



Research article

Effectiveness of water spinach (*Ipomoea reptans* Poir) in mitigating acetylcholinesterase inhibition caused by organophosphate and carbamate pesticide

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Abstract

Importance of the work: Organophosphate (OP) and carbamate (CB) pesticides pose health risks by inhibiting acetylcholinesterase (AChE). Evaluating water spinach (*Ipomoea reptans* Poir) as a remedy could provide a natural, accessible, and non-toxic remedy to counteract pesticide exposure.

Objectives: To evaluate the effectiveness of water spinach in reducing AChE inhibition caused by OPs and CBs, and to assess its toxicity using a GT-Pesticide Residue Test Kit.

Materials & Methods: Gas chromatography and mass spectroscopy (GC-MS) were used to analyze important chemical compounds. The quantity of water spinach to reduce inhibition of AChE by OP and CB pesticides was investigated using spectroscopic techniques.

Results: The sample tube colors were clearer than the control (I_0 , I_{50} = control and B1, L1, T1 = branch, leaf and top, respectively, of *I. reptans* with pesticide in the ratio of 1:1 (2.5 g:2.5 g) and B2, L2, T2 = branch, leaf and top, respectively, of *I. reptans* without pesticide, except L1 (I_{50}). Based on GC-MS, the water spinach contained alcohol functional groups, facilitating nucleophilic substitution reactions, which reduced AChE inhibition by OPs and CBs. Spectroscopic analysis showed differing efficiencies: *I. reptans* reduced OP inhibition (3 times hydrolysis) better than CB inhibition (2 times hydrolysis). *I. reptans* tea was non-toxic to human gingival fibroblast at concentrations of 0.0001–1 mg/mL.

Main finding: Water spinach consists of important compounds that could reduce the inhibition of AChE caused by OPs and CBs, as well as reducing the inhibition of OPs to a greater degree than the inhibition of CBs.

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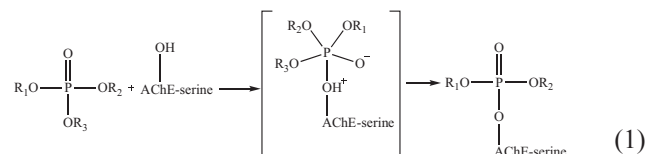
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Introduction

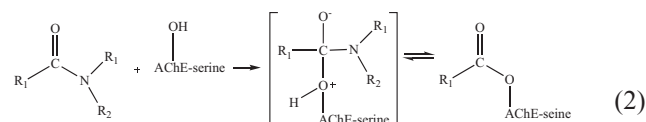
Pesticide residues in vegetables remains a major concern, with adverse implications from both economic and health perspectives (Buakham et al., 2012). OPs and CBs are widely utilized pesticides, known for their inhibitory effects on the enzyme acetylcholinesterase, commonly referred to as AChE (Hobbiger, 1961; Darvesh et al., 2008; Jantapratuang et al., 2018). It is generally accepted that AChE plays a major role in the hydrolysis of ACh (an important neurotransmitter) to acetic acid and choline (McHardy et al., 2017). AChE plays a crucial role as an enzyme within the nervous systems of animals, where its primary function is to terminate impulse transmission by rapidly hydrolyzing the neurotransmitter known as acetylcholine (Čolović et al., 2013). Inhibition of AChE can occur through the binding of organophosphate (OP) and carbamate (CB) esters, which form a covalent bond with a serine residue located in the active site of the enzyme. Modeling analysis has revealed important insights, including the influence of biotransformation on CB toxicity, the ability of OP and CB analogs to inhibit AChE, the role of hydrogen bonds in AChE inhibition and the importance of the π - π interaction with Trp86 for strong AChE inhibition (Lee and Barron, 2016). Therefore, inhibition of AChE by OPs and CBs causes an overabundance of ACh, which leads to negative effects on the nervous system (Čolović et al., 2013). Several studies have used herbs to reduce the reaction of CBs and OPs (Alkiyumi et al., 2012; Chinacarawat et al., 2012; Dua et al., 2015a, b). One of the effective plants to reduce AChE's inhibition by OPs and CBs is *Ipomoea reptans* Poir (Datta et al., 2021).

Qualitative analysis of *I. reptans* and quantitative analysis of the inhibition of the AChE enzyme have been widely studied. Gas chromatography-mass spectroscopy (GC-MS) is a suitable technique for analyzing organic compounds, with several alcohol functional groups having been identified in *I. reptans* (Shim, 2012). These alcohol functional groups are hypothesized to undergo nucleophilic substitution with CBs and OPs, potentially reducing enzyme inhibition by these pesticides. In addition to qualitative analysis, the degree of AChE inhibition by OPs and CBs has been investigated using spectroscopic methods (Beam and Hankinson, 1964; Jantapratuang et al., 2018; Karunakaran et al., 2022). Understanding the mechanism of AChE inhibition and ACh hydrolysis is essential for evaluating the quantitative analysis of AChE inhibition by OPs and CBs.

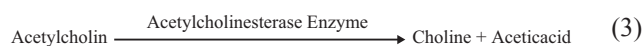
Studies have revealed that OPs act as irreversible inhibitors of AChE, forming stable AChE-OP complexes through phosphorylation of the enzyme's serine residue, while conversely, CBs form reversible AChE-CB complexes through carbonylation of the serine residue, resulting in lower stability (Karunakaran et al., 2022): as shown in Equation 1.



CBs and the serine residue of AChE can produce carbonylation and form an AChE-CB complex in a reversible reaction that has low stability (Equation 2):



AChE plays a vital role in hydrolyzing ACh (Equation 3):



AChE's role in ACh hydrolysis can be measured spectrophotometrically at wavelengths in the range 540–570 nm, implying that enzyme inhibition is correlated inversely with absorbance in that range (Karunakaran et al., 2022; Acetylcholine Assay Kit, STA-603; Cell Biolabs, Inc. USA)).

The current research aimed to identify the organic functional groups present in *I. reptans* and to examine the effects of the reaction of *I. reptans* on AChE inhibition by CBs and OPs.

Materials and Methods

Sample preparation and preliminary phytochemical screening

I. reptans collected from cropland in the Chiang Saen area, Chiang Rai province, Thailand was dried in a hot-air oven at 45°C for 24 hr or until reaching a moisture content of 5%. Subsequently, it was blended and passed through a 40-mesh sieve before being stored in a desiccator (Chattiranan et al., 2013; Čolović et al., 2013). The phytochemical screenings were conducted with three replicates. Two drops of Wagner reagent were added to 2 mL of the extract and thoroughly mixed. The formation of a reddish-brown precipitate indicated the presence of alkaloids

(Wagner, 1993). Terpenoids were detected using the Salkowski test, wherein a red-brown ring formed at the interface of solutions upon mixing 5 mL of extract with 2 mL of chloroform in a test tube, followed by the addition of 3 mL of concentrated H₂SO₄ along the sides of the test tube. (Tiwari et al., 2011; Singh and Kumar, 2017).

The procedure involved boiling 0.2 g of the extracted sample with 10% sulfuric acid for 5 min in a water bath, followed by filtration and cooling. Then, the filtrate was extracted using dichloromethane; next, 2–3 drops of 10% ammonia solution were added. The formation of a rose-pink color in the ammonia layer indicated the presence of anthraquinone (Tukiran, 2013).

A ferric chloride test was used to detect tannin, by boiling 0.20 g with 5 mL of 45% ethanol for 5 min. The mixture was cooled and filtered. A quantity of the filtrate was diluted with distilled water and added with 2 drops of ferric chloride. A transient greenish-to-black color indicated the presence of tannins (Kancherla et al., 2019).

To test for coumarin, 0.2 g of the dried sample was transferred to a 1.0 mL test tube with 50% ethanol and shaken. The filtrate from the test tube was sealed with 6 M NaOH, then shaken. A yellow solution indicated the presence of coumarin (Ayoola et al., 2008).

For the steroid test, 0.5 g of the extract was dissolved in 10 mL anhydrous chloroform and filtered. The solution was divided into two equal portions for the following tests. The first portion of the solution was mixed with 1 mL of acetic anhydride followed by the addition of 1 mL of concentrated sulfuric acid down the side of the test tube to form a layer underneath. The test tube was observed for green coloration as indicative of steroids (Ayoonla et al., 2008).

The frothing test for saponins involved combining 3 mL of the aqueous extract solution with 10 mL of distilled water in a test tube. The test tube was stoppered and vigorously shaken for approximately 5 min, followed by a 30 min period of standing to observe the formation of honeycomb froth, indicative of the presence of saponins (Ayoonla et al., 2008).

The total phenolic content of the tree parts of *I. reptans* (top, leaf, and branch) were determined using Folin-Ciocalteu assay (Deetae et al., 2012). Gallic acid (0.01–0.07 mg/mL) was prepared in methanol and used as the standard. A sample (100 μ L) was placed in a test tube followed by 1.5 mL of 10% Folin-Ciocalteu reagent dissolved in water and 100 μ L of 7.5% weight per volume Na₂CO₃. The mixture was allowed to stand in the dark for 120 min at room temperature. The absorbance at 731 nm was

measured using a spectrophotometer. Triplicate determinations were performed. The results were expressed as milligrams of gallic acid equivalents (GE) per gram of the extract compared with the standard curve of gallic acid.

The free radical scavenging capacity of different samples was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method of Yen and Chen (Yen and Chen, 1995) with slight modifications. The method involved the reaction of the antioxidants present in the extracts with the stable DPPH in 0.1 mM methanol solution. Briefly, the reaction mixture contained 300 μ L of extract of varying concentrations (10–100 μ g/mL) and 2 mL of DPPH solution. After 10 min, the change in absorbance was recorded at 517 nm in a spectrophotometer against a blank, which did not contain the extract. L-ascorbic acid was used as a positive control. The DPPH radical scavenging capacities were expressed as vitamin-C equivalent antioxidant capacity (VCEAC) in millimoles per gram of extract. The % DPPH• scavenging activity was calculated based on Equation 4:

$$\% \text{ DPPH}\bullet \text{ scavenging activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (4)$$

where A_{control} is the absorbance of the positive control and A_{sample} is the absorbance of the sample.

Toxicity survey using GT-Pesticide Residue Test Kit

Each of the separate parts of fresh *I. reptans* (top, leaf, and branch) were chopped or blended, with 5 g of the respective samples being weighed into separate sample bottles. The GT-Pesticide Residue Test Kit was used according to Gobthong (1999). Then 5 mL of solvent-1 (dichloromethane) was added into each sample bottle, shaken vigorously for 1 min and left to stand for 15 min. Next, 1 mL of extract was pipetted into a test tube and 1 mL of solvent-2 (ethanol) was added, before the mixture was evaporated in a modified warm water bath (35–36°C) by connecting the evaporating kit with the air pump and the Pasteur pipette. The end of the Pasteur pipette was inserted into the sample test tube, and the air released from the air pump into the extract was adjusted until the lower layer (solvent- 1) disappeared (sample extract). Each test tube was labeled: tube 1, cut point or unsafe; tube 2: control or not detected; tubes 3, 4, 5, 6, 7 and 8, sample extracts of top, leaf and branch with (3,4,5) and without (6,7,8) OPs and CBs, respectively. Then, 0.25 mL of solven-2 was added into each of tubes 1 and 2, while 0.25 mL of

each sample extract was added into tubes 3–8. Next, all the tubes were placed in the modified warm water bath (35–36°C). Pipette samples (each 0.5 mL) of GT-1 (acetylcholine) were added to every tube allowed to stand for 5–10 mins. Subsequently, 0.375 mL of GT-2 (acetylcholinesterase) was transferred to tube 1, while 0.25 mL was transferred to each of tubes 2–8 and allowed to stand for 60 mins, after which 1 mL of GT-3 (hydroxamine) was added to each tube. Finally, 0.5 mL of GT-4 (hydrochloric acid) and 0.5 of GT-5 (ferric chloride) were transferred into each tube. The results were observed and evaluated in three replicates.

Reducing inhibition mechanism characterization

Qualitative analysis using gas chromatography-mass spectrometry

Qualitative analysis was conducted using GC-MS (Clarus 690 gas chromatograph; PerkinElmer; USA) equipped with a Clarus SQ8T mass spectrometry detector (PerkinElmer; USA). The extracted sample was injected into a DB-5 column (30 m × 0.25 mm, 0.25 µm thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector was operated at 70–260°C with a flow rate of 6°C/min. The identification of components was based on the Willey and NIST libraries as well as comparisons of their retention indices (Wu et al., 2010).

Quantitative analysis

This research utilized the GT-Pesticide Residue Test Kit specifically designed for detecting OP and CB groups (Wattanasuwakul, 2016). Each experiment was conducted in triplicate and data were presented as mean ± SD values. Dried, blended *I. reptans* samples each weighing either 5.00 g (34.5% extraction) or 7.50 g (42.5% extraction) were placed in bottles. The samples were extracted with 10 mL and 15 mL of solvent-1 (dichloromethane), respectively, shaken and then left for 15 min. Next, 1 mL of liquid from each sample bottle was transferred into a tube, followed by 1 mL of solvent-2 (95% ethanol). The bottom solution was blown out until the tube contained only a single-layer solution (sample extract).

The samples (tubes 1–7, Table 1) were mixed with 500 µL AChE (GT-1) in a water bath at 35°C for 10 min. Subsequently, 250 µL cholinesterase (GT-2) was added and left for 60 min. Afterward, 1 mL of hydroxylamine (GT-3) and 500 µL of hydrochloric acid (GT-4) and ferric chloride (GT-5) were added to the mixture. The supernatant from the mixture,

obtained after centrifugation at 3,400 revolutions per minute, was collected for analysis of absorbance at 540 nm using an ultraviolet (UV)-visible spectrophotometer (GENESY 20; Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand).

Cytotoxicity test

The cytotoxicity test was carried out according to Vichai and Kirtikara (2006) and ISO10993-5 (2009). The dissolved *I. reptans* extract was introduced into the culture medium and centrifuged. Then, the supernatant was sterilized using filtration through a 0.2 µm membrane and diluted to the desired concentration using sterilized culture medium. Subsequently, the cytotoxicity of the *I. reptans* extract sample was tested using sulforhodamine B (Vichai and Kirtikara, 2006) and a microplate reader (Model 680; Bio-Rad Laboratories Ltd.; USA). Ultimately, in the cytotoxicity test, the survival percentage (cell viability) was calculated. The wavelength for detection was 540 nm. Cell viability as a percentage was calculated using: Cell viability = $A / B \times 100$, where A is the optical density of the sample and B is the optical density of the control.

Statistical analysis

Each experiment was conducted in triplicate, and data were presented as mean ± SD. The data on effectiveness of *I. reptans* in reducing AChE inhibition (represented by absorbance values) were analyzed using one-way analysis of variance followed by the Tukey HSD test in Statistix Version 8. Likewise, data on survival rate of human gingival fibroblasts exposed to different concentrations of *I. reptans* extract were analyzed using one-way analysis of variance followed by Tukey's range test for mean comparison. A p-value of <0.05 was considered significant.

Table 1 AChE inhibition reduction mechanisms studied

Tube	Chemical
1	AChE + ACh
2	AChE + ACh + OPs
3	AChE + ACh + OPs + <i>I. reptans</i> 34.5% extraction
4	AChE + ACh + OPs + <i>I. reptans</i> 42.5% extraction
5	AChE + ACh + CBs
6	AChE + ACh + CBs + <i>I. reptans</i> 34.5% extraction
7	AChE + ACh + CBs + <i>I. reptans</i> 42.5% extraction

Results and Discussion

Preliminary phytochemical screening

The preliminary phytochemical screening of the *I. reptans* tea revealed the presence of alkaloids, flavonoids, anthraquinones, coumarins, terpenoids and saponins, as shown in Table 2.

Table 2 Preliminary phytochemical screening of *Ipomoea reptans* extract

Phytochemical	Observation color
Alkaloids	Yellow (+)
Flavonoids	Yellow (+)
Anthraquinone	Pink (+)
Tannin	Clear (-)
Coumarins	Yellow (+)
Steroids	Clear (-)
Terpenoids	Red-brown ring (+)
Saponins	honeycomb froth (+)

The free radical scavenging capacity of the different fractions from the *I. reptans* extract samples was measured based on the DPPH radical scavenging method. The results showed higher radical scavenging in the leaves. The total phenolic content was determined using a spectrophotometric method with Folin-Ciocalteu reagent and values in the range 10–51 mg GE/g of the extract. The leaves had the highest phenolic contents (Table 3), which suggested that most of the phytochemicals were present in the leaves of *I. reptans* (Yin et al., 2013).

Table 3 Radical scavenging assays and total phenolic contents of different fractions of *Ipomoea reptans* extract

	Top	Leaves	Branch
DPPH radical scavenging activity (umol TE/g)	194.3	202.24	76.37
Total phenol content (mg GE/g)	29.17	51.5	10.5

DPPH = 2,2-Diphenyl-1-picrylhydrazyl; TE = Trolox equivalent; GE = Gallic acid equivalent.

Toxicity assessment of overall toxicity of primary pesticides

Based on the principle of the cholinesterase inhibition approach, the GT-Pesticide Residues Test Kit was selected for screening harmful residue in agricultural goods, specifically targeting OPs and CBs (Tongbor et al., 2018). Certain toxic residues can inhibit the cholinesterase enzyme at levels below 50%. These residues can be removed through washing. However, if the toxic residues inhibit the cholinesterase enzyme at or above 50%, they are categorized as highly dangerous residues. The toxicity level of the analyst sample must be

lower than the threshold that triggers poisoning, which in turn affects the performance of cholinesterase by reducing it by 50%. The color test findings indicated that all colors (B1, B2, L2, T1, T2) were significantly clearer compared to the control tube or cut point (I50), as shown in Fig. 1, except for L1.

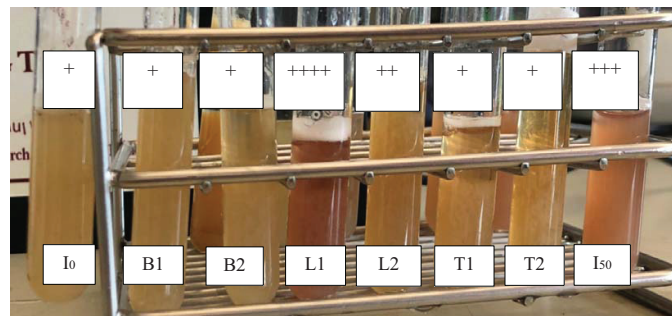


Fig. 1 Colors of sample tubes compared to control, with I₀ and I₅₀ = negative control (blank) and positive control (cut point) respectively; B1, L1 and T1 = branch, leaf, and top, respectively, of *Ipomoea reptans* with pesticide in the ratio of 1:1; and B2, L2 and T2 = branch, leave, and top, respectively, of *Ipomoea reptans* without pesticide and +, ++, +++, ++++ and +++++ = increasing levels of yellow color.

GT test kit is widely used to analyze organophosphate and carbamate residue. Evaluation of pesticide residue is to compare color of sample with control and cut point. More degree of color, more toxicity of food (ASIANMEDIC CO., LTD.). The GT-Pesticide Residual Kit revealed a significant accumulation of toxins from the OPs and CBs of the pesticide. Specifically, a substantial amount of toxins was found in the top section, whereas only a small amount of toxins was detected in the branch of the dried *I. reptans*.

The result illustrates that the degree of color change in branches and leaves (+1) is less than the degree of color change in the top color (+2). This implies that the ability to reduce toxicity in a solution of leaves and branches is more effective than in the top (Gobthong, 1999).

Investigation of reducing inhibitions mechanism

Qualitative analysis

The GC-MS analysis indicated a major chemical compound extracted from the *I. reptans* branch using water was 2,7-octadiene-1,6-diol, 2,6-dimethyl, which contains an alcohol functional group. This compound can induce a nucleophilic substitution at both the AChE CB complex and the AChE OP complex (Lee and Barron, 2016). Consequently, this reaction leads to a reduction in the inhibition process.

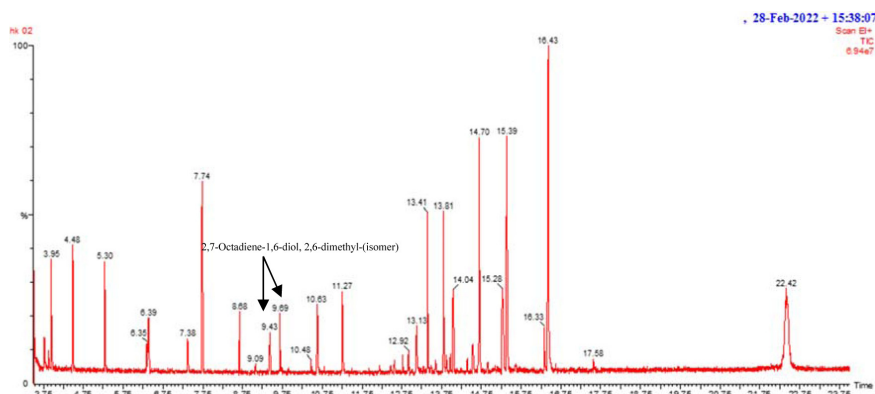


Fig. 2 Gas chromatography-mass spectroscopy chromatogram of 2,7-Octadiene-1,6-diol, 2,6-dimethyl-(isomer) in *Ipomoea reptans*

Quantitative analysis

According to the results shown in Fig. 3, hydrolysis of ACh by enzyme exhibited absorbance at 540 nm. This result was in good agreement with other studies (Chattiranan et al., 2013; Tukiran, 2013). Consequently, the hydrolysis reaction of ACh to acetic and choline correlated directly with the absorbance observed from the UV spectroscopy at 540 nm. Thus, it could be concluded that absorbance is an indirect measure of the inhibition of OPs and CBs. Specifically, high absorbance is indicative of low pesticides level, reflecting a high efficiency in reducing pesticides that inhibit AChE. Conversely, the absorbance decreased when CBs were added, as the hydrolysis of ACh was inhibited, resulting in a reduction in absorbance. When *I. reptans* was added, the absorbance increased compared to the disturbed ACh response caused by the CBs in tube 2. Since absorbance is inversely proportional to the inhibition of ACh by CBs, these findings suggest that *I. reptans* could decrease the inhibition caused by CBs. Furthermore, the greater the amount of *I. reptans* added, the more efficient the

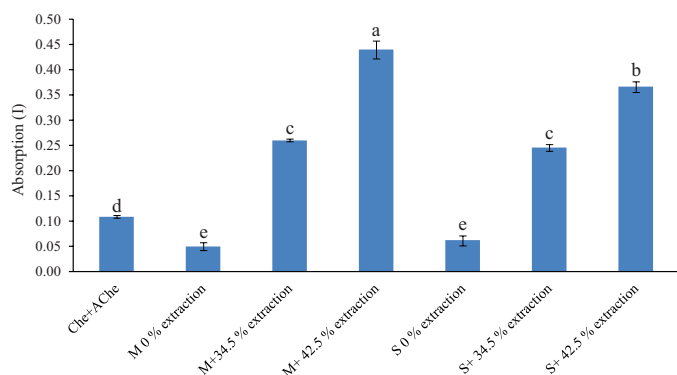


Fig. 3 Effectiveness of *Ipomoea reptans* in reducing AChE inhibition, with high absorbance reflecting enhanced hydrolysis reaction; Error bars represent standard deviation; different lowercase letters above bars denote significant ($p < 0.05$) difference; ChE- Cholinesterase, AChE -Acetylcholinesterase, M -Malathion, S-Carbaryl

reduction of AChE's inhibition by CBs. The one way analysis of variance results revealed a statistically significant effect of the inhibition caused by CBs and OPs both between groups and within groups at the < 0.05 level for the 7 groups [$F(6,14) = 718.5990, p < 0.05$].

In addition, the introduction of *I. reptans* reduced the inhibition of AChE by the OP inhibitors, as evidenced by an increase in absorbance. The absorbance reduced when the OPs AChE combined with *I. reptans*, compared to when the CB inhibitor was utilized, because the stability of the OPs complex was higher. As a result, the nucleophilic substitution reaction of the OPs was reduced. *I. reptans* had a greater capacity to mitigate the suppression of CBs compared to OPs. Furthermore, the results suggested that *I. reptans* had the capacity to reduce the hydrolysis reaction of AChE by over twice the normal amount (Lee and Barron, 2016). The results illustrated the absorbance of AChE and ACh and the reaction of ACh and AChE at 540 nm. Based on the results, the absorbance increased significantly (by 2 times) in the reaction of acetylcholine and acetylcholinesterase. It was presumed that this reaction absorbed light at 540 nm, resulting in higher absorbance due to the increased reaction between ACh and AChE. Therefore, it may be concluded that the higher absorbance was because of the lower anti-AChE activity of two pesticides.

Cytotoxicity test

The results of cytotoxicity (Table 4) show the survival rates percentage that were calculated across five different concentrations. The *I. reptans* extract demonstrated toxicity at concentrations in the range 0.0001–1 mg/mL, yet proved non-toxic for human gingival fibroblasts, with survival percentages ranging from $86.97 \pm 2.02\%$ to $92.75 \pm 4.93\%$.

Table 4 Survival rate percentages of human gingival fibroblasts exposed to different concentrations of *Ipomoea reptans* extract compared to those exposed to sodium lauryl sulfate

Sample	Concentration (mg/mL)				
	0.0001	0.001	0.01	0.1	1
<i>I. reptans</i> extract	92.23±6.29 ^a	92.75±4.93 ^a	91.70±3.50 ^a	90.64±2.65 ^a	86.97±2.02 ^a
Sodium lauryl sulfate	95.82±4.39 ^a	92.15±6.29 ^a	84.30±2.42 ^a	43.17±4.73 ^b	17.21±1.44 ^b

Mean ± SD (from four replications) in the same column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

In contrast, sodium lauryl sulfate exhibited toxicity at concentrations of 0.1 and 1 mg/mL, with survival percentages of $43.17 \pm 4.73\%$ and $17.21 \pm 1.44\%$, respectively. The one way analysis of variance results revealed a statistically significant effect of treatment on cytotoxicity survival rates, $F(9, 30) = 158.99$, $p < 0.05$. This indicates that at least one treatment group had a different mean survival rate compared to the others. The means of *I. reptans* extract and Sodium lauryl sulfate are significantly different at higher concentrations (0.1 mg/mL and 1 mg/mL), but not at the lower concentrations (0.0001, 0.001, and 0.01 mg/mL).

Conclusions

Ultimately, the initial analysis of *I. reptans* extract revealed the existence of alkaloids, flavonoids, anthraquinones, coumarins, terpenoids and saponins. The leaves of *I. reptans* had higher levels of radical scavenging activity and total phenolic content than the other components (branch and top). The hydrolysis of acetylcholine was inversely proportional to the inhibition of AChE. Thus, the hydrolysis reaction could be applied to examine the inhibition of the enzyme. Increased absorbance corresponded to a greater hydrolysis reaction, whereas a decreased inhibition reaction indicated a higher efficacy in eliminating the inhibitory effects of pesticides on the enzyme. *I. reptans* was poisonous at concentrations in the tested range 0.0001–1 mg/mL, although it did not have a harmful effect on human gingival fibroblast cells.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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