IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

INSTRUMENTAL METHOD DEVELOPMENT AND STABILITY STUDY OF AZADIRACHTIN IN HERBAL INSECTICIDE

Krutika B. Sonone[1] and Madhuri D. Game[2], Nandini J. Deshmukh[3]

Department of Pharmaceutical Quality Assurance,

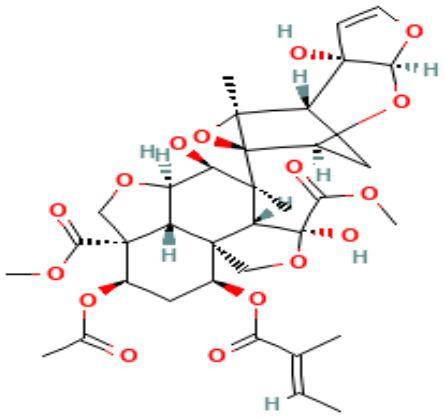
Vidyabharati College of Pharmacy, Amravati[1,2]

Abstract: A high-performance liquid chromatography (HPLC) method for the determination of azadirachtin in marketed herbal insecticide has been developed. Azadirachtin is a neutral triterpene and chemotherapeutic agent effective in controlling some pest flies in horses, stables, horns. The actual HPLC method uses an isocratic elution and UV detection. The chromatographic determination of these components is achieved using a C18 analytical column with methanol : water mixture (80;20, v/v) as mobile phase, 1 mL/min as flow rate, and UV detector at 222 nm. The analytical method developed in this work allows the quantitation of azadirachtin with precision and accuracy, establishing a lower limit of quantitation of azadirachtin. A validated stability indicating method was achieved using HPLC. The purpose of stability testing was to provide evidence on how the quality and quantity of Azaadirachtin and marketed Azadirachtin extract varies with time under the influence of various factors such as acidic hydrolysis, alkaline hydrolysis, oxidation and neutral hydrolysis.

Keywords : Azadirachtin, Analytical method development, Validation, Forced degradation, stability.

Introduction: Azadirachtin, a chemical compound belonging to the limonoid group, is a secondary metabolite present in neem seeds. It is a highly oxidized tetranortriterpenoid which boasts a plethora of oxygen-bearing functional groups, including an enol ether, acetal, hemiacetal, tetra-substituted epoxide and a variety of carboxylic esters. Azadirachtin (AZD) is a component of the Neem tree (Azadirachta indica), a native of India and a member of the Meliaceae family. The bark, leaves, fruits, and particularly the seeds are where it can be found. About 18 different chemicals were detected in the extract, however AZD was the one with the highest concentration. At least nine closely similar isomers make up AZD. There is a predominance of types A and B, with isomer A accounting for 83% and B for 16%. A chemotherapy medication called AZD is useful in preventing pest flies from landing on horses, stables, horns, and fruit. By preventing the development of eggs,

larvae, or pupae, as well as the moulting of larvae or nymphs, and by preventing mating and sexual communication, this chemical disrupts the life cycle of flies. In order to prevent fly attacks on production, sheep skin is currently treated with neem oil, an oily extract of neem. The drug residues utilised in meat and/or viscera that are imported or exported must be notified in accordance with national (Agricultural and Livestock Service of Chile, SAG) and international (European Commission) standards even though there are no limits set for them, unlike with AZD. There have been several reported analytical techniques such as UV spectroscopy, mass spectroscopy for detecting AZD, mainly in fruits. However, no chromatographic method has been developed for the estimation of Azadirachtin in herbal insecticide. The innovative aspect of this work is the development and validation of a simple approach for the detection of AZD residues in marketed herbal insecticide utilising HPLC with UV detection.



Chemical Structure of Azadirachtin

Instruments: Agilent(1100 series) software chem station with UV detector is used.

Materials: PJ Margo Pvt. Ltd. Provided the Azadirachtin. Himalaya Nutraceuticals Pvt Ltd produced Azadirachtin extract containing 2 mg Azadirachtin in 100 mg powder. The Azadirachtin extract was purchased at a nearby market. Throughout the experiment, analytical grade methanol was used.

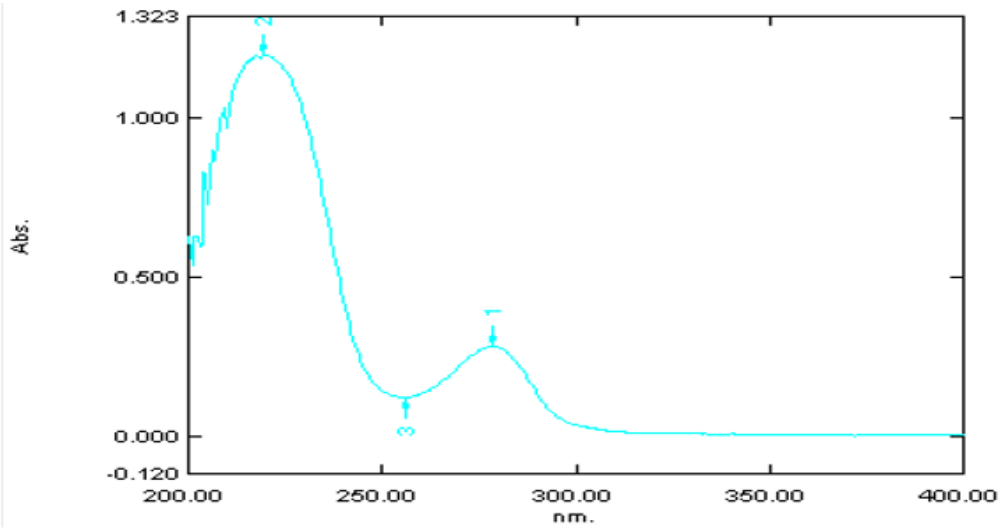
Preparation of standard solution:

Solution A: An accurately weighed quantity of about 10 mg of pure Azadirachtin was taken in 10.0 mL Volumetric flask , dissolving in methanol and volume was made up to mark (Conc.1 mg/mL)

Solution B :The aliquot portion of stock solution was diluted appropriately with same solvent to obtain a concentration 10 ug/mL, 20 ug/mL, 30 ug/mL,40 ug/mL.

Selection of detecting wavelength :

The solution B was scanned in 1 cm cell using double beam UV-visibal spectrophotometer over the range of 400-200 nm and absorbance spectra was recorded From the spectra the detecting wavelength selected for estimation of drug was 222 nm as shown in figure



Spectra for detection of wavelength of Azadirachtin at 222nm

Selection of mobile phase:

The pure drug of Azadirachtin was injected into HPLC system and run in different solvent system . Each mobile phase was allow to equilibrate with stationary phase until steady baseline was obtain .Different mobile phase like Methanol and water, acetonitryl and Methanol in various proportion were tried .Different individual solvent as well as combination of solvents were tried to get a stable peak .each mobile phase was filter through 0.45 µm nylon membrane filter and sonicated on ultra sonic bath . after several trials methanol: water (80:20) was found to be most satisfactory since it gave sharp peak with symmetry within limits and significant reproducible retention time.

Selection of chromatographic parameters :

Method was developed using YMC C18 column (250nm×4.6,5µm) coloum . Mobile phase used was methanol: water (80:20) The flow rate adjusted was 0.7 ml/min. detection was carried out at 222 nm. The mobile phase and sample were degassed by ultrasonic vibrations for 20 min and filtered through 0.45 µm nylon membrane filter .

Application of proposed method for estimation of Azadirachtin in herbal insecticide:

Preparation of sample solution: Accurately weighed marketed formulation Azadirachtin extract equivalent to 10 mg of Azadirachtin was transferred to 10.0 mL volumetric flask. The content was shaken for 10-20 minutes with HPLC grade methanol. Volume was adjusted up to mark with methanol. The solution was then filtered through 0.45 µm membrane filter. Accurately measured 1ml from above solution was further diluted with the 10 ml with methanol. (100µg/ml)

Accurately measured 4.0 mL portion of Azadirachtin extract solution was diluted to 10 mL with methanol. So as to prepare concentration of 40 µg/ml.

Equal volume (40 µL) of standard and sample solutions were injected separately after equilibrium of stationary phase .The chromatogram was recorded and the peak area was measured. The content of Azadirachtin was calculated by comparing with standard peak.

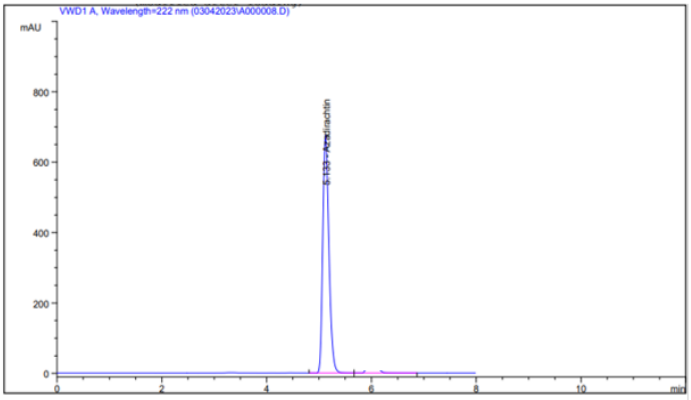


Figure. Typical Chromtogram for Standard Azadirachtin Solution (RT= 5.133)

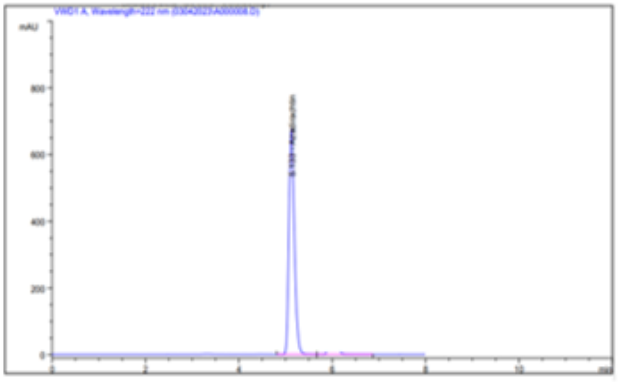


Figure. Typical Chromtogram for marketed Azadirachtin extract Solution (RT= 5.133)

Observation and Result for the estimation of Azadirachtin in herbal insecticide

Sr no	Sample	Wt of drug in mcg	Peak area	Amount found	% Label claim
1	Standard	40	5227		
2	Sample	40	5297.46	40.48	101.21
3		40	5287.81	40.4	101.22
4		40	5280.21	40.34	100.85
5		40	5263.61	40.01	100
6		40	5282.56	40.14	100.35
		Mean	**5282.33**	**40.27**	**100.72**
		SD	**12.388**	**0.197**	**0.539**
		RSD	**0.235**	**0.49%**	**0.54%**

Validation :

Validation of proposed method for Azadirachtin was carried out for the following parameters as per the ICH guidelines

A)Accuracy: Accuracy of proposed method was ascertained on the basis of recovery studies performed by standard addition method .

Recovery studies : It was carried by standard addition method

Sample solution

Accurately measured quantity of Azadirachtin equivalent to 10 µg/mL was taken in series of 50.0 mL volumetric flasks and to them known amount of Azadirachtin were added at different concentration level so as to produce solution containing 80%, 100% and 120 % of the label claim .the content of the flask were shaken with Methanol and volume were make up to the mark. The solution was filter through a 0.45µm membrane filter .An accurately measured 1.0 mL portion of each filtrate was diluted to 10.0 mL with mobile phase The amount of drug was calculated using formula as in following Table.

Observation, results and statistical data for recovery studies for Azadirachtin (80%)

Sr. no.	Concentration µgm/ml	Amount added	Area	Amount found	Amount Recovered	% Recovery
1	10	8	2550.21	17.83	7.83	99.87
2	10	8	2546.79	17.81	7.81	99.84
3	10	8	2562.18	17.91	7.85	100.00
4	10	8	2538.72	17.74	7.77	99.70
5	10	8	2540.31	17.75	7.76	99.68
		Mean	**2547.64**	**17.808**	**7.806**	**99.818**
		SD	**9.381**	**0.068**	**0.035**	**0.131**
		RSD	**0.368**	**0.386**	**0.46%**	**0.132**

Observation, results and statistical data for recovery studies for Azadirachtin (100%)

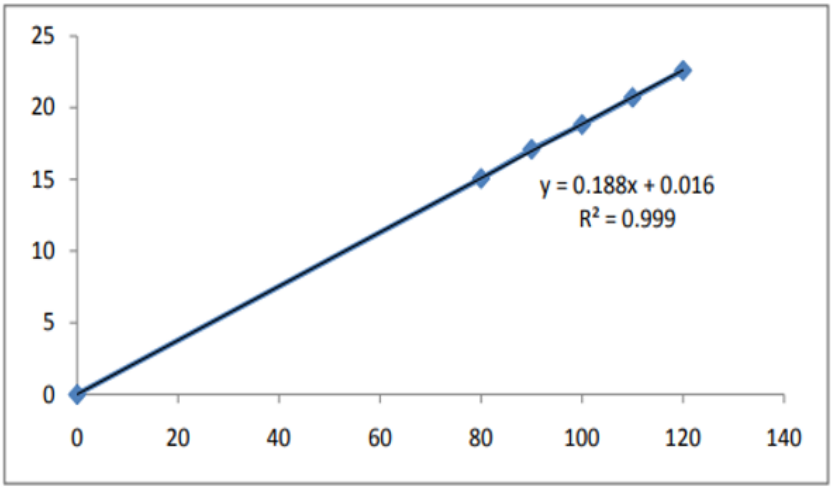
Sr. no.	Concentration µgm/ml	Amount added	Area	Amount found	Amount Recovered	% Recovery
1	10	10	2785.48	19.77	9.69	98.89
2	10	10	2782.86	19.75	9.75	99.36
3	10	10	2779.62	19.7	9.72	99.26
4	10	10	2781.21	19.71	9.71	99.15
5	10	10	2772.89	19.65	9.68	98.84
		Mean	**2780.412**	**19.716**	**9.71**	**99.1**
		SD	**4.73**	**0.0466**	**0.027**	**0.2277**
		RSD	**0.17**	**0.237**	**0.28**	**0.23**

Observation, results and statistical data for recovery studies for Azadirachtin (120%)

Sr. no.	Concentration µgm/ml	Amount added	Area	Amount found	Amount Recovered	% Recovery
1	10	12	3020.68	21.71	11.71	99.69
2	10	12	3016.26	21.68	11.68	99.63
3	10	12	3013.56	21.66	11.66	99.46
4	10	12	3021.42	21.70	11.68	99.62
5	10	12	3019.67	21.68	11.66	99.46
		Mean	**3018.31**	**21.686**	**11.678**	**99.57**
		SD	**3.312**	**0.0194**	**0.020**	**0.1056**
		RSD	**0.110**	**0.090**	**0.175**	**0.106**

B) Precision: Precision of the analytical method is expressed as the S.D or % R.S.D of the series of measurement .It was ascertained by replicate estimation of the drug by proposed method.

C) Linearity and range: Azadirachtin equivalent to 80, 90,100,110,120 % of label claim is to be taken and diluted appropriately to obtain a concentration in the range of 80% - 120 % of test concentration. So accurately measured quantity of drug equivalent to about (8,9,10,11,12) mg of Azadirachtin were taken in five different 50.0 mL volumetric flask and the procedure detailed under assay of Azadirachtin were followed. The chromatogram of resulting solution was recorded and graph was plotted as % test concentration Vs Peak area. Figure



Plot of linearity and range

AZD was found to be linear in the range of ±20 % of the test concentration.

D)The Limit of detection (LOD) of an individual analytical procedure is the lowest amount of an analyte that can be detected in given sample, which can be detected but not necessarily quantitated under the stated experimental conditions as an exact value.

Equation y=121.3x + 386.3

Slope 121.3m

Regression 0.999

Standard deviation 26.2

$$LOD = 3.3 \times \frac{SD}{Slope}$$

Where,

Sd= Standard Deviation

S= Slope of calibration curve

LOD = 0.71

E) The Limit of quantification (LOQ): The quantitation limit of an individual analytical procedure is the lowest amount of analyte that can be detected quantitatively from sample, with suitable acceptable precision and accuracy under the stated experimental conditions.

$$LOQ = 3.3 * \frac{SD}{Slope}$$

LOQ = 2.15

Based on the calibration curve the limit of Detection (LOD) and Limit of Quantitation (LOQ) for Azadirachtin were separately determined and were found to be 0.71 µg/ml and 2.15 µg/ml respectively.

Stability Study:

Stability study of Azadirachtin was done by comparing with Marketed herbal insectiside.

Marketed formulation details:

Manufactured by: Himalaya Nutaceuticals Pvt Ltd

Product name: Azadirachtin Extract

Use: Controls blackspot, powdery mildew, rust, spider, mites, aphids, whiteflies and other insect pests.

Stability Study of Azadirachtin carried out by HPLC

Stability Study of Azadirachtin (API)

Preparation of stock solution:

An accurately weighed quantity of about 10 mg of pure Azadirachtin was taken in 10.0 mL Volumetric flask , dissolving in methanol and volume was made up to mark (Conc.1000µg/ml /mL). Accurately measured 1ml from above solution was further diluted to 10 ml with methanol. (100µg/ml). Accurately measured 4.0 mL portion of Azadirachtin solution was diluted to 10 mL with methanol. So as to prepare concentration of 40 µg/ml.

Acidic and Basic Hydrolysis:

Acidic and Basic degradation of the drug involves catalyzation of ionisable functional group present in the molecule.

Study of effect of Acid and basic hydrolyis of Azadirachtin was done by taking 0.4 ml sample from the stock solution (equivalent to 40 µg/ml). In that 05 ml of 0.1 N HCL and 05 ml of 0.1 NaOH was added and volume make up with the methanol as a diluent upto 100ml. After 60 minutes resulting solution was injected and analysed.

H2O2 degradation:

Effect of Oxidation on Azadirachtin was carried out by taking 0.4 ml sample from the stock solution (equivalent to 40 µg/ml). In that 05 ml of 0.1 H2O2 was added and volume make up with the methanol as a diluent upto 100ml. After 60 minutes, resulting solution was injected and chromatogram was recorded.

Neutral Hydrolysis:

Neutral Hydrolyis of Azadirachtin was done by taking 0.4 ml sample from stock solution (equivalent to 40 µg/ml). In resulting solution 05 ml of water was added. Volume make up with the methanol as a diluent upto 100ml. After 60 minutes the solution was injected and analysed.

Amount of Azadirachtin (API) degraded in given time (60 minutes)

Sr no	Degradation	Area of standard	Area of degraded sample	%Recovery	Actual % degradation
1	Acid degradatiion	5227.62	4406.62	85	15.70
2	Basic degradation	5227.62	3859.87	74.45	13.75
3	H2O2 degradation	5227.62	4195.6	80.92	14.94
4	Neutral	5227.62	4904.41	79.16	14.61

Stability study of Marketed Azadirachtin Extract

Preparation of Stock solution:

Accurately weighed marketed formulation Azadirachtin extract equivalent to 10 mg of Azadirachtin was transferred to 10.0 mL volumetric flask. The content was shaken for 10-20 minutes with HPLC grade methanol. Volume was adjusted up to the mark with methanol. The solution was then filtered through 0.45 µm membrane filter. Accurately measured 1ml from above solution was further diluted to 10 ml with methanol. (100µg/ml). Accurately measured 4.0 mL portion of Azadirachtin extract solution was diluted to 10 mL with methanol. So as to prepare concentration of 40 µg/ml.

Acidic and Basic degradation

Study of Acidic and basic degradation of Marketed Azadirachtin extract was done by taking 0.4 ml sample from the stock solution (equivalent to 40 µg/ml). In that 05 ml of 0.1 N HCL and 05 ml of 0.1 N NaOH was added for the acidic and basic degradation respectively. Volume make up with the methanol as a diluent. After 60 minutes resulting solution was injected and analysed.

H_2O_2 Hydrolysis:

Study of effect of Oxidation of Marketed Azadirachtin extract was done by taking 0.4 ml sample from the stock solution (equivalent to 40 µg/ml). In resulting solution 05 ml of 0.1 N H2O2 was added and volume make up with the methanol as a diluent. After 60 minutes, resulting solution was injected and chromatogram was recorded.

Neutral Hydrolysis:

Neutral Hydrolyis of Marketed Azadirachtin extract was carried out by taking 0.4 ml sample from stock solution (equivalent to 40 µg/ml). In that 05 ml of water was added and volume make up with the methanol as a diluent upto 100 ml . After 60 minutes resulting solution was injected and analysed.

% Amount of Azadirachtin degraded in given time in marketed Azadirachtin extract after 60 minutes:

Sr no	Degradation	Area of standard	Area of degraded sample	% Recovery	Actual % degradation
1	Acid degradatiion	5227.62	4855.76	94.01	7.99
2	Basic degradation	5227.62	4014.74	77.72	6.60
3	H2O2 degradation	5227.62	4404.2	85.25	7.23
4	Neutral	5227.62	5052.4	97.79	8.29

Result and Discussion:

HPLC method for estimation of Azadirachtin was developed. Azadirachtin was resolved using YMC C18 (250 nmX 4.6,5µm) column using Methanol:Water as a mobile phase with a flow rate 1ml/min. UV detection were carried out at 222 nm .The retention time (RT) for Azadirachtin was 5.133 min. Azadirachtin was found to be linear in the range of ±20 % of the test concentration.. The correlation coefficient for Azadirachtin was 0.999. The result of estimation has been validated statistically and by recovery studies.

Stability study of Azadirachtin and Marketed Azadirachtin formulation was carried out successfully. It was found that Marketed Azadirachtin extract was more stable than API due to the presence of preservatives.

Conclusion:

The observations of the validation parameters such as accuracy, precision, specificity, linearity, shows that the developed method can be employed for routine analysis of azadirachtin. The analytical procedure described for assay was specific, linear, precise, accurate, and system suitable for determination of Azadirachtin in herbal insecticide. The results obtained from the validation parameters met the ICH and USP requirement as well as obeys BEER'S law.

The presented stability study provides the knowledge about possible degradation pathways and degradation of the active ingredients in marketed products and helps to elucidate the structure of the degradants. The developed method was validated according to ICH guidelines and all parameters were within limits.

This study could also be used to determine the shelf-life of the compound in the drug form.

Acknowledment:

The authors wish to express their gratitude to Vidyabharati College of Pharmacy, Amravati for providing the essential research facilities.

References:

1)Aarthy, T., Mulani, F. A., Pandreka, A., Kumar, A., Nandikol, S. S., Haldar, S., et al. (2018). Tracing biosynthetic origin of limonoids and their functional groups through stable isotope labeling and inhibition in neem tree (Azadirachta indica) cell suspension. BMC. Plant. Biol. 18:230. doi: 10.1186/s12870-018-1447-6 PubMed Abstract | CrossRef Full Text | Google Scholar

2)Abdel-Gawad, R. M. (2018). Development rate and ultrastructure changes of puparia of Megaselia scalaris (Loew) (Diptera: Phoridae) induced by azadirachtin. Egypt. Acad. J. Biolog. Sci. 11,109–120. doi: 10.21608/eajb.2018.11984 CrossRef Full Text | Google Scholar

3)Abedi, Z., Saber, M., Vojoudi, S., Mahdavid, V., and Parsaeyan, E. (2014). Acute, sublethal, and combination effects of azadirachtin and Bacillus thuringiensis on the cotton bollworm, Helicoverpa armigera. J. Insect. Sci. 14:30. doi: 10.1093/jis/14.1.30 PubMed Abstract | CrossRef Full Text | Google Scholar

4)Ahmad, S., Ansari, M. S., and Muslim, M. (2015). Toxic effects of neem based insecticides on the fitness of Helicoverpa armigera (Hübner). Crop. Prot. 68, 72–78. doi: 10.1016/j.cropro.2014.11.003 CrossRef Full Text | Google Scholar

5)Aktar, M. W., Sengupta, D., and Chowdhury, A. (2009). Impact of pesticides use in agriculture their benefits and hazards. Interdisc. Toxicol. 2, 1–12. doi: 10.2478/v10102-009-0001-7 PubMed Abstract | CrossRef Full Text | Google Scholar

6)Aljedani, D. M. (2018). Assessment of effectiveness of the imidacloprid and azadirachtin on the black watermelon bug. Int. J. Zool. Res. 14, 61–70. doi: 10.3923/ijzr.2018.61.70 CrossRef Full Text | Google Scholar

7)Amaral, K. D., Martinez, L. C., Lima, M. A. P., Serrão, J. E., and Castro Della Lucia, T. M. (2018). Azadirachtin impairs egg production in Atta sexdens leaf-cutting queens. Environ. Pollut. 243, 809–814. doi: 10.1016/j.envpol.2018.09.066 PubMed Abstract | CrossRef Full Text | Google Scholar

8) Abbott WS. 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18: 265–267. Google Scholar

9) AgriDyne Technologies, Inc. 1994. Greenhouse Grower. Floritech Report. Tough on pests, easy on crops—and the environment. AgriDyne Technologies, Inc., Salt Lake City, Utah. Google Scholar

10) Butterworth JH, Morgan ED. 1968. Isolation of a substance that suppresses feeding in locusts. Chemical Communications 1: 23–24. Google Scholar

11)Fan Y, Gan BC, Chen SL, Du CG, Yang CQ, Cui WT. 1986. The investigation and research on Tirathaba rufivena Walker of betel nut. Traditional Chinese Medicine Bulletin 11: 8–9. [In Chinese] Google Scholar

12)María Nella Gai, Christian Alvarez, Raul venegas, Javier Morales. 2011.An HPLC Method for Determination of Azadirachtin Residues in Bovine Muscle. Journal of Chromatographic Science, Volume 49.Google Scholar

13) Supriya Dubhashi 1*, V. Pranay1, M. Singaiah1, J. Satwik2, Dr. V. V. L. N, Prasad1, Dr. Prakash V Diwan. 2013.Studies on extraction and HPLC Analysis of Azadirachtin from Kernels of Neem Seed. Journal of Advanced Pharmacy Education & Research, Google Scholar

14) Parminderjit Kaur,Gurmeet Singh,Davinder Singh,Shivani Verma &Jagjeet Singh. 2017.Development and Validation of Stability Indicating UV-Visible Spectrophotometric Method for Simultaneous Estimation of Neem (Azadirachtin) and Curcumin in Pharmaceutical Tablet Dosage Form, Google Scholar

15) Subbalakshmi Lokanadhan, P Muthukrishnan, S Jeyaraman. 2017. Neem products and their applications in Biopesticides. Journal of Biopesticides, Google Scholar

16) Sara R. Fernandesa, Luisa Barreirosa, Rita F. Oliveiraa, Agostinho Cruza, Cristina Prudênciod, Ana Isabel Oliveiraa, Cláudia Pinhoa, Nuno Santosf, Joaquim Morgadog. 2019. Chemistry, bioactivities, extraction and analysis of azadirachtin: State-of-the-art. Google Scholar

17) Vishal V. Dawkar*, Sagar H. Barage, Ranjit S. Barbole, Amol Fatangare, Susana Grimalt, Saikat Haldar, David G. Heckel, Vidya S. Gupta, Hirekodathakallu V. Thulasiram, Aleš Svatoš, and Ashok P. Giri. 2019.Azadirachtin-A from Azadirachta indica Impacts Multiple Biological Targets in Cotton Bollworm Helicoverpa armigera, ACS Omega 2019, 4, 5, 9531–9541

18) Agbo, B. E., 2Nta, A. I. and 1Ajaba, M. O. 2015.A REVIEW ON THE USE OF NEEM (Azadirachta indica) AS A BIOPESTICIDE, Journal of Biopesticides and Environment Vol. 2 no.1-2, 2015,Google Scholar

19) Kanth M.S. Sundaram. 2008.Azadirachtin biopesticide: A review of studies conducted on its analytical chemistry, environmental behaviour and biological effects, Journal of Environmental Science and Health, Part B