Int.J.Curr.Res.Aca.Rev.2021; 9(11): 65-71

International Journal of Current Research and Academic Review

ISSN: 2347-3215 (Online) Volume 9 Number 11 (November-2021)

Journal homepage: http://www.ijcrar.com

doi: https://doi.org/10.20546/ijcrar.2021.911.008

Invitro Anti-Bacterial Activities of Aqueous of 80% Methanol Leaf Extract of *Stephania abyssinica* Selected Human Pathogenic Bacteria

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Abstract

Bacterial diseases are an oversized burden of morbidity and mortality globally. The emergence of antimicrobial resistance may be a major public unhealthiness worldwide, particularly in low- and middle-income countries. Therefore, screening of energetic antimicrobial drugs from herbal sources is important. Thus, this study was aimed to evaluate the antibacterial activity of medicinal plant: *Stephania abyssinica* is employed in Ethiopian traditional medicine for the treatment of stomachache, headache and ant -microbial. Dried leaves of the plant were extracted using maceration technique. The powder was soaked in methanol 1:10 solute-solvent ratio then, placed on a shaker for 72 hours. The filtered extracts were concentrated during a rotary evaporator and lyophilizer for alcoholic and aqueous extracts respectively. Antibacterial activity test was conducted against selected human pathogenic bacterial species (standard strains) using the agar-well diffusion. Furthermore, the extracted phytochemical content was identified and safety of the plant was tested on *Swiss albino* mice. Phenol, Flavonoids, terpenoids, alkaloids, steroids, saponins, tannins, steroidal glycosides and glycosides were detected in plants extract. Plants leave extracts were not toxic up to 2000mg/kg on the test mice.

Article Info

Accepted: 15 October 2021
Available Online: 20 November 2021

Keywords

waste disposal, food preparation practices, climate, viruses, bacteria, protozoa or fungi.

Introduction

Pathogenic microorganisms are microscopic infectious agents that belong either to viruses, bacteria, protozoa or fungi. The prevalence of bacterial pathogens is extremely associated with poor hygienic conditions and also the infection varies looking on the facility, waste disposal, food preparation practices, and climate. Countries like Ethiopia within which people live under poverty and tropical region microbial pathogens particularly human pathogenic bacteria are more common and affect an unlimited majority of individuals (1). The current challenge within the fight against bacterial diseases is drug-resistance. Drug-resistant bacteria become a priority not only thanks to their severity but also, they require more complex treatment and expensive diagnostic tests and even cause untreatable infections (2). To beat the matter of antibiotic resistance, screening traditional medicinal plants for the event of novel therapeutics is too much needed (3). Antimicrobial resistance (AMR) is widely observed together of the key public health concerns of the 21st century (4). Globally, the emergence of drug resistant bacterial strains has been reported and this challenge limits the effectiveness of current drugs and significantly causing failure within the treatment of infectious diseases (4). Several European

reports show that, severe infections thanks to gram-negative bacteria, including multidrug-resistant (MDR) strains that are proof against multiple classes of antibiotics are significantly increasing (4, 5).

The historical utilization of plants as a health remedy both for human and animal is centuries old. It has been recognized that plants have the capacity to combat several types of diseases in ethnoveterinary medicines, a term generally used for folk skills, beliefs, knowledge, practices, methods related to animals 'health and cure of various ailments in the rural areas (7).

Medicinal plants are widely used by all sections of the population with an estimated 7500 species of plants used by several ethnic communities (8). Plant extracts from roots, barks, stems, leaves, flowers, fruits and seeds can be used to treat different types of infections (8,9). Extract of Medicinal plant also represent an important source of drugs in the process of developing new pharmacologically active compounds (9).

Traditionally, the dried and powdered leaves of *Stephania abyysinica* are either topically applied to treat wounds or taken orally to neutralize different inflammatory conditions and Anti-Malarial Activity. The leaves are also used for the treatment of swellings and sexually transmitted disease (10, 11). Screening of energetic antimicrobial drugs from herbal sources are important as many previous studies demonstrated that herbal remedies are promising antimicrobial activities. This study was at aimed undertaking preliminary phytochemical screening, test toxicity on mice and in-vitro antibacterial activity of leaf crude extracts of these plants.

Materials and Methods

Plant Materials Collection and Identification.

Fresh leaves of *Stephania abyssinica* (local name: Kalalaa) were collected from Oromia Region at Sululta and areas. The collected leaves were filled with a bag and transported within the same day after collection.

Classified and authentication of the plant specimen was done by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, AAU and a voucher specimen (DG001) was deposited for future reference. The leaves of *S. abyssinica* were carefully cleaned to get rid of dirt and soil. The leaves were then dried at temperature under shade drying roof of MCMB and reduced to appropriate size and powdered finely using grinding machine.

Extract preparation

The plants samples were washed twice with tap water and once with sterile distilled water to eliminate adhering dust and any foreign particles and allowed to dry shade in Biomedical Research Laboratory of DMCMB at CNCS, AAU at room temperature for about 15 days. The dried samples were grinded with an electric grinder, sieved with a fine mesh of 500micrometer sieve size and stored at room temperature until considered for extraction.

The sieved powder forms of *Stephania abyssinica* (100g) were socked in 1000ml methanol 80% in separate 500ml Erlenmeyer flask on a rotary shaker (GFL, model 3020, Germany) with 120rpm at room temperature for 72 hours according to (12). The extracts were first filtered using four-fold muslin cloth then by filter paper Whatman No.1 (Whatman Ltd., England).

The final filtrates were concentrated in a rotary evaporator (BUCHI type TRE121; Switzerland) with 60rpm at a temperature of 45°C.and were further dried in oven at 45°C and the rest deposited under deep freeze for 24hours; the extracts were separated by using lyophilizer (CHRIST ALPHA1-4, Germany) at -40°C with vacuum pressure and the crude extracts were placed at -20°C until use.

Phytochemical Detection.

The methanol crude extracts of leaves of *Stephania abyssinica* were screened for the presence of bioactive compounds like phenol, flavonoid, tannins, steroids, terpenoids, steroidal glcosides, alkaloids, saponins, resin and glycosides in accordance to standard phytochemical methods.

Alkaloid Test

In this test, the extracts were dissolved in dilute (2%) Hydrochloric acid and filter with Whatsman filter paper. The filtrates were treated with Dragendorff reagent. Reddish brown precipitate was observed (13).

Glycoside Test

Two milliliters of glacial acetic acid were added to 5ml of plants extracts. Then 1 drop of 5% FeCl3 and 5ml of

conc. H2SO4 were added along the side of the test tube respectively. Brown color was formed at the junction of two layers which indicates the presence of glycosides (14).

Flavonoid Test

For this test, few drops of 10% NaOH were added to 1ml of the extracts then an intense yellow color formed. Few drops of 2% HCL were again added to yellow color mixture and allowed for a colorless change that indicated the presence of flavonoids (15).

Phenol Test

To determine the presence of phenol in the extracts, the dissolved extracts were treated with few drops of 5% glacial acetic acid and 5% $NaNO_2$ solution. The brown precipitate occurs indicates the presences of phenol in the extracts (13).

Resin Test

For this test, 2ml of distilled water was poured into 1ml of extract dissolved in acetone. Turbidity of the solution was observed to determine the presence of resin (13).

Saponin Test

To do this test, extracts solution were mixed with 20ml of distilled water and continuously shakes for 15 minutes. The formation of foam indicates the presences of saponin (13, 14).

Steroidal Glycosides Test

This test done by dissolving the 2ml of the tested extracts with glacial acetic acid and makes it cool. Add 2 drop of 5% FeCl2 and transfer it into a test tube which contains 2ml of concentrated H2SO4. The reddish-brown color was observed at the junction of two layers (16).

Steroid Test

To identify the presence of steroids in the extracts, 1ml of extracts were dissolved in 10ml of chloroform, and then 10ml of concentrated H2SO4 was added by the side of the test tube. The red color was formed in the upper layer and sulphuric acid layer showed yellow color with green fluorescence which shows the existence of steroids (16).

Tannin Test

This test is determined by adding few drops of 1% lead acetate in 2ml of stock extract solution. The formation of yellowish precipitate indicates the presences of tannins in the extracts (14).

Terpenoid Test

For this test mixing extracts solution with 2 ml of chloroform with the careful addition of concentrated H2SO4. The formation of a reddish-brown color at the interface confirmed the presence of terpenoids (12).

Test of Organisms

Bacterial Strains

Standard American Type Culture Collection (ATCC) isolates of different gram-negative and gram-positive bacterial strains were used. The standard strains used were *S. agalactie* (ATCC12386), *E. coli* (ATCC 25922), *K. pneumonia* (ATCC700609), *aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923), S. *typh*i (ATCC 13311) and listeria. The standard strains were obtained from the Ethiopian Public Health Institute (EPHI) Microbiology Department. Those bacterial strains were maintained in appropriate culture media.

Bacterial inoculum preparation

All standard bacterial strain was activated in NBA and brain heart infusion. The standard strains of *K. Pneumonia*, listeria S, agalacte, streaked on nutrient agar, *S. aureus* streaked on mannitol salt agar, *S. typhi* on S.S agar, E. coli on ethyl methyl blue (EMB) agar and the operating standard procedures. Three to five well-isolated colonies selected from an overnight culture at 37^{o}C of selective media were touched with sterilized loop of inoculator and transferred into a tube containing 5ml of sterile nutrient broth and cultured for 24 hours at 37oC.

Then the bacterial isolates were made to grow on nutrient agar (Oxoid LTD., Basingstoke, and Hampshire, England) for 24 hours at 37oC. Least three to five well-isolated colonies of the same morphological type were selected from an overnight culture at 37oC on nutrient agar plate medium and transferred into a pre-labeled tube containing 99.9% sterile physiological saline to dilute the suspension. The turbidity of the actively growing bacterial culture was adjusted optically comparable to

that of the 0.5 McFarland standards (that was prepared by adding a 0.5ml aliquot of 0.48 mol/L BaCl2 to 99.5 ml of 0.18 mol/L H2SO4 (1%v/v) through visually comparing the physiological saline inoculum tube and the 0.5 McFarland standards against a card with a white background and contrasting black lines which is resulted in a suspension containing approximately 1.5 x 108 CFU/ml for microorganism. The prepared saline culture (suspension) was used to inoculate the Mueller Hinton agar (MHA) (Oxoid LTD., Basingstoke, Hampshire, England) plates for antibacterial susceptibility testing.

Standard Antibiotics

Chloramphenicol was used as positive control for *E. coli*, *klebsiella pneumonia* and *Salmonella typhi* and Ampicillin was used for listeria, *S. aurous* and *S. agalactie* as positive control; whereas DMSO used as negative control for the antibacterial susceptibility test.

Antibacterial susceptibility test

Antibacterial activity was carried out using Agar well - diffusion method. Bacterial strains grown on nutrient agar at 37°C for 18 h were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards (10^6 Colony Forming Units (CFU)/mL). Briefly, 50µl inoculums were used to inoculate 90-mm diameter petri plates containing 25 mL Mueller-Hinton Agar (MHA), with a sterile non-toxic cotton swab on a wooden applicator. Wells with 6-mm diameter were punched in the agar and filled with 100 µl the dissolution of the organic extracts (methanol) was facilitated with the addition of dimethyl sulfoxide (DMSO) which not affected the growth of microorganisms (as shown by our control experiments). The dishes were pre incubated at 4°C for 2 h to allow uniform diffusion into the agar. After pre incubation, the plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the inhibition zone diameter observed. In addition, The DMSO used as a negative control, Chloramphenicol and Ampicillin were used as a positive control were to determine the sensitivity of the strains by the disc diffusion method the experiment was performed in triplicate (17).

Oral Acute Toxicity Determination

The test was performed according to Organization of Economic Cooperation and Development (OECD) guideline No. 420 (OECD. 2001). Twenty female Swiss albino mice weighing 20-25g were used for the toxicity test. Those mice were obtained from the animal house of the DMCMB. The mice were randomly selected, marked and distributed to permit individual identification and kept in their cages for at least 5 days prior to dosing. Then they were housed in clean cages to allow for acclimatization to the laboratory conditions. They were fed with standard mice pellet diet water and leyee's. The mice were left fasting for 3hours prior to dosing.

Following the period of fasting, the mice were weighed by sensitive digital electronics beam balance (A & D Company, Japan) and distributed in to four groups of five mice in each group. Then mouse was administered orally with the dose of 1000, 1500 and 2000mg/kg bodyweight (BW) of methanol crude extracts of plants for group one, two and three respectively and water was administered for the negative control mice group by using specially designed mice oral needle (Cadence Science Ltd., America). The extracts were delivered orally based on the average BW of the mice in each group. After the extract administration, the animals were observed within 30 minutes, 4 hours and after 24 hours checking for signs of behavioral manifestations and the mice were further checked for 14 days for any signs of acute toxicity. BW of the mice were further measured on days 7 (D7) and 14 (D14) to notice any change.

Data Analysis

Data were analyzed by using SPSS software, version 23 (IBM SPSS, United States). The results were presented as the mean ± standard deviation (SD) and statistical significance was considered at 95% confidence interval ($P<0.05$).

Results and Discussion

Yield of the Crude Extracts

In this study, the method used for the extraction achieved methanol extract yields were calculated according to (17).

$$\text{Percentage extract yield (\%)} = \frac{\text{Weight of dried extracts}}{\text{Weight of extract powder}} \text{ x } 100$$

The percentage yields of crude extracts of the leaves of *Stephania abyssinica* at concentration ratio of 100 mg powder dissolved in 1000 ml of solvents. Then weight dried extract is 17.63 g.

Table.1 Phytochemical screening of Crude extract of Stephania Abyssinica Leaves

Stephania abyssinica metabolites	Methanol extracted from leaf
Phenol	+
Alkaloids	+
Saponin	+
Glycoside	-
Tannin	+
Terpenoid	+
Steroids	-
Resin	-

Table.2 Antibacterial activity of *Stephenia abbsinica* leaf extracts

		Mean value of plant extract inhibition (mm) against pathogens					
Stephania abyssinica	**MoE dose**	*K.pneumoniae*	*E. coli*	*Agalacte*	*Listeria*	*S.aureus*	*S.typhi*
	400mg/ml	16.66±0.5	20±3.6	14.6±2.1	18±1.0	14.8±1.5	16±0.5
	300mg/ml	14.35±0.5	15±1.0	13±1.0	15.6±0.5	10.3±1.5	12±0.5
	200mg/ml	9.33±0.5	11.3±1	8.3±0.5	10.6±1.4	7.5± 0.5	8.33±1
	0.03mg/ml	24±1	27±2	26.33±1.0	26±1	27.33±1.5	24.3±1

Table.3 The effect of methanol crude extract of *S. abyssinica on* body weight of experimental mice

Test Plant	Dose	MBW±S		
Stephania abyssinica	Mg/kg	D0	D7	D7
	1000	23.600±1.1	26.60±0.5	29.6±O.5
	1500	23.40±1.1	26.40±0.5	30±1.2
	2000	24.00±0.7	27.400±0.5	30.20±0.8
	Constant	23.70±0.8	27.00±0.7	29.85±0.8

Phytochemical Detection of *Stephania Abyssinica* Leaves

Phytochemical constituents are bioactive compounds with many antibacterial activities. In this study, phytochemical detection test of leaves of *Stephania abyssinica* indicates the presence of various secondary metabolites or active compounds as shown in Table 1. Variations were also observed in the concentration of the phytochemical constituents of the plant species and the solvents used. Therefore, the phytochemicals including phenol, flavonoid, alkaloid, saponin and tannin in methanol extracts of *Stephania abyssinica* were determined whereas Resin, glycoside and steroids are absent.

Antibacterial Susceptibility Tests

In this study, the antibacterial activity of the methanol extracts of *Stephania abyssinica's* leaves were evaluated against six standard selected human pathogenic bacteria species using agar-well diffusion method. The plant extracts were shown varying degree of inhibition against the test organisms at a concentration of 400mg/ml, 300 mg/ml and 200 mg (Table 2). The methanol extracts of *Stephania abyssinica* leaves indicated considerable

antibacterial activity against standard bacterial strains. The test organisms: *E. coli, Klebsiella pneumoniae, salmonella listeria, S. aurous and agalactia* were susceptible to *Stephania abyssica* leaf. Antibacterial activity *Stephania abyssinica* on test organisms is due to the availability of different phytochemicals including phenol, flavonoid, alkaloid, saponin and tannin which had incredible antimicrobial effects of the same genus (19).

There were significant differences among the crude extracts and the positive control chloramphenicol and Ampicillin ($p<0.05$) where chloramphenicol was highly susceptible to *E. coli, Klebsiella, pneumoniae* and *Salmonella typhi* and Ampicillin were highly susceptible to *listeria, S. aurous and S. agalactie*. Likewise of *Stephania abyssinica* methanol leaf extracts indicated considerable antibacterial activity against both standard bacterial strains.

All six organisms were susceptible to methanol extract of *S. abyssinica* leaf except, *P. aeruginosa* strains were resistant to extracts. Standard *E. coli* was the most susceptible for methanol crude extract among all test organisms and *S. aurous* was least susceptible.

Results of Acute Toxicity of *Stephania abyssinica* Leaves

In this study, acute toxicity shows no mortality in the groups of mice that received water (negative control) and the groups that received 1000, 1500, and 2000mg/kg BW of the methanol extract after 24 hours. Uniformly, there was no acute toxicity with a dose level of 2000mg/kg BW of the methanol extract based on oral doses recommended by OECD guidelines 420 for testing acute toxicity.

Even after 14 days, the mice did not show any BW reduction. Table 4 shows the mean bodyweights (MBW) of the mice in the three groups before and after 14 days of treatment. There were BW gains in all treatment and control groups. No behavioral changes were observed in any of the groups. When compared to control group, BW of the treatment groups did not significantly change.

Throughout 14 days of monitoring, there was significant weight gain in both control and treatment groups ($p<0.05$). Since BW is among parameters of extract oral acute toxicity, the result of this study revealed that the leaf of *Stephania abyssinica* was non-toxic to the mice up to the dose of 2000 mg/kg.

The comparable increment of BW in both controls and treatment groups confirmed the absence of crude extracts effect on their BW change. Generally, the methanol extract of a *Stephania abyssinica* leaves were not toxic on *Swiss albino* mice up to the dose 2000 mg/kg.

The preliminary qualitative phytochemical detection of the plants confirmed presence of major bioactive molecules and the presence of those secondary metabolites explain the considerable role of the plants against the test bacterial strains. *Stephania abyssinica* plants have comparable antimicrobial activity against test bacterial strains. Among test bacterial strains E. coli was highly susceptible bacterial strain and *S. auruos* the least susceptible. In oral acute toxicity test, the mice treated with crude methanol extracts of both plants had no any clinical signs, death or BW reduction within 14 days up to oral dose of 2000mg/kg.

Acknowledgement

The authors are grateful for the help provided by Addis Ababa University and Ambo University for their chemical, material and financial support.

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How to cite this article:

Dejene Guta and Fikadu Kumsa. 2021. *Invitro* Anti-Bacterial Activities of Aqueous of 80% Methanol Leaf Extract of *Stephania abyssinica* Selected Human Pathogenic Bacteria. *Int.J.Curr.Res.Aca.Rev.* 9(11), 65-71.
doi: https://doi.org/10.20546/ijcrar.2021.911.008