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Molecular interaction of peanut proteins with some bio-pesticides: A comparative spectral study†

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Safety and quality of food supplies is an essential part of consumer protection. Due to proven toxicity of synthetic pesticides, bio-pesticides are slowly capturing the agro-economy. It is now time to evaluate the different aspects of bio-pesticides that might have effects on different agricultural products. Our interest is to find and compare the effects of some bio-pesticides on peanut proteins through molecular interaction studies using different spectroscopic methods. Peanuts are chosen as the model as they are cheap, widely available and grow in direct contact with the soil. In this work, peanut proteins arachin, conarachin II and conarachin I were extracted, purified and characterized. Interaction study of compounds having biopesticidal activity viz. azadirachtin obtained from *Azadirachta indica* and eugenol, estragole and linalool obtained from *Ocimum basilicum* are compared for their effects on peanut proteins spectroscopically. Eugenol, estragole and linalool were found to have spectral interactions with peanut proteins as observed in UV-Visible, fluorescence and CD spectroscopy. The highest interaction was found for conarachin II with estragole with a binding constant of $K = 7.75\times10^4$ M^{-1} and free energy change of –27.89 kJ. No interaction of peanut proteins was however observed with azadirachtin.

Keywords: Plant extract, bio-pesticide, peanut proteins, spectroscopic interaction.

Introduction

Considering the demand of good crop yields keeping a safe environment, biopesticides are slowly taking over synthetic organic pesticides which are used to control pests in agriculture. Biochemical pesticides are naturally occurring materials that are capable to manage pests by non-toxic means. Certain naturally occurring substances, viz. fungal and plant extracts, have been identified as biochemical/ biopesticides. Natural products under this category include insect pheromones, fermentation products and many other plant-derived products[1], which include alkaloids, terpenoids, phenolics and other secondary chemicals. Certain plant extracts are aromatic and volatile oily compounds which are obtained from leaves, stem, bark and fruit materials and are eligible to serve as alternative pesticides. Plant extracts having such properties are obtained by different techniques such as steam distillation, solvent extraction, microwave assisted extraction, ultrasound assisted extractions, and supercritical fluid extraction. Of these, hydro distillation and solvent extraction can break organic constituents, lower the yield and food quality[2]. Lucchesi *et al.*, Uquichea *et al.* and Boukroufa *et al.*, suggested that microwave extraction, ultrasound assisted extraction and supercritical fluid extraction are relatively novel techniques than the traditional ones due to their shorter extraction time, higher yields of target compounds and less solvent consumption[2–4].

One such biopesticide is clove oil eugenol, which has strong effects against insects, mites, and fungus. Eugenol also provides a knock out to hard to control pests like ticks and spiders, unlike most synthetic pyrethroids that either fail to control these pests or have resistance issues. Their primary mechanism of action is through the disruption of the cell membrane, anti-acetylcholinesterase activity and antifeedant activity[5,6]. Estragole is a natural ingredient of several herbs and spices. It is used as a flavoring agent, as a herbal medicine, as an antimicrobial against acid-compat-

†Invited Lecture.

ible food microflora. It has been observed that estragole induces hepatomas in rodents at high doses after getting metabolically converted to the DNA-reactive metabolite 1′-sulfooxyestragole[7].

Similarly, linalool (3,7-dimethyl-1,6-octadien-3-ol) is a monoterpene alcohol and occurs in the essential oils of rosewood and basil. It is known to have antifungal, and antimicrobial activities[8]. It attacks the nervous system of insects mainly by the reversible acetylcholinesterase inhibition[9]. Neem oil contains a host of antimicrobial species, but azadirachtin is known to be the major contributor towards insecticidal activity. Azadirachtin has structural similarity with the insect hormone "ecdysones" which is responsible for metamorphosis in insects. It has antifeedant properties and also leads to physiological effect in the midgut of insects, causing a reduction in their digestion. It was established that 50–100 ppm of azadirachtin has potential insecticidal effect. However, it also has a high potential to destroy the useful insects. Therefore, the interaction of these biopesticides with biomolecules present in food that come in close contact with the pesticides is worth investigating[10].

Peanut is the third most significant source of plant protein, which contributes to 11% of world's edible protein. Peanut proteins have been routinely classified as arachin and conarachin[11]. Peanuts grow in soil and so they uptake fertilizer and pesticides directly from the soil. So there is an ample possibility of interaction of peanut proteins with soil and its components.

Our aim is to study the effect of different bio-pesticides on peanut proteins, find the possibility of molecular interactions between them and to compare their effects on peanut proteins and identify those that may cause adverse effect on the protein quality.

Experimental

Materials: Sodium acetate, disodium hydrogen phosphate, acetic acid, sodium dihydrogen phosphate, ethyl alcohol, phosphoric acid, sodium hydroxide, methanol were obtained from Merck, India. Coomassie brilliant blue G (Bradford reagent), acrylamide, ammonium persulphate, TEMED (required for SDS PAGE), tris base, were procured from HIMEDIA. Ammonium sulphate and sodium chloride were obtained from SRL. Eugenol, estragole, linalool and azadirachtin were obtained from Sigma Aldrich.

Extraction of peanut proteins

Extraction and purification of conarachin II and conarachin I: Peanuts were acquired from the local market and dried under the Sun. Sundried peanuts were extracted with pet ether for 2 h and then immersed in 0.1% NaOH solution. The resulting sodium proteionate was treated with dilute H_2SO_4 until the pH of the solution came down to 5. The proteins were precipitated out as this is the isoelectric pH. Precipitated protein was centrifuged and the pellet was washed with triple distilled water to remove the salts. Peanut proteins conarachin I and conarachin II are soluble in 2% NaCl solution. So the pellet was dissolved in 2% NaCl solution to separate conarachin I and conarachin II from the crude protein part. The solution was saturated to 32% $(NH_4)_2SO_4$ and set aside at 4°C for 4 h. This saturated solution was centrifuged and the supernatant was again saturated to 42% with $(NH_4)_2SO_4$. This solution was kept again at 4°C for 4 h and then centrifuged. The clear supernatant of the solution was dialysed against 0.01 (*M*) phosphate buffer of pH 7.9 in 0.5 *M* NaCl solution (EB) to remove the sulphate ions. This pure protein solution was concentrated using a concentrating column (Pall Corporation Macrosep Advance Centrifugal Device). Using the same extraction buffer (EB), the concentrated solution was passed through the Superose-6 gel chromatography column to separate conarachin I and conarachin II. Conarachin II was eluted first for having higher molecular weight fraction, then the lower molecular weight fraction of conarachin I was eluted. The solutions were collected in 2 mL eppendorfs. UV-Visible spectra of each of the solutions in eppendorf were studied. Conarachin I and conarachin II were separated and identified by the different absorption spectra. Conarachin I and conarachin II were concentrated using the concentrating column. Concentration of conarachin II was 0.9 mg/mL and conarachin I was 0.8 mg/mL as measured by Bradford method.

Extraction and purification of arachin: Defatted peanuts were dissolved in 0.5 *M* NaCl solution in 0.01 *M* phosphate buffer of pH 7.9 and set aside at 27°C for 1 h. This solution was centrifuged at 3500×g for 30 min. $(NH_4)_2SO_4$ was added

to the clear supernatant of the solution to saturate to 18% and the solution was kept at 4°C for 2 h. This saturated solution was centrifuged to the precipitate which was further dissolved in EB. The solution was once more saturated to 9% with $(NH_4)_2SO_4$ and set aside at 4°C for 1 h. This solution was further centrifuged and the precipitate was dissolved in minimum volume of EB to get the arachin solution. The extracted arachin solution was dialysed against EB to purify the solution. The concentration of arachin (0.76 mg/mL) was measured by Bradford method.

Preparation of solutions: 0.8 mg/mL of conarachin I, 0.9 mg/mL of conarachin II and 0.76 mg/mL of arachin were used for UV-Visible and fluorescence spectroscopic study. We used 1 mM solutions of eugenol, estragole, linalool and azadirachtin for the interaction studies.

UV-Visible and fluorescence spectral study: The acidity of the solutions were maintained using the Mettler pH/ion meter (S 220-K). For spectrofluorimeter studies, a double beam Perkin-Elmer LS-55 was used with 1% attenuator. The absorption spectra were studied using Agilent 8453 diode array spectrophotometer. Absorption and emission spectroscopic technique were used to study the interaction of peanut proteins with the biopesticides. To identify the possible interactions, 0.1 mL of each of these solutions (1 mM) were mixed with 0.2 mL of protein solutions with concentrations as described above and volume was made up to 1.5 mL with 0.01 *M* phosphate buffer saline (PBS) of pH 7.9. To optimize the interaction of eugenol, estragole, linalool and azadirachtin with peanut proteins, 1 mM of each of these solutions were added to the protein solution with increasing stoichiometric ratios. The solutions were taken for absorption and emission spectral studies.

Circular dichroism (CD) spectrum: The purified conarachin I, conarachin II and arachin solutions in 0.1 *M* acetate buffer, with the aforesaid concentration (0.8 mg/mL, 0.9 mg/mL and 0.76 mg/mL respectively) were taken in a quartz cell and was treated with 1 mM solution of eugenol, estragole, linalool, and azadirachtin in various stoichiometric ratios for the CD spectrometric studies. The quartz cuvette was of 0.1 cm path-length and the temperature was maintained at 25°C. An average of three consecutive scans is taken at a scan rate of 20 nm/min with a corrected baseline.

Results and discussion

Characterization of the protein fractions:

Absorption spectroscopy: Conarachin I, conarachin II and arachin were characterized by their specific absorbance maximum (λ_{max}) at 265 nm, 275 nm and 280 nm respectively (Fig. 1). The λ_{max} values characteristics of the three proteins agree well with earlier literature reports.

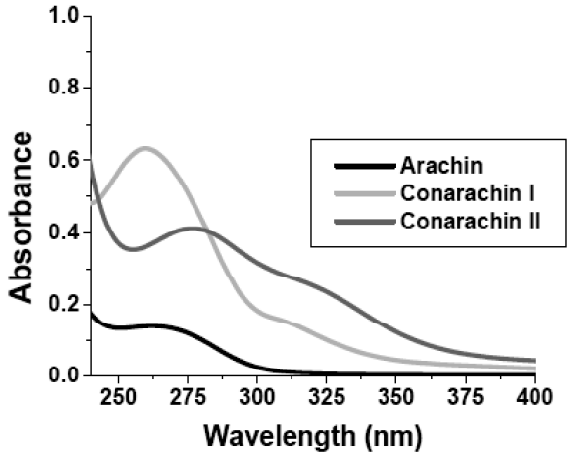

Fig. 1. Absorption spectra of peanut protein fractions conarachin II, conarachin I and arachin.

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was performed using 15% acrylamide gel in 8.5×0.6 cm tubes. Protein samples (1–5 μg) were loaded onto the column. Electrophoresis was performed in 0.02 *M* tris buffer in glycine at pH 8.3, at a constant voltage of 80 V for stacking and 100 V for redissolving for total 3 h. The gels were stained with 0.1% brilliant blue R-250 and destained by diffusion in methanol-acetic acid medium. A standard "Page ruler unstained broad range protein ladder" from Fermentas was used as molecular weight marker.

The purified proteins show characteristic molecular weight bands in the SDS PAGE gel (Fig. 2). Conarachin II and arachin show molecular weight bands at 39800, 33100, 26900, 24000, 21900, 18600, 15800 Da and 72400, 60300, 39800, 33100, 26900, 21900 Da respectively. Conarachin I shows bands at ~12000 and ~18000 Da. The SDS page patterns also agree with the literature[11].

UV-Visible and fluorescence spectral study: Conarachin I, conarachin II and arachin, show the absorption maxima at

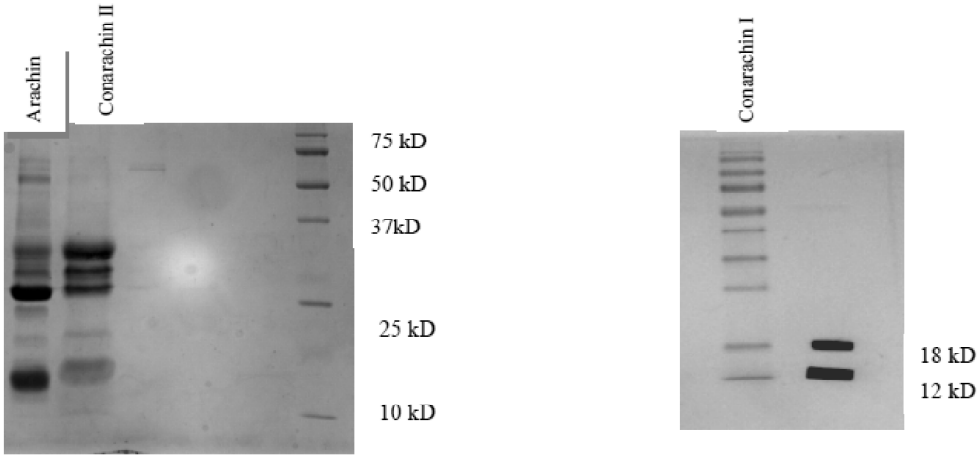

Fig. 2. Molecular weight bands of SDS PAGE showing bands of conarachin II at 39800, 33100, 26900, 24000, 21900, 18600, 15800 Da and arachin at 72400, 60300, 39800, 33100, 26900, 21900 Da and conarachin I ~12000 and ~18000 Da.

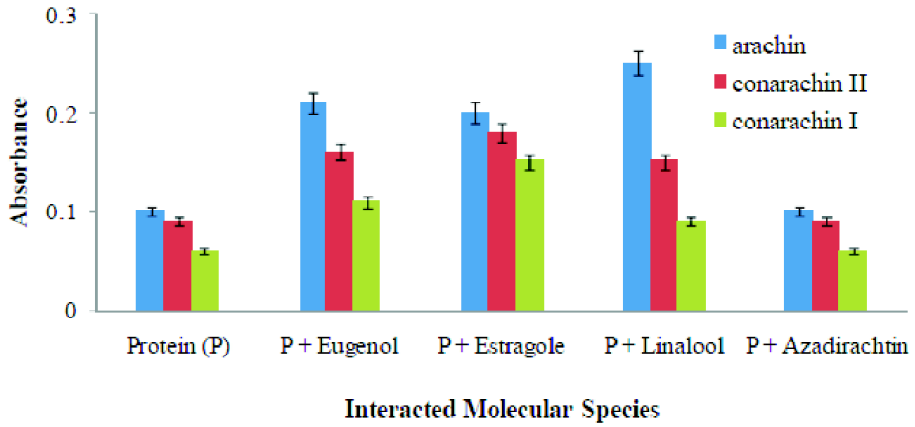

Fig. 3. UV-Visible spectroscopic interaction of arachin, conarachin II and conarachin I with 1 mM biopesticides.

265, 275 and 280 nm respectively as shown in Fig. 1. The absorbance data at these particular λ_{max} positions for the respective proteins suggest the selective interaction with eugenol, estragole and linalool (Fig. 3). With increasing concentrations of eugenol, estragole and linalool in the protein solutions a regular increase in the protein absorbance was observed for all the three proteins. Using the data obtained from absorption spectra and Benesi-Hildebrand (BH) equation the binding constant and free energy changes upon complexation were calculated[12].

$$\frac{1}{A - A_o} = \frac{1}{A_1 - A_c} + \frac{1}{(A_1 - A_o)K[M]} \quad (1)$$

Here A is the absorbance of the binary solution of biopesticide and peanut protein.

A_0 refers to the absorbance of the pure peanut protein.

A_1 refers to the absorbance value when the protein has totally interacted with the biopesticide.

$[M]$ refers to the biopesticide concentration.

K refers to the binding/association constant.

Using eq. (1) we can obtain the intercept $1/(A_1 - A_0)$ and slope $1/(A_1 - A_0)K$ of the straight line in the graph. Using the BH plot, the binding constant values (K) have been calculated. The free energy change (ΔG) values at 298 K were obtained using the equation

$$\Delta G = -RT \ln K \quad (2)$$

Binding constant (K), free energy change (ΔG) and stoichiometric ratio so obtained from the BH plot of the three peanut proteins with eugenol, estragole and linalool are tabulated in Table 1. The high K value and more negative value of ΔG indicate considerable interaction of linalool with arachin and estragole with conarachin I and conarachin II amongst the three proteins.

Table 1. Binding constant, free energy change and stoichiometric ratio of three peanut proteins-biopesticide interactions as obtained from Benesi-Hildebrand equations

Compound	Binding constant (K) M^{-1}	Free energy change (ΔG)	Stoichiometric ratio
Arachin-eugenol	$K = 1.196\times10^3$	–17.56 kJ	1:1
Arachin-estragole	$K = 0.736\times10^3$	–16.355 kJ	1:1
Arachin-linalool	$K = 1.9\times10^4$	–24.41 kJ	1:1
Conarachin II-eugenol	$K = 1.17\times10^4$	–23.21 kJ	1:1
Conarachin II-estragole	$K = 7.75\times10^4$	–27.89 kJ	1:1
Conarachin II-linalool	$K = 1.01\times10^4$	–22.84 kJ	1:1
Conarachin I-eugenol	$K = 2.41\times10^4$	–24.99 kJ	1:1
Conarachin I-estragole	$K = 3.72\times10^4$	–26.07 kJ	1:1
Conarachin I-linalool	$K = 5.96\times10^3$	–21.54 kJ	1:1

The emission intensities for conarachin I, conarachin II and arachin, were observed at wavelengths 425 nm, 365 nm and 340 nm respectively. Fluorescence spectra also support the fact that there is selective interaction of eugenol, estragole and linalool with the three peanut proteins (Figs. 4–12). With increasing eugenol and estragole concentrations emission intensity increases whereas in case of linalool a decrease is observed in intensity for all three proteins. These changes

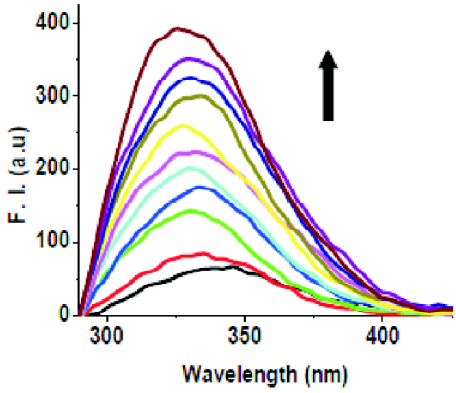

Fig. 4. Fluorescence enhancement of arachin with 1 mM eugenol.

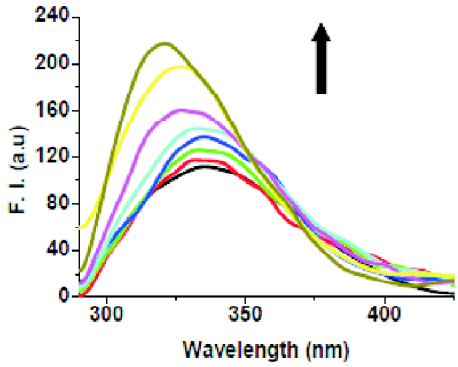

Fig. 5. Fluorescence enhancement of arachin with 1 mM estragole.

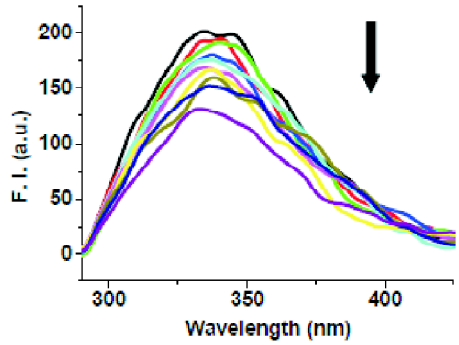

Fig. 6. Fluorescence quenching of arachin with 1 mM linalool.

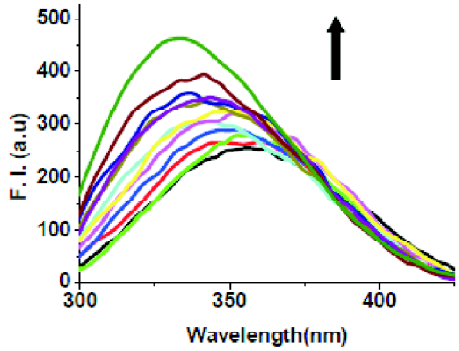

Fig. 7. Fluorescence enhancement of conarachin II with 1 mM eugenol.

indicate the interaction of the biopesticides with the three peanut proteins. No interaction was observed in UV-Visible and fluorescence spectroscopy in case of azadirachtin.

Circular Dichroism (CD) spectroscopy: CD spectroscopy gives describes the features of the secondary structure of proteins and the modifications in it upon interacting with other molecular species. The far-UV CD spectra of conarachin I,

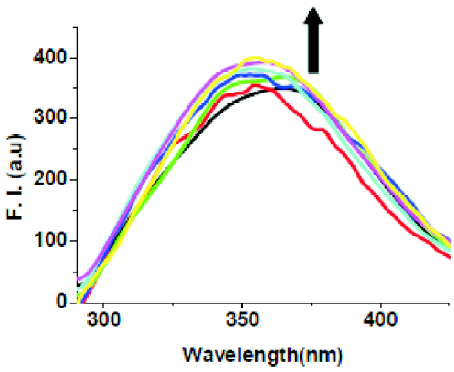

Fig. 8. Fluorescence enhancement of conarachin II with 1 mM estragole.

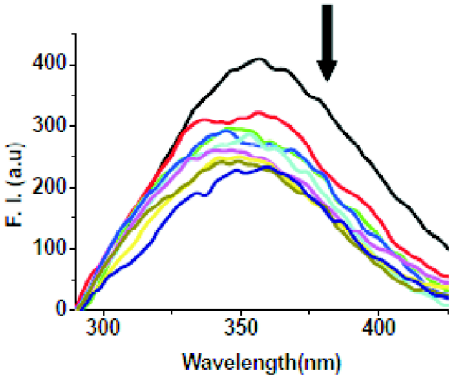

Fig. 9. Fluorescence quenching of conarachin II with 1 mM linalool.

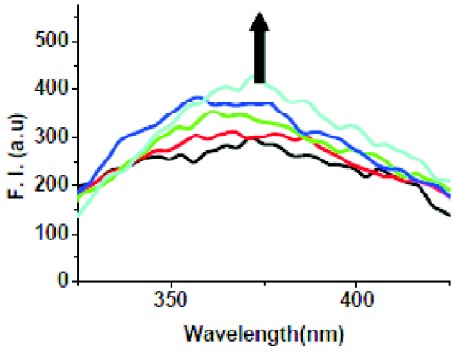

Fig. 10. Fluorescence enhancement of conarachin I with 1 mM eugenol.

conarachin II and arachin reveal a characteristic pattern owing to the α-helicity of the secondary structure with two spectral minima at ~208 nm and ~222 nm. The interaction of the three proteins with increasing concentrations of the biopesticides has been examined using the far-UV CD spectra of the proteins and the relevant spectra are displayed in Figs. 13 to 21

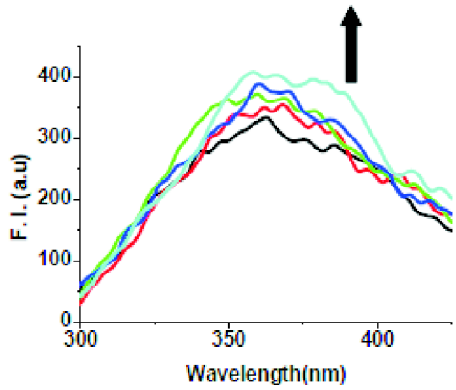

Fig. 11. Fluorescence enhancement of conarachin I with 1 mM estragole.

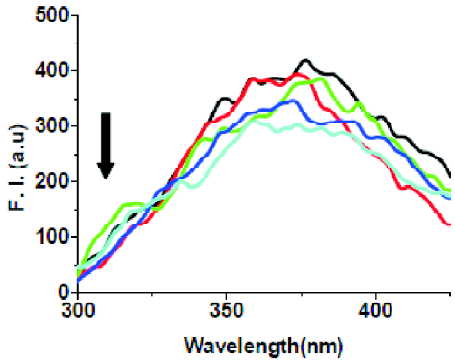

Fig. 12. Fluorescence quenching of conarachin I with 1 mM linalool.

for arachin (Figs. 13–15), conarachin II (Figs. 16–18) and conarachin I (Figs. 19–21) respectively. A distinctly decreasing ellipticity of the spectra of all the three proteins with increasing concentration of eugenol, estragole, linalool can be

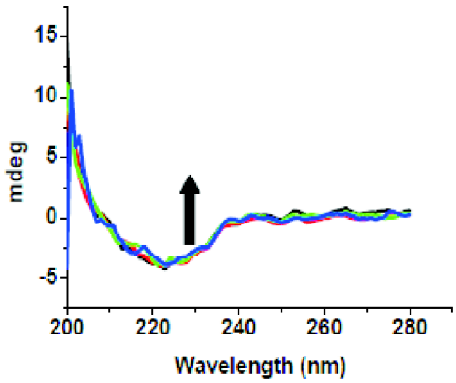

Fig. 13. CD spectra of arachin show decrease in α-helicity with increase in eugenol concentration.

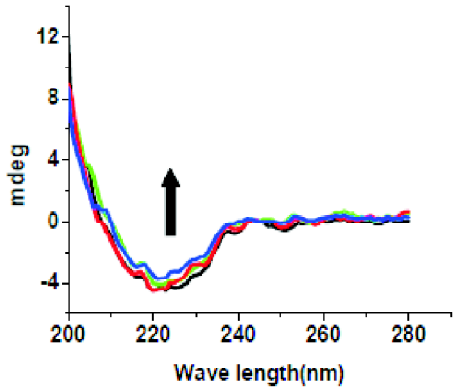

Fig. 14. CD spectra of arachin show decrease in α-helicity with increase in estragole concentration.

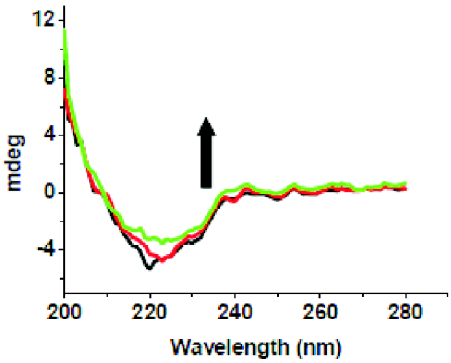

Fig. 15. CD spectra of arachin show decrease in α-helicity with increase in linalool concentration.

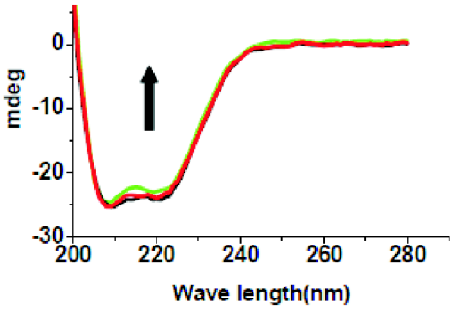

Fig. 16. CD spectra of conarachin II show decrease in α-helicity with increase in eugenol concentration.

observed in the figures without observable change in the peak positions. This designates that some alteration in the secondary protein structure must have incurred upon binding with eugenol, estragole and linalool. Diminished negative ellipticity points towards a decrease in the α-helical content

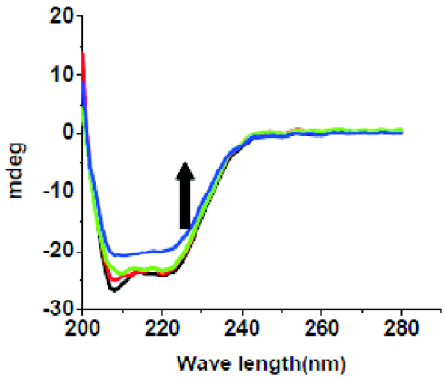

Fig. 17. CD spectra of conarachin II show decrease in α-helicity with increase in estragole concentration.

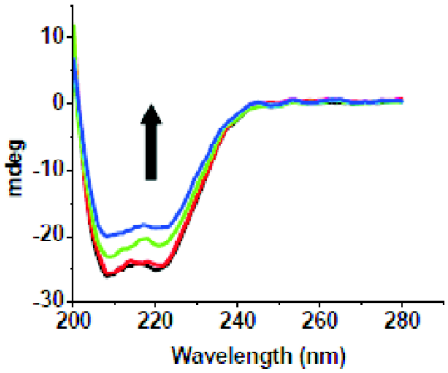

Fig. 18. CD spectra of conarachin II show decrease in α-helicity with increase in linalool concentration.

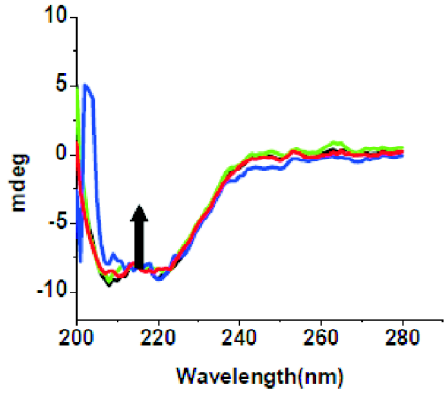

Fig. 19. CD spectra of conarachin I show decrease in α-helicity with increase in eugenol concentration.

of the protein, which advise unfolding of the constituting peptide strand. No structural modification was however observed in case of azadirachtin.

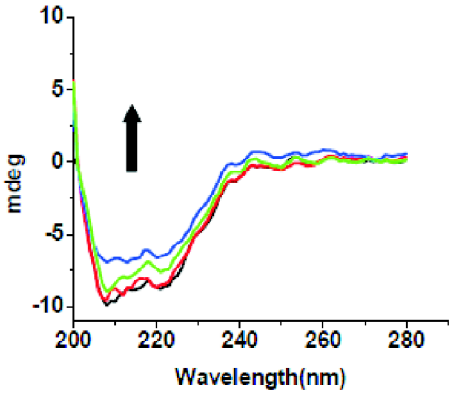

Fig. 20. CD spectra of conarachin I show decrease in α-helicity with increase in estragole concentration.

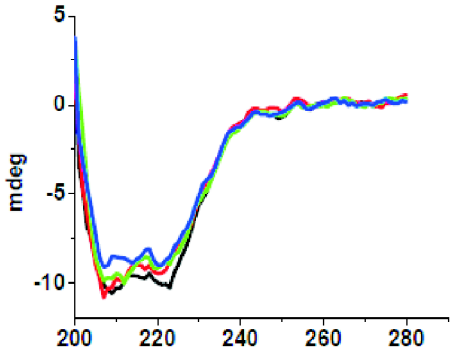

Fig. 21. CD spectra of conarachin I show decrease in α-helicity with increase in linalool concentration.

Structural modification of peanut proteins suggests the degradation of food quality of peanut proteins by the using of eugenol, estragole and linalool obtained from *Ocimum basilicum.* So it is suggested that azadirachtin can be used as a biopesticde during the cultivation of peanut crop.

Conclusion

Peanut proteins arachin, conarachin II and conarachin I were extracted, purified and characterized. Interaction of compounds having biopesticidal activity like azadirachtin obtained from *Azadirachta indica* and eugenol, estragole and linalool obtained from *Ocimum basilicum* with peanut proteins were studied. Interactions of eugenol, estragole and linalool with peanut proteins were clearly observed in UV-Visible, fluorescence and CD spectroscopy. Binding constant (*K*), free energy change (Δ*G*) values and stoichiometry for the interacted molecular species were calculated using the absorption spectral data and Benesi-Hildebrand equations. No interaction was observed with azadirachtin. The high positive value of binding constant and more negative value of free energy change suggest that the highest interaction of linalool occurs with arachin and estragole occurs with conarachin II and conarachin I amongst the three proteins. The absorption and fluorescence spectral changes were supported by CD spectra which indicated no structural modification in case of azadirachtin. Structural modification of peanut proteins suggests the degradation of food quality of peanut proteins by the using of eugenol, estragole and linalool obtained from *Ocimum basilicum.* So it is suggested that azadirachtin can be used during the cultivation of peanut.

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