Asian Journal of Pharmaceutical and Clinical Research

BIOREMEDIATION OF HEAVY METALS FROM NEEM (AZADIRACHTA INDICA) LEAF EXTRACT BY CHELATION WITH DITHIZONE

(A PROSPECTIVE AND EFFECTIVE METHOD FOR PHARMACEUTICAL INDUSTRY)

AMITAVA GHOSH[*1], PIYALI CHAKRABARTI[2], PARTHA ROY, SOMNATH BHADURY, TANUSHREE NAG AND SIMLI SARKAR

Quality assurance of the herbal formulations is the key concern of the current phytomedicinal research due to increased toxicity reports. One of the major reasons of toxicity of herbal formulations being the contamination of heavy metals in plant extracts. This guided FDA, U.S.A., to implement stringent evaluation rules for herbal formulations followed by regulations of the Indian Government for pharmaceutical industries. The aim of this research was to determine the heavy metal content of Neem (Azadirachta indica) extract and bioremediation of the polluting heavy metal ions by chelation without damaging Nimbidin, the marker compound in the present study. TLC and U.V. Spectrophotometric analysis detects the presence of Cadmium and Lead in excess to pH limits in the extract. Chelation with dithizone at different pH was the bioremediation approach for the removal of the contaminated heavy metals. The extract was analyzed in HPLC and TLC both before and after treatment with dithizone. The peak area and R_f value of Nimbidin when compared was found to show no significant deviations. This proves that dithizone has no interference with Nimbidin though it confiscated the toxic impurities. This method for heavy metal elimination is a potent industrial tool for Quality assurance of Herbal formulations.

Keywords : Bioremediation, Nimbidin, Dithizone, Chelation.

INTRODUCTION

Medicinal plants are a part and parcel of human society to combat diseases, from the dawn of civilization. Azadirachta indica A. Juss (syn. Melia azadirachtai) which is commonly called Neem[1,2], is very well known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. The compounds in Neem have been classified as isoterpinoids (triterpinoids and diterpinoids) like protomeliacins, limonoids, azadirone, and its derivatives gedunim and its derivatives, vilasinin type of compounds and C-secomeliacins such as nimbin, salanin, and azadirachtin. The other type being nonisoterpinoids include proteins and carbohydrates sulphurous compounds polyphenolics such as flavonoids and their glycosides dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. The Neem extract has undergone extensive pharmacological screening and found to have several pharmacological activities due to the presence several active constituents in it as tabulated in Table-1.[1,3,4]
Toxicity of several herbal formulations dictated stringent rules to control the quality of the herbal formulations. Heavy metal content is one of the important factors imparting toxicity[5,6] in the herbal formulations and needs to be checked and confirmed that they are well below the pH limits. Some of the hazardous heavy metals being. Lead (causing abdominal pain, convulsion, hypertension and renal dysfunction, loss of appetite, fatigue, sleeplessness, headache and vertigo).

- Arsenic (causing abdominal pain, vomiting, sensory changes, numbness and tingling, muscle tenderness, burning sensation in hand and feet, excessive darkening of skin, liver injury).
- Mercury (causing shortness of breath; metallic taste in mouth, abdominal pain, nausea, vomiting, headache, weakness, visual disturbance and hypertension).
- Cadmium (causing nausea, vomiting, abdominal pain, breathing difficulties, growth impairment, osteoporosis, loss of taste and smell, and cardio vascular disease).

As evident from above, heavy metals indeed are hazardous and several works[7] have been done to prove the severity of the heavy metal content above the prescribed WHO limit. Initiated by F.D.A., USA and now geared up by the Indian Government, the quality assurance of herbal formulations has become the key concern of the green industry. New and innovative methods are being developed to remove these heavy metals to increase the patient acceptability of the herbal drugs. Our aim lies in designing and developing one such method for the bioremediation of the Neem extract, one of the most commonly used indigenous medicinal plant. Various methods described in official books proved[7,8] that they are associated with some limitations like toxicity or lack of broad spectrum chelating action i.e. their incapability to bind with a variety of heavy metals. Among these various methods[8] used for the removal of heavy metals, chelation with dithizone was selected as the probable option. Dithizone has a number of advantages

***Corresponding author:** [*1] **Himalayan Pharmacy Institute, Majhitar Sikkim**
[2] **Dept. of Chemical Technology, University of Kolkata. e-mail:amitoli@rediffmail.com**

which can be summarized as follows:

- The same compound can be used for both identification and quantification.
- The compound does not chemically react with the main marker compound (unlike most of the other chelating agents).
- It has a wide spectrum of activity and even can chelate out the trace elements.

MATERIALS AND METHODS

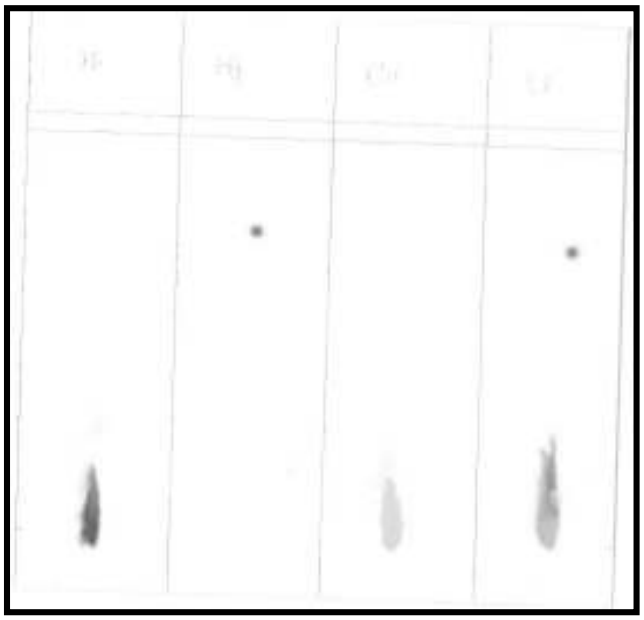
Materials

Neem leaf was purchased from the local markets of Kolkata. Dithizone of A.R. grade was obtained from S.D. Fine Chem. Ltd. Methanol Chloroform were of A.R. grade and obtained from Loba Chem. Acetonitrile and Distilled water both of HPLC grade were obtained from Loba Chem., India. All other chemicals were obtained from local purchase of A.R. and L.R. grade.

Detection of heavy metal content in Neem

The powdered dry neem leaf was mixed with distilled water in the proportion of powder: water (1:8) & filtered in order to remove the unwanted materials, fibers etc. The solution was then subjected to analytical tests to detect the undesirable elements or impurities.[9, 25]

Thin layer chromatography was performed to confirm the presence of the heavy metals. Comparison of the R_f value of the herbal extract and heavy metals proved the presence of lead cadmium and mercury (Figure 1). The specifications of the process are as follows:



A- Lead, B – Mercury, C- Cadmium, H – Herbal Extract

FIGURE 1. TLC profile of the leaf extract and the heavy metals

Stationary Phase	Silica Gel 60 F_{254}
Mobile Phase:	Benzene
Duration of Run:	40 min for 10 cm
R_f value of sample A	0.316
R_f value of sample B	0.769
R_f value of sample C	0.143
Temperature	25^0C

U.V. spectrophotometric analysis

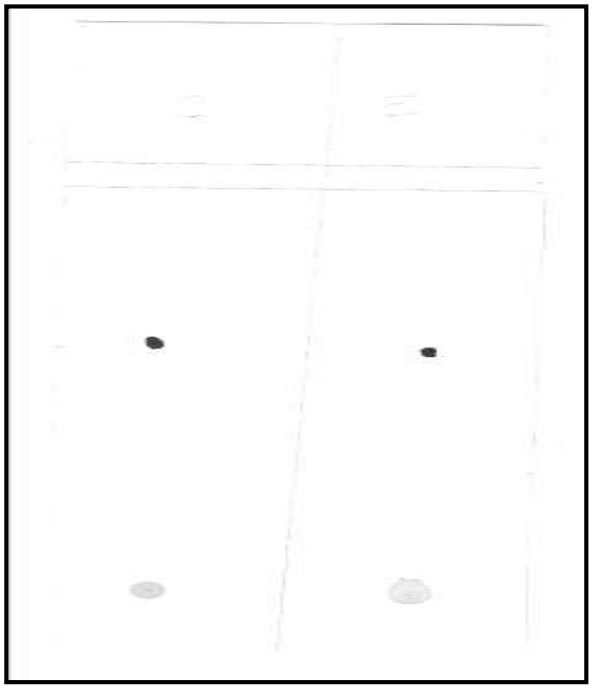
The spectrophotometric analysis (standard curve method) was used for quantification of the herbal extract. Standard curves were plotted for lead, cadmium, mercury, and arsenic. Specific wavelengths were used for each heavy metal (like 490 nm and 510 nm were the official wavelengths for the determination of cadmium and lead respectively). The absorbance hence obtained was compared with the standard curve by using SHIMADZU UV-VIS PharmSpec 1700 U.V. spectrophotometer.

Removal of the heavy metals

Nimbidin (an active constituent of Neem extract) was chosen as the marker compound. From the analytical studies, it transpires that the heavy metal impurities, namely, cadmium and lead, needs priority action for their removal. So, the work plan was designed to facilitate the elimination of these two hazardous elements in improving the quality of the Neem based products. The reagents used for this purpose was dithizone (0.005%) in chloroform. Trial &

FIGURE 2. TLC profile of Neem extract both before and after dithizone treatment.

(A =before dithizone treatment, B= after dithizone treatment.)



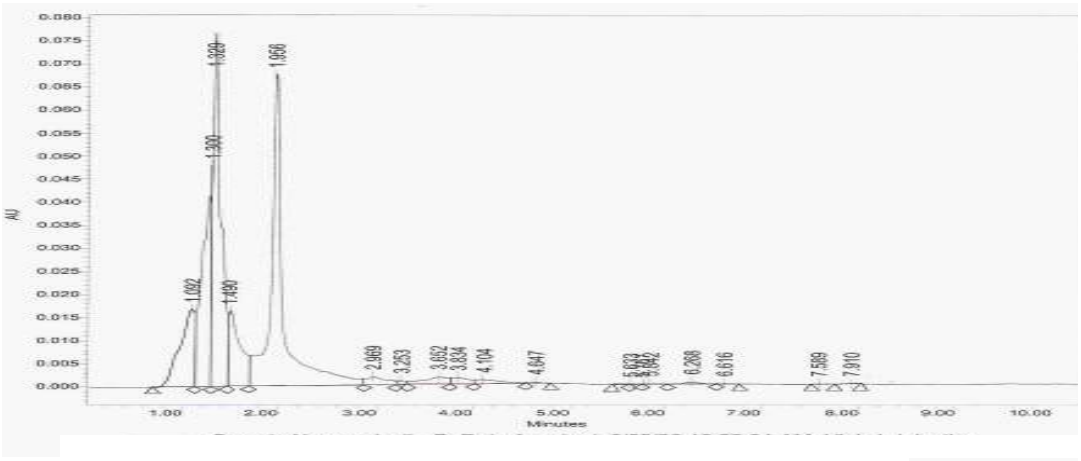


FIGURE 3. HPLC chromatogram of Neem extract before Dithizone treatment.

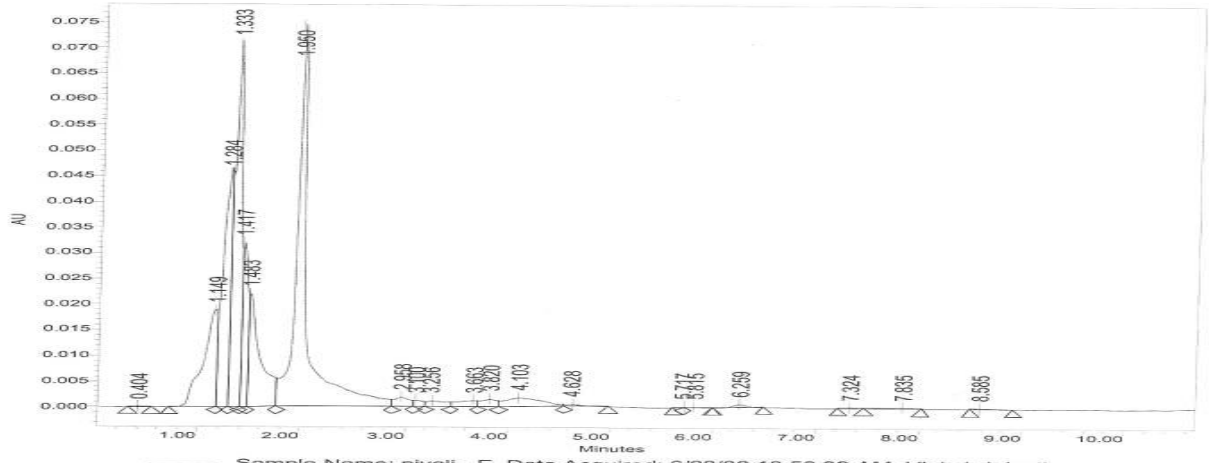


FIGURE 4. HPLC chromatogram of Neem extract after Dithizone treatment

error method was resorted for the purpose of volumetric quantification of the reactants as also the respective pH values (Table.2). Dithizone was added quantitatively to avoid the occurrence of any untreated reagent.[8-10]

Marker Detection and it's Interference study with Dithizone.

Thin layer chromatography

The TLC analysis followed the following specifications (Figure 2)[9-11]

Extract loaded	2 ml
Mobile phase	chloroform:menthol (97 : 3)
Stationary phase	silica gel 60 f_{254}
Extraction media	methanol
R value of sample – a	0.527
R^f value of sample – b	0.520
Temperature	25^0C

High Performance Liquid Chromatography (HPLC)

Based on reversed-phase high performance liquid chromatography and dual ë-absorbance detector a HPLC method was developed to permit the rapid qualitative and quantitative analysis of Nimbidin and related terpinoids from neem (*Azadirachta indica)* (Figure 3 and 4, Table 3). The instrument used was SHIMADZU LC-20AT and detector SPD-20A . The specifications of the process can be stated as follows[10-11]

Mobile phase	20 % Acetonitrile
Mode	Isocratic
Flow rate	1ml/min
UV ë value	220 nm

RESULTS AND DISCUSSIONS

The TLC[12] performed (**FIG. 1**) for the identification of the heavy metals involved the principle of comparing the R_f values of the standard metal solutions and the herbal

TABLE- 1 Pharmacological activities of some bioactive components of Neem[2,3, 4,5]

Neem compound	Biological activity
Nimbidin	Anti-inflammatory Antiarthiritic Antipyretic Hypoglycemic activity Antigastric ulcer Spermicidal Antifungal Antibacterial Diuretic
Sodium nimbate	Anti-inflammatory
Nimbin	Spermicidal
Nimbolide	Antibacterial Antimalarial
Gedunin	Antifungal Antimalarial
Mahmoodin	Antibacterial
Gallic acid, epicatachin and catechin	Anti-inflamatory and immunomodulatory
Margolone, margolonone and isomargolonone	Antibacterial
cyclictrisulphide and cyclic tetrasulphide	Antifungal

TABLE- 2 Required pH for removal of heavy metals from the extract.

Amount of herbal extract used.	Amount Of reactants [Dithizone (0.005%)] in Chloroform	Impurities	pH	Inference
10 ml	17.5ml	Lead	6-7	Complexation reaction occurs in neutral medium
		Cadmium	10-12	Complexation reaction occurs in alkaline medium
		Mercury	3.5-4	Complexation reaction occurs in acidic medium.

extract. Quantification of the estimation was done using U.V.Spectrophotometric analysis[6] which establishes the presence of Lead and Cadmium[4, 11] well beyond the WHO limit.

The heavy metals traced were as follows

Lead (16.53 ppm; WHO limit 10 ppm)
Cadmium (2.43 ppm, WHO limit 0.3)
Mercury (< .10 ppm, WHO limit 1)
Arsenic (< 0.50 ppm, WHO limit 10)

After the chelation of the heavy metals, further confirmation studies were performed to establish that the marker compound remained unaffected with the chelating agent.

TLC[12] and HPLC[13] were the methods (Table 2) used for the quantitative and qualitative confirmation for the non interference of dithizone with the marker compound. From the TLC (Figure 2) it can be inferred that since the R_f value of the marker compound did not show considerable deviation(R_f value before dithizone treatment 0.527 and after dithizone treatment 0.520), Nimbidin can be said to remain unaffected during the chelation of the heavy metal impurities.

HPLC chromatogram (Figure 3 and 4, Table 3) showed there was no significant change in the mean area (A) and the retention time (RT) of the chromatogram both before (A= 97528.663 and RT= 4.181), and after (A= 88727.576 and RT = 3.804) the dithizone treatment. From the two HPLC chromatograms it is reconfirmed that the marker compound, Nimbidin, remained unaffected by chelation, although the toxic heavy metals were removed from the Neem extract.

TABLE- 3 HPLC chromatogram analysis before after Dithiazone treatment.

S. no.	R_t before Dithiazone treatment (in minutes)	R_t after dithiazone treatment (in minutes)	Mean area before dithiazone treatment (Vsec)	Mean area after dithiazone treatment (Vsec)
1.	1.092	0.404	183609	280
2.	1.3	1.149	307664	173192
3.	1.329	1.284	406185	290105
4.	1.490	1.333	145715	304452
5.	1.956	1.417	597837	110306
6.	2.696	1.483	25656	182367
7.	3.253	1.950	6976	580716
8.	3.652	2.958	31062	18400
9.	3.834	3.100	18522	7897
10.	4.104	3.256	19318	15185
11.	4.637	3.663	2684	17313
12.	5.633	3.820	298	15155
13.	5.767	4.103	604	44517
14.	5.842	4.628	1382	5521
15.	6.268	5.717	6632	107
16.	6.616	5.815	758	861
17.	7.89	6.259	138	5738
18.	7.910	7.324	498	269
Mean	4.181	3.804	97528.663	88727.576
Standard deviation	2.227	2.440	172019.016	151355.463

Easy and convenient methods like the present study[14] can be utilized by the pharmaceutical industry to meet stringent laws regarding quality assurance of herbal drugs.

ACKNOWLEDGEMENTS

We are thankful to the officials of University of Kolkata for providing us with the necessary library facility and instruments whenever required. We also would like to thank Himalayan Pharmacy Institute officials for their support in instrumentation and experimentation work for the completion of this project.

REFERNCES

1. Biswas K., Chattopadhyay I., Banerjee K.R., and Bandhopadhyay U. Biological Activities and Medicinal Properties of Neem (*Azadirachta indica*).Current Science. 2002 ;82(11):1336-1345.
2. Bhargava K.P.; Gupta M.B.; Gupta G.P.; and Mitra C.R. Antiinflamatory activity of saponins.Indian J. Med. Res. 1970; 58:724-730.
3. Tidjani M.A.; Dupont C. and Wepierre . Perspectives on ethno-phytotherapy of "Yoruba" medicinal herbs and preparations. J. Planta Med.Phytother. 1989; 23:259-266.
4. Ray A., Banerjee B.D. and Sen P. Modulation of humoral and cell-mediated immune responses by Azadirachta.1996; 34:698-701.
5. Murty K.S., Rao D.N., Rao D.K. and Murty L.B.G. Effect of *Azadirachta indica* leef extract on serum lipid profile. Indian J. Pharmacol. 1978; 10:247-250.
6. Garg G.P., Nigam S.K. and Ogle C.W. Neem: the divine tree : *Azadirachta indica.* Planta Medica.1993; 59: 215-217.
7. Despande V.Y., Mendulkar K.N., and Sadre N.L. Male antifertility activity of *Azadirachta indica* in mice. J. Postgraduate Med.1993; 59:215-217.
8. Badani L., Deolankar R.P., Kulkarni M.M., Nagsampgi B.A., and Waugh U.V.Biological activities and medicinal properties of neem. Indian J. Malariol. 1987; 24:111-117.
9. Chopra I.C., Gupta K.C., and Nair B.N. Biological activities and medicinal activities of neem.Indian J. Med. Res. 1952; 40:511-515.
10. Badam. L., Joshi S.P., Bedekar, S.S. Antibacterial finishing of polyester/cotton blend fabrics using neem (*Azadirachta indica*): A natural bioactive agent. J. Commn. Diseases. 1999; 31:79-90.
11. Balasenthil S., Arivazhagan S., Ramchandran C.R., and

Nagini S. Protective effects of ethanolic Neem leaf extract. J. Pharmacol. 1999; 67:189-195.

12. Bhanwara, S., Singh J., and Khosla P. Antinociceptive activity of Azadirachta Indica (neem) in rats. Indian J. of Physiol. Pharmacol. 2000; 18:17-21.
13. Rao A.D., Devi K.N., and Thyagaraju K.J. Ayurtox for body detoxification. Enzyme Inhib. 1998; 14:85-86.
14. Singh S.D., Junnarkar A.Y., Reddi G.S. and Singh K.V. Effect of *azadirachta indica* (neem) on the growth pattern of dermatophytes. Fitoterapia. 1987, 58, 235-238.
15. S.Ponnusankar, K.Santhi Dhanraj, Nancy Jacob, and B.Jacob; Safety Measures with Herbals; Indian J.Pharm.Educ; 2005,37 (2); 84-87.
16. Government of India, Ministry of Health and Family Welfare, Indian Pharmacopoea, 1996, The Controller of Publications, Civil Lines, Delhi-110054, A-62 – A-64.
17. http://ewes-clib.ice.mpg.de/fulltext.
18. www.gwydir.demon.co.uk/jo/minerals/metals.htm
19. www.webexibits.org/pigments/indivi/i/32mineral/greenockite.jpg.
20. www.lef.org/LEFCMS/aspx
21. Farnsworth N.R., Aberele O, Bingel As, et.al; Medicinal Plants in Therapy, Bull. WHO 63 (b), 1985, 965-981.
22. Arele O, Natures Medicinal bounty: don't throw it away, World Health Forum, 14(4), 1993, 390-395.
23. Drew A.K., and Meyers S.P., Safety Measures in Herbal Medicine: implications for health professions, MJA, 166, 19(5), 1987, 538-547.
24. Jeffery G.J., Basset J., Mendham J. and Denney R.C.; Vogel's Textbook of Quantitive Chemical Analysis; John Willey and Sons Inc, New York; 2001 5th edition; pp216-235, 309-327,and 708-726
25. Kircher J.G.; Thin Layer Chromatography; John Willey, New York; 1978, 2nd edition
26. Mukherjee.Pulok K., Evaluation of Indian Traditional medicine, DIJ, 35(2), 2001, 623-632
27. Tyler V.E.; Herbal Medicines in America, Plant med., 53 (1), 1987, 1-4.
28. Flora S.J., Flora J., Saxena G. and Mishra M; Arsenic and lead induced free radical generation and their reversibility following chelation; (Noisy-le-grand); 2007 Apr 15; 53(1):26-47. www.pubmed.com
29. Aposhian HV, Maiorino RM, Gonzalez-Ramirez D, Zuniga-Charles M, Xu Z, Hurlbut KM, Junco-Munoz P, Dart RC, Aposhian MM.; Mobilization of heavy metals by newer, therapeutically useful chelating agents; 1995 Mar 31;97(1-3):23-38. www.pubmed.com.
30. Danscher G, Finn-Mogens, and Haug S Depletion of metal in the rat hippocampal mossy fibre system by intravital chelation with dithizone; 1971; 28(3):211-9; www.pubmed.com.