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Research Article

Pharmacognostical standardization of leaves of *Azadirachta indica* – An effective panacea for all disease

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ABSTRACT

Azadirachta indica A. Juss. (Meliaceae) commonly called Neem in India and is nature's drugstore due to thousands of medicinal properties associated with it. It is one of two species in the genus *Azadirachta*, and is native to India and Burma, growing in tropical and semi-tropical regions. Present investigation highlights the standardization of *Azadirachta indica* leaf from Garhwal Himalayas. The various parameters applied (WHO Guideline) were ash value, acid insoluble ash, water & alcohol extractive values, loss on drying, pH, volatile matter, heavy metal content, phytochemical investigation, chromatographic profile, spectroscopic profile, heavey metal content and microscopic and macroscopic description. The quantitative determination of marker component was also done by HPTLC studyusing azadirachtin as standard.

Keywords: *Azadirachta indica;* Pharmacognostical Standardization; HPTLC; Marker Component.

INTRODUCTION

Azadirachta indica (AI) is a tree in the mahogany family Meliaceae and useful herb for internal and external disease management. AI is well known, in India and its neighboring countries for more than 2000 years, asone of the most versatile medicinal plants having a wide spectrum of biological activity. Every part of the tree has been used as traditional medicine for house- hold remedy against various human ailments, from antiquity (Chopra et al, 1958; Kritikar et al, 1975; and Chatterjee et al, 1994). More than 135 compounds have been isolated from different parts of AI and sev- eral reviews have also been published on the chemistry and structural diversity of these compounds. Individual AI tree may vary in chemical make-up because of ge- netic and environmental factors. The studies carried by various scientists in different passage of time have proved the natural variability in percentage content of the phytochemicals (Ermel et al, 1986; Rengasamy et al, 1993; and Kumar 1995).

In Indian herbal market in place of genuine crude drug, botanical drugs having similar appearance is being sold. *Saraca asoca* (Ashoka) is adulterated by the bark of *Polyalthea longifolia* instead of genuine bark. The *Cassia articulata* L. leaf adulterates Cassia *acutifolia* (Senna leaves), which do not have any medicinal value (Rawat, 2004). Such adulteration and substitutions leads to the poor quality of herbal products. To over- come this problem need for quality control of herbal medicines is realized by WHO and strict standardization parameters were putted forward in form of various national pharmacopoeia and monographs of medicinal plants. Standardization of raw drug includes passport data of raw plant drugs in respect of taxonomic identification, authentication, botanical description,and chemical composition (Pushpangadan, 2004). The development of these traditional systems of medicines with the perspectives of safety, efficacy and quality will help not only to preserve this traditional heritage but also to rationalize the use of natural products in the health care.

Keeping this view in mind the present investigation was aimed to screen leaves of AI, from Garhwal Hima- laya, for various parameters suggested by WHO for standardization (figure 1) and the experimental values were matched with already established reports (if any). Moreover, HPTLC standardization was done using azadirachtin as a marker component.

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MATERIAL AND METHODS

Reagents and Chemicals

All the reagents and chemicals used for testing were of analytical grade and purchased from Rankem Laboratories Ltd, New Delhi; Loba Chemie, Mumbai; and Merck Chemicals, Mumbai.

Plant Materials

The fresh matured leaves of AI were procured from Dr. Shushila Tiwari Herbal Garden, Muni-Ki-Reti, Rishikesh in the month of September. The species was authenticated from Botanical Survey of India, Northern Circle, Dehradun, India and a voucher specimen (113502) was deposited in BSI, Dehradun India. With appropriate storage condition the plant material was weighed and processed for standardization.

Botanical Description

Macroscopic description

The freshly procured leaves of AI were spread into a thin plastic sheet and investigated different organolep- tic features by repeated observations.

Microscopic determination

Microscopic studies were done by preparing a thin hand section of midrib and lamina region of AI leaf. Good sections were collected and placed on a grease free microscopic slide along with a drop of glycerin : water (1:1). The sections were covered with clean and dry cover slips and observed under compound micro- scope of 40x magnification. The observed microscopic patterns are sketched as figure 2 and 3.

Extractive values & preliminary phytochemical studies

Different extracts, of well dried and crushed 2.5kg leaves, of AI were prepared by hot percolation method using Soxhlet apparatus (Mukherjee 2002). The extraction was done with various solvents viz., petroleum ether, chloroform, acetone, methanol and water in increasing order of their polarity. Each time before extraction with the next solvent of higher polarity the powdered drug was dried in hot air oven below 50^0C for 10 minutes. Each extract was concentrated by distillation of the solvent which was recovered subsequently. The concentrated extract was evaporated to dryness till constant mass and extract obtained with each solvent was weighed and percentage yield recorded as extractive value. The individual extract was then subjected to qualitative detection of Phytoconstituents (Peach et al, 1995; Evans 1994).

Quantitative analysis to determine the physicochemical constant

The fresh and dried leaves of AI was subjected to physicochemical studies such as loss on drying (LOD), total ash value, acid insoluble ash, volatile oil, pH, foreign matter, water and alcohol soluble extractives were determined as per the Indian Pharmacopeia (Anonymous, 2002; 2003; 2003; 2005).

Ultra – Violet spectrophotometric study on the drug preparing the tincture

The ultra violet spectra of the drug are a plot that exhibit how much electromagnetic radiation is absorbed at each frequency. This study thus involves the measurement of absorption of light in ultra-violet region (200 – 800nm) and hence reveals the profile of phytoconstituents present (Willard et al, 1965). 1g of powdered drug was dissolved in 20ml deionized water and the resultant tincture studied under UV-Vis spectrophotometer (SYSTRONICS).

Chromatographic fingerprinting

TLC study

The fluorescence studies are based on the behavior of provided drug and its extract under fluorescence light. For this a TLC study was conducted with the aqueous extract of *AI.* One dimensional ascending method by using standard protocol as per Indian Pharmacopeiawas followed (Anonymous 1996). The TLC plate after development of chromatogram was placed inside the UV-chamber and viewed in daylight, short (254nm) and long (365nm) ultraviolet radiation.

HPTLC Study

HPTLC has been used for quantitative analysis of azadirachtin, as marker compound, in *AI* leaves extract. Chromatography was performed in aluminum plates coated with 0.2mm layer of silica gel 60 F245, using mobile phase ethyl acetate : benzene in a proportion of 7 : 3 (v/v) after the saturation of the chamber (30 ± 5^0C) 10 min prior to the experiment. The absorption maxima during densitometry scanning were obtained to be 366nm. The test and standard preparation was done with ethanol and 5 spots of standard 10, 12, 14, 16, 18µg/ml and a spot of test 18µg/ml was tested for the presence of azadirachtin in aqueous extract of AI leaves. The percentage of azadirachtin in the sample was found to be 2.27%, and the Rf value was observed to be 0.59. These results are indicative of the excellent reliability, reproducibility, accuracy and precision of the method. (Figure 4)

Determination of micro-organism

Medicinal plant material normally carries a great number of bacteria and mould, often originating in soil. Presence of micro-organism can be hazardous tohealth if absorbed even in very small amount. For the safe use of the plant drug, microbial screening was done for individual raw materials and checked whether total aerobic count, total yeast and mould count are within the prescribed WHO limits (Patwardan, Feb 2005).

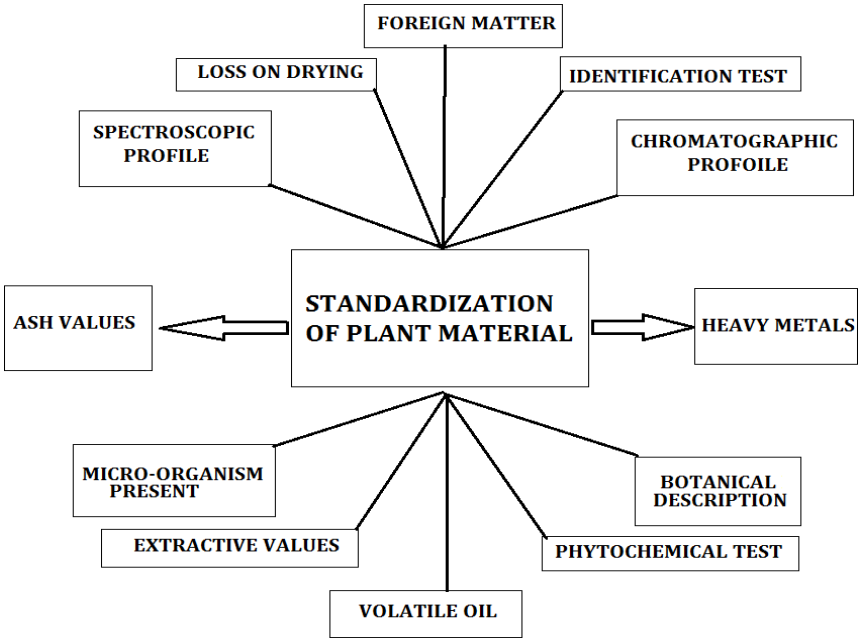


Figure 1: Standardization parameters suggested by WHO (12 Parameters)

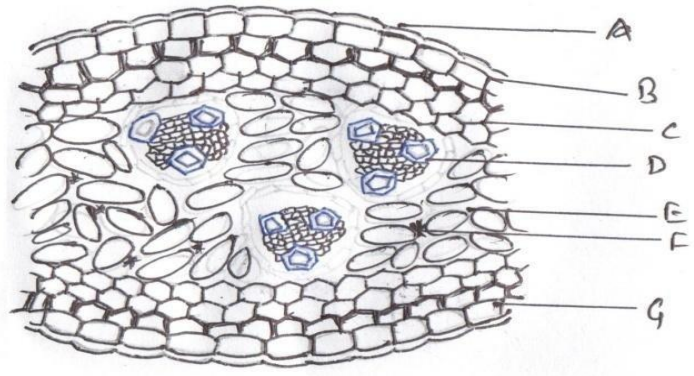


Figure 2: T.S. of Leaf through midrib A. Cuticle; B. Upper Edpidermis; C. Collenchyma; D. Vascular Bundle; E. Parenchyma; F. Ca-crystals

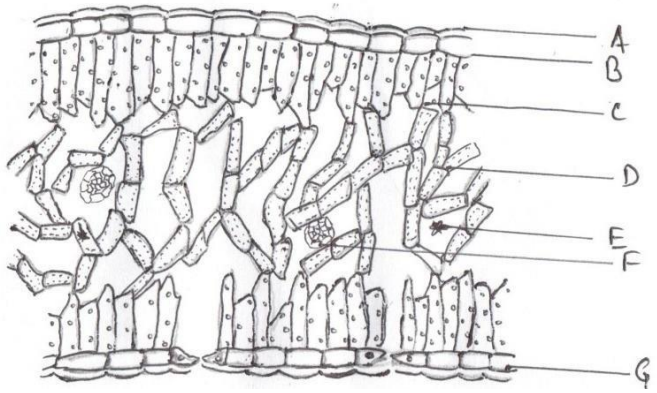


Figure 3: T.S. of leaf through lamina A. Cuticle; B. Upper Epidermis; C. Palisade; D. Spongy parenchyma; E. Ca-oxalate crystal; F. Vein; G. Lower Epidermis

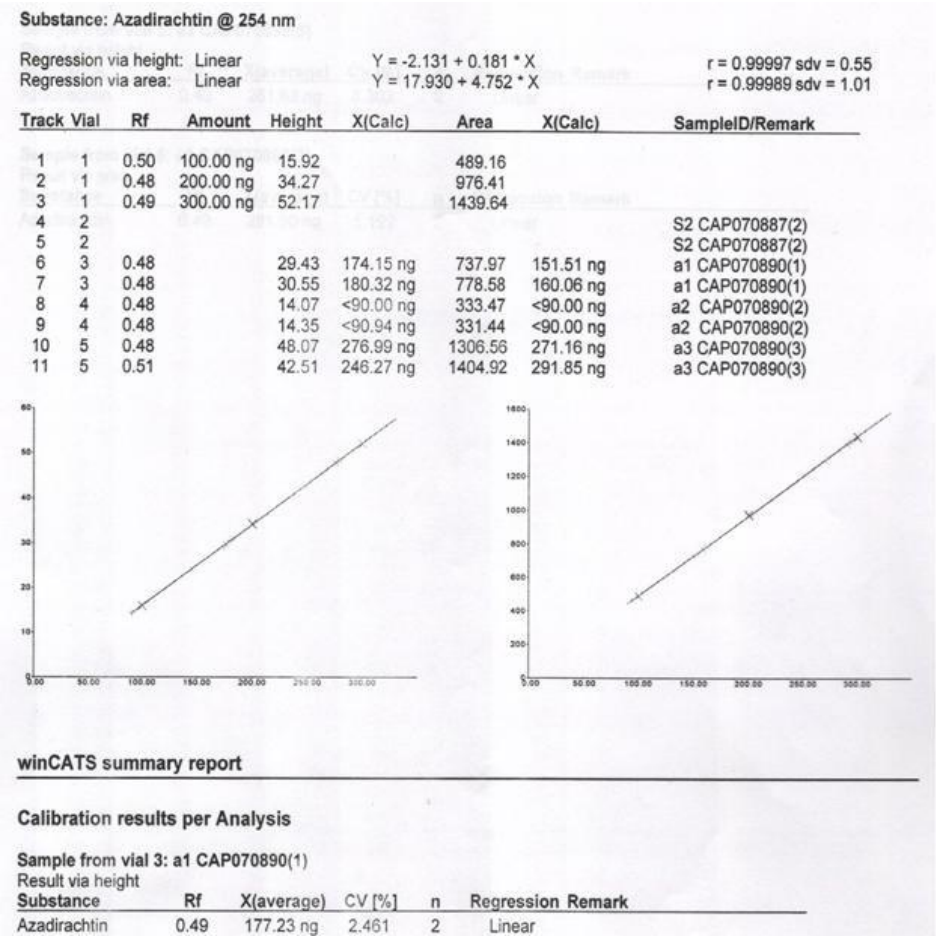
Determination of heavy metal content

The presence of heavy metal even in a trace amount found to be hazardous through a number of clinical studies. So before using any raw material in botanical medicine it must be screened for the heavy metal content. Determination of heavy metal was done by the procedure suggested by WHO (Anonymous 2005).

RESULT AND DISCUSSION

Macroscopic Description

The leaves of AI are compound, reticulate, exstipulate, serrate, lanceolate, 1.0 – 2.0cm wide and 15 – 25cm long. It is green to yellowish green in colour, the odour



Substance: Azadirachtin @ 254 nm

Regression via height: Linear Y = -2.131 + 0.181 * X r = 0.99997 sdv = 0.55
Regression via area: Linear Y = 17.930 + 4.752 * X r = 0.99989 sdv = 1.01

Track	Vial	Rf	Amount	Height	X(Calc)	Area	X(Calc)	SampleID/Remark
1	1	0.50	100.00 ng	15.92		489.16		
2	1	0.48	200.00 ng	34.27		976.41		
3	1	0.49	300.00 ng	52.17		1439.64		
4	2							S2 CAP070887(2)
5	2							S2 CAP070887(2)
6	3	0.48		29.43	174.15 ng	737.97	151.51 ng	a1 CAP070890(1)
7	3	0.48		30.55	180.32 ng	778.58	160.06 ng	a1 CAP070890(1)
8	4	0.48		14.07	<90.00 ng	333.47	<90.00 ng	a2 CAP070890(2)
9	4	0.48		14.35	<90.94 ng	331.44	<90.00 ng	a2 CAP070890(2)
10	5	0.48		48.07	276.99 ng	1306.56	271.16 ng	a3 CAP070890(3)
11	5	0.51		42.51	246.27 ng	1404.92	291.85 ng	a3 CAP070890(3)

winCATS summary report

Calibration results per Analysis

Sample from vial 3: a1 CAP070890(1)
Result via height

Substance	Rf	X(average)	CV [%]	n	Regression	Remark
Azadirachtin	0.49	177.23 ng	2.461	2	Linear	

Figure 4: Calibration curve and Quantitative data of HPLC

is characteristic and taste is bitter. Arrangement of leaves on main branch is alternate and opposite.

Microscopic Determination

When transverse section was cut through midrib showed a biconvex outline, and epidermis at both surface (upper and lower) which was covered with thick cuticle. Below epidermis 4 to 5 layers of collenchyma were present. The stele was composed of a group of vascular bundle, a single vascular bundle toward lower surface while 2 to 3 toward upper surface. Rest of the cells around stele was parenchymatous thin walled cells having secretary cells and rosette calcium oxalate crystals. Phloem cells were non-lignified and crystals were present in the phloem region.

When transverse section was cut through lamina a dorsiventral structure was exhibited having epidermis on both sides. It also exhibited elongated thin wall cells, covered with thick cuticle layer. Stoma was present at lower surface only of the epidermis. Single layered palisade cells present above the 5 to 6 layer thin walled spongy parenchymatous cells transverse by several numbers of veins. Although a few cells of spon- gy parenchymatous contains calcium oxalates.

Extractive values & preliminary phytochemical studies

The result of extractive value is present in table 1 which reveals that highest extraction is obtained with water. The result of phytochemical study indicates the presence of different chemical compounds such as fixed oil, saponins, steroids, saponin, tannin, alkaloids, carbohyderates, phenolic compound, tannin and glycosides (table1). These phytochemicals could be responsible for treating different ailment.

Quantitative analysis to determine the physicochemical constant

The present finding of quantitative analysis to determine the physicochemical constant like loss on drying (5%), total ash value (7%), acid insoluble ash (0.9%), alcohol soluble extractive (6%), water soluble extractive (12%) and foreign matter (1.2%). These values are in limit as compared with the early report[16]. There was 0.32% of volatile oil and the pH of the water soluble extractive was observed to be 7.5. There is no previous report to confirming these values (table2).

Ultra – violet spectrophotometric study on the drug preparing the tincture

A very dilute solution of *AI* leaves in water (about 10mg/10ml) shows absorption maxima at 200, 261 and 312nm respectively. There is no earlier report of ultraviolet spectrophotometric studies on the subjected drugs.

Table 1: Percentage yield and Phytoconstituents of different extracts of *Azadirachta indica* leaves

Tests	Pet. Ether Extract	Chloroform Extract	Acetone Extract	Methanol Extract	Aqueous Extract
Yield (%)	15.5	12.8	14.5	18.5	23.1
Steroids	++	++	–	–	–
Phenolics	–	–	–	++	++
Fixed oil	++	–	–	–	–
Alkaloids	–	–	++	++	++
Glycosides	–	–	–	++	++
Saponins	–	++	–	–	–
Tannins	–	++	–	++	++
Carbohydrates	–	–	++	++	++

++ present; – absent

Table 2: Result of Qualitative analysis of Physicochemical Constant

Test	Experimental Yield	Earlier Reports	Reference	Protocol
Physicochemical Analysis				
Loss on drying (moisture content)	5%	NE	1	Quality Control Methods for Medicinal Plant Materials – WHO[2]
Foreign matters	1.2	NMT 2.0%		
Ash content	7%	NMT 10%		
Acid insoluble ash	0.9%	NMT 1.0%		
Alcohol soluble extractive	6%	NLT 13.0%		
Water soluble extractive	12%	NLT19.0%		
pH	7.51	NE		
Volatile oils	0.32%	NE		
Heavy Metal Analysis				
Lead (Pb)	0.5 ppm	10ppm	1	-do-
Cadmium (Cd)	0.2 ppm	0.3ppm		
Arsenic (As)	0.9 ppm	5ppm		
Microbiological Analysis				
Total viable aerobic count	Absent	$< 10^4$cfu g-1	1	-do-
Total Fungal count	Absent	$< 10^2$ cfu g-1		
Standardization By Marker Compound				
Quantity of Azadirachtin	2.27%	NE		HPTLC

CHROMATOGRAPHIC FINGERPRINTING

TLC study

TLC study was conducted with the aqueous extract of *AI*. It shows under UV light (366nm) one fluorescent zone at Rf 0.34. On exposure to iodine vapour four spots appear at Rf 0.34, 0.58, 0.74 and 0.98 (all yellow).On spraying with vanillin sulphuric acid reagent and heating the plate for ten minute, three spot appears at Rf value 0.13, 0.33, and 0.56 and on spraying with nin- hydrine only one spot at Rf 0.89(violet) appears. There are no earlier reports available on this study of aqueous leaf extract of *AI*.

HPTLC study

The percentage of azadirachtin, determined through HPTLC study, in the aqueous extract was found to be 2.27%.These results are indicative of the excellent reliability, reproducibility, accuracy and precision of the method. There are no earlier reports available on this study of aqueous extract of *AI* leaves extract.

Determination of micro-organism

Result of quantitative determination of microbial contamination in raw material is summarized in table 2.

Determination of heavy metal content

Trace amounts of heavy metal As, Cd, and Pb were determined by standard procedure suggested by WHO and values were found to be in limit.[17] Result is shown in table 2.

CONCLUSION

AI is used in management of various chronic disease and very popular medicinal plant of most of the herbal medicinal systems. The structure its leaf is very common and can be adulterated very easily with the leaf of many other medicinal plants. The standardization is

thus a very crucial part of establishing its correct identity. The present study could therefore serve as important data for proper identification, collection and investigation of the AI leaves. Moreover few parameters evaluated in this study do not have any previous reports hence these may be further crossed evaluate for the inclusion in Herbal Pharmacopeia and standards must be established.

However, the variation on reported values and estimated values in the present study may be expected due to the various ecological factors. The habitat of earlier studies and present study are different. The results of present study and early reports are shown in table 1 & 2.

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