Research Article

Pharmacological evaluation and quantification of bioactive metabolites in *Bauhinia variegata* Linn.

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Abstract

Objective: Evaluation and Quantification of Bioactive Metabolites in *Bauhinia variegata Bauhinia variegata* Linn. is well known herbal medicine in Ayurvedic and traditional system of medicine. **Material and methods:** Quantification of lupeol and oleanoleic acid have been done by HPTLC using solvent system toluene: ethyl acetate: formic acid (7:3:1). In-vitro antibacterial and antifungal activity of ethanol and water extract has been done, to evaluate the pharmacological potential of *Bauhinia variegata*. **Results and conclusion:** Lupeol and oleanoleic acid was resolved at R_f 0.76 ± 0.03 and 0.85 ± 0.04 by pre chromatographic derivatization with Anisyldehyde sulphuric acid. The amount of lupeol and oleanoleic acid was found to be 0.0007 and 0.0013 mg/ml in respectively. Considerable antioxidant activity in both the plants were observed showing IC_{50} values of 0.420±0.011 by DPPH method. As compared to ethanol extracts and water extracts of the plants showed stronger growth inhibitions against *Salmonella, Staphylococcus, Pseudomonas, E. coli, Total coliform*. Ethanolic extract are more effective compared to water extract. Plant derived medicines have made significant contribution towards human health and need to extend further for activity guided extraction/isolation of the active chemical moiety.

Keywords: *Bauhinia variegata*, HPTLC, Lupeol, Oleanoleic acid, DPPH, antimicrobial

Introduction

Crude drugs are derived from natural sources like plants, animals and minerals. *Bauhinia variegata* (Family: *Leguminosae*) are the Ayurvedic plant, known as "*Kanchnar*" in hindi, are widely used in folk medicine in different tropical and subtropical regions of the world (Anonymous 1948; Day, *1998*). About 12 species of this genus are found in India. *B. variegata* is also used in traditional system of medicine as antidiabetic (Folin and Ciocalteu, 1927) antimalarial, antimicrobial, treatment of allergic disorders, inflammation, ulcer, and tumor diseases. Other species of these plants has been used to treat allergies, anxiety colds colitis, constipation, depression, diabetes, hypertension, skin problems, and ulcers [Gupta et al., 1980]. The bark powder of the plant is used as a major ingredient in herbal tonic "Kanchanr guggul" an Ayurvedic medicine (Kumar et al., 2012).

Previously reported phytochemical constituents in the leaves of *Bauhinia variegata* are β-sitosterol, tannins, kaempferol-3-glucoside, amides, carbohydrates, reducing sugars, crude protein, vitamin C, fibers, calcium, phosphorus, rutin, quercetin, quercitrin, apigenin, apigenin -7-O-glucoside, dotetracont-15-en-9-ol and heptatriacontan-12,13-diol (Pachouri et al 2005; Lino et al., 2004).

The present study deals with phytochemical and pharmacological investigation in leaves part of *B. variegata* along with quantification of triterpenoids through HPTLC. According to our knowledge, there are very few studies are presently available that documents the biological activities of *B. variegata*. So, in this study, our main goal is to evaluate possible chemical groups, and biochemical activities such as antioxidant, antibacterial and antifungal activity to validate its use in folkloric treatments. Previously quantification of lupeol, and oleanolic acid was not reported in arial part of *B. variegata.* This study may serve a basis for their use in ayurvedic medicinal preparations.

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Materials and Methods

Plant material

Bauhinia variegata was collected in June 2013 from Rajeev Gandhi College of Pharmacy, Bhopal, Madhya Pradesh (India). Sample was authenticated and voucher specimens were deposited in institute's herbarium. Collected sample were washed, shade dried and powdered for further studies.

Chemicals and reagents

Lupeol (> 94% w/w), Oleanoleic acid (> 99% w/w), 1-1-diphenyl-2-picrylhydrazyl (DPPH), α-amylase was purchased from Sigma-Aldrich. Other solvents and chemicals (A.R grade) viz. aluminum chloride, sodium carbonate, Folin's reagent, methanol, ethyl acetate, toluene, formic acid were procured from SD Fine Chemicals, Mumbai, India.

Physico-chemical analysis

Air dried leaves material was used for the quantitative determination of loss on drying, total ash, acid insoluble ash, alcohol and water soluble extractive values, according to standard procedure of Indian Pharmacopoeia and WHO/QCMMPM (Manandhar, 2002).

Preparation of plant extracts

The plant material was manually screened for any impurities and dried in shade, followed by drying in hot air oven at 30˚C and grinded (electric grinder) to fine powder (40 mesh). The powdered sample, 10 gm leaves (Lf.) was treated with petroleum ether for removal of fatty impurity and then subjected for extraction in methanol. Samples were continuously stirred for 6 hrs, followed by standing time of 18 hrs at room temperature and then filtered (Whatman No. 1 filter paper). The extraction process was repeated, till complete extraction and the pooled extract were concentrated under vacuum in a rotatory evaporator (Buchi rotavpour, Switzerland) at 40°C. The extract was finally freeze dried and stored at 4°C for further use. Extractive value of *B. variegata* is 310mg.

High Performance Thin Layer Chromatography

Preparation of working solution (s)

The working solution of standards viz. Lupeol and oleanolic acid (1 mg/ml) and samples (10 mg/ml) was freshly prepared in methanol. For calibration a stock solution of 1 mg/mL, each of lupeol and oleanolic acid were diluted in same solvent to obtain a four working solutions in concentration ranging from 2 – 8 µg/ml. The solutions were filtered through a 0.45 µm Millipore membrane filter (Pall, USA) before application.

HPTLC conditions

High Performance Thin Layer Chromatography is used for separation of the components present in mixture, both quantitatively as well qualitatively (Pawar et al., 2011). For quantitative analysis about 10µl sample was applied using Camag 100 ml sample syringe (Hamilton, Switzerland) on pre-coated plates with silica gel 60F $_{254}$ of 0.2 mm thickness as 6 mm-wide bands positioned 10 mm from the bottom and 15 mm from side of the plate, using CAMAG LINOMAT V automated TLC applicator with nitrogen flow providing a delivery speed of 150nl/s from application syringe. Following sample application, layers were developed in a CAMAG twin trough glass chamber which was pre-saturated with mobile phase toluene: ethyl acetate: formic acid (7: 3: 1 v/v). After development of the plate, it was dried and then derivatized with anisaldehyde-sulphuric acid and scanned at 680nm with a TLC scanner (WINCATS 1.3.2, CAMAG).

Antioxidant activity

Total flavonoid and phenolic content was estimated (Dwivedi and Tripathi, 2018) and expressed in terms of mg/g of QE (Quercetin Equivalent) and mg/g GAE (Gallic Acid Equivalent) based on calibration curve of standard(s) viz. Quercetin and Gallic acid respectively. The radical scavenging potential was analyzed via DPPH radical scavenging assay.

Antimicrobial and Anti fungal activity

Preparation of Methanolic extract

The Methanolic extract of drug by soaking 7.5 g of drug powder in 150 ml of 95% methanol. The mixture was allowed to stay for 72hrs in dark away from direct sun light. It was stirred at 12hrs. The resulting solution was filtered using Whatman filter paper 01.Than the filtrate was evaporated in a shallow dish to dryness. The dried powdered of extract was scratched off the dish and dissolved in small amount of methanol. This solution was used as antimicrobial agent in the test.

Preparation of hot water extract

Hot water extract of the drug sample was prepared by dissolving 7.5g of powdered drug in 150ml of distill water for 4hrs. It was than further extracted using the soxlet apparatus for 2hrs. The resulting infusion was filtered using Whatman filter paper 1. The filtrate was then subjected to evaporation till dryness. The dried powdered of extract was scratched off the dish and dissolved in small amount of distill water. This solution was used as antimicrobial agent in the test.

Preparation of inoculum

An inoculum of *Salmonella, Staphylococcus, Pseudomonas, E. coli* and total coliform was prepared from contaminated water by culturing them in nutrient broth, lactose broth etc. 10ml of inoculum was diluted with 10ml

of the sterilized distilled water just before inoculation. Similarly, mixed fungal culture was used to prepare a fungal inoculum.

Preparation of media

100ml of following media were prepared for the protocol and autoclaved.

Microorganism	Enrichment media used	Code of media
Salmonella	Xyline -lysine deoxycholate agar	M031
Staphylococcus	*S. aureus* broth + agar	M464
Pseudomonas	Cetrimide agar	M024
E. coli	Brilliant green agar	M016
Total coliform	Violet red bile agar	M011

Inoculation

1ml of diluted inoculums was poured in a sterilized petridish. Than about 15ml of autoclaved liquefied media was poured and mixed well. Than the plate were allowed to solidify in a refrigerator for about 2hrs. After that wells were bored on the solidified agar plates with the help of sterile cork borer. 50µl of drug extract was poured into the wells and disk of known antibiotics (Amoxyclave, Chloramphenicol) were kept on the agar surface. All the plates were allowed to stand at room temperature for 1hrs so that the drug diffuses in the agar, than all the plates were incubated at 37ºC for 24hrs. In case of fungal culture, Clotrimazole was used as known antibiotic. The fungal culture plates were incubated at 25ºC for 72hrs. After completion of incubation period the plates were observed for antimicrobial activity and the diameter of zone of inhibition of growth of microorganism was measured (Tiwari et al., *2013*).

Results and discussion

Pharmacological Evaluation and Quantification of Bioactive Metabolites of *Bauhinia variegata* Linn is carried out and total phenolic and flavanoid content and Calibration curve of lupeol and oleanoleic acid were given in table 1and

Table 1. Total phenolic and flavonoid content in *Bauhinia variegata* Linn.

Species	Total phenolic content (mg/g)*GAE	Total Flavonoid content (mg/g)*QE
	y = 0.265x + 0.016 **R^2 = 0.975**	**y = 81.37x - 0.080** **R^2 = 0.981**
Bauhinia variegate Linn.	3.757±0.057	3.98 ±0.0057

*mean ± S.D, n=3

Table 2. Calibration parameter of Lupeol and Oleanoleic acid

Parameters	Lupeol	Oleanoleic acid
Linearity range (mg/ml)	2-8	2-8
R_f	0.76	0.85
Regression eq.	y = 1823.1x + 3315.3	y = 2128.3x + 6610.7
R^2	0.9815	0.9936
Slop	1823.055	2128.34
Intercept	3315.3	6610.75
Average	12430.58	17252.45
Standard deviation	4751.338	5512.999
Standard error	792.1664	0.25283
LOD (µg mL−1)	8.6	8,5
LOQ (µg mL−1)	26.06	25.9

Table 3. In vitro antibacterial analysis of methanolic extract of *Bauhinia variegata* (leaves) compared to known antibiotics: measurement of zone of inhibition (mm)

S. No.	Organism	Zone of inhibition of known antibiotic		Zone of inhibition of drug extract
		Amoxyclav AMC -30	Chloramphenicol C -30	
1	*E. coli*	Nil	25mm	24mm
2	*Pseudomonas*	11mm	22mm	19mm
3	*Salmonella*	Nil	30mm	24mm
4	*Staphylococcus*	23mm	21mm	14mm
5	Total coliform	Nil	23mm	22mm

Table 4. In vitro antibacterial analysis of hot water extracts of Bauhinia variegata (Leaves) compared to known antibiotics: measurement of zone of inhibition (mm)

S. No.	Organism	Zone of inhibition of known antibiotic		Zone of inhibition of drug extract
		Amoxyclav AMC -30	Chloramphenicol C -30	
1	*E. coli*	Nil	25mm	24mm
2	*Pseudomonas*	11mm	22mm	19mm
3	*Salmonella*	Nil	30mm	24mm
4	*Staphylococcus*	23mm	21mm	14mm
5	Total coliform	Nil	23mm	22mm

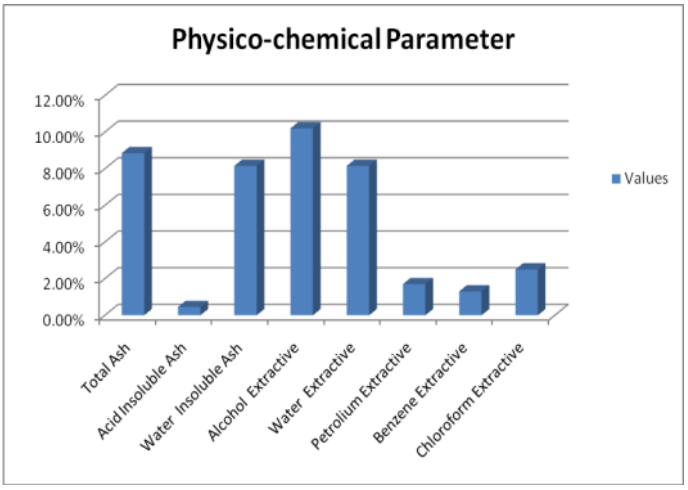

Figure 1. Physico-chemical evaluation of *Bauhinia variegata* Linn.

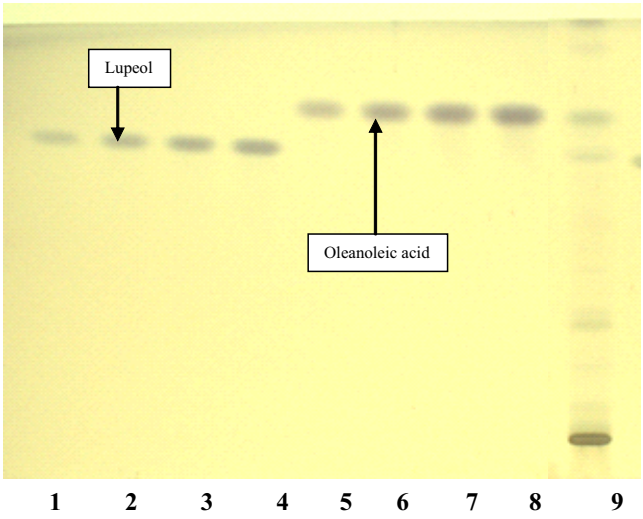

Figure 2. HPTLC fingerprint Chromatograph of *Bauhinia variageta.* **Abbreviation:** 1-4: Lupeol, 5-8: Oleanoleic acid, 9: *Bauhinia variageta*

Table 5. In vitro antifungal analysis of methanolic and hotwater extract of *Bauhinia variegata* (Leaves) as compared to known antibiotics: measurement of zone of inhibition (mm)

S. No.	Organism	Methanolic Extract			Hot Water Extract		
		Zone of inhibition of known antibiotic Clotrimazole CC - 10	Zone of inhibition of drug extract		Zone of inhibition of known antibiotic Clotrimazole CC - 10	Zone of inhibition of drug extract	
			I	II		I	II
1	Mixed fungal culture	21mm	16mm	21mm	28mm	Nil	Nil

2 respectively. In vitro antibacterial analysis of Bauhinia variegata Linn in methanolic, water and hot water extract results were given in table 3-4 respectively. Physico-chemical evalution and HPTLC fingerprint of evaluated Bauhinia variegata Linn were shown in figure 1 & 2. Purity spectra of lupeol oleanoleic acid, 3D densitometry spectra and calibration curve of oleanoleic acid & lupeol were shown in Figure 3–6 respectively. In vitro antibacterial analysis of methanolic, hot water and hot water:methanolic extract of Bauhinia variegata Linn (Leaves) compared to known antibiotics (Zone of inhibition against E.coli, Zone of inhibition against Pseudomonas, Zone of inhibition against Salmonella, Zone of inhibition against Staphylococcus and Zone of inhibition against total Coliform) were shown in figure 7-8 respectively.

It is important that plant should be properly identified and characterized for their physical and chemical

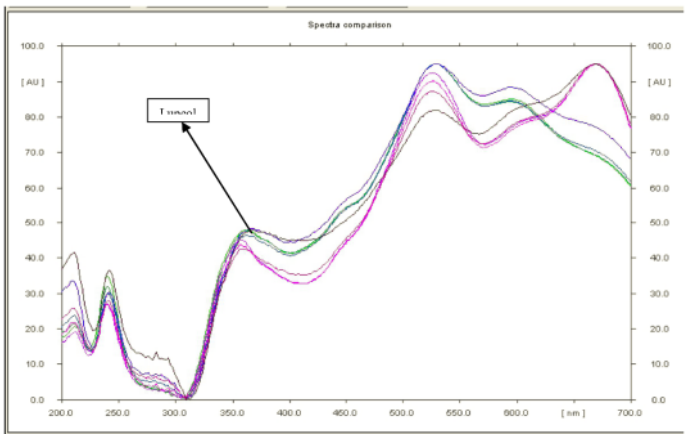

Figure 3. Purity spectra of Lupeol and Oleanoleic acid

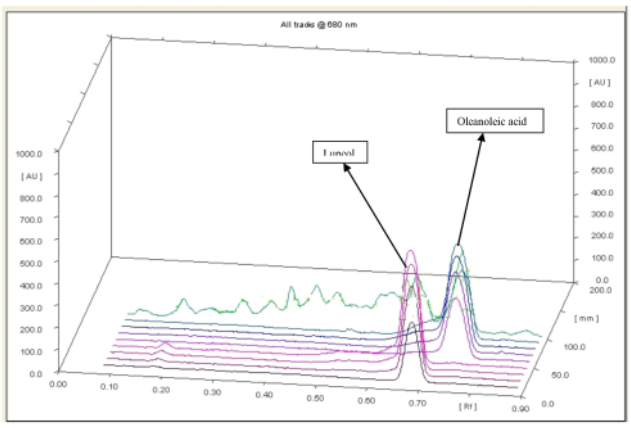

Figure 4. 3D densitometry spectra of *Bauhinia variegata.* **Abbreviation:** 1-4: Lupeol, 5-8: Oleanoleic acid, 9: *Bauhinia variageta* (From bottam to top)

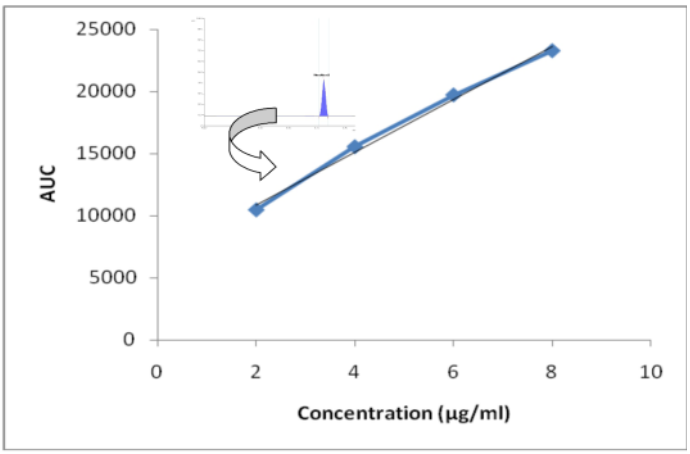

Figure 5. Calibration curve of Oleanoleic acid

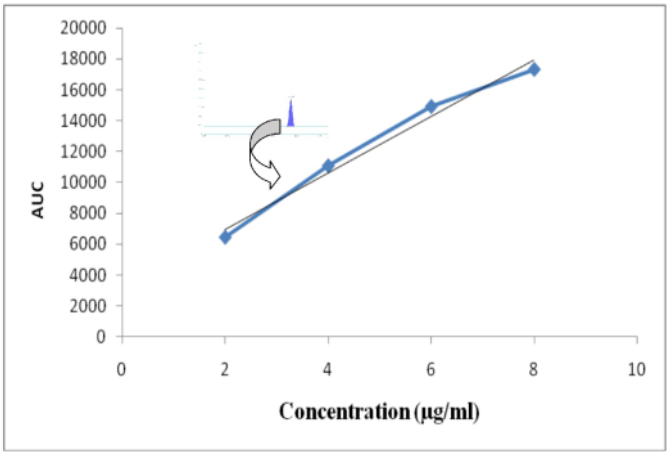

Figure 6. Calibration curve of Lupeol

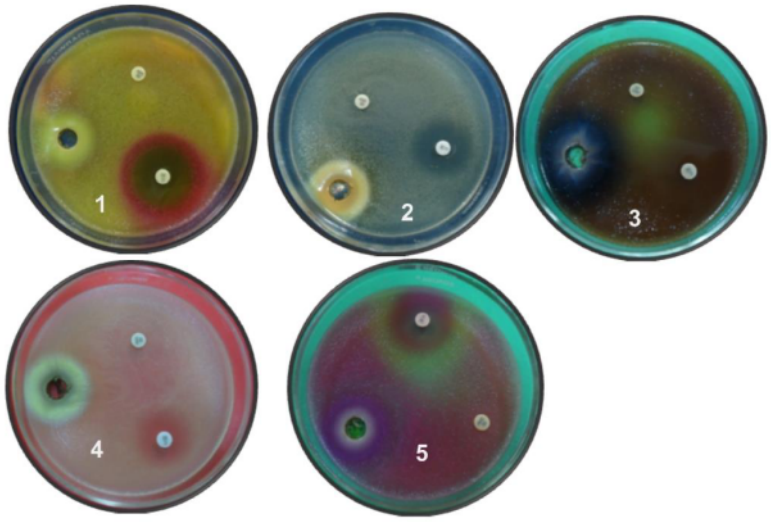

Figure 7. In vitro antibacterial analysis of methanolic extract of *Bauhinia variegata* Linn (Leaves) compared to known antibiotics. 1. Zone of inhibition against *E.coli*, 2. Zone of inhibition against *Pseudomonas,* 3. Zone of inhibition against *Salmonella.* 4. Zone of inhibition against *Staphylococcus,* 5. Zone of inhibition against *total Coliform*

characteristics. Therefore a control on their quality should be enforced. *Bauhinia variegata* Linn is one of that plants which is being scientifically researched. The aim of our study is to provide scientific evidence concerned to the medicinal values of these unexplored plants.

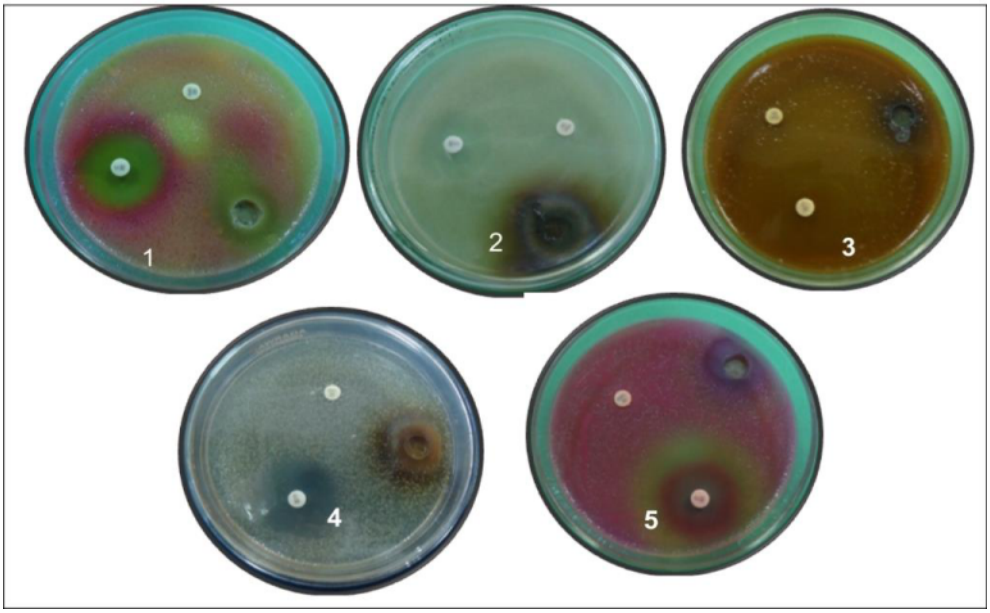

Figure 8. *In vitro* antibacterial analysis of hot water extract of *Bauhinia variegata* Linn (Leaves) compared to known antibiotics. 1. Zone of inhibition against *E.coli*, 2. Zone of inhibition against *Pseudomonas,* 3. Zone of inhibition against *Staphylococcus,* 4. Zone of inhibition against *Salmonella.* 5. Zone of inhibition against *total Coliform.*

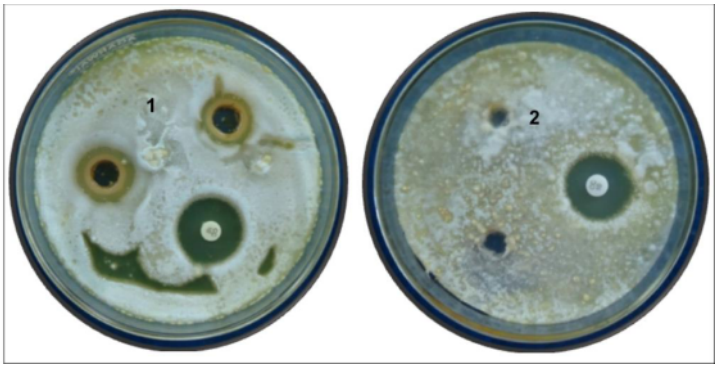

Figure 9. *In vitro* antibacterial analysis of methanolic and hot water extract of *Bauhinia variegata* Linn (Leaves) compared to known antibiotics. 1 Methanolic extract of *Bauhinia variegata* showing zone of inhibition against mixed fungal culture, 2. Hot water extract of *Bauhinia variegata* showing no zone of inhibition against mixed fungal culture

Standardization tests were performed as per API Standard. Air dried leaves material was used for the determination of loss on drying, total ash, acid insoluble ash, water soluble and alcohol soluble extractive value. HPTLC quantification of targeted biomarker viz. lupeol and oleaolic acid has been done on the basis of calibration curve. Four dilutions of standard were used in concentration range of 2-8 µg/ml. TLC plate has been developed by using mobile phase toluene: ethyl acetate: formic acid (7:3:1) at R_f 0.76 and R_f 0.85 respectively. The amount of lupeol and oleanolic acid was found to be 0.0007 and 0.0013 mg/ml in *B. variagata* (Lf.). Densitometry scanning has been done at 680nm.

Antioxidant activities of plant were calculated by three models having different mechanism of action to analyze the potentiality of species as antioxidants. Polyphenolic content *viz*. total phenolic and flavonoid content were estimated (Table 1). Free radical scavenging activity of DPPH is most widely used for screening of medicinal plants having anti oxidant activity. The mechanism however, well evident is due to de-colorization of DPPH through electron donated by anti oxidant compound. The scavenging effect of DPPH radical by methanolic extract of *B. variagata* was concentration dependant and potentially varied for samples and standards (Ascorbic acid, quercetin and rutin). Ascorbic acid exhibits maximum inhibition of 77.57 %, which is followed by quercetin and rutin having inhibition of 72.43% and 71.48 % respectively. *Bauhinia variegate* has IC_{50} value 0.420±0.011mg/ml (Table 2). Methanolic and hot water extract of drug was found to show a significant antibacterial effect against *Salmonella, Staphylococcus, Pseudomonas, E.coli* and total coliform; methanolic being more effective

than water extract(Table 4). Methanolic extract was also found to possess antifungal activity but it was not significant in hot water extract. The higher antibacterial activity of the alcoholic extracts as compare to aqueous extracts might be due to the lack of solubility of active constituents in aqueous solution. It is a good antibacterial agent and can be used in future as antibiotics.

Conclusion

In conclusion, the results obtained from phytochemical analysis will be helpful in the identification, standardization and quality control of the drug. Ethanolic extract of plant show very good exhibition of antioxidant and antimicrobial potential.

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Conflicts of interest: Not declared.

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