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Original Research Article

ANTIMICROBIAL SCREENING OF *HETEROPHRAGMA ADENOPHYLLUM* EXTRACTS AND EFFECTS OF LIGHT IRRADIATION

Muhammad Shoaib Akhtar*, Sajid Bashir and Nabeela Tabbasum Sial

Department of Pharmacology, Faculty of Pharmacy, University of Sargodha, Sargodha-40100, Pakistan

SUMMARY

Medicinal plants have considerable widespread use as crude drugs in the primary health care of communities. Various plants have already been tested for their antimicrobial activity to discover new antimicrobial agents. In this investigation antimicrobial activities of aqueous and methanolic extracts of *Heterophragma adenophyllum* leaves and seeds were screened as these have been used since centuries in traditional medicine for the treatment of skin and urinary tract infections and also as antidiarrheol and antidiabetic agents. Preliminary phytochemical screening of *Heterophragma adenophyllum* leaves showed presence of saponins, alkaloids and glycosides while *H. adenophyllum* seeds contained flavonoids, alkaloids, glycosides, tannins and saponins. Testing of antimicrobial activity of aqueous and methanolic extracts of the plant was conducted at the concentrations of 1 and 2mg /disc against three gram-positive strains including *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus epidermidis* (ATCC 12228) and two gram-negative strains viz. *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 27853) by disc diffusion method. The *H. adenophyllum* leaves showed maximum activity against *S. aureus* as the zone of inhibition was 22.37 ± 0.32 mm but aqueous extract of the leaves showed no activity against *S. epidermidis*. The methanolic extract of *H. adenophyllum* seeds showed maximum activity against *E. coli* producing 16.33 ± 0.31 mm zone of inhibition, but showed no activity against *S. epidermidis*. The aqueous extract of *H. adenophyllum* seeds showed maximum activity against *S. aureus* and *S. epidermidis* with 22.13 ± 0.09 mm and 23.43 ± 0.35 mm zones of inhibition. Furthermore, data obtained showed that there was a significant variation in the zones of inhibition produced by the test samples of the plant parts exposed to sunlight nakedly or wrapped in aluminum foils and those kept in cupboard when compared with controls kept unirradiated.

Keywords: Antimicrobial effect, *Heterophragma adenophyllum*, light irradiation

Corresponding Address: Muhammad Shoaib Akhtar, Department of Pharmacology, Faculty of Pharmacy, University of Sargodha, Sargodha-40100, Pakistan. Email: drmsakhtar@gmail.com

INTRODUCTION

Antimicrobial agents like antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. The available antibiotics have become less and less effective over the past

few decades against certain infections not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria. Thus it has become essential to develop newer antimicrobial drugs with lesser resistance [1]. Control of microorganisms is critical for the prevention and treatment of infectious diseases. A vast majority of synthetic antibiotics control the growth and development of microorganisms effectively but they are highly toxic at their optimum dosage level. Among the proposed strategies, antimicrobial screening of medicinal plants offer the potential for developing new and potent broad spectrum antibiotics [2].

The plant derived antibiotics have already played important role to cure several serious diseases. Approximately 60-80% of the total population depends on traditional medicines to cure common illnesses and the medicinal plant drugs continue to play a central role in the healthcare systems of large proportions of the world's population, particularly in developing countries, where herbal medicine has a long and uninterrupted history of use [3]. According to World Health Organization (WHO), up to 80% of the population in Africa depends on traditional herbal medicine for primary health care, accounting for around 20% of the overall drug market [4]. The popularity of such plants in these communities owes largely to their local availability and price affordability [5] which also confirms their effectiveness.

The current state of development of new antimicrobial drugs is not encouraging with only a few new ones being licensed in recent years [6, 7]. This mismatch between the slow development of new drugs and the fast emergence of resistant strains makes the future management of infectious diseases look bleak. As an alternative and perhaps a sustainable option, attempts to improve the efficacy of available antibiotics, particularly the older and cheaper ones have been suggested [8].

Heterophragma adenophyllum leaves and seeds have antimicrobial folkloric use. However, as far as ascertained, no detailed scientific study was carried out to assess the antimicrobial activity of the indigenous medicinal plants. Therefore, present study was conducted to determine the antimicrobial activity of aqueous and methanolic extracts of leaves and seeds of this plant and to also to observe the effects of light irradiation on the activity.

MATERIAL AND METHODS

Plant materials

The plant tested in the study was *Heterophragma adenophyllum* (Beeripatta) and its leaves and seeds were collected from campus of Sargodha University. They were got identified and authenticated by the Botany Department of the University. The plant parts were shade dried and powdered finely with a Chinese herbal grinder. The powdered material was stored in well closed cellophane bags at 4 °C in a refrigerator.

Bacterial strains

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus epidermidis* (ATCC 12228) were obtained from a standard microbiology laboratory in Lahore.

Inoculum preparation

Inoculums were prepared by transferring a large no. of bacterial cells from bacterial cell culture to test tube having 10ml nutrient broth and incubated for 24 hours at 37°C. The tubes were shaken periodically to accelerate growth [9].

Chemicals used

Acetone (Merck, Darmstadt, Germany), Mueller Hinton Agar (Oxide, USA), Nutrient Agar (Oxide, USA), Methyl red (Merck, Darmstadt, Germany), Nutrient Broth (Oxide, USA), Commercial antibiotics discs (Oxide, Basingstoke, Hampshire, England), HCl (Merck, Darmstadt, Germany) and Methanol (Merck Chemical Co, Darmstadt Germany) were obtained.

Preparation of plant extracts

Methanolic extract of the powdered leaves and seeds of *Heterophragma adenophyllum* were prepared by cold maceration. The filtrates were concentrated in rotary evaporator and were then air dried and the percentage yield was 13.76% [10]. For preparation of aqueous extract, 500g of powdered *Heterophragma adenophyllum* leaves and seeds were soaked in distilled water for 24 hrs. After filtration these were frozen dried in lyophilizer and percentage yield was 13.9% [11].

Antimicrobial susceptibility testing

The antimicrobial assay for plant extract(s) against different bacterial strains was conducted by disk diffusion method [12]. Filter paper discs prepared from Whatman No.1 having diameter of 6mm were loaded with 1mg and 2mg of dried extract per disc. Discs were dried and sterilized [13]. 0.1ml of culture (3×10^8 cells/ml) according to McFarland's solution as standard was spreaded over Mueller Hinton Agar. Filter paper discs were placed over the surface of media with flamed forceps pressed gently to ensure contact. The plates were placed for 2hrs in refrigerator to ensure prediffusion of the extract into the surface of the agar. The plates were incubated at 37°C overnight. Commercial antibiotics such as gentamicin, streptomycin, ciprofloxacin and tobramycin were used as positive control. After incubation period the antibacterial activity was determined by measuring the zones of inhibition in mms i.e. diameter of the paper disc plus diameter of zone.

Determination of the effects of irradiation on the antibacterial potentials of plant extracts:

The effect of sunlight on the activity of methanolic and aqueous plant extracts was determined. Each extract divided into three samples in test tubes. Two of the test tubes were placed under the sun with one tube wrapped completely with aluminum foil and the other bare. The third tube was kept in a dark cupboard. Test tubes were placed in sunlight for an average of 10 hrs per day over a period of 28 days. Unirradiated samples in methanol and water (UMC and UAC) were used as controls (14). Antibacterial activity of irradiated and unirradiated samples will be evaluated.

Statistical analysis

The triplicate data were subjected to ANOVA (Analysis of variance) test and Means $\pm$ SEM were calculated. Student t-test was performed to evaluate the significance of the data. Probabilities of < 0.05 were considered statistically significant.

RESULTS

Phytochemical screening of *Heterophragma adenophyllum* leaves

Heterophragma adenophyllum leaves powder was tested for the presence of tannins and phenolics, saponins, glycosides, flavonoids and alkaloids. Its leaves showed positive test for saponins, tannins, alkaloids, glycosides and showed negative results for the flavonoids, Fehling's test for reducing sugars in glycosides and also negative test for anthraquinones as summarized in Table 1.

Table 1: Phytochemical constituents and identification tests for *Heterophragma adenophyllum* leaves

Test	Observations	Inferences
1. Frothing test for saponins	Thick persistent froth for 10 minutes	Saponins present
2. Dragendroff reagent test	Presence of colored ppt.	Alkaloids present
3. Ferric chloride test for tannins	Appearance of green color	Tannins present (hydrolysable tannins present)
4. Keller kiliani test for glycosides	Brown ring appears at junction of two solutions.	Glycosides present
5. Test for anthracene derivatives	No pink color	Absence of anthracene derivatives
6. Test for flavonoids HCl test	Yellow color soln. does not turns colorless with HCl	Flavonoids absent

Phytochemical screening of *Heterophragma adenophyllum* seeds

Heterophragma adenophyllum seeds powder was tested for the presence of tannins and phenolics, saponins, glycosides, flavonoids and alkaloids. The seeds showed positive test for saponins, tannins and phenolics, flavonoids, cardiac glycosides and showed negative results for Fehling's test for reducing sugars in glycosides, alkaloids and also negative test for anthraquinones as given in table 2.

Antimicrobial activity of *Heterophragma adenophyllum* leaves after light irradiation

Antimicrobial activity of *Heterophragma adenophyllum* leaves extract (aqueous and methanolic) was determined by using disc diffusion method of antimicrobial susceptibility testing and also effects of light irradiation on three samples that were placed in sunlight compared with unirradiated aqueous and methanolic samples. Tables 3 and 4 give the zones of inhibition (ZOI) of aqueous and methanolic extract irradiated and unirradiated samples

Table 2: Phytochemical constituents and identification tests for *Heterophragma adenophyllum* seeds

Test	Observations	Inferences
Frothing test for saponins	Thick persistent froth for 10 minutes	Saponins present
Dragendroff reagent test	No presence of colored ppt.	Alkaloids absent
Ferric chloride test for tannins	Appearance of green color	Tannins present (hydrolysable tannins present)
Keller kiliani test for glycosides	Brown ring appears at junction of two solns.	Glycosides present
Test for anthracene derivatives	No pink color	Absence of anthracene derivatives
Test for flavonoids HCl test	Yellow color soln. turns colorless with HCl	Flavonoids present

Table 3: Zones of inhibition of the methanolic extracts of *Heterophragma adenophyllum* leaves (irradiated and unirradiated samples)

Zones of inhibition in mm (Means $\pm$ S.E.M)					
Sample	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. epidermidis</i>
MES	18.2 $\pm$ 0.15	6.1 $\pm$ 0.09	5.1 $\pm$ 0.10	6.1 $\pm$ 0.12	14.1 $\pm$ 0.06
MWS	19.2 $\pm$ 0.08	6.3 $\pm$ 0.09	5.2 $\pm$ 0.22	6.2 $\pm$ 0.12	14.3 $\pm$ 0.15
MC	21.5 $\pm$ 0.29	6.2 $\pm$ 0.09	6.2 $\pm$ 0.06	6.1 $\pm$ 0.12	16.3 $\pm$ 0.15
UMC	22.3 $\pm$ 0.32	7.0 $\pm$ 0.15	6.7 $\pm$ 0.25	7.1 $\pm$ 0.57	17.1 $\pm$ 0.12
P-value	0.00*	0.16*	0.00*	0.00*	0.00*

Values are means of three determinations $\pm$ S.E.M; * - Significant (P < 0.05)

MES: methanolic extract exposed to sunlight

MWS: methanolic extract wrapped in aluminium foil exposed to sunlight

MC: methanolic extract placed in cupboard

UMC: unirradiated sample of methanolic extract

Table 4: Zones of inhibition of the aqueous extracts of *Heterophragma adenophyllum* leaves (irradiated and unirradiated samples)

Zones of inhibition in mm (Means $\pm$ S.E.M)					
Sample	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. epidermidis</i>
AES	13.1 $\pm$ 0.12	10.3 $\pm$ 0.12	7.2 $\pm$ 0.09	7.4 $\pm$ 0.17	Nil
AWS	12.1 $\pm$ 0.09	11.4 $\pm$ 0.09	7.4 $\pm$ 0.03	7.5 $\pm$ 0.06	Nil
AC	12.4 $\pm$ 0.18	12.3 $\pm$ 0.09	9.0 $\pm$ 0.15	8.4 $\pm$ 0.19	Nil
UAC	20.1 $\pm$ 0.17	12.8 $\pm$ 0.32	9.7 $\pm$ 0.44	9.0 $\pm$ 0.15	Nil

Values are means of three determinations $\pm$ S.E.M, and P < 0.05

AES: aqueous extract exposed to sunlight

AWS: aqueous extract wrapped in aluminium foil exposed to sunlight

AC: aqueous extract placed in cupboard

UAC: Unirradiated sample of aqueous extract

Antimicrobial activity of *Heterophragma adenophyllum* seeds after light irradiation

Antimicrobial susceptibility testing by disc diffusion method of *Heterophragma adenophyllum* seed extract (aqueous and methanolic) was performed against five bacterial strains *S. aureus* (ATCC 25923), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 27853), *B. subtilis* (ATCC 6633) and *S. epidermidis* (ATCC 12228). Tables 5 and 6 showed the zones of inhibition (ZOI) of aqueous and methanolic extract irradiated and unirradiated samples.

Table 5: Zones of inhibition of the methanolic extracts of *Heterophragma adenophyllum* seeds (irradiated and unirradiated samples)

Zones of inhibition in mm (Mean $\pm$ S.E.M)					
Sample	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. epidermidis</i>
MES	10.2 $\pm$ 0.12	15.5 $\pm$ 0.12	5.1 $\pm$ 0.06	8.3 $\pm$ 0.18	Nil
MWS	10.2 $\pm$ 0.10	15.5 $\pm$ 0.21	5.0 $\pm$ 0.07	8.2 $\pm$ 0.12	Nil
MC	11.1 $\pm$ 0.12	16.1 $\pm$ 0.09	5.4 $\pm$ 0.06	8.6 $\pm$ 0.12	Nil
UMC	10.6 $\pm$ 0.06	16.3 $\pm$ 0.31	5.3 $\pm$ 0.10	9.3 $\pm$ 0.23	Nil
P-value	0.01*	0.03*	0.00*	0.00*	

Values are means of three determinations $\pm$ S.E.M; * - Significant (P < 0.05)

MES: methanolic extract exposed to sunlight

MWS: methanolic extract wrapped in aluminium foil exposed to sunlight

MC: methanolic extract placed in cupboard

UMC: unirradiated sample of methanolic extract

Table 6: Zones of inhibition of the aqueous extracts of *Heterophragma adenophyllum* seeds (irradiated and unirradiated samples)

Zones of inhibition in mm (Mean $\pm$ S.E.M)					
Sample	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. epidermidis</i>
AES	14.4 $\pm$ 0.24	9.6 $\pm$ 0.15	7.0 $\pm$ 0.09	9.0 $\pm$ 0.09	21.5 $\pm$ 0.26
AWS	20.6 $\pm$ 0.12	9.1 $\pm$ 0.17	6.9 $\pm$ 0.12	9.2 $\pm$ 0.15	21.6 $\pm$ 0.12
AC	21.8 $\pm$ 0.12	10.7 $\pm$ 0.09	7.6 $\pm$ 0.28	10.3 $\pm$ 0.07	22.8 $\pm$ 0.12
UAC	22.1 $\pm$ 0.09	11.0 $\pm$ 0.20	7.8 $\pm$ 0.25	10.5 $\pm$ 0.35	23.4 $\pm$ 0.35

Values are means of three determinations $\pm$ S.E.M, and P < 0.05

AES: aqueous extract exposed to sunlight

AWS: aqueous extract wrapped in aluminium foil exposed to sunlight

AC: aqueous extract placed in cupboard

UAC: Unirradiated sample of aqueous extract

Comparative antimicrobial activity of standard antibiotics with plants extract(s)

Antimicrobial activities of standard antibiotics for like streptomycin, gentamicin, tobramycin and ciprofloxacin were compared with the unirradiated plant extracts (methanolic and aqueous), summarized in Table 7. The standard antibiotic discs were also placed on the Mueller-Hinton agar plates containing bacterial culture in the same manner as the discs impregnated with plant extracts placed on plates in the triplicate manner.

Table 7: Antimicrobial activity of plants compared with standard antibiotic discs

Micro-organism	Zones of Inhibition of Antibiotics in mm (Mean $\pm$ S.E.M)		<i>H.adenophyllum</i> Leaves Me/Aq	<i>H. adenophyllum</i> Seeds Me/Aq
<i>S. aureus</i>	Gentamicin (10ug)	30 $\pm$ 0.03	22.37 $\pm$ 0.32	10.60 $\pm$ 0.06
	Streptomycin(10ug)	34 $\pm$ 0.04	13.17 $\pm$ 0.17	22.13 $\pm$ 0.09
<i>E. coli</i>	Gentamicin (10ug)	29 $\pm$ 0.05	7.07 $\pm$ 0.15	16.33 $\pm$ 0.31
	Ciprofloxacin(5ug)	31 $\pm$ 0.07	12.87 $\pm$ 0.32	11.00 $\pm$ 0.20
<i>P. aeruginosa</i>	Tobramycin (10ug)	34 $\pm$ 0.03	6.77 $\pm$ 0.25	5.30 $\pm$ 0.10
	Ciprofloxacin(5ug)	30 $\pm$ 0.06	9.70 $\pm$ 0.44	7.87 $\pm$ 0.25
<i>B. subtilis</i>	Gentamicin (10ug)	28 $\pm$ 0.01	7.13 $\pm$ 0.57	9.33 $\pm$ 0.23
	Ciprofloxacin(5ug)	30 $\pm$ 0.03	9.07 $\pm$ 0.15	10.57 $\pm$ 0.35
<i>S. epidermidis</i>	Gentamicin (10ug)	32 $\pm$ 0.03	17.13 $\pm$ 0.12	Nil
	Streptomycin(10ug)	35 $\pm$ 0.01	Nil	23.43 $\pm$ 0.35

DISCUSSION

In the present study, it has been observed that the extracts of the tested plant possess significant antibacterial activity against certain gram negative and gram positive pathogenic bacteria. The results are in agreement with the earlier studies [14; 15; 16].

In our study, antimicrobial activity of the methanolic and aqueous extracts of *Heterophragma adenophyllum* have showed marked variation in sensitivity against pathogenic bacteria three of which were gram-positive such as *S. aureus*, *B. subtilis*, *S. epidermidis* and two were gram-negative i.e. *E. coli* and *P. aeruginosa*. The activity was comparable to some standard antibiotics including streptomycin, gentamicin, tobramycin and ciprofloxacin. The variation in the effectiveness of the extracts against different microorganisms would be due to difference in the chemical composition of the extracts. In the disc diffusion method reduction in bacterial growth is determined on the basis of zone of inhibition around the disc. An inhibition zone of 14mm or more has been considered to be high antibacterial activity [17]. In our study high antibacterial activity was thus produced by the test plant extracts.

The methanolic extracts of *H. adenophyllum* leaves showed maximum zones of inhibition of 22.37 $\pm$ 0.32 mm and 17.13 $\pm$ 0.12 mm against *S. aureus* and *S. epidermidis*, respectively (Table 6), while the methanolic extracts of its seeds produced highest activity against *E. coli*, i.e. 16.33 $\pm$ 0.31 mm mean zone of inhibition as summarized in Table 7. The aqueous extract of *H. adenophyllum* leaves produced maximum activity against *S. aureus* and *E. coli* with zones of inhibition of 20.17 $\pm$ 0.17 mm and 12.87 $\pm$ 0.32 mm, respectively (Table 7), while the aqueous extract of its seeds produced highest activity against *S. aureus* and *S. epidermidis* with mean zones of inhibition 22.13 $\pm$ 0.09 mm and 23.43 $\pm$ 0.35 mm, respectively (Table 6).

Phytochemical analysis of *H. adenophyllum* leaves have showed the presence of saponins, alkaloids, tannins (Table 1), while its seeds contain flavonoids in addition to the saponins, tannins and glycosides (Table 2). Xu-Hx and Lee 2001 [18] have reported that strong antibacterial activity of some plants is due to flavonoids which are active against several microorganisms. They have been found to be active *in-vitro* to be effective against a wide

array of microorganisms [19]. However, tannins and saponins have also been found to contribute to compounds the antibacterial properties. Many plants rich in alkaloids have been shown to possess some antimicrobial activity against a number of microorganisms [20]. There were, therefore, differences in antimicrobial activity of plants due to different concentration of their constituents.

In the present study, the aqueous extract of *H. adenophyllum* leaves and methanolic extract of its seeds did not produce any antimicrobial activity (Table 6, 7) against *S. epidermidis*. It may be possible that some plant species lack the antimicrobial constituents which may be responsible for antimicrobial activity or do possess those constituents but not in sufficient quantity to produce the effect. The aqueous and methanolic extracts showed clear cut color changes when exposed to sunlight irradiation. The color of the samples before irradiation was bright-green but it changed to light-green due to sunlight exposure. However, change in color is not considered as determinant of decomposition. It is a sign that process of decomposition has started [21]. The photochemical degradation has been reported to occur as a result of improper or inadequate storage and distribution of the products leading to their deterioration and decomposition resulting in reduced bioactivity [22]. Photo-degradation is a major form of instability not only for certain allopathic drugs but also for the herbal products. In the case of herbal preparations the situation is further complicated due to the multi-component nature of the products and the presence of unknown chemical structures [23; 24; 25].

In the present study the results shown in Table 7 suggested that plant extracts produced less zones of inhibition than the test standard antibiotics. There was a significant variation in the zones of inhibition of samples that were exposed to sunlight directly, wrapped in aluminium foil and placed in cupboard as compared to unirradiated control sample. Our results have also revealed that sunlight has not caused significant photo-deterioration. Therefore, it is recommended to conduct further activity-oriented fractionation studies to isolate active principle(s) of this indigenous medicinal plant showing considerable antimicrobial activity.

The development of antimicrobials from higher plants seems rewarding as it would lead to the discovery of new therapeutic agents or lead compounds required for the treatment of resistant infections. Plant-derived antimicrobials would have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobial agents [26]. However, detailed pharmacological and phytochemical studies are still needed to prove the real utility of the medicinal plant and to isolate the active principles and determination of their mechanism(s) of action. In conclusion, it has become evident that aqueous and methanolic extracts of the plants used in the study possess good antibacterial activity against some pathogenic organism *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. epidermidis*. The results also revealed that samples with unirradiated condition possess more antibacterial activity than irradiated samples against pathogenic organisms. The study has also suggested that there is need to educate traditional medicine practitioners on the need to protect their herbal products from direct sunlight as most of them expose their products in open markets in an attempt to attract customers. The organisms tested are known causative agents for skin diseases, urinary tract infections and abdominal diseases. Therefore, the results obtained have supported the use of the plant in ethno-medicine for the treatment of various infections. The present study can ultimately help in identification of novel/lead natural compounds which may lead to development of new antimicrobial agents from these plants.

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