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EVALUATION OF GROWTH INHIBITORY ACTIVITY OF PHYTOCONSTITUENTS OF BLACK MYROBALAN (FRUIT OF *TERMINALIA CHEBULA RETZ.)* AGAINST UROPATHOGENIC *ESCHERICHIA COLI*

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ABSTRACT

Terminalia chebula Retz. has been extensively used in Ayurveda, Unani and Homoeopathic medicines and has become a cynosure of modern medicine. Antibacterial potential of black myrobalan (fruits of *Terminalia chebula Retz*.) against uropathogenic *Escherichia coli* has already been evaluated and reported by us. In the present study, antibacterial potential of phytoconstituents (total phenolics, flavonoids and carotenoids) fractionated from aqueous and ethanol extracts of black myrobalan against uropathogenic *Escherichia coli* was assessed by *in vitro* growth inhibitory assay methods. It was observed that ethanol extract of the plant material contained high concentration of phytoconstituents and exhibited more strong antibacterial potential against the test strain, when compared with its aqueous counter parts. MIC values revealed that the test strain was more susceptible towards phenolics than other phytoconstituents (flavonoids and carotenoids) evaluated. These results strongly document the beneficial effects of phenolics fractionated from ethanol extract of black myrobalan against uropathogenic *Escherichia coli* and reinforce the importance of ethnomedical approach as a potential source of bioactive compounds for the treatment of urinary tract infections (UTIs).

Key words : Black myrobalan, Phenolics, Flavonoids, Carotenoids, Uropathogenic *Escherichia coli*, Antibacterial potential

INTRODUCTION

Terminalia chebula Retz. (*Combretaceae*) is called the 'King of Medicine' in Tibet

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and is always listed first in the Ayurvedic Materia Medica because of its extraordinary powers of healing. The dried fruit of *Terminalia chebula Retz* also called black myrobalan has traditionally been used in the treatment of asthma, sore throat, vomiting, hicoccough, diarrhoea, bleeding piles, gout, heart and bladder diseases[1]. It has antioxidant and free radical scavenging properties[2]. A herbal formulation containing *Terminalia chebula* Retz. fruit under the name 'TRIPHALA' is a very popular traditional medicine for the treatment of chronic disorders[3]. Fruit of *Terminalia chebula Retz.* is effecive against cancer cells[4] and *Hellicobactor pyroli*[5]. It is also useful as anticaries agent [6], in dermal wound healing[7], improving gastrointestinal motility[8], anaphylactic shock[9] and in diabetes mellitus[10]. Antibacterial activity of aqueous and ethanol extracts of *Terminalia chebula Retz.* fruits against uropathogenic *E. coli* has been reported by us[11]. Literature reveals that ethanol and aqueous extracts from plants are potential sources of antimicrobial agents[12] and selection of crude plant extracts for screening programmes has the potential of being more successful as an initial step than the screening of pure compounds isolated from natural products[13]. In the present study, possible antibacterial potential of phytoconstituents of black myrobalan against uropathogenic *Escherichia coli* has been evaluated. The active phytoconstitents having strong antibacterial activity *in vitro* may be of value in the design of further studies to unravel novel treatment strategies for urinary tract infections caused by *Escherichia coli*.

EXPERIMENTAL

Materials and methods

Collection of plant materials

Fresh matured fruits of *Terminalia chebula* were collected locally and were identified and authenticated by a botanist. The seedless fruits were dried under shade and powdered.

Preparation of plant extracts

Aqueous extract

The dried powder of *T. chebula* fruits (100 g) were maintained at 60°C for 3 h in sterile distilled water. The resulting suspensions were filtered and evaporated to dryness at room temperature in a steady air-current.

Ethanol extract

T. chebula fruits powder (100 g) were placed in a Soxhlet extractor containing 70%

of ethanol. The solvent was removed in a rotary evaporator and the crude extract was dried at room temperature in a steady air-current. The resulting extracts were then kept at 4°C in air-tight jars for further studies.

Phytochemical study : The phytochemical study was designed to estimate total phenolics, flavonoids and carotenoids present in the aqueous and ethanol extracts of black myrobalan.

Isolation and estimation of total phenolics

The phenolics were extracted as described by Sun et al[14]. Briefly, 5 g of the sample mixed with 25 mL of 80 : 20 methanol : water and homogenized for 1 h. The mixture was centrifuged for 10 min at 500 x g and the clear supernatant was collected. The procedure was repeated with another 25 mL of solvent and the supernatants were combined and dried completely and stored at – 80°C until use.

Total phenolics were determined by Folin Ciocalteu reagent[15]. A dilute extract (0.5 mL of 1 : 10 g/mL^{-1}) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (5 mL, 1 : 10 diluted with distilled water) and aqueous Na_2CO_3 (4 mL, 1 M). The mixtures were allowed to stand for 15 min and the total phenolics were determined by colorimetry at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg L^{-1} solutions of gallic acid in methanol : water (50 : 50 v/v). Total phenolics were expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference standard.

Isolation and estimation of flavonoids

Isolation of flavonoids were done using the protocol suggested by Harborne (1975)[16]. Briefly, 5 g of the sample was acid hydrolysed with 10 mL of 1 (N) H_2SO_4 at 70°C for 1 h and neutralized with 0.5 mL of 10 (N) NaOH. To this 5 mL of ethyl acetate was added, shaken well and the ethyl acetate portion was collected. This was repeated thrice and the ethyl acetate was pooled and evaporated to dryness.

Aluminium chloride colorimetric method was used for flavonoids determination[17]. The extract (0.5 mL of 1 : 10 g mL^{-1}) in methanol was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured spectrophotometrically at 415 nm. The calibration curve

was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg mL^{-1} in methanol.

Isolation and estimation of carotenoids

Extraction and estimation of carotenoids was performed by protocols suggested by Narayanaswamy and Palanisamy[18]. Briefly, 5 g of the sample was homogenized in 20 mL of acetone using a morter and pestle and filtered using Whatman No. 1 filter paper. The extraction was repeated until free from pigments. The filtrate was pooled and partitioned with an equal quantity of peroxide free ether, thrice using the separating funnel. The ether phase containing carotenoids was evaporated and the residue was dissolved in 1 mL ethanol. Subsequently, 0.1 mL of 60% KOH was added and partitioned twice in peroxide free ether. The ether was evaporated and the residue was dissolved in 0.5 mL ethanol, before measurement of carotenoids. The extract was measured at 450 nm for absorbance and the carotenoid present were estimated using the formula

$$C = D \times V \times \frac{F}{2500}$$

Where C is the total carotenoids, D is the absorbance at 450 nm in a 1 cm cell, V is the volume of original extract in mL, F is the dilution factor and 2500 is the average extinction coefficient of the pigments. The carotenoids were expressed as µg/g sample.

Microbial analysis

Microbial strain

The microorganism used in this study was uropathogenic *Escherichia coli* (L. C. I.). This uropahogenic *E. coli* was procured from the Department of Bacteriology and Serology, School of Tropical Medicine, Kolkata, India and maintained in nutrient agar medium.

Formulation of extracts

Crude extracts and phytoconstituents were diluted with either 5% dimethylsulphoxide (DMSO) or sterile ditilled water to a final concentration of 100 mg/mL.

Bacterial dilution

The test bacterial strain was inoculated in Mueller Hinton Broth (Oxoid) medium

and incubated for 3- 6 hours at 35°C in a shaker water bath until the culture attained the turbidity of 0.5 McFarland Unit. The final inoculum size was adjusted to 5 x 10^5 cfu/mL.

Sensitivity test

Disk diffusion assay

Antibacterial activity of aqueous and ethanol extracts of black myrobalan was evaluated by disk diffusion assay method[19]. The Mueller-Hinton Agar (MHA; Oxoid) plates were prepared by pouring 15 mL of molten media into sterile petriplates. The plates were allowed to solidify for 5 minutes and 1 mL (5 x 10^5 cfu/mL) inoculum suspension was swabbed uniformly on MHA plates. Sterile paper disks (6 mm in diameter) was saturated with 10 µL of the reconstituted crude extracts and phytoconstituents, allowed to dry and was introduced on the upper layer of seeded MHA plates and allowed to diffuse for 1 h. The plates were incubated at 37°C for 24 h. At the end of incubation, inhibition zones formed around the disc were measured to the nearest millimeter (mm). Gentamycin sulphate (1 µg/disc) was used as experimental positive control and 5% DMSO as negative control. The tests were performed in triplicate for the microorganism evaluated and the final results were presented as the arithmetic average.

Minimal inhibitory concentration (MIC) evaluation

The minimal inhibitory concentrations (MICs) of the extracts and phytoconstituents against the test strain were determined by macrobroth dilution assay method following NCCLS, 1993 guidelines[20]. Two-fold serial dilutions of extracts and phytoconstituents (3.12-100 µg/mL) were prepared in tubes with Mueller- Hinton Broth (Oxoid) as diluent. Duplicate tubes of each dilution were seeded with test organism (100 µl) to the standard concentration (5 x 10^5 cfu/mL). Two-fold serial dilution of gentamycin sulphate (125 – 512 µg/mL) was used as experimental positive control. The tubes were incubated at 37°C for 24h. The least concentration of the drug showing a clear zone of inhibition was taken as the MIC.

RESULTS AND DISCUSSION

Table 1 shows that both aqueous and ethanol extracts of black myrobalan contained high concentration of phenolics followed by flavonoids and carotenoids.

Table 2 shows that both the crude extracts (aqueous and ethanol) as well as their phytoconstituents exhibited varying degrees of inhibitory action against the test strain and ethanol extract was found to be more potent than aqueous extract. Besides, phenolics were

more potent against the test strain than flavonoids and carotenoids as evidenced from their IZD and MIC values.

Table 1. Phytochemical constituents of aqueous and ethanol extracts of black myrobalan

T. chebula extract	Phenolics (mg/g)	Flavonoids (mg/g)	Carotenoids (μg/g)
Aqueous extract	182.0 ± 3.2	32.7 ± 3.4	204.5 ± 5.6
Ethanol extract	304.5 ± 5.3	62.5 ± 4.8	372.2 ± 4.7

Values are average of triplicate experiments ± S. D.

Table 2. Inhibition zone diameter (IZD) and minimal inhibitory concentration (MIC) of crude extracts and phytoconstituents of black myrobalan against uropathogenic *Escherichia coli*

Treatment	Inhibition zone diameter (IZD) (mm)		Minimal inhibitory concentration (MIC) (mg/mL)	
	Aqueous	Ethanol	Aqueous	Ethanol
Crude extract	++	+++	50	25
Total phenolics	++	+++	25	12.5
Flavonoids	+	++	100	50
Carotenoids	+			
Gentamycin (Positive control)	+++		4	
5% DMSO (Negative control)	-		ND	

(-) : Absence of inhibition; (+) : IZD (1 mm-7 mm); (++) : IZD (8 mm-11 mm); (+++) : IZD (12 mm or more). ND : Not done.

Values are average of triplicate experiments.

Urinary tract infections (UTIs) are the serious health problem affecting millions of people each year. Infections of the urinary tract are the second most common type of infections in the body. *Escherichi coli* causes about 80% of urinary tract infections in adults. Other bacteria that causes urinary tract infections include *Staphylococcus saprophyticus* (5 to 15% of cases) *Chlamydia trachomatis* and *Mycoplasma hominis* [21, 22]

It has been reported by several researchers that phenolics, flavonoids and carotenoids are the most commonly found phytoconstituents in the plant extracts that possess antimicrobial activities along with other medicinal properties [23, 24].

In the foregoing findings, the greater antibacterial activity (Table 2) and high concentration of phytoconstituents of ethanol extract in comparison with its aqueous counter parts (Table 1) might possibly be due to the lesser solubility of the active constituents in aqueous solution, resulting in less antibacterial effect on the bacterial isolates tested. The reason for the test bacterial strain being more sensitive towards total phenolics than flavonoids and carotenoids is not clear right now. It may be due to the presence of high amount of tannins[25], which is soluble both in alcohol and water and exhibits antimicrobial activity [26].

Thus, strong antibacterial activity exhibited by the phenolics fractionated from ethanol extract of black myrobalan over others both in disc diffusion and macrobroth dilution assay methods against the uropathogenic *E. coli* support the use of *Terminalia chebula Retz.* fruits for UTIs therapy and warrants further studies both on the extract and/or its chemical constituents to pinpoint the findings. This report may serve as a footstep in this aspect.

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REFERENCES

1. K. R. Kirtikar and B. D. Basu, Ind. Medicinal Plants, Lm Basu, Allahabad (1935).
2. H. Y. Cheng, T. C. Lin, K. H. Tu, C. M. Yang and C. C. Lin, Biol. Pharm. Bull., **26**, 1331 (2003).
3. S. S. Juss, Ind. Med. Gazette, **131 (6),** 194 (1997).

4. A. Saleem, M. Husheem, K. Harkonen and K. Pihlaja, J. Ethnopharmacol., **81**, 327 (2002).
5. F. Malekzadeh, H. Ehsanifar, M. Shahamat, M. Levin and R. R. Colwell, Int. J. Antimicrob. Agents, **18**, 85 (2001).
6. A. G. Jagpat and S. G. Katkera, J. Ethnopharmacol., **68**, 299 (1999).
7. L. Sugina, S. Sing, P. Sivakumar, P. Sampath and G. Chandrakasan, Phytother. Res., **16**, 227 (2002).
8. M. D. Tamhane, S. P. Thorat, N. N. Rege and S. A. Dahanukar, J. Postgrad. Med., **43**, 12 (1997).
9. T. Y. Shin, H. J. Jeong, D. K. Kim, S. H. Kim, J. K. Lee, A. K. Kim, B. S. Chae, H. W. Kang, C. M. Lee, K. C. Lee, S. T. Park, E. J. Lee, J. P. Lim, H. M. Kim and Y. M. Lee, J. Ethnopharmacol., **74**, 133 (2001).
10. M. C. Sabu and R. Kuttan, J. Ethnopharmacol., **81**, 155 (2002).
11. R. R. Chattopadhyay, S. K. Bhattacharyya, C. Medda, S. Chanda, S. Dutta and N. K. Pal, Pharmacog. Mag., **3 (11),** 212 (2007).
12. T. H. Chung, J. C. Kim, M. K. Kim et al., Phyther. Res., **9**, 429 (1995).
13. I. T. Kusumoto, T. Nakabayashi, H. Kida, H. Miyashiro and M. Hattori, Phytother. Res., **9**, 180 (1995).
14. J. Sun, Y. F. Chu, X. Wu and R. H. Liu, J. Agric. Food Chem., **50**, 7449 (2002).
15. S. Mcdonald, P. D. Preuzler, M. Autolovich and K. Robards, Food Chem., **73**, 73 (2001).
16. J. B. Harborne, Biochemical Systematics of Flavonoids, in the Flavonoids, J. B. Harbornae and H. Mabry (Eds), Academic Press, New York, (1975) Pp. 1056.
17. C. Chang, M. Yang, H. Wen and J. Chern, J. Food Drug Anal., **10**, 178 (2002).
18. P. Narayanaswamy and A. Palanisami, Exp. Biol., **29**, 1165 (1973).
19. A. W. Bauer, W. M. Kirby, J. C. Sherris and M. Turck, Am. J. Clin. Pathol., **45**, 493 (1966).
20. National Committee for Clinical Laboratory Standards (Nccls). Methods for Dilution in Antimicrobial Susceptibility Tests. Approved Standard, M2-M5, Nccls, Villanova, Pa, (1993).
21. W. E. Stamm and T. M. Hooton, N. Eng. J. Med., **329**, 1328 (1993)
22. B. A. Lipsky, Annals. Int. Med., **110**, 138 (1989).

23. N. S. Alzoreky and K. Nakahara, Int. Food Microbiol., **80**, 223 (2003).

24. H. Mahanom, A. H. Azizah and M. H. Dzulkifly, Mal. J. Nutr., **5**, 47 (1999).

25. L. J. Juang, S. J. Sheu and T. C. Lin, J. Sep. Sci., **27 (9),** 718 (2004).

26. K. T. Chung, T. Y. Wong, C. I. Wei, Y. W. Huang and Y. Lin, Crit. Rev. Food. Sci. Nutr., **38 (6),** 421 (1988).

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