List of Publications

1. Estimation and Isolation of Azadirachtin-A from Neem (*Azadirachta indica* A. Juss) seed kernels by using preparative HPLC method.

   P. T. Deota, **P. R. Upadhyay**, K. B. Patel, K. J. Mehta, B. V. Kamath and M. H. Mehta

   J. of Liquid Chromatography & Related Technologies, 23 (14), 2225-2235, (2000).

   # Cited in "Current Awareness in BIOMEDICINE High Performance Liquid Chromatography" (SUBIS, Sheffield Academic Press England, Vol. 19, No. 21, November 2000)

2. Effect of some Ultraviolet light absorbers on Photo-stabilization of Azadirachtin-A (Part: I)

   P. T. Deota, **P. R. Upadhyay**, K. B. Patel, K. J. Mehta, A. K. Varshney, and M. H. Mehta

   Natural Product Letters, 16 (5), 329-334, (2002)

3. Effect of some Ultraviolet light absorbers on Photo-stabilization of Azadirachtin-A in solution (Part: II)

   P. T. Deota, **P. R. Upadhyay**, and V. B. Valodkar

   Natural Product Research, 17 (1), 21-26, (2003)

J. LIQ. CHROM. & REL. TECHNOL., 23(14), 2225–2235 (2000)

ESTIMATION AND ISOLATION OF AZADIRACHTIN-A FROM NEEM [*AZADIRACHTA INDICA A. JUSS*] SEED KERNELS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An improved reverse phase high performance liquid chromatography (HPLC) procedure has been described to estimate azadirachtin-A content in crude methanolic extracts of neem seed kernels using valley-to-valley integration techniques. The present paper highlights a detailed study of extraction, estimation, and purification of azadirachtin-A present in neem seed kernels. We also present, herein, our results of Soxhlet extraction of Aza-A from the defatted neem seed cake as a function of time, which is found to be more efficient than the conventional extraction procedure.

INTRODUCTION

The versatility of the neem tree, a species native to India, is growing in tremendous potential for human use.[1] *Azadirachta indica A. Juss* (Neem tree), an indigenous tree of Indo-Pakistan subcontinent, belongs to the family Meliaceae and is widely distributed in Asia, Africa, and other tropical parts of the world.[2] The active compound of the neem seed extracts that has been isolated is a mixture of seven structurally related tetranortriterpenoids known as azadirachtin-A to azadirachtin-G.[3a] Among them, azadirachtin-A (Aza-A) ($C_{35}H_{44}O_{16}$) [Figure 1], which shows promising biological activity, is present in large proportion (~85 %).[3a,4] It has also been shown to be non-mutagenic, biodegradable, and non-toxic to mammals,[5] but sensitive to air, sunlight, heat, and moisture.[6]

In a synthetic endeavor to develop more potential compounds based on Aza-A, we required an efficient method for its isolation from kernels and estimation. A survey through literature, however, revealed that there existed no such method which would give us exact amounts of purity of Aza-A after extraction and isolation.[7-10] It was, therefore, thought to develop an analytical methodology for our purpose mentioned above.

At the outset it appeared to us that the analytical methodology for the quantization of Aza-A was still in developmental stage as compared to established synthetic pesticides. Due to its thermal instability and possible adsorption on the stationary phase, a gas chromatographic method is unsuitable for the analysis of Aza-A.[6] The preferred method for the estimation of Aza-A is by HPLC-UV.[7-13] A number of analytical and preparative HPLC-UV methods have been reported for isolation and estimation of Aza-A in neem seed kernels.[7-13] However, they involve either a complex procedure[7b,8,11] or do not report the initial and final purity of the azadirachtin samples[8,10,13] or were not sensitive.[9]

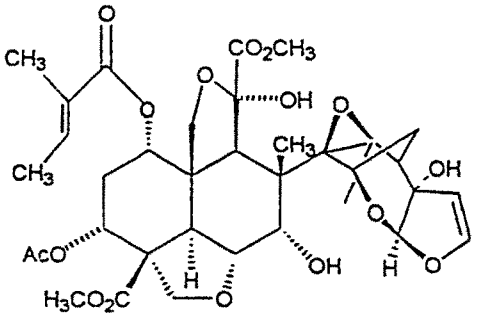

Figure 1. Structure of Azadirachtin.

In this paper, we describe a simplified extraction procedure for Aza-A from neem seed kernels and a quantitative analytical HPLC-UV method for its estimation. Several HPLC-UV determinations showed that Aza-A was always obtained as a distinct peak from other components in methanolic extracts of seed kernels. Hence, we have developed a preparative purification method of Aza-A (up to 91%) from a very complex mixture obtained from plant source

EXPERIMENTAL

Plant Material

The seeds developed over a period of four months from July to October were collected from Kheda region, Gujarat, India. The soft husk was removed from the seeds using a decoating machine.

Solvents

All the solvents (both commercial as well as HPLC grade) were obtained from Qualigens, Glaxo India Ltd. and filtered through a Millipore filter (0.45 μm). Water (18Ω) HPLC grade was purified by Elga Water Purification System Life Science Maxima, and filtered as above. Extractions were carried out at room temperature with an overhead mechanical stirrer for different time intervals unless otherwise mentioned.

Standard Azadirachtin-A

Analytical grade standard Aza –A was obtained from Sigma Chemical Co. A 1000-ppm stock solution of the standard Aza-A in methanol (0.5 mg/0.5 mL) was prepared and stored between 0 - 4°C. Aliquots of the solutions were diluted with mobile phase prior to use, to give working solutions containing Aza-A in the range of 31.25 to 250 ppm (15.62 –125 μg/0.5 mL), which on injection gave a distinct peak with retention time (t_R) of 6.95 minutes (Figure 2A) under the specified conditions.

Extraction and Enrichment

Extraction of the kernel powder was carried out by the method of Schroeder and Nakanishi[5] with some modifications. The changes made were that extraction with ethanol, vacuum liquid chromatography, and flash chromatography were omitted to make the process simpler and more efficient.

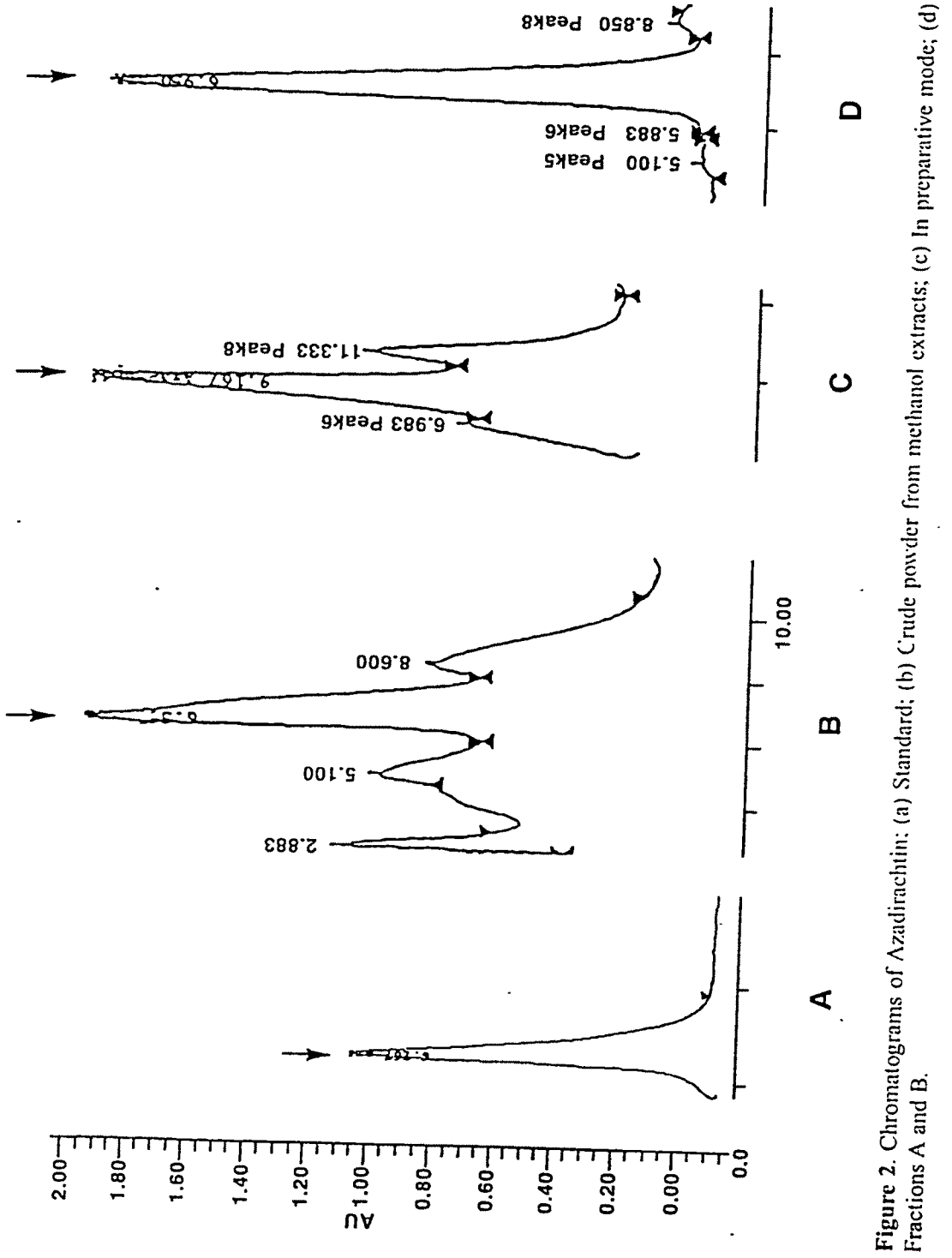

Figure 2. Chromatograms of Azadirachtin: (a) Standard; (b) Crude powder from methanol extracts; (c) In preparative mode; (d) Fractions A and B.

Finely ground powder of neem seed kernel (500 g) was extracted with hexane (1.0 l) for 30 minutes (Figure 3). The hexane extract was filtered and the process repeated with fresh hexane three more times. The pooled hexane extracts were concentrated under reduced pressure in a rotary evaporator between 45-50°C, which yielded fatty oil (A) (40-45%) having 0.0235% Aza-A (w/w of oil) as determined by following the procedure of Sundaram *et al.* The dry defatted cake (B, 250 g) was then extracted with methanol (6 × 500 mL).

The six methanolic extracts were analyzed for Aza-A content by analytical HPLC-UV method (*vide infra*). These combined extracts were then concen-

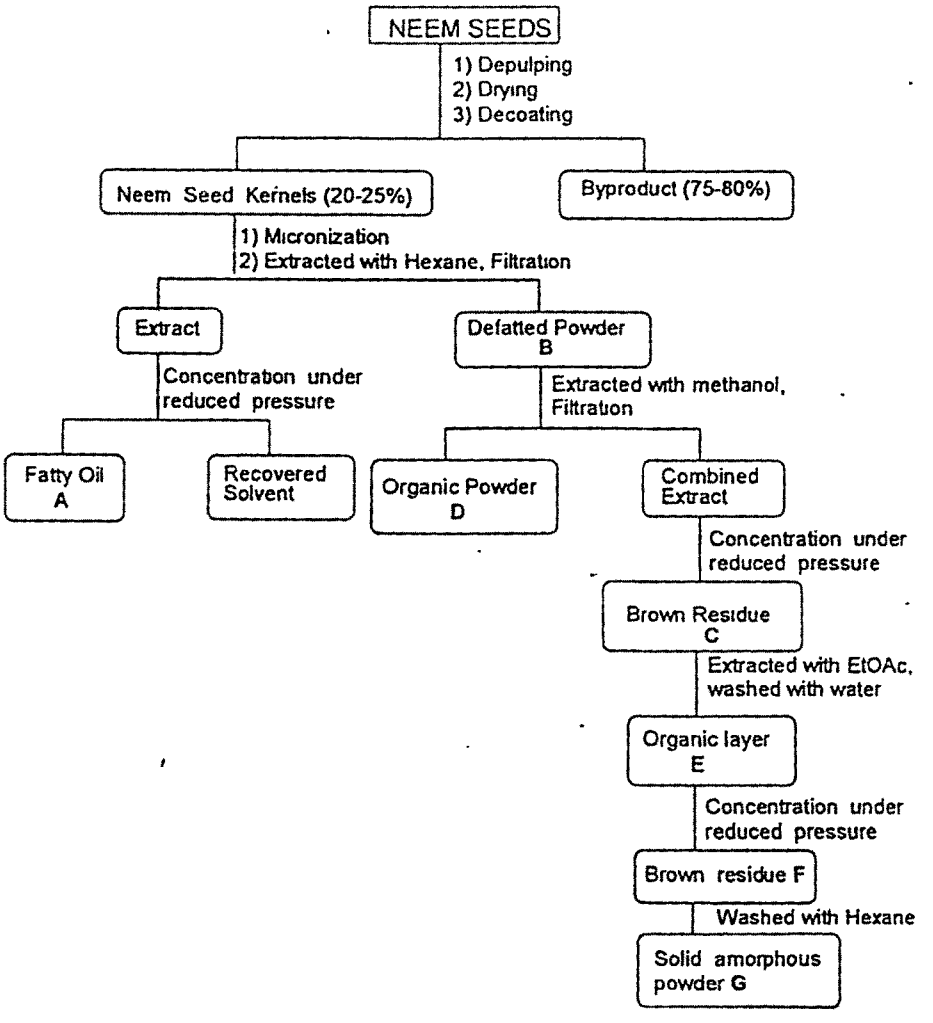

Figure 3. Flow chart of Neem Seed Extraction Procedure.

trated under vacuum, after filtration, to give a brown residue (C, 200 mL) and organic powder (D). The brown concentrated residue containing Aza-A was further extracted with ethyl acetate (300 mL) for 30 minutes to give (E). This was then washed with distilled water (150 mL) to remove water-soluble compounds such as carbohydrates, proteins, and minerals followed by passing it through a column of anhydrous sodium sulfate (17mm id × 45cm). The column was rinsed with ethyl acetate (25mL) which was added to (E).

It was then concentrated under reduced pressure to furnish a brown residue (F), which was washed with hexane (4 × 15 mL) to give a dark brown amorphous solid (G, 6.5 g). A weighed portion of this solid was dissolved in methanol for the estimation of Aza-A content in it using analytical HPLC-UV method (*vide infra*). It should be mentioned here that the extraction procedure reported herein, is much shorter and economical than the one reported earlier.[7b]

Soxhlet Extraction

Finely ground powder of neem seed kernels (500 g) was extracted with hexane (1.0 L). The defatted solid cake (B, 230 g) obtained by following the above procedure was subjected to Soxhlet extractions for Aza-A enrichment using methanol (500 mL). These experiments were carried out for 24 and 72 hours independently and their Aza-A contents were analyzed by HPLC-UV method (*vide infra*) at the interval of 4 h.

High Performance Liquid Chromatography

Analytical as well as preparative HPLC-UV studies were carried out on a Waters LC-4000 system linked with chromatography software Millennium 2010. The HPLC system was equipped with a Flow controller (Waters model No: 600), a tunable absorbance UV Detector (190-600 nm, Waters model No: 486), a guard column, Rheodyne injector 7010, and an automatic fraction collector. The instrument employed an automatic degassing system and dual pump heads with common drive flows. The Millennium 2010 provided the chromatograph, calibration curve, peak area, area %, and retention time (t_R) etc.

Analytical HPLC-UV was carried out on a reverse phase C_{18} Bondapak column (3.9mm id × 300 mm) with 10 µm particle size and 125Å pore size. The solvent system consisting of methanol: water (60:40 v/v) was run isocratically at a flow rate of 1 mL/min and an average pressure of 1500-2000 psi for an injection volume of 20 µL. The peak corresponding to Aza-A was detected at 217 nm. Detected peaks were integrated (valley-to-valley) and retention time was recorded using Millennium 2010, set at peak width of 30.00, retention window 5%, and threshold 25. The chromatographic run time was 30 minutes.

The brown amorphous solid (1.0 g, 9.14% purity) obtained after concentration of organic layer (F), was dissolved in methanol and filtered through a Nylon 66 filter (0.45 µm removal rating and 0.13 mm diameter). This solution (1 mL) was then injected into a preparative reverse phase C_{18} Bondapak (19 mm id · 300mm) column, with 15-20 µm particle size and 125 Å pore size, and eluted isocratically with methanol: water (60:40 v/v). The peak was detected at 217 nm under 400-500 psi column pressure at a flow rate of 15 mL min. Under these conditions Aza –A peak appeared at 9.16 min during the preparative run. When the Aza-A peak ascended from the baseline, the eluent was collected until the peak was at maximum (Fraction 'A') and the peak descended to the baseline again (Fraction 'B'). The presence of the Aza-A in fractions 'A' and 'B' was confirmed by the analytical HPLC-UV (*vide supra*). The less polar compounds could be removed by eluting the column with pure methanol for 10 to15 min at a flow rate of 20 mL/min. Restabilization of the column was done with methanol: water (60:40 v/v) for another 20 - 25 min.[10]

RESULTS AND DISCUSSION

Enrichment by solvent extraction and partition was carried out to prepare neem fractions rich in azadirachtin (Figure 3). The extraction of Aza-A with methanol was found to be more efficient, when the ground neem seeds were defatted with hexane. Hexane extracts removed neem oil having Aza-A content of 0.0235% (w/w of oil).[10]

For complete recovery of Aza-A, the defatted powder (B) was extracted six times with methanol at room temperature.[11] Methanol extractions removed the traces of oil left with kernels along with Aza-A, soluble sugars, amino acids and proteins. To ensure near-complete extraction of Aza-A from defatted neem cake, we applied the present HPLC-UV method, which proved to be very useful. Our results showed negligible quantities of Aza-A (< 0.0041%) left in the defatted neem seed cake after six extractions.

The Aza-A content in the six methanolic extracts ranged from 0.21 to 0.0041%. An analytical HPLC-UV method was used to estimate Aza-A content in crude powder. For reverse phase HPLC-UV method, aqueous methanol (60:40 v/v) was suitable for determination and separation of Aza-A giving sharp peaks and good resolution without much baseline drift. The retention time (t_R) of crude Aza-A in our system was 6.9 min (Figure 2B). The concentration of Aza-A in the crude methanol extracts and in the solid powder (G) was determined from the calibration curve obtained by plotting of peak area versus Aza-A concentration, by injecting 20 µL of standard Aza-A solutions in triplet, ranging in concentration from 31.25 to 250 ppm. From the HPLC-UV analysis and calculations, the percentage purity of Aza-A in crude powder was found to be 9.14%, the same in seed kernels was found to be 0.11% and in fruits 0.029%. Our results are in close agreement with those reported in literature.[14,19]

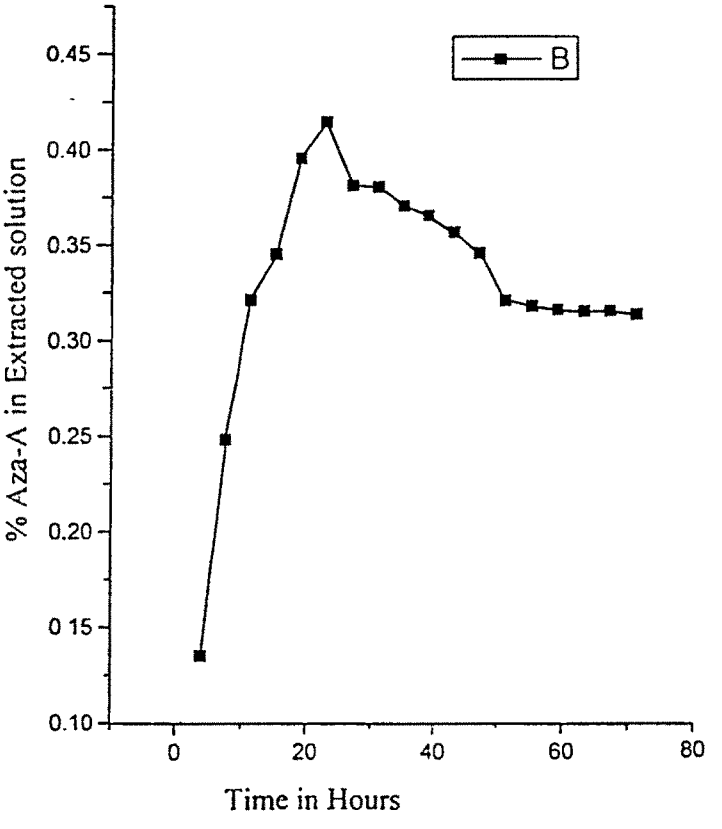

Figure 5. Plot of % Aza-A extracted vs. time (72 hrs) by Soxhlet method.

six extractions with methanol) thus showing the former method to be more efficient. The HPLC-UV results and Figures 4 and 5 showed that in Soxhlet extraction, the amount of Aza-A in the extracted solutions increased up to 24 h initially and decreased gradually, threafter, perhaps due to decomposition of Aza-A.

CONCLUSIONS

The present study describes an improved reverse phase HPLC method, for the quantification of Aza-A in neem seed kernels and its purification of single peak purity using the preparative HPLC-UV method. In addition, a short and economical extraction procedure of Aza-A from kernels is presented. The HPLC-UV results show that the Soxhlet method, which gives a higher percentage of extracted Aza-A, is preferable over a conventional one. Other applications of this procedure could include the determination of Aza-A in degraded neem formulations, which is the subject of our future study.

The preparative HPLC-UV method used in this study was chosen on the basis of the results obtained from analytical HPLC-UV. Each 1mL injection of methanolic solution containing ~9.14% Aza-A content in the residue and each run was completed in 60 min yielding 5-10 mg of 88 to 90% pure Aza-A. Seed kernels (500 g) gave 6.5 g of crude powder containing 9.14% Aza-A. From 1g of this powder, 90 mg of the 88 to 90% pure Aza-A was isolated. The retention time (t_R) of Aza-A in our system was 9.16 min (Figure 2C). Fractions 'A' and 'B' (*vide supra*) were found to contain approximately equal amounts Aza-A (Figure 2D). IR, UV, and PMR spectra of the sample of Aza-A purified by this method were found to be in good agreement with those reported in the literature.[14-15]

In Soxhlet extraction experiments, Aza-A content was determined by analytical HPLC-UV method by taking samples of extracted solutions at 4 h interval for total period of 24 and 72 h, independently. The HPLC-UV results showed that the enrichment of the Aza-A in the extracted solutions by Soxhlet method (0.449%) was higher than the normal extraction method (0.386%, after

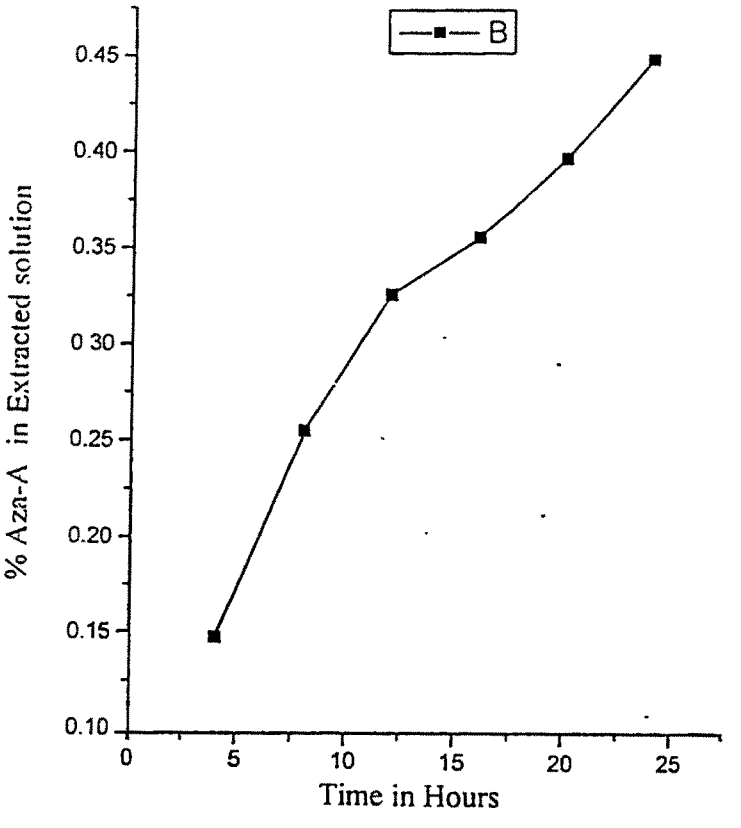

Figure 4. Plot of % Aza-A extracted vs. time (24 hrs) by Soxhlet method.

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EFFECT OF SOME ULTRAVIOLET LIGHT ABSORBERS ON PHOTO-STABILIZATION OF AZADIRACHTIN-A

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The effect of photo-stabilization of Azadirachtin-A (Aza-A) was examined when exposed to sunlight and ultraviolet light in the presence of four structurally different ultraviolet stabilizers namely 4-aminobenzoic acid, 2,4-dihydroxybenzophenone, 4,4′-dihydroxybenzophenone and phenyl salicylate The percentages of Aza-A recovered at different time intervals from slides exposed to different light conditions with and without UV stabilizers as well as kinetic studies indicated that the addition of phenyl salicylate in methanolic solution of Aza-A (in 1 . 1 mole ratio) provides the best photo-stabilization of Aza-A molecule among the four UV stabilizers studied

Keywords Ultraviolet light, Photo-stabilization, Aza-A

INTRODUCTION

Last decade has witnessed a flurry of activity concerning the application of biopesticides in crop protection due to the increasing awareness for safe and green environment [1,2]. Neem (*Azadirachta indica* A. Juss), which is an indigenous tree of Indo-Pakistan subcontinent belonging to the family Meliaceae, widely distributed in Asia, Africa and other tropical parts of the world, has been on the forefront of this activity [3]. Azadirachtin-A (Aza-A, $C_{35}H_{44}O_{16}$) is the major constituent in neem seed extracts (about 85%) and shows considerable promise in controlling several agricultural insects by acting as an antifeedant, as well as growth and molt inhibitor, eventually causing their death [4–8]. It is toxic to target pests and non-toxic to mammals [9,10].

Recently we have reported an efficient and simple method for its estimation and isolation from seed kernels [11]. Aza-A is highly photolabile, either breaking down or isomerising in sunlight, probably due to the presence of sensitive functionalities like

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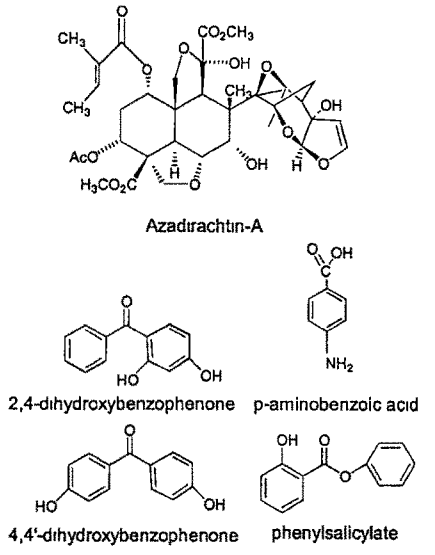

FIGURE 1 Structures of Azadirachtin-A and four UV absorbers

π-electrons, ester linkages and epoxide ring [12–16]. Degradation of the insecticide in sunlight is the major limitation encountered in its use in agriculture, because the insecticide should remain intact on the leaves so as to provide the insect enough time to ingest the material causing death of the insect. Therefore, Aza-A molecule should be photo-stabilized by addition of UV stabilizers for its effective use. It should be noted that the stabilizer itself must not be destroyed during long-term exposure and should have low volatility and chemical inertness to the substrate or additives. The UV stabilizers can either absorb light preferentially to prevent photo-excitation of the pesticide molecule or quench the already excited pesticide molecule by different energy transfer or charge transfer mechanisms thus extending the life span of the pesticide [17]. UV stabilizers such as *p*-aminobenzoic acid, and its esters, substituted hydroxybenzophenones and their derivatives have been used to reduce its photolytic decomposition by ultraviolet radiation, to extend its environmental life [18–19]. However, the effect of different UV stabilizers on its photo-decomposition under sunlight, which is required for the purpose of field applications was not studied earlier. Present study describes the effect of four structurally different UV stabilizers namely *p*-aminobenzoic acid, 2,4-dihydroxybenzophenone, 4,4′-dihydroxybenzophenone and phenyl salicylate (Fig. 1) on the photo-stabilization of Aza-A precoated on glass plates. The plates were exposed to ultraviolet radiation and sunlight with and without UV stabilizers. Control experiments were also performed by keeping the precoated plates in dark.

EXPERIMENTAL

Materials and Methods

Isolation of Aza-A from neem seed kernels by preparative HPLC method and determination of its purity by analytical HPLC method were done by following the procedure

developed by our group recently [11]. All the UV stabilizers purchased from Lancaster, England and HPLC-grade solvents purchased from Glaxo (Qualigens) India Ltd. were used without any purification.

HPLC Instrumentation

The HPLC used in the study was Waters (LC-4000) analytical cum preparative HPLC equipped with a variable wavelength detector (190–600 nm, Model 486), flow controller and Millennium 2010 software. The instrument employed an automatic degassing system; dual solvent system and dual pump heads with common drive, which gave stable and reproducible flows. The Millennium 2010 provided the chromatogram, percent area, and retention time (R_t), for each peak. Detailed descriptions of HPLC procedures are given in our recent publication [11].

Standard Solutions

Solutions of Aza-A (5.8 mg/5mL, 88.58% pure) were prepared along with the four UV stabilizers in the mole ratio of 1 : 1 (Aza-A : UV stabilizer) and 1 : 0 (no UV stabilizer) in ethyl acetate. The solutions were stored in amber-coloured bottles between 0–4°C and the Aza-A content in each was determined by HPLC [11].

Irradiation Experiments

Standard solutions (100 µl each containing 116 µg of Aza-A) of pure Aza-A with and without each UV stabilizers, were applied onto surface of glass slides (each of $75 \times 25\,mm^2$, 1 mm thick) as a thin film of uniform thickness by using a Hamilton syringe. Slides with each UV stabilizers were containing equimolar solutions of Aza-A and the UV stabilizers. The solvent was evaporated at room temperature. Ten such precoated slides each with and without UV stabilizers, were kept in a chamber providing ultraviolet radiation (254 nm, Chromline India, low-pressure mercury lamp) from a distance of 21 cm from the source. Another ten such precoated slides each with and without UV stabilizers, kept in a glass-chamber to protect them from dust, were exposed to sunlight ($\sim$300–1100 nm, open sky in May–June 1999, noon with no cloud cover over the study area, grid reference 22° 18′ N; 73° 13′ E, $37 \sim 40 \pm 0.2$°C) incident upon the glass surface during the study period. Remaining ten such precoated slides without any UV stabilizer were kept in a dark chamber under similar experimental conditions which served as a control.

Extraction and Analysis

Two glass slides each with and without UV stabilizers were removed from the respective chambers at intervals of 6 h up to a total period of 30 h of exposure to ultraviolet light/sun light. These glass slides were then rinsed with methanol (2×2 mL), filtered through a nylon-66 filter media (0.45 µm removal rating, 0.13 mm diameter) and analyzed for their remaining Aza-A content by analytical HPLC [11]. Control samples were collected at the same intervals and extracted and analyzed similarly.

RESULTS AND DISCUSSION

The percentage remaining of Aza-A recovered at different intervals of time (in hours) for the five solutions (methanolic solutions of Aza-A and four UV stabilizers) from different slides exposed to different light conditions with and without UV stabilizers are shown graphically in Figs. 2 and 3 respectively.

The dissipation half-life values (in hours) and rate constant values (in hours^{-1}) for degradation of Aza-A under ultraviolet radiation and sunlight were obtained using first order kinetic equation [20]. The values of dissipation half-life (DT_{50}) and rate constant (K) of Aza-A with and without four UV stabilizers are given in Tables I and II respectively. DT_{50} values (in hours) for Aza-A in the presence of four UV stabilizers under ultraviolet radiation and sunlight were found to be higher as compared to those obtained for Aza-A alone (Table I). Similarly rate constant values (in hours^{-1}) for Aza-A in the presence of four UV stabilizers under ultraviolet radiation and sunlight were found to be lower as compared to those obtained for Aza-A alone (Table II). The DT_{50} values for Aza-A mixed with phenyl salicylate were found to increase 2.6 and 2.5 fold as compared to Aza-A alone under ultraviolet radiation and sunlight respectively. The addition of 2,4-dihydroxybenzophenone, increased its photo-stability nearly twofold, while 4,4'-dihydroxybenzophenone and *p*-aminobenzoic acid provided moderate degree of photo-stabilization to Aza-A in ultraviolet radiation and sunlight Photo-stabilization of Aza-A by different stabilizers appears to be due to competitive energy absorption of UV photons by the stabilizer molecules, which cause degradation of Aza-A molecules.

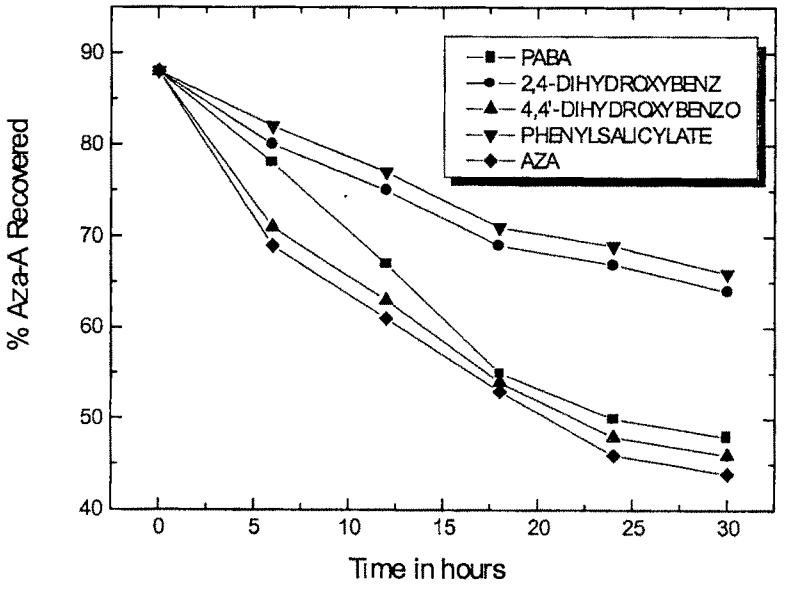

FIGURE 2 Recovery of Aza-A after irradiation of the mixture of Aza-A and different UV stabilizers under ultraviolet radiation.

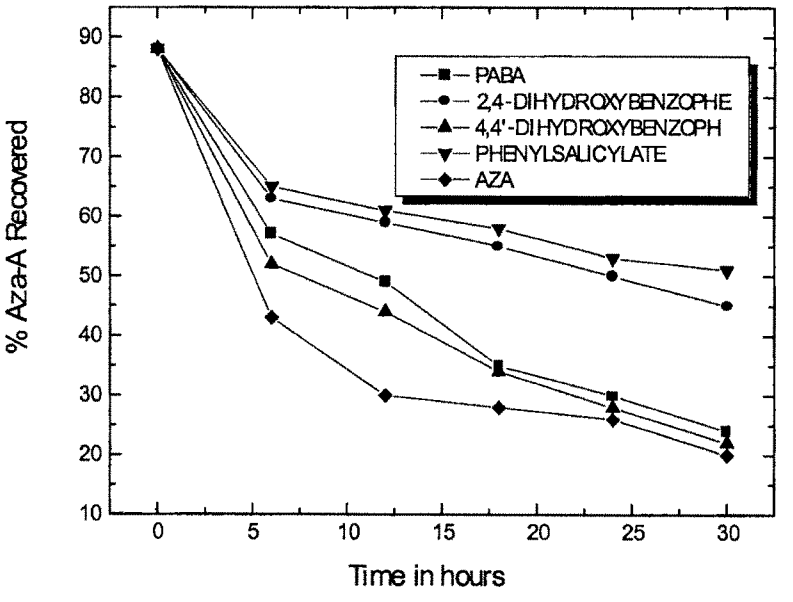

FIGURE 3 Recovery of Aza-A after irradiation of the mixture of Aza-A and different UV stabilizers under sunlight

TABLE I Dissipation half-life (in hours) of Aza-A in the presence of different UV stabilizers (Aza-A/UV stabilizer 1 . 1)

Sr No	*Light*	*Aza-A alone (no UV stabilizer)*	*UV stabilizers*			
			4,4'-dihydroxy-benzophenone	*p-amino-benzoic acid*	*2,4-dihydroxy-benzophenone*	*Phenyl-salicylate*
1	UV	22.64	24 75	30 13	50.21	58 72
2	Sunlight	9.24	11.82	13 07	21 00	23 10

TABLE II Rate constant values (in hours^{-1}) of Aza-A in the presence of different UV stabilizers (Aza-A/ UV stabilizer 1 1)

Sr No	*Light*	*Aza-A alone (no UV stabilizer)*	*UV stabilizers*			
			4,4'-dihydroxy-benzophenone	*p-amino-benzoic acid*	*2,4-dihydroxy-benzophenone*	*Phenyl-salicylate*
1	UV	0 030	0.028	0 023	0.013	0 011
2	Sunlight	0.015	0 014	0.012	0 009	0.007

CONCLUSION

The data presented here on the percentage recovery of Aza-A after irradiation under different light conditions, suggests that the addition of phenyl salicylate (in 1 : 1 mole ratio) can provide excellent photo-stabilization of azadirachtin molecule in neem based agro formulations.

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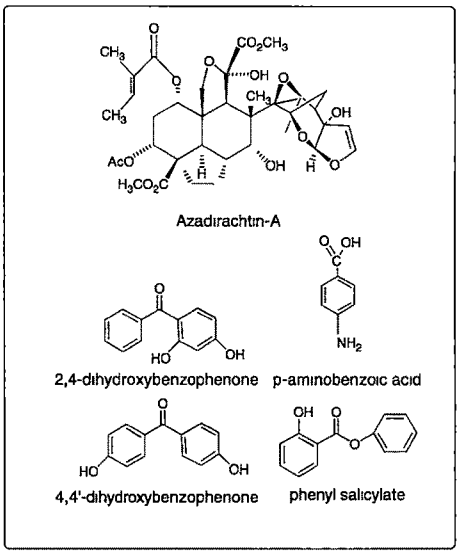

FIGURE 1 Structures of Azadirachtin-A and four UV absorbers

Aza-A is highly photolabile, either breaking down or isomerizing rapidly in the presence of sunlight, due to the presence of sensitive functionalities like π-electrons, ester linkages and epoxide ring [6–10]. Degradation of the insecticide in the sunlight is the major limitation encountered in its use in agriculture, because the insecticide should remain intact on the leaves so as to provide the insect enough time to ingest the material causing death of the insect. Therefore Aza-A molecule should be photostabilized by addition of UV absorbers for its effective use.

In the previous paper, we have reported the effect of different UV absorbers on the photostabilization of Aza-A precoated on glass plates (solid phase) [11]. The plates were exposed to ultraviolet radiation and sunlight in the presence and absence of UV absorbers. The data on the percentages recovery of Aza-A after irradiation under different light conditions in the presence and absence of UV absorbers as well as kinetic studies, suggested that the addition of phenyl salicylate (in 1 : 1 mole ratio) provides excellent photostabilization of Aza-A molecule in solid phase among the four UV absorbers studied [11].

This result prompted us to explore the photostabilizing effect of these UV absorbers on Aza-A in solution. Thus, the methanolic solutions of Aza-A with and without UV absorbers were irradiated individually in the presence of ultraviolet radiation using a high pressure mercury vapor lamp in an immersion-well type of reactor. Results of this study are presented here under.

MATERIALS AND METHODS

Chemicals

Isolation of Aza-A from neem seed kernels by using preparative HPLC method and determination of its purity by analytical HPLC method were done by following the

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EFFECT OF ULTRAVIOLET LIGHT ABSORBERS ON PHOTOSTABILIZATION OF AZADIRACHTIN-A IN SOLUTION (PART: II)

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The effect of photostabilization of azadirachtin-A (Aza-A) was examined in solutions when exposed to UV radiation, in the presence of four structurally different UV absorbers namely, *p*-aminobenzoic acid, 2,4-dihydroxybenzophenone, 4,4′-dihydroxybenzophenone and phenyl salicylate. The percentages of Aza-A recovered from the solutions after 6 h exposed to UV radiation in the presence and absence of UV absorbers indicated that the order of stabilization of Aza-A by these absorbers was similar to that obtained in the solid phase experiments in accordance with our previous observations. It is observed that the addition of phenyl salicylate in Aza-A (in 1 : 1 mole ratio) provides the excellent photostabilization of Aza-A molecule in solid phase as well as in solution among the four absorbers studied.

Keywords: Photostabilization; Azadirachtin-A; UV radiation; UV absorber

INTRODUCTION

In recent years, the interest in agriculture pest control in the developed as well as developing countries has shifted from the use of conventional, synthetic insecticides towards more specific, environmentally acceptable biopesticides [1,2]. Azadirachtin is one of the most promising biopesticide isolated from the neem tree (*Azadirachta indica*, A. Juss) of Indian origin. Azadirachtin-A (Aza-A, Fig. 1, $C_{35}H_{44}O_{16}$) is the major constituent in the neem seed extracts (85 % approx.) and showing high insecticidal activity [3]. It is shown to be potent insect antifeedant, antimalarial and insect growth inhibitor amongst many other biological activities [4]. Moreover, Aza-A has also been reported to be biodegradable, less toxic and non-mutagenic to mammals [5].

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procedure developed by our group recently [12]. All the UV stabilizers purchased from Lancaster, England and HPLC grade solvents purchased from Glaxo (Qualigens) India Ltd., were used without any purification [11].

HPLC Instrumentation

Detailed descriptions on the type and model of HPLC instrument used, column type, detector and related instrument parameters, procedures for peak area and analyte quantification, type of mobile phase used and the measurement of retention time of Aza-A, etc. are given in the previous papers [11,12].

Standard Solutions

Standard solutions of pure Aza-A (2.5 mg / 5 mL) were prepared along with the four individual UV absorbers (*vide supra*) in the mole ratio of 1 : 1 (Aza-A : UV absorber) and 1 : 0 (no UV absorber) in methanol. The solutions were stored in amber colored bottles between 0–4°C and the Aza-A content in each was determined by analytical HPLC [12].

Spectroscopic Measurements

UV–Visible absorption spectra (200–400 nm) of methanolic solutions of analytical grade Aza-A and the four UV absorbers were measured using a Perkin-Elmer Lambda spectrophotometer and are reproduced in Fig. 2(A–E).

Irradiation Experiments

The standard solutions of pure Aza-A prepared as above with and without individual UV absorbers in methanol (5 mL), placed in a pyrex immersion-well type of a photochemical reactor were irradiated individually using a high-pressure mercury vapor lamp (HPMV, 250 W, Bajaj India) for 6 h. The irradiated solutions were then filtered through a nylon-66 filter media (0.45 μm removal rating, 0.13 mm diameter) and diluted with methanol (25 mL) and analyzed for the remaining Aza-A content by analytical HPLC. Detailed procedures of the HPLC analysis of Aza-A are given in our previous paper [12]. Control sample was irradiated and analyzed similarly.

RESULTS AND DISCUSSION

The percentages remaining of Aza-A recovered from the solutions after 6 h of exposure to UV radiation are shown in Table I. After completion of the experiment (6 h, post-treatment), the residual amounts of Aza-A in the presence of three UV absorbers e.g. *p*-amino benzoic acid, 2,4-dihydroxybenzophenone and phenyl salicylate on exposure to UV radiation were found to be 1.50%, 1.88% and 2.53% respectively. In case of pure Aza-A (no UV absorber) following six hours of exposure to UV radiation, HPLC chromatogram (Fig. 3) showed disappearance of the peak corresponding to Aza-A and appearance of a number of unresolved peaks. Similar results were obtained when Aza-A was irradiated in the presence of 4,4′-dihydroxybenzophenone (not shown). The comparison of percentages of Aza-A recovered from

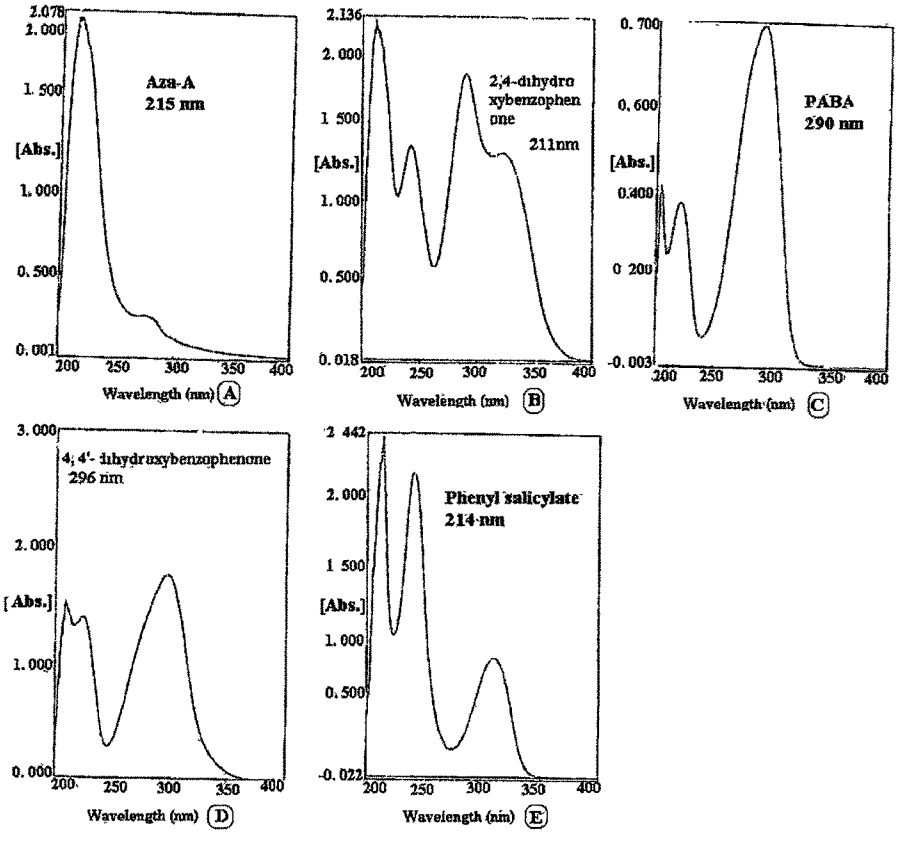

FIGURE 2 UV–Visible spectra of pure Aza-A and the four UV absorbers.

TABLE I Percentage recovery of Aza-A in presence and absence of UV absorbers on exposure to UV radiation (Aza-A : UV absorber, 1 . 1 mole ratio)

Sr. no.	*Samples*	*% Recovery*
1	Aza-A (no UV absorber)	–
2	*p*-Amino benzoic acid	1.50
3	2,4-Dihydroxybenzophenone	1.88
4	4,4′-Dihydroxybenzophenone	–
5	Phenyl salicylate	2 53

the solutions after exposing to UV radiations for 6 h in the presence and absence of different UV absorbers indicated that the addition of phenyl salicylate in Aza-A (in 1 : 1 mole ratio) provides excellent photostabilization of Aza-A molecule in solution.

Absorption Spectra of Aza-A and UV Absorbers

Figure 2(A–E) shows the absorption spectra of pure Aza-A and four UV absorbers in methanol. An examination of the UV spectra of pure Aza-A and phenyl salicylate

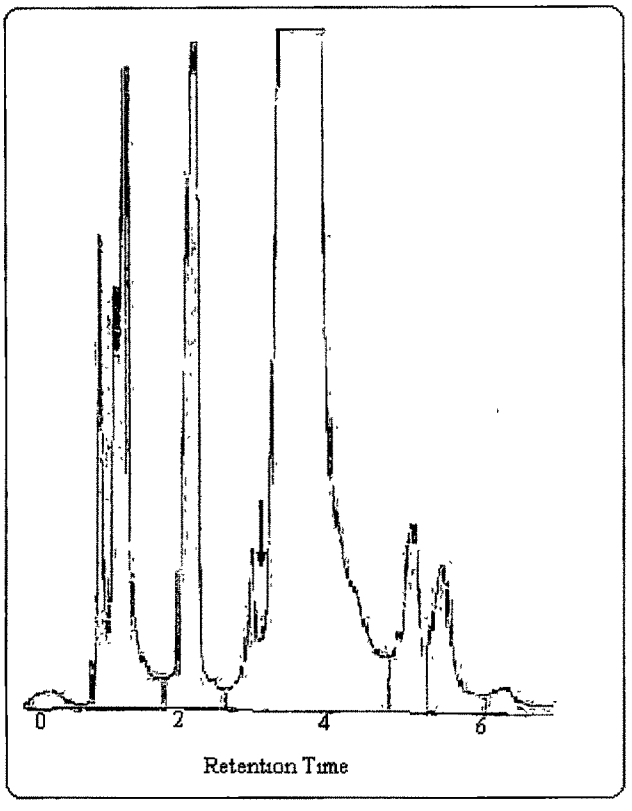

FIGURE 3 Analytical HPLC chromatogram of Aza-A after exposure to UV radiation for 6 h Arrow indicates peak position of non irradiated Aza-A.

(Fig. 2A and E) shows that both the molecules absorb strongly near 215 nm and 214.20 nm respectively. Better photostabilization of Aza-A by phenyl salicylate molecule appears to be due to competitive energy absorption of UV photons which cause degradation of Aza-A molecule by the absorber molecule. The λ_{max} of 2,4-dihydroxybenzophenone (Fig. 2B, 211 nm) is somewhat different from that of Aza-A, which causes relatively less efficient stabilization of Aza-A molecule. While, *p*-aminobenzoic acid and 4,4′-dihydroxybenzophenone showed only marginal effect in stabilizing Aza-A as they showed λ_{max} at 290 nm and 296 nm (Fig. 2C and D) respectively, and small absorption peak near 215 nm. Evidently their ability to capture the photons at 215 nm is low and hence decrease in their capacity to stabilize UV-labile Aza-A.

Although, it was also suggested earlier by Sundaram and Curry [2] in similar experiments, that the photostabilization could be due to either intermolecular energy transfer or competitive absorbtion of UV photons by the absorber, however in our opinion, the later possibility seems more likely. In our previous paper, we reported the effect of four structurally different UV absorbers on the photostabilization of Aza-A precoated on glass plates (solid phase). The plates were exposed to UV radiation and sunlight in presence and absence of UV absorbers. The data on the percentages

recovery of Aza-A after irradiation under different light conditions at intervals of 6 h up to a total period of 30 h with and without UV absorbers as well as kinetic studies suggested that the addition of phenyl salicylate in (1 : 1 mole ratio) provides excellent photostabilization of Aza-A molecule in the solid phase among the four absorbers studied [11]. Our results of solution phase obtained as above indicated that the order of stabilization of Aza-A by these absorbers was similar to that obtained in solid phase in accordance with our previous observations [11]. These results indicate that the addition of phenyl salicylate (in 1 : 1 mole ratio) can provide excellent photostabilization of azadirachtin in neem based agro formulations.

Acknowledgements

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