Assessing the Effectiveness of Saponins from Alfalfa (*Medicago sativa* L.) to Mitigate Cypermethrin Residues in Apples

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Summary: Pesticide residues on fruits and vegetables are of major health concern around the world. Some of these pesticide residues are extremely toxic and can become a major causative factor for various diseases such as cardiovascular disorders (CVDs), lung, endocrine, and nervous system damage, as well as the circulatory system, and reproductive system problems. This study was aimed at investigating the effectiveness of saponins isolated from alfalfa (*Medicago sativa* L.) seeds for mitigating cypermethrin residues on apples (*Malus domestica* Borkh.) in comparison to tap water, citric acid, and baking soda. Cypermethrin concentration applied to apples was 1 ml/L. After washing the apples with varying concentrations of different washing solutions, analysis for cypermethrin residues was performed using a UV/VIS spectrophotometer at a wavelength of 535 nm. The maximal removal of residues recorded for baking soda, tap water, and citric acid was 92.98, 72.50, and 74.59 % respectively. Saponins exhibited a maximum of 13.90 % of residual removal which was not as effective as other washing agents.

Keywords: Cypermethrin, Sodium carbonate, Citric acid, Saponins, Apple.

Introduction

Fruits and vegetables, as part of a healthy and varied diet, play a vital role in the improvement of health outcomes for humans owing to being excellent sources of total sugars, various polyphenols, antioxidants, dietary fibers, micronutrients such as minerals including calcium, potassium, sodium, phosphorus, magnesium, and iron, as well as vitamins such as vitamins A, and C, and B complex vitamins [1-3], and therefore, many potential health benefits [4]. In developing countries, farmers use various pesticides, often injudiciously, during the maturity stages of fruit and vegetable plants in order to obtain higher yields, manifesting in the form of pesticide residue deposits on the surface of fruits and vegetables, a probable pathway for causation of various adverse health effects in the consumers such as birth defects, cancers, and neurodevelopmental disorders [5-6].

In this study, the residues of cypermethrin (CYM) were investigated and analyzed on apples sourced locally from various markets. CYM is a highly active pyrethroid insecticide and has been used for more than 40 years as an agrochemical in large-scale commercial agricultural applications across the world, and being a contact and digestive insecticide, is effective against fruit and leaf-eating Coleoptera and Lepidoptera in fruits, vegetables, cotton, tobacco, vines, and other crops [7]. CYM is a synthetic analog of terpenoid pyrethrins derived from Dalmatian chrysanthemum (*Chrysanthemum cinerariaefolium* Vis.), and has the potential to cause neurotoxicity in humans [8]. The proposed acute oral LD_{50} value of CYM for rats is 251 mg/kg [7, 9].

Various studies have revealed that fruits and vegetables may contain pesticide residues at levels above the maximum residue limits (MRLs) as per the Codex Alimentarius standards [10-12]. In this regard, various homemade washing solutions such as sodium carbonate, sodium bicarbonate (baking soda), acetic acid, and aqueous solutions of sodium chloride are frequently used for washing fruits and vegetables [13]. It was shown that pesticide residues readily degrade in these acidic and alkaline homemade washing solutions [1, 13-14]. Similarly, Abdullah *et al*. [29] demonstrated the effectiveness of acetic acid, citric acid, and hydrogen peroxide as soaking/washing solutions for minimizing pesticide residues in various vegetable species [29]. Furthermore, various modern techniques such as cold plasma technology, ozonation, and high hydrostatic pressure (HHP), etc. have also exhibited improved effectiveness in the elimination of pesticide residues. However, due to high maintenance and installation costs associated with the techniques, the application of these techniques has been fairly limited in the fruits and vegetable processing operations [5].

Besides the homemade washing agents already discussed, biological extracts have also been investigated for mitigation of pesticide residues in both fruits and

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vegetables. For instance, the extracts from ginger, garlic, radish, and lemon showed 73.04, 65.89, 60.68, and 68.68% respectively of CYM residues removal in spinach [1]. Similarly, another study utilized the extracts from *Albizia amara* and *Acacia concinna* demonstrated excellent reduction ability against residues of organophosphorus pesticides (dichlorvos, dimethoate, malathion, and chlorpyrifos) on tomato samples without altering their nutritional and sensory attributes [30]. Rhamnolipid biosurfactant (JBR425) has also been investigated as a cleaning agent for cypermethrin residues in cabbage, and exhibited significant reduction potential [31]. Similarly, a recent study utilized saponins extracted from food byproducts (soybean and camellia seed cakes) for removal of pesticide residues in field-sprayed fruits and vegetables, and reported significant dislodgement of pesticide residues by way of saponin-assisted aeration washing [32]. In the present study, alfalfa (*Medicago sativa* L.) was used for the isolation of saponins, which have traditionally been used as detergents and biosurfactants [15]. However, studies involving biological extracts and biosurfactants as washing agents for removal of pesticide residues in fruits and vegetables are very limited, and present an interesting avenue for exploring and investigating biosurfactants as a natural, safe, and cost-effective solution for mitigating pesticide residues in various types of plant produce.

Saponins are highly specialized phytochemicals and represent a broad group of biomolecules, primarily consisting of a complex mixture of glycosides formed from high molecular weight triterpenes, with hederagenin, zahnic acid, bayogenin, medicagenic acid, and soyasapogenols A and B as the predominant aglycones, and one or more sugar chains (glycone) attached to the aglycone component. Saponins are well-known biosurfactants with favorable attributes such as lower toxicity, are eco-friendly, and biodegradable, and are easily adaptable than the other types of surfactants [16-18]. These biomolecules also possess viable health benefits, such as antitumor, antibacterial, and antiviral activities. The present research, therefore, was aimed at developing an effective washing method using biological agents for removal of pesticide residues in fruits and vegetables.

Experimental

Sample collection

Alfalfa seeds (*Medicago sativa* L., family Fabaceae) were used in this study, while apples were purchased from the local markets of Lahore, Pakistan. CYM standard (purity 94%) was provided by Four Brothers Pvt. Limited. Oleanolic acid (purity 95%) standard was purchased from Biosynth Carbosynth Limited UK. All reagents used in the research study were of analytical grade or maximum purity. Moreover, distilled water was used throughout the experiment.

Extraction and quantification of alfalfa seeds

Extraction was achieved using the methodology devised by [19]) with minor modifications. Alfalfa seeds were finely ground using a multipurpose food processor (Anex, model AG-3051). Subsequently, the plant material was soaked in 40% ethanol (1:5), and subjected to continuous agitation for 48h at ambient temperature. After this, the soaked material was centrifuged (Eppendorf centrifuge, model 5810 R Hamburg, Germany), and the supernatant was collected. The solvent was evaporated (50°C at 30 hPa) using a rotary evaporator (model WEV-1001L, Daihan Scientific, Korea). The remaining solution was stored at 4°C for further use [19]. Saponin contents were determined by using the methodology outlined by [20]) with minor modifications. A standard stock solution of Oleanolic acid (2.4 mg/mL) was prepared in ethanol. Appropriate dilutions (100-1600 µg/mL) of standard stock solutions were prepared in water. Initially, standard, blank (EtOH), and extraction solution (0.25 mL) were taken in a 10 mL volumetric flask, followed by 8% vanillin (w/v) in ethanol (0.25 mL), and 72% sulfuric acid (v/v) in water was added to it (Table-1) and incubated in a water bath (model HH-S4) at 60°C for 15 mins with continual shaking at different time intervals. After this, the solution was cooled in water at room temperature for 5 mins. The absorbance was measured at 560 nm wavelength using UV/VIS spectrophotometer (model UV 1100) [20].

Qualitative verification of saponins was achieved by placing the plant material in a test tube, followed by the addition of distilled water, and then vigorous shaking for 2 mins [21-22].

Table-1: Standard curve (oleanolic acid) setup procedure.

Components	Reagent Blank (mL)	Standards (mL)	Samples (mL)
Extraction Solvent	0.25	-	-
Standard	-	0.25	-
Plant Sample	-	-	0.25
Vanillin 8%	0.25	0.25	0.25
Sulphuric acid 72%	2.50	2.50	2.50

Preparation of cypermethrin standard stock solution

1 mg/mL stock solution of CYM was prepared in ethanol. The stock solution was appropriately diluted with distilled water for the preparation of standard

working solutions. For simulation of acidic and alkaline conditions, aqueous solutions of hydrochloric acid (3%), and sodium hydroxide (20%) were used. 0.225 g of sodium nitrate was dissolved in water for the preparation of nitrate solution (0.15%), which was then diluted up to 100 mL with distilled water. Likewise, 2 mL of aniline was dissolved in 70 mL of ethanol for the preparation of 2% aniline solution, which was then diluted up to 100 mL with distilled water [7].

Preparation of calibration graph of cypermethrin

From the standard stock solution of CYM, 3 to 30 µg portions were taken in a 25 mL volumetric flask, and 1 mL of sodium hydroxide was added to it. For achieving complete hydrolysis, the solution was kept for 15 mins at ambient temperature. After that, 1 mL of 3% hydrochloric acid was added along with 0.15% nitrite solution and shaken well. 1 mL of aniline solution (2%) was added and diluted to 25 ml with distilled water. The formation of azo dye was recorded to take place after 45 mins. The absorbance was measured at 535 nm using a UV spectrophotometer (

Fig. 1) [7].

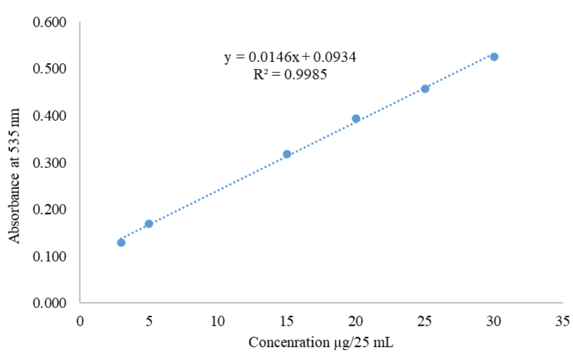


Fig. 1: Absorbance spectra of CYM. The result shows good linearity with coefficient of determination of 0.99.

Washing of apples

1 mL/L concentration of CYM in water was applied to apples [23]. Unwashed samples were used as control, whereas varying concentrations (1, 2, and 3%) of sodium bicarbonate (baking soda), citric acid, and alfalfa seeds extract along with tap water were used for washing of apples. Thorough hand rinsing of apples with distilled water was followed by air drying before the experiment to remove surface contamination such as dust particles. Pesticide solution was sprayed by using a spray bottle on the surface of the apples. The apples were then air-dried for 30 mins. Apples were first dipped in washing solutions for 5, 10, and 15 mins, followed by gentle rinsing with 150 mL of distilled water. The washed apples were subsequently subjected to air drying at ambient temperature for 10 mins [13].

Sample preparation for analysis of pesticide residues

The peel of the apple was removed and finely chopped using a knife to facilitate analysis. Then, 5 g of sample was taken in a beaker, and 25 mL of the solvent [acetone and petroleum ether (1:1)] was added to it for extraction of CYM. This solution was kept for 1.5h with shaking at regular intervals. The solution was filtered by using filter paper and evaporated at 50°C to achieve a volume of 3 mL, and which was further diluted to 25 mL with pure ethanol. From this diluted solution, 3 mL was taken in a 25 mL volumetric flask, and observations were made till the color developed under ideal conditions. The absorbance was measured at 535 nm using a spectrophotometer [7].

Statistical analysis

The experiment was performed in triplicates and results were presented in the form of mean ± standard deviation (SD). Standard curves of CYM and oleanolic acid were generated using standardized tools of Microsoft Excel.

Results and Discussion

Quantification of Saponin

Following the procedure described in Table-1, the standard curve was obtained with linearity (R^2 = 0.9952) (

Fig2), and then the saponin contents were quantified using the regression equation. The saponin content of the sample was expressed in mg of standard equivalents per liter of plant sample (mg SE L^{-1}) [20].

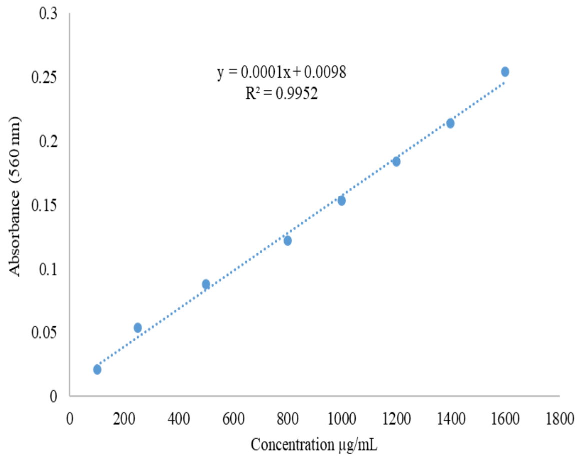
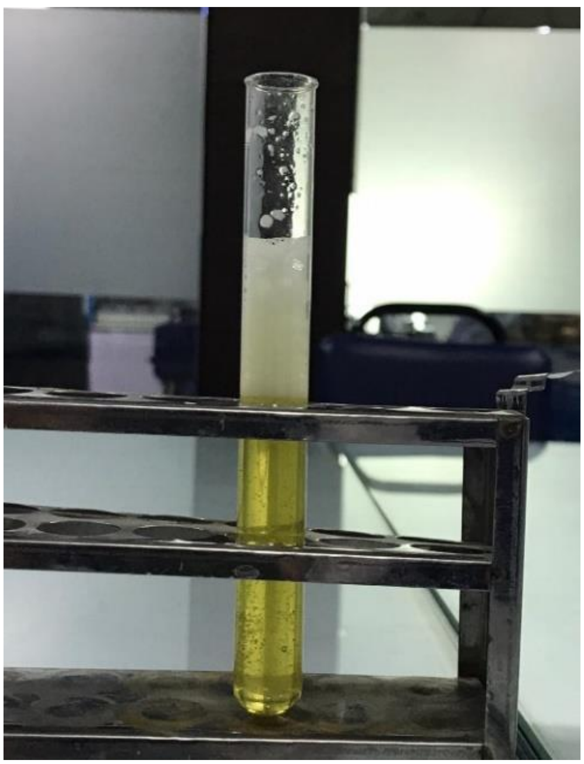
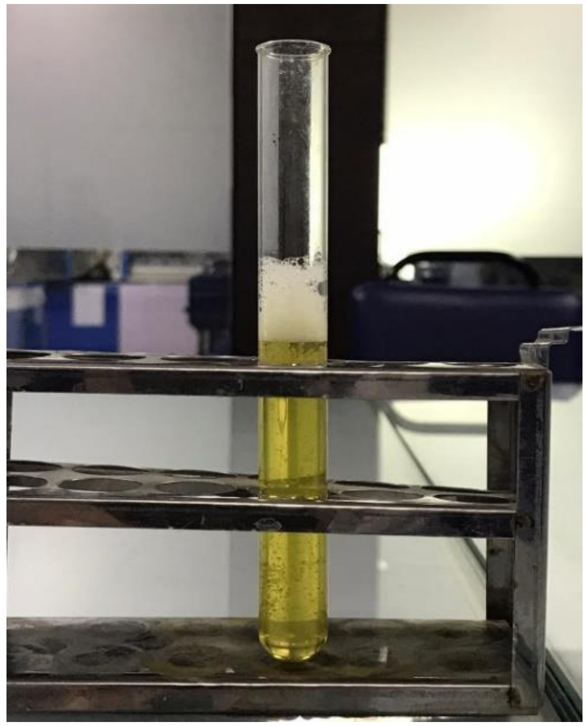


Fig. 2: Standard curve of oleanolic acid. The result shows good linearity with coefficient of determination of 0.99.



(A)



(B)

Fig. 3: Qualitative presentation of saponins after immediate shaking (A) and after 15 min of shaking (B).

Qualitative verification of saponin was achieved by placing the plant material in a test tube, filled with distilled water, and the mixture was vigorously shaken for 2 min. The formation of stable and persistent foam for 15 min (

Fig. 3) indicated the presence of saponins [21-22].

Degradation of CYM residues

This study was carried out to evaluate the effectiveness of saponins as biosurfactant cleaning agents for mitigation of pesticide residues in apples, as well as other conventional homemade washing solutions from a comparative perspective. Alfalfa plant contains saponins which have strong surface-active properties which can be used as a new washing agent. 1 mL/L concentration of cypermethrin in water was applied to apples. Moreover, unwashed samples were used as control whereas varying concentrations (1, 2, and 3%) of baking soda, citric acid, and alfalfa seeds extract along with tap water was used for washing of apples at three different times (5, 10, and 15 min) to observe the residual removal of CYM on apples. Baking soda was more effective in reducing pesticide residues as compared to tap water, citric acid, and saponins (

Table). Furthermore, results showed that increasing trends were observed by increasing the concentration of washing solutions, as well as washing time (Figure 4).

After washing of apples with tap water for 5 mins, 8.53 ± 0.027% of degradation was observed in CYM residues. Washing for 10 mins and 15 mins showed 21.68 ± 0.020% and 72.5 ± 0.035% of residual degradation respectively. Enhanced levels of residual removal were observed upon increasing the concentration and time.

After treating with 1% baking soda (pH 8.1) for 5, 10, and 15 mins, 18.09 ± 0.037%, 75.04 ± 0.045%, and 79.68 ± 0.023% of CYM residues were removed respectively. 2% baking soda (pH 8.2) demonstrated 27.06 ± 0.034%, 78.03 ± 0.023%, and 84.16 ± 0.058% of CYM removal after 5, 10, and 15 mins of washing respectively. Moreover, 3% baking soda (pH 8.2) exhibited 37.37 ± 0.048%, 85.95 ± 0.030%, and 92.98 ± 0.031% of CYM residual degradation after 5, 10, and 15 mins of washing respectively. Furthermore, enhanced levels of residue removal were observed upon increasing the concentration of the washing solution, as well as washing time.

Table-2: Effects of treatments on reduction of CYM residues in apples.

Treatments	**Washing time**		
	5 min	**10 min**	**15 min**
Tap water	**08.53 ± 0.027%**	**21.68 ± 0.020%**	**72.5 ± 0.035%**
Baking soda 1%	**18.09 ± 0.037%**	**75.04 ± 0.045%**	**79.68 ± 0.023%**
Baking soda 2%	**27.06 ± 0.034%**	**78.03 ± 0.023%**	**84.16 ± 0.058%**
Baking soda 3%	**37.37 ± 0.048%**	**85.95 ± 0.030%**	**92.98 ± 0.031%**
Citric acid 1%	**10.92 ± 0.054%**	**38.57 ± 0.042%**	**53.37 ± 0.016%**
Citric acid 2%	**22.43 ± 0.044%**	**45.45 ± 0.041%**	**57.70 ± 0.038%**
Citric acid 3%	**31.49 ± 0.038%**	**63.98 ± 0.021%**	**74.59 ± 0.054%**
Alfalfa seed extract 1%	**07.32 ± 0.035%**	**08.97 ± 0.041%**	**09.42 ± 0.031%**
Alfalfa seed extract 2%	**09.72 ± 0.037%**	**10.31 ± 0.039%**	**10.76 ± 0.038%**
Alfalfa seed extract 3%	**11.21 ± 0.039%**	**12.11 ± 0.050%**	**13.90 ± 0.032%**

*(% residual removal ± SD)

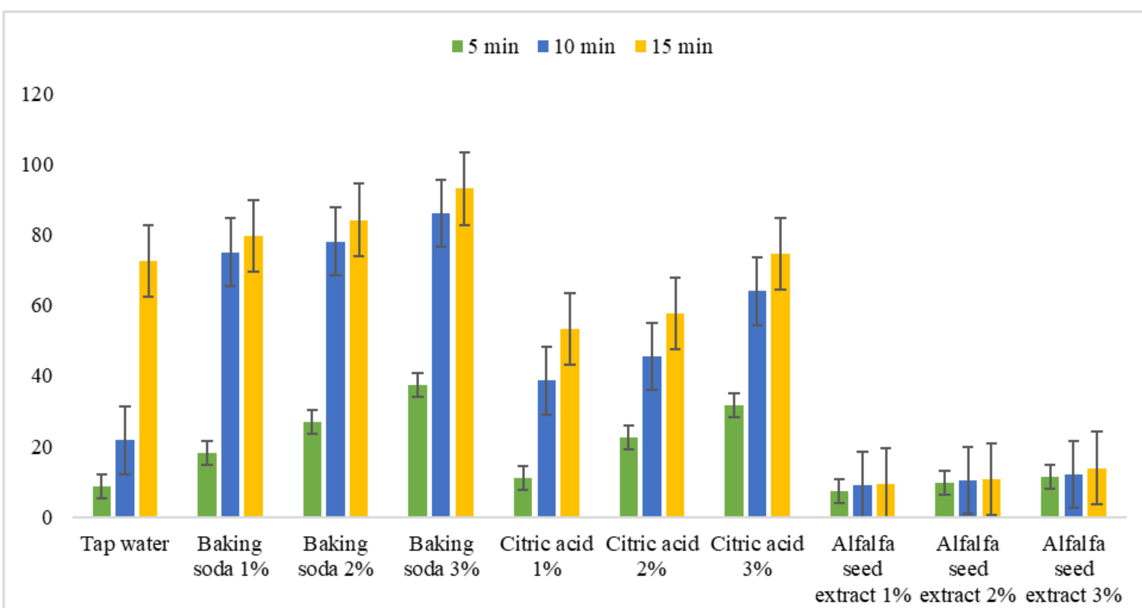


Fig. 4: CYM residual removal (%).

After treating with 1% citric acid (pH 2.3) for 5, 10, and 15 mins, 10.92 ± 0.054%, 38.57 ± 0.042%, and 53.37 ± 0.016% of CYM residues were removed respectively. For 2% citric acid (pH 2.1) showed 22.43 ± 0.044%, 45.45 ± 0.041%, and 57.7 ± 0.038% of CYM removal after 5, 10, and 15 mins of washing respectively. While 3% citric acid (pH 2.0) exhibited 31.49 ± 0.038%, 63.98 ± 0.021%, and 74.59 ± 0.054% of CYM residual degradation after 5, 10, and 15 mins of washing respectively. Overall, the results demonstrated, as is evident in the case of washing with tap water, and baking soda as well, that increased levels of residue removal were observed upon increasing the concentration and time.

After treating with 1% saponin solution (pH 5.3) for 5, 10, and 15 min, 07.32 ± 0.035%, 08.97 ± 0.041%, and 09.42 ± 0.031% of CYM residues were removed respectively. 2% saponin solution (pH 5.4) exhibited 09.72 ± 0.037%, 10.31 ± 0.039%, and 10.76 ± 0.038% of CYM removal after 5, 10, and 15 mins of washing respectively. While 3% saponin solution (pH 5.5) exhibited 11.21 ± 0.039%, 12.11 ± 0.050%, and 13.90 ± 0.032% of CYM residual degradation after 5, 10, and 15 mins of washing respectively. Results indicated that there was no significant improvement in residue removal upon increasing the concentration of saponin solution and time.

The extent of removal of pesticide residues is directly associated with the pH of the washing solution, whereby alkaline conditions have been shown to accelerate the degradation of pesticide residues [14, 24-25]. The alkalinity of tap water (pH 7.5) therefore, might have aided in the degradation of CYM residues. Overall results demonstrated that an increasing trend with respect to the degradation of CYM residues was observed when washing times were increased.

The possible reasons for this enhanced washing efficiency can be attributed to the fact that CYM (a non-systemic insecticide) residues readily degraded in the presence of baking soda, thereby improving the physical removal strength and potency of washing [13]. Therefore, it can be implied from the results that the removal rate of pesticide residues has a direct correlation with the pH of the washing solution. As mentioned earlier, pyrethroid pesticides are fairly unstable and readily degrade in alkaline solutions [14]. Likewise, CYM can easily hydrolyze in an alkaline solution [24-27]. Furthermore, an increasing trend in the degradation of CYM residues was observed when the concentrations of the washing agent, as well as washing time, were increased.

Similarly, the residues of pyrethroid insecticides exhibit greater degradation in the acidic mediums. Acidic solutions such as citric, and the acetic acid act as chelating agents which aids in the removal of the pesticide residues [1]. Moreover, a similar increasing trend in the degradation of CYM residues was observed when the concentrations of the washing agent, as well as washing time, were increased.

Saponins being surfactants (reduce surface tension) due to the presence of a water-soluble sugar (glycone) chain and lipid-soluble aglycones (amphiphilic nature) [15] were thought to be effective in reducing the residues of CYM. However, results showed that alfalfa seed extract containing saponins was not effective in reducing the CYM residues. Alfalfa seeds contain low concentration of saponins compared to other plants such as licorice, yucca, Quillaja bark, sugar beet (leaves) and ginseng etc. This could be a possible reason that alfalfa seeds were not as effective as other washing solutions used in this study. There is a need for further studies to evaluate the effectiveness of other saponin-containing plants in order to find a more improved and safe washing alternative for fruits and vegetables.

The results of this study showed similarities with the findings of Soliman [28]), who reported that tap water could be used to reduce levels of pesticide residues. The results also exhibited a strong agreement with studies by Wu *et al*. [14]), and Yang *et al*. [13]), whereby the researchers reported gradual degradation of pesticide residues when the washing time was increased. Moreover, alkaline solutions (sodium bicarbonate/baking soda) were found to be more effective in mitigating the pesticide residues.

Conclusion

The present work demonstrated the effectiveness of various washing solutions including tap water, sodium bicarbonate/baking soda, citric acid, and saponins from alfalfa seeds for mitigation of pesticide residues in apples. In general, a gradual reduction in the level of CYM residues was observed upon increasing the concentration of washing solutions as well as washing time. Among all washing solutions, sodium bicarbonate/baking soda recorded the best results in terms of mitigating the residue levels. However, further research involving saponins from other plants could be beneficial in finding a more improved and safe washing alternative for fruits and vegetables.

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