

## Species Sensitive Electrochemical Method for Enrichment of Azadirachtin-A from Neem Seed

Kirti Thakur and K.S. Pitre\*

Department of Chemistry, Dr. H.S. Gour Vishwavidyalaya, Sagar (M.P.) 470003 India

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### Abstract

Azadirachtin the active principle of *Azadirachta-indica J. Juss.* (Neem) ranges from 0.3 to 0.6 percent. A simplified procedure, when compared to established methods, has been developed to enrich azadirachtin-A to 50 percent using minimum possible steps without resorting to chromatographic techniques. It involves additional partition with  $\text{CCl}_4$  before final extraction which removes most of nimbin, salanin and allied triterpenoids. Enriched product finds immense utility in preparing formulations with high azadirachtin concentration.

DCP and DPP polarographic methods have been developed for the qualitative as well as quantitative analysis of azadirachtin in plant product and its formulation (Neemgold®). Azadirachtin produces a well defined polarographic wave/peak in 0.1M Tetramethyl ammonium bromide at pH  $3.3 \pm 0.1$  with  $E_{1/2}/E_p = -1.48\text{V/SCE}$ .

The wave/peak height is found to be proportional to the concentration of Azadirachtin. The developed procedure was used for the analysis of Azadirachtin in extract of *Azadirachta indica* (seeds) and market formulation. Statistical treatment of the observed voltammetric data revealed high accuracy and good precision of determination.

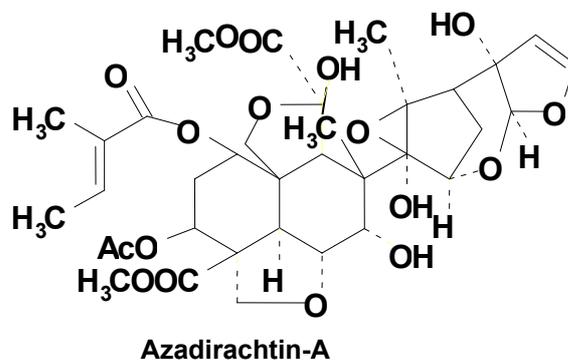
**Keywords:** Azadirachtin-A; Neem; Neemgold; DCP - DPP Analysis.

### Introduction

Azadirachtin ( $\text{C}_{35}\text{H}_{94}\text{O}_{16}$ ), a tetranortriterenoid from the neem tree (*Azadirachta-indica A Juss.*) was first isolated by Butterworth and Morgan<sup>[1]</sup>, it is hormonally active in insects<sup>[2,3]</sup> and a powerful antifeedant<sup>[4]</sup> and growth disruptive agent, It is ovipositional of deterrent<sup>[5,6]</sup> and an excellent nematicide<sup>[7,8]</sup>. It has therefore aroused world wide academic and industrial interests. More than hundred compounds have been isolated so far from various parts of *A. indica* and related Meliaceae species. These compounds and extracts of *A. indica* have potent and specific effects against various insect pests<sup>[9]</sup>, but none has biological activity comparable to Azadirachtin, Formulation From neem kernel extract standardised in terms of Azadirachtin content, hold promise for use in plant protection, with no adverse effects on the environment The Purity of Azadirachtin is difficult to accomplish due to the complexity and similarity in structure of compounds found in the seeds and foliage of neem tree. Although Azadirachtin is present in Neem kernel quantities ranging from 0.3 to 0.6 percent its isolation<sup>[1,10]</sup> is

\* Corresponding author. E-mail: kirtit24@gmail.com

tedious and time consuming involving partitions; Column chromatography; preparative TLC; Preparative HPLC<sup>[11]</sup>, HPLC and LC/MS<sup>[12]</sup>, reversed phase column chromatography, Despite these methods, there is still shortage of pure material available. The best technique so far purification and identification of Azadirachtin, is polarography. The present paper deals with enrichment of Azadirachtin, using simple laboratory techniques like solvent extraction.



The authors have therefore developed DCP and DPP methods for the analysis of Azadirachtin in samples of plant origin and its formulations (Neemgold<sup>®</sup>). The work has been supplemented by UV, Preparative HPLC and FT-IR spectral Studies. The results of which have been reported in this paper.

## Materials and Methods

### *Extraction of Azadirachtin A from Neem seeds*

A suspension of 100gm of ground neem seed kernel in 500ml of hexane was stirred at 40°C for 2h and filtered. The hexane extract was concentrated in vacuum. The defatted mass was then extracted with 500ml methanol in the same manner as was done in case of n-hexane extraction. The extract was concentrated under vacuum to yield dark brown semisolid. The methenolic extract was dissolved in 50ml of 90 percent aqueous methanol and partitioned twice with 25ml hexane to remove remaining oil and other non-polar compounds. The aqueous layer was then diluted with water to make it (1 : 1) Methanol : water saturated with sodium chloride and partitioned twice with (1 : 1) 50ml of the following solvents (I & II):

- I Ethylacetate and dichloromethane, and
- II Carbontetrachloride, extraction was followed by ethylacetate and dichloromethane.

The organic layers were azeotroped with isopropanol and rotary evaporated in vacuum and dissolved in minimum amount of ethylacetate and dropped in hexane under constant stirring to yield a light yellow to whitish coloured enriched dry amorphous powder yield and purity of the fractions obtained by adopting the above procedures (I) and (II) are presented in table 1 and 2, respectively.

**Table 1:** Extraction of methenolic extract with different Solvents

Solvent	Yield, g/kg	Azadirachtin Percent	Pure azt. g/kg
I – Ethyl acetate	12.19	17.12	2.08
II-Dichloromethane	5.00	15.78	0.79

**Table 2:** Extraction in different Solvents after CCl<sub>4</sub> Partition.

Solvent	Yield, g/kg	Azadirachtin Percent	Pure azt. g/kg
I – Ethyl acetate	5.8	35.5	2.1
II-Dichloromethane	3.2	33.0	1.6

### *Thin layer chromatography*

Normal phase analytical TLC was performed on 3×5 cm silica gel (0.5 mm thickness) coated glass plates activated at 60°C for 12h. The plates were developed in solvent system (ethyl acetate). The developed plates were air dried, sprayed with vanillin : sulphuric acid : ethanol (3g :1.5 ml : 100ml) reagent and heated to 100°C. The compounds were detected by the characteristic colours of the spots : Azadirachtin (green), Nimbin (violet to black) and Salanin (blue).

## **Experimental**

### *Chemicals and Reagents*

The chemicals used were of Anal R grade. Whereas, Azadirachtin was obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Stock solution of (0.1M) tetramethyl ammonium bromide was prepared by dissolving its required amount in double distilled water. (0.05 mM) Azadirachtin Solution was prepared by dissolving the requisite amount in methanol and water (1 : 1).

### *Apparatus*

The DCP and DPP, Studies were carried out on an Elico (India) micro processor based polarographic analyzer, model CL-362. The polarographic cell consisted of an electrode assembly having a dropping mercury electrode, a coiled platinum wire electrode and a saturated electrode. The capillary characteristics of the DME had a  $m^{2/3} t^{1/6}$  value of  $2.5 \text{ mg}^{2/3} \text{ Sec}^{-1/2}$  at 60cm effective height of mercury column. A Systronics digital  $\mu\text{pH}$  meter model - 361 was used for the pH measurements. The UV Studies were carried out on an Elico (India) SL 164 Double beam UV-Vis spectrophotometer.

### *Polarographic determination of Azadirachtin - A:*

A known concentration of Azadirachtin was taken in a polarographic cell having 5ml of (0.1M) tetramethyl ammonium bromide (TMAB). The Volume of analyte was made upto 50ml with methanol and water (1 : 1). The pH was adjusted to  $3.3 \pm 0.1$  with dilute Solution of NaOH/HCl, Polarogram was then recorded.

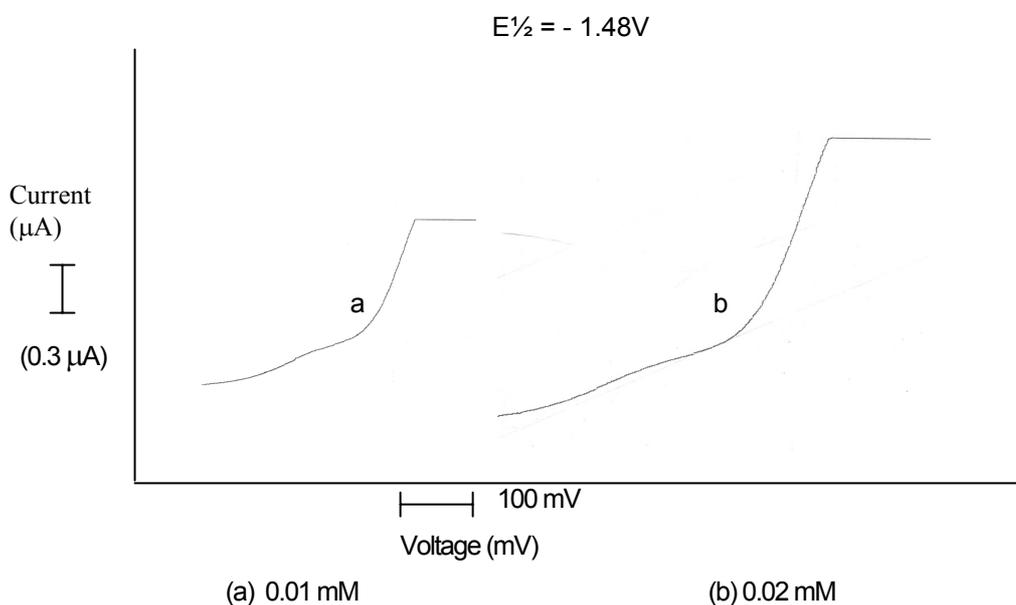
Azadirachtin produces a well defined Polarographic response in TMAB solution. The Wave /peak height of the polarogram was found to be proportional to Azadirachtin concentration. Calibration curve was obtained by taking different known concentrations of Azadirachtin in tetramethyl ammonium bromide supporting electrolyte under identical experimental conditions as discussed above and recording the polarograms and plotting  $i_d/i_p$  VS. Azadirachtin concentration curve.

#### *Determination of Azadirachtin-A in neem extract*

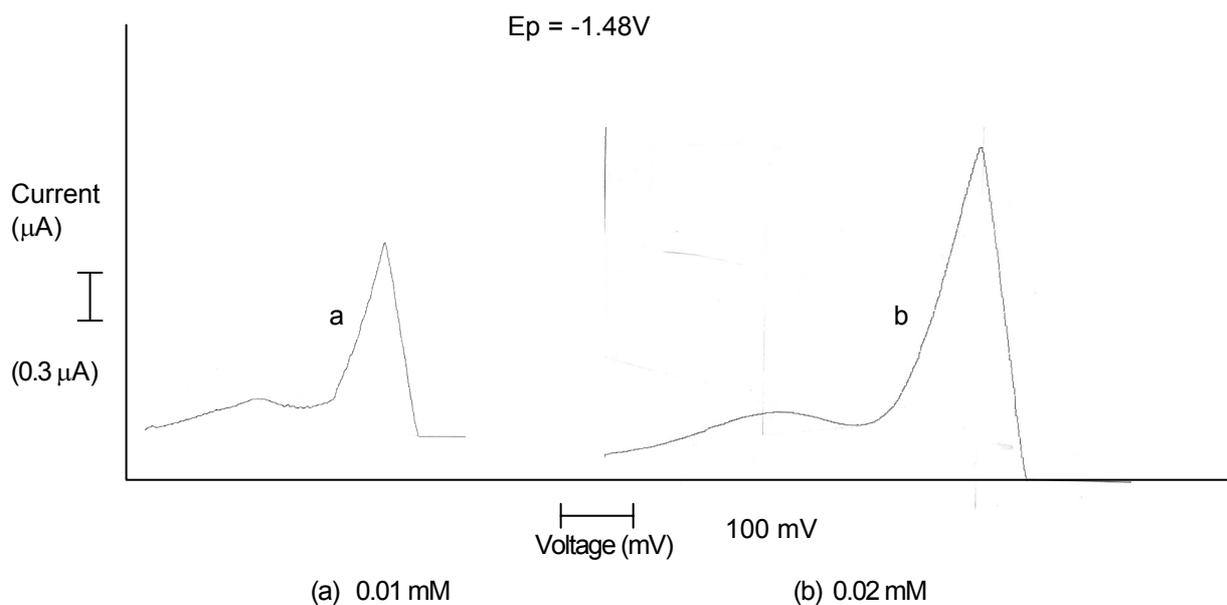
For the determination of Azadirachtin in neem extract a known weight (0.022g) of extract was diluted with (10ml) methanol and water. 1ml of this Solution was mixed with 5ml, (0.1M) Tetramethyl ammonium bromide and final volume of analyte was made upto 20ml with methenol and distilled water (1 : 1). The pH of the test solution was adjusted to  $3.3 \pm 0.1$  with dil HCl/NaOH Solution and its polarogram was recorded as discussed earlier.

### Results and discussion

The direct current polarogram (DCP) and Differential pulse polarogram (DPP) Figure 1 and 2 of the authentic sample Solution of Azadirachtin in tetramethyl ammonium bromide (0.1M) at pH  $3.3 \pm 0.1$  produced a well defined polarographic wave/peak with  $E_{1/2}/E_p = -1.48$  vs, SCE, indicating the presence of Azadirachtin in the sample.



**Figure (1):** Direct current polarograms of Azadirachtin-A in 0.1 M tetramethyl ammonium bromide at pH =  $3.3 \pm 0.1$



**Figure 2:** Differential pulse polarograms of Azadirachtin-A in 0.1 M tetramethyl ammonium bromide at pH = 3.3 ± 0.1

To ascertain as to whether the wave/peak is due to Azadirachtin a known amount of standard solution of Azadirachtin was added to the analyte and polarogram was recorded under above experimental conditions. An increase in wave /Peak height of the polarogram due to Azadirachtin was observed without any change in half wave/Peak potential, thus, confirming the presence of Azadirachtin in the sample and reconfirming the possibility of an accurate qualitative and quantitative determination of Azadirachtin in the sample.

Figures 3a and 4a show the DC and DP Polarograms for Azadirachtin content in *Azadirachtin-Indica* (Neem) whereas, figures 3b and 4b show the DCP and DPP for the Azadirachtin content in neemgold<sup>®</sup>, a market formulation.

Each of these figures, clearly shows a well defined polarographic response with  $E_{1/2} / E_p = -1.48$  vs. SCE for the presence of Azadirachtin in the sample. Method of external spiking was used for the analysis of Azadirachtin in the extracted samples from *Azadirachtin-indica* and neemgold<sup>®</sup>.



samples was proved by the percentage recovery (table 3) and standard deviation of the data. The percentage recovery was always found to be above 99.5% and the standard deviation never exceeded 0.01.

Thus confirming the reliability of the analysis. On the basis of the observed polarographic data the concentration of Azadirachtin in neem (methanol-Ethylacetate extract) and neemgold® are found to be 2080 mg/kg and 300 mg/L respectively.

The percentage of Azadirachtin in market formulation was determined by developed method which is in close agreement with that reported by the manufactures (i.e. 300 ppm).

*Analysis of Azadirachtin–A in Neemgold®*

The DC and DP Polarograms of Azadirachtin content of Neemgold produced polarograms with reduced wave/peak heights as compared to that observed with blank. The reduction in wave/peak height in the first sample solution may be explained on the basis of matrix effect. However, on further increase in concentration of Azadirachtin-A in Neemgold resulted in proportionally increased wave/peak heights.

Method of spiking was used for the determination of Azadirachtin content of Neemgold sample. The results have been depicted in table (3).

**Table 3:** Azadirachtin–A (ppm)<sup>a</sup> in neem methenolic extract and Neem-gold.

Sample	Added (µg)	Found	%R	S.D
Neem (Methenolic Extract)	-	2080	99.95	0.002
	0.021	-		
Neemgold (Market formulation)	-	300	99.37	0.001
	0.020	-		
	-	318		

a=Results are average of five determinations.

R% = Recovery percent.

S.D. = Standard deviation.

The work has been supplemented by FTIR Screening of the extracted and market formulation samples. Azadirachtin exhibits absorption bands show in table (4), similar FTIR signals were also observed for authentic Azadirachtin sample. Thus confirming the presence of Azadirachtin in the extracted samples from Neem. The results have been depicted in table (4).

**Table 4:** Azadirachtin–A, FT-IR results

S.No.	FT-IR signals	Assignment
1	1043 cm <sup>-1</sup>	C-O stretching (alkyl vinyl ether and ester)
2	1350 cm <sup>-1</sup> & 1609 cm <sup>-1</sup>	C-O stretching (sec. Alcohol)
3	1450 cm <sup>-1</sup>	C-H bending (Alkane)
4	1651 cm <sup>-1</sup>	C=O stretching (Carbonyl gp.) and C-C stretching (Alkene disubstituted)
5	1740 cm <sup>-1</sup>	C-O stretching (Ester)
6	2480 cm <sup>-1</sup>	C-H stretching (Alkane)
7	2877 cm <sup>-1</sup>	OH stretching (Bonded)
8	3422 cm <sup>-1</sup>	O-H stretching (Polymeric association)

## Conclusion

On the basis of the observed data and ongoing discussion it could be concluded that the developed polarographic procedure could be successfully used for the accurate analysis of Azadirachtin in plant extract and industrial samples i.e. Pharmaceutical formulation.

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