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RESEARCH ARTICLE

PHARMACOGNOSY

ANTI MICROBIAL ACTIVITY OF ASHWAGANDHA, SHUNTHI AND SARIVA AGAINST VARIOUS HUMAN PATHOGENS: AN INVITRO STUDY

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ABSTRACT

The different plant extracts were initially screened for their anti microbial activity and then, were extracted in different solvents. The extracts were tested against two Gram positive and four Gram negative organisms by using the standard disc diffusion method. Phytochemical analysis were also carried out that included Assay of Withanoloides for *Withania somnifera* (Ashwagandha) and Assay of Tannin and Saponin for *Hemidesmus indicus* (Sariva) and Assay of Gingerol in *Zingiber officinale* (Shunthi Rhizome). The actives of *Zingiber officinale* were also confirmed by the HPLC method. They were also screened for the presence of both Heavy metals and microbial analysis by standard protocols.

KEYWORDS

Anti microbial activity, HPLC, disc diffusion method, Mc Farland's turbidity, heavy metal, microbial analysis.

INTRODUCTION

Ashwagandha belongs to the family Solanaceae and has a characteristic odour and is bitter in taste.[1] certain antibacterial activity of Ashwagandha has been reported.[2-3] whereas *Zingiber officinale* (Shunthi) belongs to the family *Zingiberaceae* and have an aromatic odour and pungent taste and spicy [4-5].They also show anti microbial and anti oxidant activities[6]. Shunthi consists of dried roots. It is acrid in taste and the odour is similar to vanillin.[1] The chloroform and ethanol (95 %) extracts were reported to possess good anti fungal activity against *Aspergillus.niger* and weak anti bacterial activity against *E.coli*, *S.aureus* and *P.aeruginosa.* The root bark showed antioxidant activity. The root extract demonstrated inhibitory activity against *Mycobacterium leprae* in mice.

The plant extracts were tested for their anti bacterial activity and were also screened for Phytochemical parameters like Assay of Saponin for *Hemidesmus indicus* (Sariva) and Assay of Gingerol in *Zingiber officinale* (Shunthi Rhizome). The actives of *Withania somnifera* and *Zingiber officinale* were also confirmed by the HPLC method and the peaks obtained were compared with that of a standard. They were also screened for the presence of both Heavy metals and microbial analysis by standard protocols.

1 MATERIAL AND METHODS

1.1 Collection of the material*:* All the extracts were procured from a standard supplier in and around India and were stored in suitable conditions.

1.2 Test Organism: All the clinical strains were procured from NCIM, Pune that included two gram positive organisms and 4 gram negative organisms as stated below.

Bacillus subtilis (ATCC 6633, NCIM 2063), *S.aureus* (ATCC 6358, NCIM 2079), *E.coli* (NCIM NCIM: 2065; ATCC: 8739), *P.aeruginosa* (ATCC 9027, NCIM 2200), *K.pneumoniae* and *Salmonella* (NCIM 2257, NCTC 6017).

Immediately they were sub-cultured by inoculating a loopful in Nutrient Broth (Hi Media, M002) and then incubated at 35-37°C for 18-24 hours. They were then streaked onto Nutrient agar (Hi Media, MM 012) plates and the plates were inverted and incubated at 35-37°C for 18-24 hours. They were then stored at 4°C till use.

2 Extraction of active principle:

2.1 Aqueous Extraction: 10 g of the extract was weighed accurately and dissolved in 100 ml of distilled water taken in a 250 ml round flat bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to cool down to room temperature and filtered using a Whatman Filter paper no 1. The filtrate was then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and different aliquots were dissolved in 5 ml sterile water and were stored at 4°C till used for further analysis.

2.2.1 ***Methanolic Extraction:*** 10 g of the extract was weighed accurately and dissolved in 100 ml of methanol solution taken in a 250 ml round flat bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to cool down to room temperature and filtered using a Whatman Filter paper no 1. The filtrate was

then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and different aliquots were dissolved in 5 ml sterile water and were stored at 4°C till used for further analysis.

2.3 ***Chloroform extraction:*** 10 g of the extract was weighed accurately and dissolved in 100 ml of chloroform solution taken in a 250 ml round flat bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to cool down to room temperature and filtered using a Whatman Filter paper no 1. The filtrate was then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and different aliquots were dissolved in 5 ml sterile water and were stored at 4°C till used for further analysis.

2.4 ***Ethyl acetate Extraction:*** 10 g of the extract was weighed accurately and dissolved in 100 ml of ethyl acetate solution taken in a 250 ml round flat bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to cool down to room temperature and filtered using a Whatman Filter paper no 1. The filtrate was then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and different aliquots were dissolved in 5 ml sterile water and were stored at 4°C till used for further analysis.

3*. Preparation of Mc Farland standard Turbidity Standards*

Mc Farland standards were prepared by adding specific volumes of 1% sulphuric acid and 1.174% barium chloride. Mac Farland 0.5 standard were used in this study, which contains 99.5ml of 1% sulphuric acid and 0.5 ml of 1.174% barium chloride. Solution is dispensed into tubes comparable to those used for inoculum preparation, which were sealed tightly and stored in dark at room temperature. The Mc Farland 0.5 standard provides turbidity comparable to a bacterial suspension containing $1.5x10^8$ cfu/ml (NCCLS 1993)

4. In Vitro Anti Bacterial Study:

The modified agar-well diffusion method of Cappuccino and Sherman (1999) was employed to study the antibacterial activity of the plant extracts. 3.7% of Muller Hinton Agar was mixed with hot distilled water and autoclaved at 15 lb pressure for 15 minutes. After autoclaving, it was allowed to cool to 45°C-50°C. Then the medium was poured into sterilized Petri dishes with a uniform depth of approximately 4 mm. The agar medium was allowed to solidify and then stored at 4°C till used for further analysis.

4.1 Disc Diffusion (Kirby–Bauer) Method

The disc diffusion test was done for each isolate on Mueller-Hinton agar. The turbidity of the broth was adjusted according to 0.5 Mc Farland standards by adding sterile saline. A sterile cotton swab was saturated by dipping into standardized bacterial culture. Lawn culture of the test strain was prepared by swabbing to give a uniform inoculum to the entire surface. The plates were allowed to dry, after which wells were bored in the middle of the well with the help of a cork borer and 0.1 ml of the sample was loaded into the well. The plates were first incubated at 25° C for 30 minutes and then shifted to 37°C for 18 – 24 hours. After incubation the plates were examined and zone of inhibition were measured. All the tests were carried out in triplicates and their mean value was calculated. To screen the anti bacterial activity against the tested organisms a standard was used which was antibiotic amoxycillin (5 mg/ml) which showed a good zone of inhibition against the tested organism.

5. RESULTS

Sample Concentration (500 mg/ml)

Table 1
The mean zone of inhibition exhibited by the test organism against Withania somnifera (Ashwagandha)

Sr No	Name of the Organism	Aqueous Extraction	Methanolic Extraction	Chloroform Extraction	Ethyl acetate Extraction
1	*E.coli*	No ZOI	17.66 mm ±0.622 mm	No ZOI	No ZOI
2	*P.aeruginosa*	No ZOI	15.33 mm ±0.622 mm	No ZOI	No ZOI
3	*Bacillus subtilis*	No ZOI	No ZOI	No ZOI	No ZOI
4	*S.aureus*	No ZOI	15.33 mm ±0.622 mm	No ZOI	No ZOI
5	*K.pneumoniae*	No ZOI	No ZOI	No ZOI	No ZOI
6	*Salmonella*	15.66 mm ±0.622 mm	No ZOI	No ZOI	No ZOI

ZOI: *Zone of Inhibition*

Table 2
The mean zone of inhibition exhibited by the test organism Hemidesmus indicus (Sariva)

Sr No	Name of the Organism	Aqueous Extraction	Methanolic Extraction	Chloroform Extraction	Ethyl acetate Extraction
1	*E.coli*	15 mm ±0.605 mm	20.3 mm ±0.622 mm	8 mm ±0.816 mm	No ZOI
2	*P.aeruginosa*	12 mm ±0.577 mm	No ZOI	20 mm ±0.816 mm	No ZOI
3	*Bacillus subtilis*	19 mm ±0.622 mm	21 mm ±0.816 mm	18.3 mm ±0.622 mm	12 mm ±0.622 mm
4	*S.aureus*	19.6 mm ±0.622 mm	21.3 mm ±0.622 mm	14.3 mm ±0.848 mm	No ZOI
5	*Salmonella*	19.6 mm ±0.622 mm	23.3 mm ±0.622 mm	No ZOI	No ZOI

ZOI: *Zone of Inhibition*

Table 3

The mean zone of inhibition exhibited by the test organism Zingiber officinale (Shunthi)

Sr No	Name of the Organism	Aqueous Extraction	Methanolic Extraction	Chloroform Extraction	Ethyl acetate Extraction
1	*E.coli*	18.66 ±0.816 mm	19 mm ±0.577 mm	13.66mm ±0.605 mm	No ZOI
2	*P.aeruginosa*	21.3 mm ±0.605 mm	22.3 mm ±0.622 mm	No ZOI	25.3 mm ±0.846 mm
3	*Bacillus subtilis*	23.3 mm ±0.605 mm	30.3mm ±0.605 mm	No ZOI	25 mm ±0.816 mm
4	*S.aureus*	14.6 mm ±0.622 mm	19.3mm ±0.232 mm	No ZOI	16 mm ±0.577 mm
5	*Salmonella*	13.3 mm ±0.622 mm	8.6 mm ±0.816 mm	15.3 mm ±0.622 mm	16.3 mm ±0.622 mm

ZOI: *Zone of Inhibition*

6. Microbial Analysis

Microbial analysis was carried out for all the three extracts as per procedure of Indian pharmacopoeia 2007 and WHO Guideline. It included total bacterial count, Total yeast and mould Count, Presence of *Escherichia coli,* Presence of *Salmonella abony,* Presence of *Pseudomonas aeruginosa,* and Presence of *Staphylococcus aureus.* Pure culture of *Escherichia coli* (NCIM: 2065; ATCC: 8739), *Salmonella abony* (NCIM: 2257 NCTC: 6017), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6358). Obtained from NCIM Pune were used as control. The media used for the microbial limit test were of HiMedia Pvt. Ltd.[7-8]

The results are as shown in Table 4

Table 4

Microbial Analysis Report of Withania somnifera (Ashwagandha), Hemidesmus indicus (Sariva) and Zingiber officinale (Shunthi)

Sr No	Name of the Sample	Limit	Ashwagandha	Sariva	Shunthi
1	Total Bacterial Count	NMT 10^7 cfu/g	15 x 10^2 cfu/g	25 x 10^2cfu/g	38 x 10^2 cfu/g
2	Total Fungal Count	NMT 10^5 cfu/g	Absent	Absent	Absent
3	*E.coli*	Absent	Absent	Absent	Absent
4	*Salmonella abony*	Absent	Absent	Absent	Absent
5	*Pseudomonas aeruginosa*	Absent	Absent	Absent	Absent
6	*Staphylococcus aureus*	Absent	Absent	Absent	Absent

7. Heavy Metal Analysis

Accurately weigh 2 g of the sample in a kjendal flask. An acid mixture of HNO_3:$HClO_4$ (4:1) was added in the flask and heated continuously till the solution becomes colorless. The sample was then transferred to a 25 ml volumetric flask and volume was made up with distilled water. A reagent blank was synchronously prepared accordingly to the above procedure. The standard of Lead (Pb), Cadmium (Cd), Arsenic (As) and Mercury (Hg) were prepared as per the protocol in the manual and calibration curve developed for each of them. The sample were analyzed for the presence of Pb, Cd, As, and Hg using atomic absorbance spectrophotometer (AAS) 6300 (by SHIMADZU) [9]

The results are as shown in Table 5

Table 5
Heavy Metal Analysis of the three Extracts

		Heavy Metal (ppm)		
Sr No	**Extracts**	Ashwagandha	Sariva	Shunthi
1	Lead (10 ppm)	1.203	1.067	1.605
2	Cadmium (0.3 ppm)	0.055	0.067	0.054
3	Arsenic (10 ppm)	0.121	0.378	0.240
4	Mercury (1 ppm)	0.056	0.048	0.071

8. *Phytochemical Analysis:*

Phytochemical evaluation were carried out including different tests like Assay of Alkaloids, Assay of Saponin and Assay of Gingerol.[10-12]

The results are as shown in Table 6

Table 6
Phytochemical Analysis of all the three extracts

Sr No	**Name of the Sample**	**Assay**	**Result**
1	*Withania somnifera* (Ashwagandha)	Assay of Alkaloids	0.60 %
2	*Hemidesmus indicus* (Sariva)	Assay of Tannin	10.58 %
		Assay of Saponin	15.56 %

9. High Performance Liquid Chromatography (HPLC)

It is a chromatographic technique that is used to separate a mixture of compounds. HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The pump provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better

separation on columns of shorter length when compared to ordinary column chromatography.

9.1 HPLC of Zingiber officinale (Shunthi):

Chromatogram:

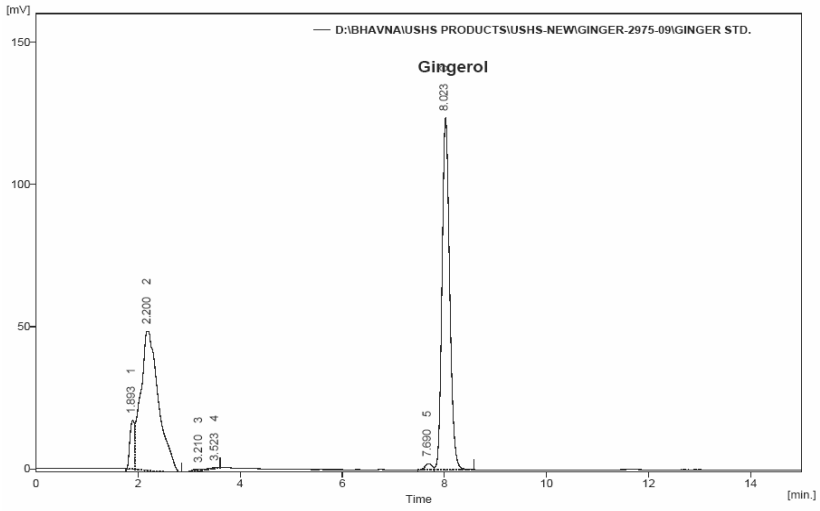

Figure 1

Reference Standard of 6 – Gingerol

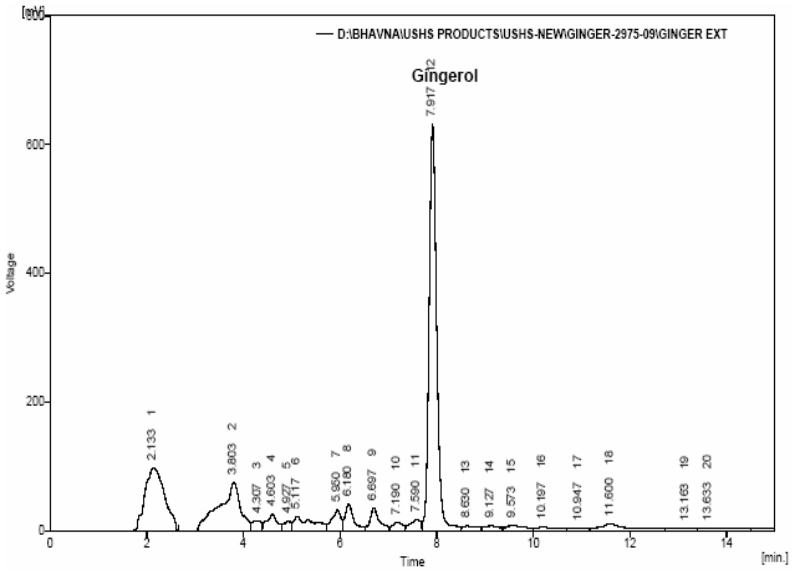

Figure 2

Extract of Zingiber officinale

Result of HPLC: The Extract of *Zingiber officinale* (Figure 2) showed 5.56 % result of 6- Gingerol with comparison to the reference standard that is depicted in the (figure). The purity of the reference standard was however known to be 98.6 %

CONCLUSION

The study thus concluded that the three extracts were shown to have anti microbial activity and a good amount of the active was present in the sample. Ayurveda since time immemorial has always known to show tremendous efficiency and the three extracts that have been studied above are used in day to day life and have tremendous medicinal values as well.

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