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Physio-chemical properties and Antimicrobial Activity of Triphala Masi

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Abstract

Triphala is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal plants *Terminalia chebula, Terminalia belerica* and *Phyllanthus embelica*. Triphala is also main ingredient of Triphala Masi and practiced very less which is unexplored. In consideration of all above the present study was planned to make an attempt to evaluate the pharmaceutical analysis and evaluation of antimicrobial activity of Triphala Masi. The Triphala Masi was prepared by heating at 450 °C for 150 min. The physical values like total ash, acid insoluble ash, water-soluble ash, loss on drying, pH value and loss on drying were determined. The organoleptic characters of prepared Triphala Masi were studied. The prepared Masi were black colour with charcoal like taste and burnt smell. Total ash, Acid insoluble ash, water soluble ash and loss on drying of Triphala Masi fulfill the standards criteria. pH value suggested to be acidic for Triphala Masi. Triphala Masi exhibited a broad-spectrum antimicrobial activity and inhibited the growth of *S. aureus* and *E. coli*. The above findings of pharmaceutical, analytical profile and antimicrobial study of Triphala Masi indicates that the formulation was stable and has effective antibacterial activity.

1 Introduction

Presently Ayurveda is consisting of huge number of formulations to accomplish its objectives. Masi is one of such preparation and employed for controlled of various diseases. Masi is a solid dosage form in which bulk of raw material is reduced to a greater extent by application of a certain quantum of energy. As a result of this treatment hidden chemical constituents become prominent and/or new chemical moieties are formed which are therapeutically active. Due to thermal degradation or decomposition thermo labile constituent are lost. Therapeutically active organic and inorganic chemical constituents can be prepared by simple heat treatment in a controlled manner. The black color indicates high percentage of carbon and oxides. Non-specific odor and charcoal like taste may be attributed to oxides, inorganic elements and carbon[1-4].

The antibiotics available for the bacterial infection have developed resistance against bacteria. The novel drug delivery system or development of novel formulation is best option to overcome this problem. Masi Kalpana is an important pharmaceutical preparation mentioned in Ayurveda Pharmaceutics. Masi is a dosage form producing higher extent of therapeutic effect compared to other Ayurvedic dosage form due to application of a certain quantum of energy. As a result of this treatment hidden chemical constituents become prominent and/or new chemical moieties are formed which are therapeutically active[4,5].

Triphala is a traditional Ayurvedic herbal formulation consisting of *Terminalia chebula, Terminalia bellerica* and *Emblica officinalis*. Triphala is used in Ayurvedic medicine to treat a variety of conditions like headache, dyspepsia, blood purifier, constipation, liver conditions, ascites, skin disease, intermittent fever, leucorrhoea etc.

Triphala in Ayurvedic system of medicines widely used in different dosage forms in different conditions. Among them Kashaya it is used for vrana prkshalana, netra prakshalana etc indicating about its bactericidal action[6-8].

Triphala is also main ingredient of Triphala Masi and practiced very less which is unexplored. In consideration of all above the present study was planned to make an attempt to evaluate the pharmaceutical analysis and evaluation of antimicrobial activity of Triphala masi.

2 Materials and Methods

2.1 Collection of the drug

The raw drugs required for the preparation of medicine was procured from locality of Bangalore, Karnataka.

2.2 Authentication of the drug

The authentication of the raw drug was done at the Rasashastra and Bhaishajya Kalpana P.G. department laboratory, Ramakrishna Ayurvedic Medical Collage, Hospital and Research Center, Yalahanka, Bangalore.

2.3 Preparation of Triphala Masi

The Triphala Masi was prepared at Rasashastra and Bhaishajya Kalpana P.G. department laboratory, Ramakrishna Ayurvedic Medical Collage, Hospital and Research Center, Yalahanka, Bangalore. The Triphala Masi was prepared with classic reference (Table 1). The plant material used in preparation of Triphala Masi demonstrated in table 1.

Table 1: Details of the raw drug used

Common Name	Botanical name	Part used	Parts purchased
Harad	*Terminalia chebula*	Fruits	Amruth Keasri Pharmacy, K R Market, Bangalore
Bahera	*Terminalia bellerica*	Fruits	Amruth Keasri Pharmacy, K R Market, Bangalore
Amla	*Emblica officinalis*	Fruits	Amruth Keasri Pharmacy, K R Market, Bangalore

Place of pre-processing of the drug like cleaning, drying was done at Rasashastra and Bhaishajya Kalpana P.G. department laboratory, Ramakrishna Ayurvedic Medical Collage, Hospital and Research Center, Yalahanka, Bangalore.

The dried fruits of *Terminalia chebula*, *Terminalia bellerica* and *Emblica officinalis* were taken and made into powder called as churn. Further 150 gm churn of *Terminalia chebula*, *Terminalia bellerica* and *Emblica officinalis* individually weighed and were mixed to form Triphala churn. The Triphala was heated in closed silica crucibles in a muffle furnace. Triphala was heated from 30 °C to 450 °C higher temperature by continuously increasing temperature to 10 °C/min. Then it was filtered with cloth and preserved in the glass containers[9, 10].

2.4 Analytical study

2.4.1 Organoleptic parameters[11,12]

Organoleptic (i. e., impression on the organs) refers to evaluation by means of the organs of sense and the odor, taste etc. of the drugs. The macroscopic characteristics of the drug include:

- Appearance
- Colour
- Consistency
- Odour
- Taste

2.4.2 Physico-chemical analysis[13-20]

The physical values like total ash, acid insoluble ash, water-soluble ash, loss on drying, pH value and TLC were determined.

Determination of total ash value

Accurately weighed about 3 gms of air dried powdered drug was taken in a tared silica crucible and incinerated by gradually increasing the temperature to make it dull red hot until free from carbon. Cooled and weighed, repeated for constant value. Then the percentage of total ash was calculated with reference to the air dried drug.

Determination of acid insoluble ash value

The ash obtained as directed under total ash was boiled with 25 ml of 2N HCl for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water, ignited and weighed, then calculated the percentage of acid insoluble ash with reference to the air dried drug.

Determination of water soluble ash value

The total ash obtained was boiled with 25 ml. of water for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

Loss on drying

About 1.5 gm of powdered drug was weighed accurately in a tared porcelein dish which was previously dried at 105 °C in hot air oven to constant weight and then weighed. From the difference in weight, the percentage loss of drying with reference to the air dried substance was calculated.

Determination of pH

1 ml of sample was taken and mark up to 10 ml with distilled water, stirred well and filtered. The filtrate was used for the experiment. Instrument was switched on. 30 minutes time was given for warming pH meter. The pH 4 solution was first introduced and the pH adjusted by using the knob to 4.02 for room temperature 30 °C. The pH 7 solution was introduced and the pH meter adjusted to 7 by using the knob. Introduced the pH 9.2 solution and checked the pH reading without adjusting the knob. Then the sample solution was introduced and reading was noted. Repeated the test four times and the average reading were taken as result.

2.5 Preparation of aqueous extract of Triphala Masi

The powder of Triphala Masi packed well in soxhlet apparatus and extracted with distilled water until the completion of the extraction. The extract was filtered while hot, and the solvents were removed by distillation and the last traces of solvent being removed under reduced pressure. The aqueous extracts were stored in refrigerator for further experimental work.

Phytochemical investigation[13-20]

Qualitative chemical tests were performed to determine the presence of alkaloids, carbohydrates, cardiac glycosides, polyphenols, saponins, tannins and terpenoids.

2.6 Thin layer chromatography (TLC)[21-26]

The drug sample was prepared with the respective solvent ethanol and distilled water and made up to 10 ml in different test tubes. Then the samples were taken in a capillary tube and it was spotted in preparative TLC plates coated with silica gel G. The plates were developed in TLC chamber previously saturated with different solvent systems. The different spots developed in each solvent system were identified by means of detecting agent and the Rf value are correspondingly calculated.

2.7 Anti-microbial study

The aqueous extracts of Triphala Masi was prepared and stored in refrigerator for further experimental work.

2.7.1 Test microorganisms

Pure chemical isolates of *Staphylococcus aureus* and *Escherichia coli* were used for studies.

2.7.2 Culture medium and inoculum preparation

High sensitivity testing agar (Hi-Media) was used for checking antimicrobial activity of extract against *S. aureus* and *E. coli*. The microbial strains were cultured on the slants in the sterilized Laminar Air Flow from the pure culture. These cultured slants were incubated at 37 °C for bacterial growth for 2–3 days. High sensitivity testing agar was mixed at a concentration of 23.4 g/1000 ml in distilled water and autoclaved at 121 °C for 15 min. A loop full from pure culture of a bacterial strain was mixed in the 10 ml of Nutrient broth medium and incubated at 37 °C overnight and the activated culture was used for streaking onto the agar plates for antimicrobial sensitivity.

2.7.3 Agar well diffusion assay

The antibacterial activity of the aqueous extract of Triphala Masi at different concentration (100 µg/ml and 200 µg/ml) was determined by Agar well diffusion assay. The standard drug Ciprofloxacin (25 µg/ml) was used to evaluate antibacterial activity. 2.34 gm of high sensitivity testing agar was dissolved in 100 ml of distilled water and autoclaved at 121 °C for 15 min. Before transferring this medium in sterilized petri plates, it was allowed to cool and then was poured into the petri plates and allowed to solidify.

After this, it was inoculated with activated culture using sterile cotton swabs. And the wells were created using sterile agar borer and the wells were filled by adding 25 µl of extract using micropipette and were incubated at 37 °C for 12–24 h. Three replicates were carried out for extract against the test organisms. After incubation, the diameters of the zones of inhibition (ZOI) were measured in millimeters, and the mean values were tabulated[27-31].

2.7.8 Minimum inhibitory concentration (MIC)

The extracts, which showed antibacterial activity in the agar well diffusion assay, were subjected to the MIC assay. Serial two-fold dilutions of the extracts were prepared in nutrient broth with different concentrations (ranging from 200 to 0.39 and 100 to 0.39 mg/ml). An equal volume of inoculum (1 ml) was added in all the tubes. The tubes (total volume 2 ml) were incubated at 37 °C for 18 h. The MIC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of turbidity. Solvent blanks and positive controls were also included. All tests were performed in triplicate[30, 31].

2.8 Statistical analysis

Values are mean ± SEM of three replicates in results for physicochemical analysis.

Results were analyzed using one way analysis of variance (ANOVA) followed by the Dunnett's test by using statistical software package, Graph Pad Prism; version 7 for antibacterial activity.

3 Results and Discussions

3.1 Triphala Masi

Table 2 demonstrated that about 6% drug loss during powder of plant materials.

Table 2: Preparation of sample for Triphala

Plants	Weight (gm) before powder	Weight (gm) after powder
Terminalia chebula	150.0	140.21
Terminalia bellerica	150.0	139.16
Emblica officinalis	150.0	141.05

Table 3 shows that there was drug loss about 13.6 during preparation of Triphala Masi.

Table 3: Preparation of sample for Triphala Masi

Triphala	Wt. (gm) before treatment	Wt. (gm) after treatment	Wt. loss
T.chebula (125 gm): *T.bellerica* (125 gm): *E. officinalis* (125gm)	375.0	361.4	13.6 gm

The colour of Triphala Masi was changes on heating the Triphala at various temperatures (Fig 1).

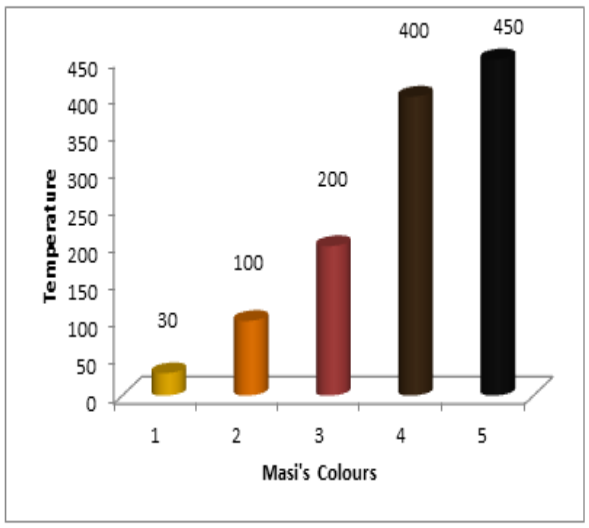

Fig 1: Graphical representation of colour changes during preparation of Triphala Masi

Triphala Masi was obtained, when Triphala Churn was heated slowly, at lower temperature (generally below 450 °C). If heating is continued further at higher temperatures (above 450 °C) it forms Bhasma (White ash).

Mashi is an intermediate product of Bhasma in which unlike Bhasma, both organic and inorganic constituents are present.

For preparation of Triphala Masi sample in muffle furnace, 150 minutes were required and temperature given was 400 °C. Minimum six hours were required for self cooling.

Masi is a dosage form in which bulk of raw material is reduced to a greater extent by application of a certain quantum of energy. As a result of this treatment hidden chemical constituents become prominent and/or new chemical moieties are formed which are therapeutically active.

Due to thermal degradation or decomposition thermo labile constituent are lost. Therapeutically active organic and inorganic chemical constituents can be prepared by simple heat treatment in a controlled manner. The black color indicates high percentage of carbon and oxides. Non-specific odor and charcoal like taste may be attributed to oxides, inorganic elements and carbon.

3.2 Organoleptic properties

Table 4 shows the black colour of Masi with charcoal like taste and burnt smell products.

Table 4: Organoleptic Properties of Triphala Masi

Parameters	Characteristics
Color	Black
Appearance	Powder form
Touch	Smooth
Taste	Charcoal like taste
Odour	Burnt smell

Observations

Weight of Triphala Masi - 361.4 gms

Weight loss - 13.6 gms

3.3 Analytical study

3.3.1 Total Ash

It is the criteria for identity or purity of drugs. Total ash is inclusive of physiological ash derived from plant tissue and non physiological ash consists of residue of the extraneous matter such as sand, soil etc. adhering to the herb itself. Total Ash value of Triphala Masi was 3.4±0.62% w/w (Table 5). The total ash value of Triphala Masi was low in number which refers the less amount of non physiological Ash refers to purity of the drug.

3.3.2 Ash value

Water soluble Ash and acid insoluble ash also has been shown

less value for of Triphala Masi (0.46±0.06% w/w and 1.7±0.36% w/w) (Table 5). Less acid insoluble ash value refers less adherent dirt and sand particles. This can be used as criteria for quality control purpose.

Table 5: Analytical result of Triphala Masi

Parameters	Triphala Masi (% w/w)	Mean±SD
Total ash	3.2	3.4±0.62
	4.1	
	2.9	
Acid insoluble ash	1.6	1.7±0.36
	2.1	
	1.4	
Water-soluble ash	0.46	0.46±0.06
	0.53	
	0.41	
Loss on drying	0.91	0.87±0.04
	0.82	
	0.89	

Values are mean ± SD of three determinations

3.3.3 Loss on drying

Triphala Masi (0.87±0.04% w/w) have lower value of loss on drying (Table 5). This test is to detect the moisture and volatile content in the sample. Due to low loss on drying value, formulation might less susceptible to microbial contamination.

3.3.4 pH

Mean pH value of Triphala Masi was shown in acidic nature (Table 6). It was facilitated the absorption of drug at the stomach level. The results obtained in pH were found under the limits of Ayurvedic Pharmacopoeia of India.

Table 6: Showing pH value of Triphala Masi

Parameters	Triphala Masi	Mean±SD
pH	5.5	5.5±0.60
	4.9	
	6.1	

Values are mean ± SD of three determinations

3.4 Phytochemical screening

Preliminary phytochemical investigations of the extracts of Triphala Masi revealed the absence of chemical constituents (Table 7). The findings confirm that the chemical constituents are thermolabile and lost during heat treatment for preparation of Triphala Masi.

Table 7: Phytochemicals present in Triphala masi extracts

Phytoconstituent		Aqueous extract
Alkaloids	Dragendorff's test	-
	Hager's test	-
	Mayers	-
	Wagners	-
Glycosides	Legal's test	-
	Keller killiani test	-
	Baljet test	-
	Keller-Killiani test	-
	Borntrager's test	-
Carbohydrates	Molish test	-
	Benedict's test	-
	Fehling's test	-
Tannins and Phenolic compound	5%$Fecl_3$ solution	-
	Lead acetate sol.	-
	Bromine water	-
	Potassium ferric cyanide and ammonia sol.	-
Flavonoids	Shinoda test	-
Steroid test	Liebermann burchard test	-
	Salkowski test	-
Protein	Biuret test	-
	Ninhydrin test	-
Fat and oil test	Saponification test	-
	Spot Test	-

3.5 TLC

TLC photo documentation of Triphala Masi explains the presence of total 1 spots also the R_f value – 0.71 (Table 8). Suggested the presence of flavonoids in the sample which confirms the genuenity of drug.

3.6 Antibacterial activity

The diameter of the inhibition of zone of Triphala Masi on gram positive at different concentrations, by disc diffusion method,

was determined to access its antibacterial effect.

The Triphala Masi at the dose of 100 mg/ml and 200 mg/ml demonstrated 4.3±0.85 and 11.7±1.34 mm ZOI for *S. aureus*, respectively. The ciprofloxacin exhibited zone of inhibition of 14.8±1.35 mm for *S. aureus* (Table 9).

Table 8: TLC studies of Triphala Masi

Solvent system	No. of spots	R_f value
Hexane: $CHCl_3$: Ethanol (4:2:4)	1	0.71

Table 9: Antibacterial activity of Triphala Masi for *S. aureus*

Sample	Zone of inhibition (mm)	
	S. aureus	**Mean±SD**
Control (water)	0.00	0.00
Triphala Masi 100 mg/ml	5.2	4.3±0.85
	3.5	
	4.3	
Triphala Masi 200 mg/ml	13.2	11.7±1.34
	11.3	
	10.6	
Standard, Ciprofloxacin (25 µg/ml)	13.5	14.8±1.35
	14.9	
	16.2	

Values are mean ± SD of three determinations

The MIC value for Triphala Masi 100 mg/ml, Triphala Masi 200 mg/ml and Ciprofloxacin were 6.25, 3.125 and 1.562 for *S. aureus*, respectively (Table 10).

In general, the aqueous extracts of Triphala Masi exhibited a broad-spectrum antimicrobial activity against gram positive and gram negative. It inhibited the growth of *S. aureus* and *E. coli*. In agar diffusion method, the extract inhibited the growth of *S. aureus* and *E. coli*. More inhibitory zone was observed for the strains *E. coli*.

The *S. aureus* and *E. coli* that presented resistance to certain tested antibiotics, showed good susceptibility to the extracts of Triphala Masi. Variations of susceptibilities and resistance existed among same microorganisms to the same antibiotics.

Triphala Masi is widely used in the treatment of *grahani* (sprue), *pravahika* (dysentery) and *raktatisara* (diarrhea accompanied with blood) and is likely to be activity against the organisms associated with gastrointestinal tract. The zone of inhibition against gastrointestinal organisms such as *S. aureus* and *E. coli* confirms that Triphala Masi is having profound antibacterial effect on these two organisms and hence effective in mitigating dysentery.

Table 10: Determination of Minimum inhibitory Concentration for *S. aureus*

Sample	MIC (mg/mL)	Methodology
Triphala Masi 100 mg/ml	6.25	Microbroth dilution technique using Culture Medium: Potato dextrose broth for *S. aureus.* Sample test concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390 mg/ml.
Triphala Masi 200 mg/ml	3.125	
Standard, Ciprofloxacin (25 µg/ml)	1.562	

This variation observed in the activity among the market samples may be due to one or more reasons such as differences in composition of the product, differences in methods of manufacture, differences in methods of storage, differences in geographical origin and season of plant parts collected, lack of standards for formulations and lack of adequate documentation of production and testing.

From this perspective, it is of utmost importance to standardize classical Ayurvedic formulation and establish standards of testing to ensure uniformity and consistency in its activity and effectiveness. The method described may be used as the standard method for standardization of each batch of Triphala Masi, which ensures consistency and uniformity in the quality of the product.

5 Conclusion

The present study was planned to prepare Triphala Masi is used for treatment of various diseases. The prepared Masi were black colour with charcoal like taste and burnt smell. The values of analytical properties namely total ash, acid insoluble ash, water soluble ash, and loss on drying of Triphala Masi fulfill the standards criteria. pH value suggested to be acidic for Triphala Masi.

Triphala Masi exhibited a broad-spectrum antimicrobial activity against gram positive and gram negative. It inhibited the growth of *S. aureus* and *E. coli*. The above findings of pharmaceutical, analytical profile and antimicrobial study of Triphala Masi indicates that the formulation was stable and has effective antibacterial activity.

5 Conflict of interest

We declared that we have no conflict of interest.

6 Author contributions

RS and JKB have carried out the research work in the laboratory. RS compiled and analyzed the data of present work. Both authors approved the final manuscript.

7 References

1. Pal M. The Tridosha Theory. Anc Sci Life 1991;10:144–55.
2. Bhanu Prakash. Use of Metals in Ayurvedic Medicine.Pdf. Indian J Hist Sci 1997;32.
3. Sharma S. Rasa Tarangini. Fifth edi. Motilal Banarasi Das, Varanasi; 1994.
4. Patwardhan K. The history of the discovery of blood circulation: unrecognized contributions of Ayurveda masters. AJP Adv Physiol Educ 2012;36:77–82.
5. Hankey A. Ayurvedic Physiology and Etiology : Ayurvedo Amritanaam.The Doshas and Their Functioning in Terms of Contemporary Biology And Physical Chemistry. J Altern Complement Med 2001;7:567–74.
6. Anonymous. The Ayurvedic Formulary of India. Second edi. Ministry of Health and Family Welfare, Government of India, New Delhi; 2003.
7. Arun N, Kadibagil VR, Ganti BY. Various dosage forms of Ayurveda. Unique Journal of Ayurvedic and Herbal Medicines. 2014; 2(4): 20-23.
8. Biradar YS, Jagatap S, Khandelwal KR, Singhania SS. Exploring of Antimicrobial Activity of Triphala Mashi—an Ayurvedic Formulation. eCAM 2008; 5(1): 107–113.
9. Biradar YS, Jagatap S, Khandelwal KR, Singhania SS. Exploring of Antimicrobial Activity of Triphala Mashi—an Ayurvedic Formulation eCAM 2008;5(1)107–113.
10. Bhatt G, Kaur H, Harisha CR, Galib R, Prajapati PK. Pharmacognostical Profiles of Triphala Masi Prepared at Different Levels of Temperature. International Journal of Ayurvedic Medicine, 2016, 7(2), 100-103.
11. Khandelwal KR, Kokate CK, Gokhale SB. Practical Pharmacognosy. Pune; Nirali Prakashan; 1996. 149p 7.
12. Wallis TE. Text book of Pharmacognosy. 5ed. New Delhi; CBS Publishers & Distributors; 2002. 210-215p.
13. Quality standard of Indian medicine, CCRAS, volume 9th, 2012.p.26 - 28.
14. Honward. VS, Handbook of standardization of Ayurvedic Formulation.1sted, Varanasi: Chaukahambha Orientalia; 2012.p.54.
15. Honward.VS, Handbook of standardization of Ayurvedic Formulation.1sted, Varanasi: Chaukahambha Orientalia; 2012.p.60 – 63
16. Ayurvediya RasShastra, Siddhinandan Mishra, chaukhamba Orientalia, 9th Ed., 1999; 99. 11.
17. Ayurvediya Rasashastra, Chandrabhushan Jha, chaukhamba Orientalia, 1st Ed., 1994; 333.
18. WHO Publication of Quality Control Methods of Medicinal plant materials. 1998; 10-21.
19. Divakar MC. Plant Drug Evaluation - A laboratory guide. C.D. Remedies, Coimbatore. 1996; 1-100.
20. Brain KR, Turner TD. The practical evaluation of phytopharmaceuticals, Wright Science technica, Bristol. 1975; 81-82.
21. Stahl E. Thin layer chromatography, Toppan Co. Ltd., Tokyo. 1969; 308-309.
22. Skoog D, West D, Holler J. Fundamentals of Analytical Chemistry. Fifth edition. Saunders College Publishing. A division of Holt, Rinehart and Winston, New York-Chicago-San Fransico. 1988; 894.
23. Chatwal G, Anand S. Instrumental methods of chemical analysis, Himalaya Publishing House, Bombay. 1984; 311.
24. Sharma BK. Instrumental methods of chemical analysis, Goel Publishing House, Merrut. 1979; 623.
25. Zweig G, Sharma J. Handbook of Chromatography, Vol. II, CRC Press, Ohio, USA. 1976; 173.
26. Lederer E, Lederer M. Chromatography. Elsevier, London. 1957; 22, 36, 41.
27. Perez C, Paul M, Bazerque P. Antibiotic assay by agar-well diffusion method. Acta Bio Med. 1990; 25: 113-115.
28. Gupta AK, Ahirwar NK, Shinde N, Choudhary M, Rajput YS, Singh A. Phytochemical Screening and Antimicrobial Assessment of Leaves of Adhatoda vasica, Azadirachta indica and Datura stramonium. UK Journal of Pharmaceutical and Biosciences. 2013; 1(1): 42-47.
29. Cordell GA, Quinn-Beattie ML, Farnsworth NR. The potential of alkaloids in drug discovery Phytother. Res., 15 (2001), pp. 183–205.

30. Paech K, Tracey MV. Modern Methods of Plant Analysis, 3, Springer Verlag, Berlin (1995), pp. 626–654.

31. Rıos J, Recio M. Medicinal plants and antimicrobial activity J. Ethnopharmacol., 100 (2005), pp. 80–84.