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Original Research Article

In Vitro Phytochemical Analysis for Combating Urinary Tract Infection with *Andrographis Paniculata*

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ABSTRACT

Urinary tract infections (UTIs) are among the most common infections with an increasing resistance to antimicrobials. They are responsible for more than 8.1 million visits to physicians per year and about five percent of all visits to primary care physicians. Approximately 40 percent of women and 12 percent of men will experience at least one symptomatic urinary tract infection during their lifetime. The prescribed antibiotics are now show resistance against UTI causing bacteria and this is the major problem in the treatment of in- and out-patients. The consequences of antibiotic resistance include higher mortality and greater morbidity. The drug resistance can endanger the life of any patient in either an acute or chronic infection. The UTI samples were procured from the pathology labs. The pathogenic bacteria isolated from these samples included are *E.coli, E. faecalis* and *P. aeruginosa*. The antibiotic sensitivity pattern was studied for all clinical isolates by Bauer-Kirby method. All bacteria showed resistance to more than one antibiotic. The leaf extracts of *Andrographis paniculata* were checked for the antibacterial activity against all the clinical isolates. The plant showed great potential as antibacterial agent to fight against urinary tract infections.

Key words: *Andrographis paniculata;* multi drug resistance; urinary tract infection

INTRODUCTION

The use of plant or its part in treatment has been an ancient practice and is an important component of healthcare system in India. The use of herbs to treat disease is almost universal. The World Health Organisation (WHO) estimated that 80% population of Asian and African countries presently use herbal medicine for primary healthcare. Considerable research has been carried out on pharmacognosy, chemistry, pharmacology and clinical therapeutics of Ayurvedic medicinal plants.

In general, bacteria have the genetic ability to transmit and acquire resistance to drug [1]. The antibiotic resistance is the major problem in the treatment of in- and out- patients [2]. The

consequences of antibiotic resistance include higher mortality and greater morbidity. Considerable attention has been put on control of the use of antibiotics, understand the genetic mechanism of resistance [3, 4], the development and use of plant derived medicines as they are safe, effective and no resistance against them has been reported till date.

Various studies and research is underway to investigate the antimicrobial potency of medicinal plants [5]. Many reports have showed the effectiveness of traditional herbs against microorganisms [6]. A. paniculata has been used traditionally for digestive complaints, infections including leprosy, pneumonia, tuberculosis, gonorrhoea, syphilis, malaria, cholera, etc. It has also been studied for the treatment of upper respiratory tract infection [7], cardiac function [8].

MATERIALS AND METHODS

Plant collection and processing

Leaves of *Andrographis paniculata* were collected from Nagpur City (M.S., India). The plant was identified with a voucher number 9038 from Botany Department, RTM Nagpur University, Nagpur.

The leaves were washed with water and shade dried. The completely dried leaves were macerated in a mechanical grinder to yield a fine powder. This dried powder (30 g) was extracted in a Soxhlet apparatus using solvents of increasing polarity, i.e. 300ml each of Petroleum ether, Chloroform, Acetone, Methanol and Water. To compare the antibacterial potency, same quantity of powder (30 g) was cold extracted with 50% aqueous-methanolic solvent system. This extract was then evaporated at 40-50^{0}C on a hot plate to a final volume of 30ml.

Clinical isolates

100 urine samples were procured from different pathology labs in North-West region of Nagpur city. The collected urine samples were diluted in 1:100 ratio and streaked on UTI-agar plate (Himedia, FL031). The single isolated colony was used for further biochemical tests [3] to identify and characterise the bacteria. Twenty four strains of *Escherichia coli*, thirty two strains of *Enterococcus faecalis* and thirty three strains of *Pseudomonas aeruginosa* were identified. These were tested against standard antibiotics (Himedia, Mumbai) to get the antibiotic sensitivity pattern. Antibiotics which have been used for the current study are represented in Table 1.

Table 1. Name of antibiotics

S.No.	Name of antibiotics	Abbreviation	Concentration/disc	Catalogue number (Himedia)
1.	Ampicillin	A	5mcg	SD002
2.	Amoxycillin	Am	30mcg	SD076
3.	Chloramphenicol	C	30mcg	SD006
4.	Ciprofloxacin	Cf	5mcg	SD060
5.	Co-trimoxazole	Co	25mcg	SD010
6.	Cephalexin	Cp	30mcg	SD048
7.	Gatifloxacin	Gf	5mcg	SD737
8.	Kanamycin	K	30mcg	SD-017
9.	Norfloxacin	Nx	10mcg	SD057
10.	Ofloxacin	Of	5mcg	SD087
11.	Penicillin-G	P	10mcg	SD028

12.	Pefloxacin	Pf	5mcg	SD070
13.	Streptomycin	S	10mcg	SD031
14.	Sparfloxacin	Sc	5mcg	SD162
15.	Tetracycline	T	30mcg	SD037

Antibiotic Susceptibility Testing

All clinical isolates of *E.coli, E. Faecalis* and *P. aeruginosa* were tested for susceptibility to 15 different antibiotics using Bauer-Kirby method [9]. The single colony from the UTI agar plate was inoculated in 10ml of LB media and incubated at 37^0C for 14-16 hr McFarland's turbidity standard of 0.5 which in turn was equal to 1.5×10^8cells/ml. This culture was then used for antibiotic susceptibility testing. The plates were examined for zone of inhibition and recorded as sensitive, intermediate and resistant [10] from zone size interpretive chart (Himedia).

Preparation of plant extract and phytochemicals analysis

The extracts of the plant obtained by Soxhletion with 5 subsequent solvents and by cold extraction were analysed for qualitative estimation of phytochemicals [11]. By comparing the qualitative phytochemicals estimation, it was found that cold extraction gave better results than hot extraction and hence the extract obtained upon cold extraction was used for quantitative estimation of phytochemicals [12-15].

Quantitative estimation of phytochemicals

1) Determination of phenols

Total phenolic contents were determined by the Folin-Ciocalteau method [16]. 1000μg extract + 1mL Folin-Ciocalteu reagent + shake + wait for 3 min + 3mL 2% Na2CO3 + keep for 2 hr. Read absorbance at 760 nm. Gallic acid was used as standard (1-10µg). The total phenolic contents were expressed as mg of Gallic acid equivalents (GAE)/g of plant material.

2) Determination of alkaloids

5g sample + 200 ml 10% acetic acid in ethanol + cover it and incubate for 4hr + filter. Take this extract + keep on boiling water bath to remain one fourth original volume + drop wise ammonium hydroxide until complete precipitation + allow the solution to settle + collect precipitate + wash with dilute NH4OH + filter. Take residue, dry it and weigh [17].

3) Determination of flavonoids

A. 10g sample extracted with 100mL 80% aqueous methanol + filter through Whatmann filter paper no. 42 (125mm). Transfer filtrate into crucible + evaporate + weigh.

B. 0.5 ml each plant extracts + 1.5 ml of methanol + 0.1 ml of 10% aluminium chloride + 0.1 ml of 1 M potassium acetate + 2.8 ml of distilled water + keep at room temperature for 30 minutes. Read at 415 nm with UV/Visible spectrophotometer. Total flavanoids contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg/ ml in methanol [15].

4) Determination of saponins

20g sample + 100mL 20% aqueous ethanol + heat over hot water bath for 4hr (550C) + filter + take residue and again extract with 200mL 20% ethanol + keep in water bath (900C) to reduce up to 40mL + transfer the concentrate into a 250 ml separator funnel + 20 ml of diethyl ether + shake vigorously + recover the aqueous layer while discard ether layer + repeat the purification process + 60 ml of n-butanol + wash twice with 10 ml of 5% aqueous

sodium chloride + heat the remaining solution in a water bath. After evaporation, dry the samples to a constant weight [12-15, 18].

5) Determination of anthocyanidin

1mL of sample or catechin standard solutions (50-300 mg/L) + 2.5mL of 1% (w/v) vanillin in methanol + 2.5mL of 9.0 N HCl in methanol + incubate at 30°C for 20 min + take absorbance at 500 nm [19].

6) Determination of tannin:

50µL extract + make up to 7.5mL with water + 0.5mL Folin-Denis reagent + 1mL Na2CO3 + make up to 10mL with water. Read at 700nm [20].

Preparation of discs for antibacterial activity

The Whatmann filter 1 was cut into 6mm diameter circle. 20mg of plant the extract obtained by hot and cold extraction was put onto the disc and air dried. The completely dried discs were used for antibacterial assay by Bauer-Kirby method [9].

Antioxidant activity

The antioxidant activity of all extracts obtained by hot and cold extraction method was investigated by FRAP assay [17-20].

Thin layer chromatography (TLC)

Slurry of silica gel G (2.5g) was prepared in distilled water (5ml) and poured over a glass plate to form a thin layer. Calcium sulphate dihydrate (0.5g) was used as binder. These prepared plates were air dried and activated at 100-120^{o}C for 30min. The extracts were spotted (15µL) over an activated plate 1cm above from the bottom. The spotted plates were kept in a previously saturated developing chamber containing mobile phase and allowed to run 3/4th of the height of the prepared plate [21]. Mobile phase and spraying agents which have been used in TLC are represented in Table 2.

Table 2. Mobile phase and spraying agent for respective phytochemicals

Phytochemical	Mobile Phase	Spray reagent	Colour develops	Reference
Alkaloids	Toluene:Acetone:Ethanol:Ammonia 40 : 40 : 6 : 2	Dragendorffs reagent	Orange-brown	20
Anthraquinone	Hexane : Ethyl acetate 9 : 1	10% Alc. KOH	Pink	21
Tannins	Toluene : Acetone : Formic acid 60 : 60 : 10	0.1% $FeCl_3$	Green	21
Phenol	Chloroform : Methanol : Water 5 : 4 : 2	1% $FeCl_3$ in methanol	Dark blue	19

RESULTS AND DISCUSSIONS

Table no 3-10 and Fig. 1-5 summarizes the results of the study. In the current study, the phytochemicals from *Andrographis paniculata* were extracted with five successive solvents by Soxhlation (petroleum ether, chloroform, acetone, methanol and water) and cold extraction (50% methanol). The evaluation of results obtained upon qualitative phytochemical analysis of hot and cold extracts showed that most of the bioactive compounds might be heat labile and are best extracted with cold maceration. Hence, the extracts obtained by cold maceration were used for quantitative estimation of phytochemicals. *A. paniculata* was found to be a rich source of saponins

(116.8 %) and alkaloids (5.00%). The plant was observed to contain antioxidant properties as well. The extract obtained by cold maceration showed highest antioxidant activity (371.82 per ascorbic acid equivalent). The plant was tested for antibacterial activity on account of its reported traditional uses in the treatment of diseases caused by uropathogenic bacteria. The microorganisms utilized in this study were multidrug resistant *E.coli*, *E. faecalis and P. aeruginosa*. The results showed that *A. paniculata* has a bio-therapeutic antibacterial potential. It was also found that the extract obtained by cold maceration has more potent antibacterial activity compared to Soxhlet extracts.

Table 3. Yield of extracts by Soxhlet and cold extraction

	Solvent extract	Colour	Nature	Weight (g)	% yield
Soxhlet extraction	Petroleum ether	Green	Semisolid	2.014	6.71
	Chloroform	Dark green	Solid	1.763	5.87
	Acetone	Dark green	Semisolid	1.029	3.43
	Methanol	Dark green	Semisolid	1.932	6.44
	Aqueous	Chocolate brown	Solid	2.738	9.12
Cold extraction	50% methanol	Greenish brown	Liquid	30 ml	----

Table 4. Qualitative estimation of phytochemicals

Tests	**Hot Extraction**					**Cold Extraction**
Solvents	PE	C	A	M	Aq	50 % Methanol
			Sterols			
Salkowski's test	-	-	+	+++	+	++++
Liberman test	-	-	+	+++	+	++++
Liberman Burchard	-	-	+	+++	+	++++
			Alkaloids			
Dragendorf's test	-	-	-	+++	+++	++++
Mayer's reagent	-	-	-	+++	+	++++
Wagner's test	-	-	-	+++	+++	++++
Hager's test	-	-	-	++	+	++++
Tannic acid test	-	-	-	+++	++	++++
Scheibler's test	-	-	-	++	+	++++
			Saponins			
Foam test	++	-	-	+++	++++	++++
Flavinoids	-	-	-	+	+++	++++
			Cardiac Glycosides			

Keller-Killiani test	+	-	++	++	+	++++
Legal's test	+	-	++++	++	+	+++
Cyanogenetic glycosides						
Grignard's test	-	-	-	-	-	----
Anthroquinones						
Bortranger's test	-	-	-	-	-	-
Tannins						
Ferric Chloride test	-	-	-	++	+++	+++
Lead acetate test	-	-	-	+	++	+++
Potassium dichromate test	+			+	+++	+++
Gelatin solution test	-	-	-	+	++	+++
Bromine water test	-	-	-	++	+	+++
Phenols						
Ferric Chloride test	-	-	-	+	+++	++++
Nitric acid test	-	-	-	+	+++	++++
Phthalic acid test	-	-	-	+	+++	++++
Proteins						
Biuret test	-	-	-	++	++	++++
Xanthoproteic test	-	-	-	++	++	++++
Millon's test	-	-	-	++	++	++++
Amino acids						
Ninhydrin test	-	-	-	++	+++	++++
Carbohydrates						
Molisch test	-	-	-	++	++	++++
Barfoed's test	-	-	-	++	++	++++
Fehling's test	-	-	-	++	++	++++

PE= Petroleum ether; C=Chloroform; A=Acetone; M=Methanol; Aq=Water

Table 5. Quantitative estimation of phytochemicals

S.No.	Phytochemical	% Yield
1	Alkaloid	5.00
2	Flavanoids	0.54
3	Phenol	0.0157
4	Tannin	0.258
5	Anthocyanidin	0.169
6	Saponins	116.8

Table 6. Antioxidant activity per ascorbic acid equivalent

Soxhlet extraction					Cold extraction
Concentration (µg)					
PE	**C**	**A**	**M**	**Aq**	**50% methanol**
38.75	37.00	45.00	71.75	333.75	371.82

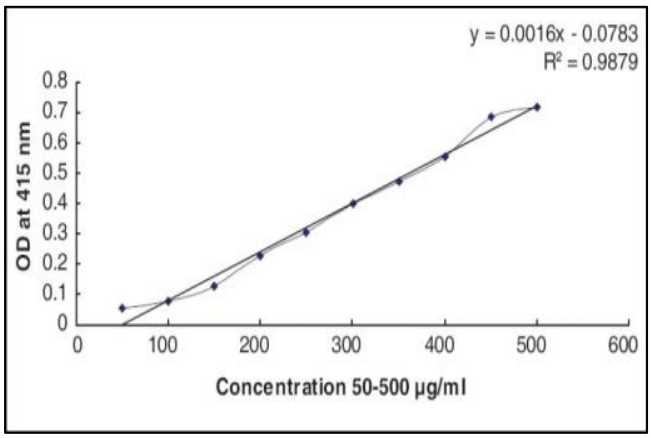


Fig.1 Standard quercetin curve for flavanoid estimation [17]

Flavanoid concentration as quercetin equivalent per gram of plant material

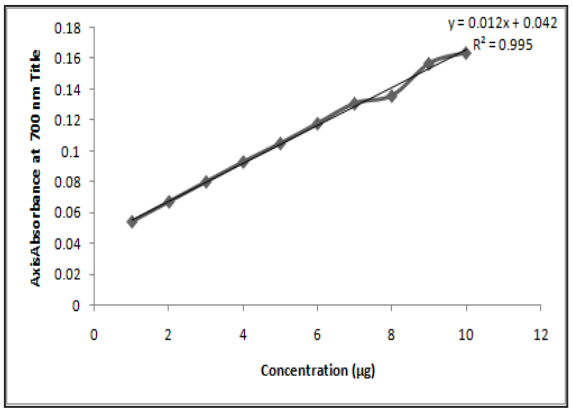


Fig.2. Standard Gallic acid curve for phenol estimation

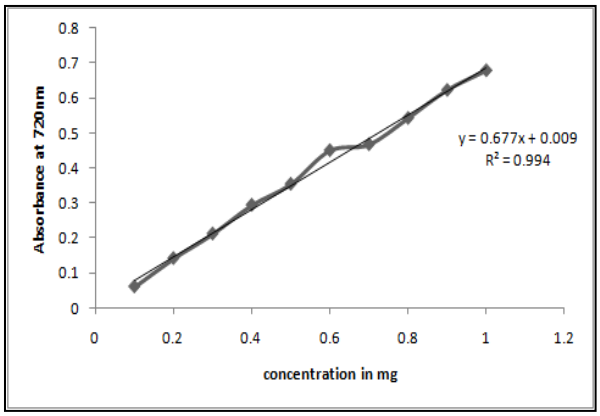


Fig. 3 Standard tannic acid curve for tannin estimation

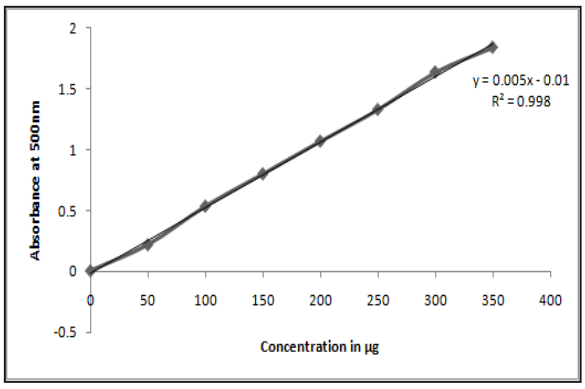


Fig. 4. Standard catechin curve for anthocyanidin estimation

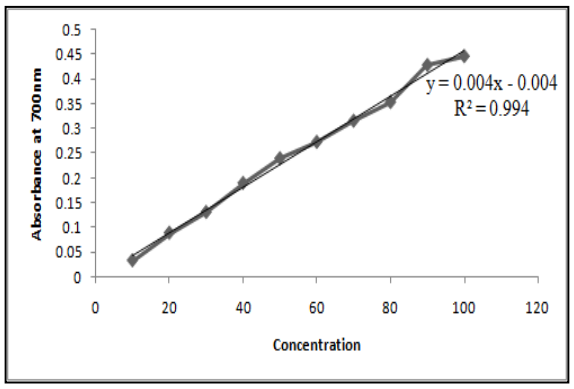


Fig. 5. Standard ascorbic acid curve for antioxidant assay

Concentration of anthocyanidin per catechin equivalent per gram of plant material

Antibacterial activity:

Effect of methanolic and aqueous extract on multi drug resistant clinical isolates

Table 7. Soxhlet extract

Sr. No.	Clinical isolates	Methanolic extract (mg)					Aqueous extract (mg)					Antibiotic Resistance Pattern
		20	40	60	80	100	20	40	60	80	100	
		13	14	16	18	21	11	13	15	19	23	A; Am; C; Cf; Co; Cp; Gf; K; Nx; Of; P; Pf; S; Sc; T
1	1 EC											
2	2 EC	14	15	17	18	20	12	13	14	17	20	A; Am; Cf; Co; Cp; K; Nx; P; Pf; S; Sc; T
3	3 EC	15	16	18	19	21	13	14	16	17	18	A; Am; Cp; P
4	4 EC	16	17	18	19	21	14	15	16	18	21	A; Am; Co; Cp; P; S; T
5	5 EC	15	17	18	19	20	13	14	15	17	18	A; Am; C; Co; Cp; K; Nx; P; S; Sc; T
6	1 EF	11	12	13	14	15	12	13	14	16	17	A; Am; C; Cf; Co; Cp; Gf; K; Nx; Of; P; Pf; S; Sc; T
7	2 EF	13	14	15	17	18	13	14	15	17	19	A; C; Cf; Co; Cp; Gf; K; Nx; Of; P; Pf; S; Sc; T
8	3 EF	14	15	17	19	21	13	14	17	18	21	A; Cp; P;T;
9	4 EF	12	13	15	18	20	12	13	15	17	23	A; Am; Cp; P;T;
10	5 EF	12	14	16	18	20	13	14	15	17	21	A; Am; P
11	1 PA	14	15	16	17	19	12	15	17	18	20	A; Am; C; Co; Cp; Gf; K; Of; P; S;T
12	2 PA	12	13	14	15	17	12	13	16	18	19	A; Am; C; Cf; Co; Cp; Gf; K; Nx; Of; P; Pf; S; Sc; T
13	3 PA	14	15	16	18	19	11	12	14	15	17	A; K; P; Sc
14	4 PA	12	13	14	16	18	<10	15	18	19	23	Co; K; Nx; P; Pf
15	5 PA	12	13	14	16	17	13	14	16	18	19	P

Clinical isolates 1EC-5EC: *E.coli*, 1EF-5EF: *E. faecalis*, 1PA-5PA: *P. aeruginosa*

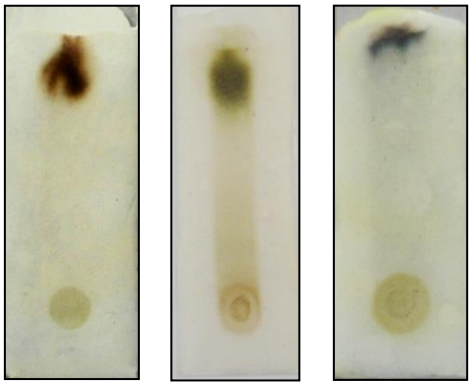
Table 8. Cold extract (bacteria with the resistance pattern same as above)

Sr. No.	Clinical isolates	Aq-Methanolic extract (mg)				
		20	40	60	80	100
1	1 EC	14	15	17	20	24
2	2 EC	13	14	18	21	23
3	3 EC	16	17	19	21	25
4	4 EC	17	19	21	23	27
5	5 EC	15	16	18	22	24
6	1 EF	13	14	16	18	19
7	2 EF	14	15	16	18	20
8	3 EF	15	17	19	21	23

9	4 EF	14	16	19	23	25
10	5 EF	14	15	17	19	23
11	1 PA	15	16	18	21	23
12	2 PA	14	15	17	19	21
13	3 PA	14	15	16	17	21
14	4 PA	13	15	17	21	27
15	5 PA	15	16	18	20	22

Table 9. MIC

Sr. No.	Clinical isolates	Soxhlet extraction				Cold extraction (mg)	
		Methanolic extract (mg)		Aqueous extract (mg)			
1	1 EC	12		10		6	
2	2 EC	11	11.8 (Mean)	10	10.2	6	5.8 (Mean)
3	3 EC	12		11	(Mean)	5	
4	4 EC	12		10		6	
5	5 EC	12		10		6	
6	1 EF	10		9		7	
7	2 EF	11	10.2 (Mean)	9	8.8 (Mean)	7	7.2 (Mean)
8	3 EF	10		8		8	
9	4 EF	10		9		7	
10	5 EF	10		9		7	
11	1 PA	14		12		6	
12	2 PA	12	13.2 (Mean)	12	12.2	6	5.8 (Mean)
13	3 PA	14		12	(Mean)	5	
14	4 PA	14		13		6	
15	5 PA	12		12		6	



Alkaloids Tannin Phenol

Fig. 6: Photographs of thin layer chromatography plates

Table 10. Rf values

Phytochemical's Rf values			
Alkaloids	**Tannins**	**Phenol**	**Anthraquinone**
0.81	0.77	1.06	-----

CONCLUSION

From all the results of phytochemicals analysis, it was found that cold maceration gave better extraction of phytochemicals as compared to Soxhlet extracts. This may be due to bioactive phytochemicals being heat labile. The antibacterial analysis of leaf extract showed that *Andrographis paniculata* exhibits significant antibacterial activity. The phytochemicals extracted by cold maceration produce higher concentration of bioactive phytochemicals which are responsible for

combating multi drug resistant human pathogens that causes urinary tract infection. The minimum inhibitory concentration was found to be less in cold extracts as compared to hot extracts. This shows that cold extract has more potent antibacterial activity than hot extracts. Therefore this plant can be further investigated with respect to toxicity studies. The phytochemicals from this plant can be envisaged to be a good novel drug against urinary tract infection causing pathogens after thorough study.

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