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Development of bioanalytical parameters for standardization of *Azadiracta indica*

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ABSTRACT

Objective: To develop noval bioanalytical parameters for standardization of *Azadiracta indica (A. indica)* extract. **Methods:** In the present investigation, preliminary phytochemical analysis, total phenol, flavonoid, solubility test, heavy metal analysis, antimicrobial study and quantitative analysis through HPTLC method were performed. **Results:** Preliminary phytochemical analysis showed the presence of carbohydrate, tannin, steroid, triterpenoid, glycoside, saponin and flavonoid in the extract. Loss on drying, total ash and solubility in water was found to be 4.60%, 5.61% and 91.0%. Total flavonoid and phenol content was found to be 1.8% and 14.08% w/w. The content of quercetin, rutin and gallic acid in *A. indica* was found to be 0.93%, 1.22% and 0.33% w/w respectively. Further the level of heavy metal content and microrganism were found to be in the safe level. **Conclusions:** In future, these bioanalytical parameters could be used as important tool for the standardization of *A. indica* extract.

1. Introduction

Azadirachta indica (A. indica) belonging family Meliaceae has been well known in the traditional system of medicine for more than 2000 years as one of the most versatile medicinal plants having a wide range of biological activity [1]. The neem tree (*A. indica*) is native to the Indian subcontinent and grows in many countries of the world [2]. According to the literature, the plant *A. indica* has about 35 biologically active compounds, such as limonoids, azadirone, azadirachtin, nonisoprenoids, aminoacids, polysaccharides, polyphenolics, and flavonoids. [3, 4]. The seeds contain bitter principles such as azadirachtin, nimbin, nimbidin, salannin, salannol, vilasinin and flaxinellone [5]. Some useful products such as antimalarials, spermicidals, antituberculosis agents, antipyrrhetics, antiviral drugs, antiseborrhoeics, antiallergic medicines, antienzymic, and antifungal agents, have been extracted from the *A. indica* [6]. *A. indica* have antiseptic, antifungal, antibacterial, antipyretic, anti-malarial, anti-diabetic and anti-fertility properties [5]. The extract from bark, leaves, fruits, root and oil have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children [7]. The aqueous extract of bark is reported to have beneficial effect on gastric hypersecretion and gastroduodenal ulcer. A dental gel formulation containing neem extract has been reported to reduce oral infections, plaque index, and bacterial count [8].

The neem is a bitter tonic herb that reduces inflammation and clears toxins, while promoting healing and improving all body functions. It has parasitic, insecticidal spermicidal properties [9]. Uses of plant material to treat sexual disorder is a long back history in the different system of medicine and it was practiced by different type of vaidyas and traditional healer in almost all the countries in the world, like China, India, Egypt, Rome and Greek [10]. The

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Juice of fresh green leaves of *A. indica* was believed to suppress "Kam vasna" (desire for sex) so peoples such as saints and "sanyasees" consumed it for the same purpose. During last three decades, neem has attracted the scientific community of both national and international scenario [11]. The injection of neem oil into the vas deferens has been successfully tested as an alternative to surgical vasectomy. Various forms of neem have been studied as potential reversible male contraceptives. Male mice fed water crushed with fresh neem leaves impregnated fewer female mice and had smaller average litter sizes. Contraceptive tablets made from neem extract are currently being used in India [12]. From the above mentioned information, it was concluded that *A. indica* have various pharmacological activities and used in the various marketed formulation, so it is necessary to standardized the *A. indica* plant extract before consumption. In the present investigation we have tried to develop some phytochemical parameters which could be useful for the standardization of *A. indica* extract.

2. Materials and methods

Crude plant extract of *A. indica* was procured from Garlico Herbal Concentrate (M.P.), India. A phytochemical screening was conducted on the *A. indica* extract using standard qualitative methods to confirm the presence of different phytoconstituents [13, 14]. The presence of phytoconstituents in the extract was also analysed through TLC analysis [15]. Study of parameters such as solubility in water, loss on drying, heavy metal and microbiological analysis were also performed as per method of IP, 1996 and WHO guidelines [16, 17]. Total phenol and flavonoid contents were also determined according to the standard methods [18, 19]. The quantification of rutin, quercetin and gallic acid in *A. indica* were determined through HPTLC techniques. The analytical parameters for the estimation of rutin, quercetin and gallic acid in the extract are as follows:

Analysis: Estimation of rutin, quercetin and gallic acid in *A. indica* extract.

HPTLC Plate: HPTLC Precoated plates Silica Gel Merck 60F254.

HPTLC Syringe: 100 μL Hamilton (Bonadzu, Switzerland)

Sample application: CAMAG Automatic TLC Sampler III

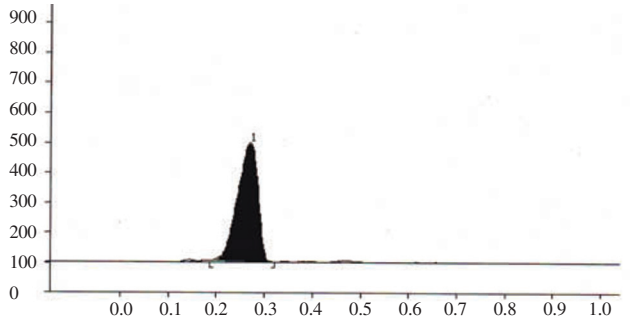
Development mode: Ascending

Scanning mode: CAMAG TLC scanner 3 with Cats software

Analytical conditions: Temperature (25±2) ℃, relative humidity 40%

3. Results

Preliminary phytochemical analysis showed the presence of carbohydrate, tannin, steroid, triterpenoid, glycoside, saponin and flavonoid in the extract. Moreover the test of the crude extract was found to be characteristic bitter. TLC analysis showed three prominent spots [R_f (0.41, 0.73, 0.89)] in ethyl acetate: methanol: (50:50) solvent system. However in the fingerprint analysis in ethyl acetate: formic acid: glacial acetic acid: H_2O (100:11:11:26) solvent system through HPTLC method showed five prominent spots with respective R_f (0.36, 0.47, 0.70, 0.83 and 0.98) and maximum peak are (19.99%, 7.07%, 17.79%, 9.10% and 46.04%). Loss on drying, total ash and solubily in water were found to be 4.60%, 5.61% and 91.0%. Total flavonoid and phenol content was found to be 1.8% and 14.08%. Further level of different heavy metal such as lead, arsenic, mercury and cadmium were found to be under the limit. Microbiological assay was also performed in the current task and result showed that *Escherichia coli* and salmonella was found to be absent whereas total bacterial count and yeast & moulds contents were found to be 550 and 46 CFU/GM. For quantitative analysis through HPTLC techniques, optimization of solvent system was done. Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) was found to be suitable solvent system for quercetin and rutin quantification whereas toluene: ethyl acetate: formic acid (7:5:1) was found to be most suitable solvent system for gallic acid quantification. The content of quercetin, rutin and gallic acid in *A. indica* was found to be 0.93% w/w, 1.22% w/w and 0.33% w/w respectively. The respective HPTLC chromatogram of rutin, quercetin, gallic acid and *A. indica* extract are presented in the Figure 1-3 and Figure 4-5.

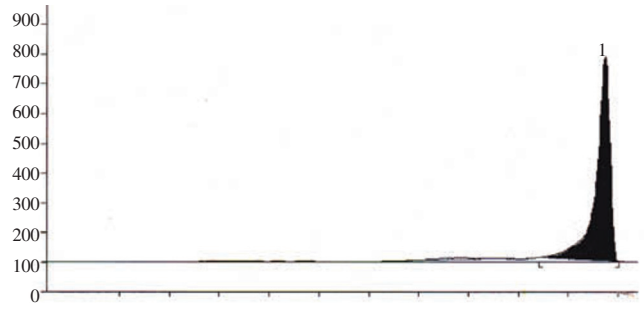


Wavelength:365nm [R_f]
Track:2,noise level: 0.473AU,raw data file:DPQ
U4.06 S/N:0612A004 CAMAG SOFTWARE (c) 1998 SCANNER 3:061121
Track 2,Analysis b: rut4mcl

Peak	start		max			end		area	
#	R_f	H	R_f	H	[%]	R_f	H	F	[%]
1	0.17	0.0	0.27	395.6	100.00	0.32	0.0	11308.0	100.00

Total height =395.6 total area =11308.0

Figure 1. HPTLC chromatogram of standard rutin.

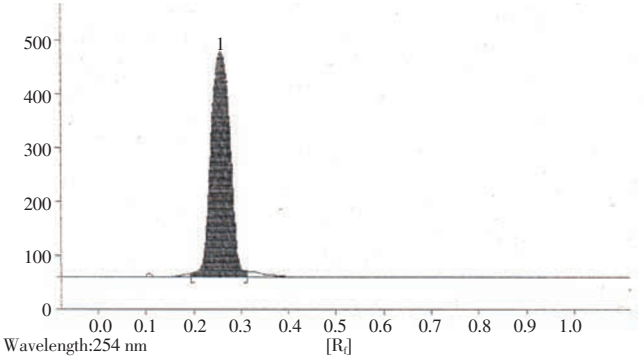


Wavelength:365nm [R_f]
Track:1,noise level: 0.473AU,raw data file:DPQ
U4.06 S/N:0612A004 CAMAG SOFTWARE (c) 1998 SCANNER 3: 061121
Track 1,Analysis a: qut4mcl

Peak	start		max			end		area		
#	R_f	H	R_f	H	[%]	R_f	H	F	[%]	
1	0.90	0.0	0.97	682.7	100.00	1.00	0.0	13018.4	100.00	

Total height =682.7 total area =13018.4

Figure 2. HPTLC chromatogram of standard quercetin.

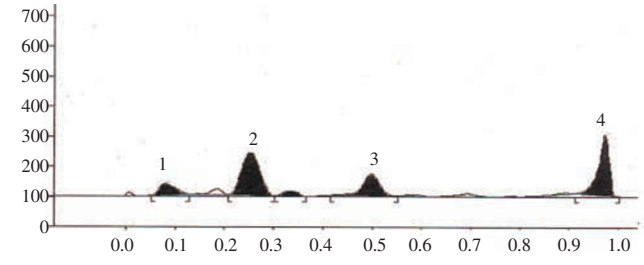


Wavelength:254 nm [R_f]
Track:1,noise level: 0.351AU,raw data file:DINGA
U4.06 S/N:0612A004 CAMAG SOFTWARE (c) 1998 SCANNER 3: 061121
Track 1,Standard level 1

Peak	start		max			end		area	
#	R_f	H	R_f	H	[%]	R_f	H	F	[%]
1	0.21	6.0	0.26	417.3	100.00	0.31	10.1	14786.9	100.00

Total height =417.3 total area =14786.9

Figure 3. HPTLC chromatogram of standard gallic acid.

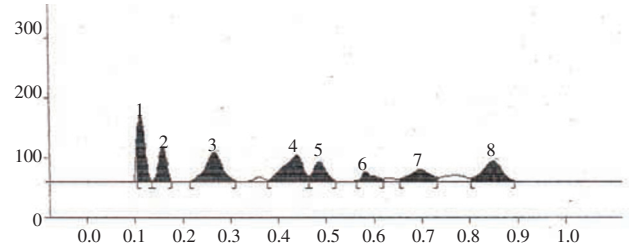


Wavelength:365nm [R_f]
Track:1,noise level: 0.473AU,raw data file:DPQ
U4.06 S/N:0612A004 CAMAG SOFTWARE (c) 1998 SCANNER 3: 061121
Track 1,Analysis a: qu4mcl

Peak	start		max			end		area	
#	R_f	H	R_f	H	[%]	R_f	H	F	[%]
1	0.05	0.0	0.08	37.2	7.85	0.13	0.0	812.9	8.70
2	0.18	0.0	0.21	142.2	29.99	0.30	0.0	3474.2	37.19
3	0.30	0.0	0.34	21.2	4.47	0.37	0.0	383.0	4.10
4	0.42	0.0	0.50	72.5	15.29	0.55	0.0	1661.9	17.79
5	0.91	0.0	0.97	201.0	42.40	1.00	0.0	3011.0	32.23

Total height =682.7 total area =13018.4

Figure 4. HPTLC chromatogram of *A. indica* extract.



Wavelength:254 nm [R_f]
Track:3,noise level: 0.351AU,raw data file:DINGA
U4.06 S/N:0612A004 CAMAG SOFTWARE (c) 1998 SCANNER 3: 061121
Track 3,Analysis b: AZAD 15 Mcl

Peak	start		max			end		area	
#	R_f	H	R_f	H	[%]	R_f	H	F	[%]
1	0.11	88.7	0.11	110.8	300.20	0.14	1.1	1319.7	16.22
2	0.14	1.1	0.16	59.6	16.25	0.18	0.4	842.0	10.35
3	0.22	0.4	0.27	48.5	13.20	0.31	1.2	1469.8	18.07
4	0.38	2.2	0.44	44.4	12.10	0.47	8.7	1531.9	18.83
5	0.47	8.7	0.49	33.0	8.99	0.52	0.6	731.0	8.99
6	0.56	0.8	0.58	16.9	4.60	0.62	4.0	355.4	4.37
7	0.65	4.1	0.69	19.9	5.41	0.73	6.4	729.3	8.97
8	0.80	5.2	0.85	33.9	9.24	0.90	1.2	1155.1	14.20

Total height =367.2 total area =8134.3

Figure 5. HPTLC chromatogram of *A. indica* extract.

4. Discussion

Physicochemical and phytochemical analysis was generally performed to ensure the identity, purity and quality of the drug. These parameters are also used to check the genuine nature of the crude drug, thus it plays an important role in preventing the possible steps of adulteration [20]. The presence of different phytoconstituents such as flavonoid, tannin, saponins, alkaloid and glycoside in the phytocohemical tests justifies their therapeutic potential [21-23]. Phytoconstituents obtained from natural sources have been gaining importance in the day by day due to the health promoting activity. So it is necessary to check the quality safety and efficacy of herbal drugs before its consumption [24, 25]. Phytochemical standardization plays an important role to ensure the quality safety and efficacy of the herbal drug. In the last few decades, an HPTLC technique has gained much popularity for standardization of the herbal drugs and formulations. Analysis of several samples simultaneously using a small quantity of marker compound and mobile phase with very less time is the major advantage of HPTLC technique [26]. HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a wide range of fields such as medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis[27]. Quality evaluation and standardization of the herbal preparation is a fundamental requirement of industry and other organization dealing with ayurvedic and herbal products. According to WHO guidelines, an herbal product needs to be standardized with respect to safety before releasing it into the market [28].

These bioanalytical parameters can be utilizes for the

simultaneous analysis of different phytoconstituents present in the *A. indica* plant material. In future, this information may be useful as a standard to identify and to differentiate from its adultrants and other related species.

Conflict of interest statement

We dedare that we have no conflict of interest.

Acknowledgments

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