *Eugenia calycina* leaf extract against *Ae. aegypti* (199.3 μg/mL), which was reported to show high toxicity against the third instar compared to Hela cells (240.3 μg/mL) (Silva et al., 2021).

5. Conclusion

The hexane extract of the two plants tested individually or in combinations resulted in a promising larvicidal potential against *Cx. pipiens* larvae. Tested singly, the hexane extract of *Cinnamomum burmannii* was revealed to be the most active compared to *Syzygium aromaticum* against mosquito larvae. However, the blending of the two hexane extracts of the two plants showed a synergistic effect when applied to mosquito larvae. This makes it a promising candidate for developing a new eco-friendly larvicidal agent in the larvae breeding sites.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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