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Original Article

In vivo antimalarial and antidiabetic activities of *A. indica* leaf extracts from Ethiopia, with in vitro antimicrobial activity and essential oil characterization

Ermias Sissay Balcha¹, Mulugeta Mengistu^{1*}, Fanueal Belayneh², Adugna Abdi Woldesemayat³, Mesfin Tafesse Gemedat³, Ebrahim Mamma Abda³, Musa Mohammed Ali¹

¹School of Medical Laboratory Science, College of Medicine and Health Sciences, Hawassa University, Hawassa, Sidama Regional State, Ethiopia

²School of Public Health, College of Medicine and Health Sciences, Hawassa University, Hawassa, Sidama Regional State, Ethiopia

³Department of Biotechnology, College of Biological and Chemical Engineering, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

* Corresponding author: ysnmss@yahoo.com

Abstract

Background: *A. indica* is the most versatile medicinal plant. It is known as “the village pharmacy”. The plant is well known for its broad spectrum of biological activity in India and various countries throughout history by many different human cultures. The present study was undertaken to determine the antimalarial and antidiabetic properties of the leaf extracts of *A. indica* grown in Ethiopia when treated *in vivo*. This work has also concerned with determining essential oil composition and an antimicrobial activity of the plant *in vitro*.

Methods: Leaf extracts were prepared using three different selected solvents. Standard and clinical isolates were treated with extracts of the leaves of *A. indica* using agar well diffusion method. The antimalarial and antidiabetic tests were conducted *in vivo* in mice. Phytochemical screening was done using various chemical tests and the volatile oil constituents were determined using Gas chromatography–mass spectrometry (GC-MS).

Results: *In vivo* antimalarial activity studies showed 85.23%, 69.01% and 81.54% suppression of parasitemia for 70% ethanol, acetone and water extracts respectively. The extracts collected from the leaves also showed reduced blood sugar level in alloxan induced diabetic mice. In addition, the solvent extracts were shown to have inhibitory effect on the growth of microorganisms under the study. The minimum inhibitory concentration (MIC) ranged from 850 to 1050 µg/ml. The phytochemical investigation of the ethanol extracts showed the presence of secondary metabolites. Seventeen compounds (mainly sesquiterpenes) that represent 75.45% of the essential oil were characterized by GC/MS analysis.

Conclusion: The findings of this study demonstrate that leaf extracts of *A. indica* cultivated in Ethiopia retained biological activities comparable to those reported for plants grown in their native habitat. This suggests that the Ethiopian agroecological conditions do not adversely affect the plant's bioactive properties and support its potential as a reliable source of biologically active compounds for therapeutic and pharmaceutical applications. In addition, phytochemical investigation and GC-MS analysis of volatile oil constituents showed comparable results to those presented in India and elsewhere.

Key words: *A. indica*, *in vivo*, antimalarial activity, antidiabetic activity, alloxan, mice, phytochemical screening

Introduction

The use of medicinal plants by human being is an ancient practice, perhaps as old as human being himself (1). Even in the presence of allopathic medicine, different populations in the world have, to a large extent, the belief in herbal medicine, possibly due to the assumption that herbal remedies are safer and cheaper than the expensive treatments by Western practitioners (2). Over 100 pure chemical substances isolated from higher plants are used in modern medicine for the same or related medicinal use with the original traditional therapeutic claims (3). Traditionally used medicinal plants also served as sources of starting material (lead compounds) for the development of more complex synthetic compounds (1-3). Plant-based medicine offers therapies that do not even exist in conventional medicine, such as silymarin, a fraction containing three flavanolignins from the fruits of *Silybum marianum* to protect liver (4).

Perhaps no other botanical comes closer to the definition of a panacea than *A. indica* (commonly known as neem) a tropical evergreen native to India for more than 2000 years. Findings of recent investigations revealed a dramatic increase worldwide interest in *A. indica*, and many products are now manufactured with this

miraculous herb. For instance, many standardized, commercial preparations are on market, e.g. neem tea bags (Neem & Muarubaini 25s tea bags) in Kenya and capsules from fractionated leaf extract (Iracarp) in Nigeria (5). Every part of this fascinating plant has been used to treat hundreds of different maladies and is included in the pharmacopeia of a range of traditional medicine systems worldwide (6). It has been used for the management of rheumatism, leprosy, chronic syphilitic, intestinal helminthiasis, respiratory disorders, constipation and sores and also as a general health promoter (7). The leaf extracts of *A. indica* showed superior anti-malarial and anti-hyperglycemic activity; its importance has been published, 1992, by the US National Academy of Sciences entitled 'Neem – a tree for solving global problems'(8). The plant-based extracts have also been evident to control the microbial diseases (9). In addition, neem contains essential oil, which is used to treat a range of skin infections, as well as a variety of molecules that have the ability to interfere with a variety of pathological illnesses (10). Chemical characterization studies identified more than 300 neem compounds from various parts of the plant which are considered the potential for safeguarding the health of animals and humans (11).

Although there are several reports on the immense biological activities and chemical composition of *A. indica* worldwide, there has been a research gap on studying the plant biological activities based on modern scientific investigations in Ethiopia. Traditional medicines are used by around 80% of the Ethiopian population to meet their healthcare needs (12). Therefore, the present work aims at investigating the biological activities of the leaf extracts of *A.indica* specifically *in vivo* antimalarial and antidiabetic effect and *in vitro* antimicrobial activity. The present study also undertakes to examine the chemical constituents of the plant through phytochemical screening and GC/MS analysis of volatile oil.

Materials and methods

Collection of plant materials

All necessary permits were obtained for the described study from national and regional authorities. The leaves of the plant were collected from different localities of Ethiopia to access neem tree (*A. indica* leaves) resources. The catapult method, which is a technique used to administer substances orally to small laboratory animals, particularly rodents such as mice and rats, during oral gavage when a feeding needle or gavage tube is used, was used to collect fresh and mature leaves of *A. indica* from the selected sample sites. An

experienced botanist from Haramaya University was employed to collect the samples. The plants were identified taxonomically by Mr. Melaku Wondafrash and confirmed using morphological and anatomical techniques. The leaves of the plant were authenticated at the National Herbarium, Department of Botany, Addis Ababa University and voucher specimens (voucher number **E0012**) were deposited. The leaf samples were washed thoroughly in running tap water, air-dried under a shed at room temperature and made into powder using pestle and mortar. The powdered plant materials were packed in plastic bags for about 15 days until extraction.

Extraction of plant materials

The powdered *A. indica* leaves, 100 g, were macerated with 100 ml of 70% ethanol overnight and then extracted with 70% ethanol in a Soxhlet extractor, filtered out using a Whatman No. 1 filter paper. The filtered extract was concentrated under vacuum in a rotary evaporator to remove ethanol at a temperature not exceeding 60°C and 30% of water was removed using Lyophilizer (freeze dryer) (13). The dried extract has a 13.5% (w/w) percentage yield and it was kept in a tightly closed amber colored bottle in a refrigerator at 4°C until used for the experiment. The same

procedure was followed to prepare the aqueous and acetone extracts.

Extraction of essential oil

The essential oil was distilled by hydro distillation of the freshly collected leaves in a Clevenger type apparatus. Usually 200 g of the leaves of *A. indica* was chopped and dissolved in a flask containing 1.5 L of distilled water. The extraction was started by heating the flask for 3 h and condensation was carried out continuously (14). The distilled essential oil was recovered, dried in anhydrous sodium sulphate, filtered and the solvent was removed by a rotary evaporator. The oil was kept in a refrigerator at 4°C till GC/MS analysis was conducted.

Preparation of test solution

The acetone and 70% ethanol extracts of *A. indica* were solubilized in 5% Tween 80 in distilled water. The hot water extract was found to be freely soluble in water. However, for the *in vitro* antimicrobial activity the acetone and 70% ethanol extracts were dissolved in dimethyl sulfoxide.

Acute toxicity test

The crude leaf extracts of *A. indica* were assessed for their toxicity in non-infected male Swiss albino mice weighed 30 grams and aged between 6 to 8 weeks as per the standard guideline of the Organization for Economic Cooperation and Development

(OECD) (15). The animals were divided randomly into five groups of five mice per cage. Four test groups and a 'negative control, received 0.2 ml 5% Tween 80 (vehicle). They were acclimatized, weighed and then fasted over night while only taking water (16). Animals in the first four groups were dosed orally (0.2 ml) with 1000, 2000, 3000 and 5000 mg/kg of the 70% ethanol, acetone and aqueous *A. indica* leaf extracts respectively. The dose levels of the extracts were selected based on previously established methods for the determination of acute toxicity test for plant extracts (17,18). The extract was given to the starved mice. The animals were then observed continuously for 1 hour, intermittently for 4 hours, and over a period of 72 hours for toxicity signs such as a change in physical appearance (lacrimation, hair erection, weakness in movement), behavioral changes and death according to the standard toxicity study guideline of OECD (15,17). Standard mice pellets (food) were given after four hour of administration of the extracts. Throughout the experiments, all animals were treated humanely according to the criteria outlined by international guidelines for the care and use of laboratory animals (19).

In vivo antimalarial tests

The protocol for animal studies was approved by the Institutional Animal

Experiments Ethics Review Committee of Addis Ababa University. A 4-day suppressive standard test was used as it was designed by Peters and adopted by Fidock (20) and *P. berghei* Anka strain that most widely infects rodents was used to infect mice. In-house bred mycoplasma free male Swiss albino mice weighing 30 g each were obtained from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia. For the study, a donor mouse was anesthetized with sodium pentobarbital and then infected with a rising parasitemia of 20 % *P. berghei* and blood was collected from the auxiliary vessel. The collected blood was then diluted with trisodium citrate and saline solution so that each 0.2 ml contained approximately 10^7 infected red blood cells.

The test mice were allowed to acclimatize to the laboratory environment under a controlled temperature of 20°C for 3 days before being subjected to the experiments. The experiments were carried out in a quiet laboratory setting with ambient illuminations and a temperature close to one in the animal sanctuary. The test animals were anesthetized with sodium pentobarbital and inoculated intraperitoneally 0.2 ml of a 1:4 dilution of blood from the donor mouse in saline and trisodium citrate. The inoculum contained about 10 million parasites per gram body weight that was expected to produce a

steadily rising infection in mice. The *P. berghei* infected mice were then weighed and randomly divided into six groups of five animals each. Animals in groups I, II, III and IV received 200, 400, 600 and 800 mg/kg/day of 70% ethanol extract of *A. indica* leaves respectively. Animals in group V were used as negative control and received only the vehicle (5% Tween 80) while the sixth group was used as a positive control, received the standard antimalarial drug, chloroquine, 25 mg/kg/day. The animals were dosed orally using an oral feeding tube for four consecutive days, commenced three hours after infection i.e. from day zero (D0) to the day three (D3) (17,21). The total volume administered was 200 μ L. The same procedure was used for the acetone and aqueous extracts of the plant. The dose levels of the extracts were selected and applied based on the previously established traditional methods of usage of plant extracts (18).

Thin smears of blood films were prepared on the fifth day (D4) using blood samples collected from the tail of each mouse under mild sodium pentobarbital anesthesia. The blood specimen was fixed with methanol and stained with the conventional Gemsa staining method for parasitemia count. The microscope had an Ehrlich's eyepiece and a nose diaphragm showing about 100 red blood cells per field.

The parasite count was outsourced for the reliability of the data and carried out by an experienced blinded technician. The percentage parasitemia and percentage chemo-suppression were calculated using

$$\% \text{ Parasitemia} = \frac{\text{Number of infected RBC}}{\text{Number of infected RBC} + \text{number of non-infected RBC}} \times 100\%$$

$$\% \text{ Suppression} = \frac{\text{Parasitemia in negative control} - \text{parasitemia in study group}}{\text{Parasitemia in negative control}} \times 100\%$$

It was anticipated clinically that mice in negative control groups might die, thus animals health and behavior were monitored every hour to reduce potential pain or distress with euthanasia especially when mice have demonstrated weakness in body movement and some other severe behavioral changes. The intent is to end a study before the development of pain or distress, as is emphasized in the OECD document (15). No mice were died before the meeting criteria for euthanasia. The researchers were well trained and able to make impartial judgments about an animal's well-being and ensured a proper outcome for both the animals and the study.

In vivo antidiabetic tests

Twenty five male albino mice were kept fasting for 24 hours with water and made diabetic by a single intraperitoneal injection (a volume of 1 mL/kg) of freshly

the formula given below (20). Parasitemia is the quantitative content of parasites in the blood. The animals in all the groups were also monitored for their survival for 15 days.

prepared alloxan monohydrate solution (20 mg/kg body weight). Alloxan interferes with the glucose metabolism by damaging pancreatic beta cells, resulting in a rise in blood glucose levels. It was prepared by weighing according to individual animal weight and solubilized with 0.5 mL sodium citrate at pH 4.5. Blood glucose level was checked before alloxanisation and 48 hours after alloxanisation. Mice were considered diabetic when the blood glucose level was raised beyond 200 mg/100 ml of blood (22). The animals were weighed and randomly divided into five treatment groups of five mice in each group. The mice were treated with the plant extracts after two days of alloxan injection. Two of the groups received 600 mg/kg and 1000 mg/kg of the 70% ethanol extract of *A. indica* leaves. The third group, diabetic untreated, received the vehicle (5% v/v Tween 80 in distilled water)

and was used as negative control and the fourth group is served as positive control and received the standard antidiabetic drug (glibenclamide, 10 mg/kg suspended in 2% v/v Tween 80 solution) (23). The fifth group did not get any treatment (normal healthy controls). The mice were monitored for 21 days and blood samples were collected from the tail vein under mild sodium pentobarbital anesthesia. Blood glucose level was measured on day 0 (pre-treatment), day 1, day 7, on day 14 and 21 after application of extracts as per the established method (24). The same procedure was used for the acetone and aqueous extracts of the plant.

Bacterial inoculum preparation

Standard strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC27853) and undesignated strains of *Streptococcus pyogenes* (clinical isolate) were used during this study. These organisms were grown on Mueller Hinton agar. Isolated colonies (4-5) from 18-24 hours old bacterial culture were obtained (to minimize the risk of picking bacteria that had lost their resistance) with a sterilized spatula and sub cultured to a tube with 3 ml Mueller Hinton broth by shaking until it achieves turbidity of concentration 10^8 cfu/ml which is almost equivalent to 0.5 MacFarland standards (commercially available and prepared by

adding 0.5 ml of 0.048 M BaCl₂ to 99.5 ml of 0.36 M NH₂SO₄).

Anti-bacteria activity assay

A standardized inoculum (1×10^8 cfu /ml = 0.5 McFarland standards) of the organisms (*S. aureus*, *P. aeruginosa*, *E. coli* and *S. pyogenes*) was applied onto the surface of sterile Mueller Hinton agar plates (20 cm in diameter). A sterile cotton swab spreader was used for the even distribution of the inoculums. Agar well diffusion method was used to evaluate the antibacterial activity of the plant extract (26). Four plugs were removed from each agar plate using a sterile cork borer to produce an 8 mm diameter hole. To each hole, 100 μ l of 250, 500, 1000, 2500 and 4000 μ g/ml solutions of the acetone, 70% ethanol and water extracts were added and allowed to diffuse at room temperature for 20 min. The plates were then incubated at 37°C for 24 hours and then examined for zones of inhibition which was measured if any. Mueller Hinton agar with 100 μ l of inoculums served as positive control and the 3% dimethylsulfoxide (DMSO), was used to dissolve the extracts and served as a negative control. 0.3% Ciprofloxacin was used as a standard drug during the test. The experiment was carried out in triplicate for each extracts and test organism.

Anti-fungal activity assay

The fungal susceptibility test was carried out using standardized inoculums (1×10^8

cfu/ml = 0.5 McFarland standards) of *Candida albicans* and *Aspergillus niger*. Clinical isolates of *C. albicans* and *A. niger* were introduced onto separate surfaces of sterile Sabouraud Dextrose Agar (fungal media) keeping other experimental conditions similar to that of bacterial susceptibility testing. Plates for the antifungal susceptibility testing were incubated at room temperature and examined for zones of inhibition after 48 hours. Ketoconazole was used as a standard drug during the experiment. Each treatment consists of three replicates and was repeated at least twice.

Determination of MIC against test organisms

The MIC values of the extracts was determined by serial dilution of 70 % ethanol extract from initial concentrations of 4200 µg/ml. Screw cap test tubes were prepared and labeled 1 through 6. Two milliliters of test solution (4200 µg/ml) was added to the first test tube and 1 ml of Muller-Hinton broth was added to the rest. One milliliter of the test solution was transferred to the second tube. The contents of this tube were mixed thoroughly and using a different pipette 1 ml of the mixture from the second test tube was transferred to the third tube. The dilution continued in the same way up to test tube 5, using different sterilized pipettes to prevent the exchange of carryover of test solutions.

One milliliter of the test solution was then removed from test tube 5 and discarded. The sixth tube which served as a negative control, received no test solution. The final concentrations of the plant extracts after dilution were 2100, 1050, 525, 262.5 µg/ml consecutively. One milliliter of standardized inoculums containing 1×10^8 cfu/ml of *E. coli*, *P. aeruginosa*, *S. aureus* or *S. pyogenes* was added to each tube. Similar serial dilutions were done starting with 3500 µg/ml of the 70 % ethanol extract to get extract concentrations of 1750, 875, 437.5, 218.75 µg/ml. The contents of the tubes were incubated at 37°C for 24 hours. Two control tubes were maintained for each test batch. These included a negative control (tube containing extract and the growth medium without inoculums) and positive control (the tube containing the growth medium, DMSO and the inoculum). The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as MIC. The MIC against fungi was also determined by preparing serial dilution of the acetone, 70 % ethanol and water extracts as described above in Sabouraud dextrose broth and inoculating with a standard fungal inoculum (1×10^8 cfu/ml) of clinical isolates of *C. albicans* or *A. niger*. The resulting mixture was incubated at room temperature for 48 hours. The concentration

which showed no visible growth after 48 hours of incubation was taken as the MIC(27). The same procedure was repeated for the acetone and water extracts. The experiment was done in triplicate for each extract and organism.

Phytochemical screening

The ethanol extracts of the plant were screened for the presence or absence of main secondary metabolite groups such as alkaloids, flavonoids, saponins, tannins, steroidal and polyphenolic compounds. The screening was conducted using the following reagents and chemicals: Alkaloids with Dragendorff's and Mayer's reagent; steroids with chloroform/sulfuric acid and acetic anhydride/sulfuric acid solutions; polyphenolic compounds with the use of Ferric chloride and Potassium ferrocyanide; flavonoids with Ethyl acetate, 10% Lead acetate and Sodium hydroxide; tannins with the use of ferric chloride, formaldehyde, hydrochloric acid and modified iron complex solutions and saponins with ability to produce stable foam. These were carefully identified by characteristic color changes using standard procedures (28).

Gas chromatography (GC) - Mass spectrum (MS) Conditions

One micro liter of the volatile oil sample was injected in to the GC using an automatic injector in a split less condition. Chromo pack Cp-SiL5 (fused silica, 30 m x

0.32 mm i.d. with a film thickness of 0.25 μ m) capillary columns were used during the investigation. The carrier gas was helium (5psi) at 50 ml/min through the injector and a 30 cm/s through the column. The injector temperature was 220°C for the analysis. The GC column temperature program was 40-220°C at a rate of 3°C/min and a hold up at 220°C for 3 min. A Varian star 3400c x gas chromatograph coupled with a varian saturn 3 mass spectrometer was used for the analysis. The MS detector was set at 175°C and a mass range of 40-400 (m/z) was recorded. All mass spectra were acquired in EI mode. The compounds in volatile fractions were identified by the use of a combination of mass spectrum data base search (Terpenoids library, 2013), the relative retention index (Data base of essential oils, 2006) and comparison of mass spectra in literature (29).

Data analysis

The results of the study are expressed as mean $\pm$ standard deviation. Comparisons among the control and treatment groups were made using two way analysis of variance (ANOVA) and group means were considered to be significantly different at $P < 0.05$. Data were statistically evaluated using Statistical Package for the Social Sciences (SPSS) version 20.0 software.

Results

Acute toxicity studies

In the present study, the leaf extracts of *A. indica* which were tested in mice orally did not show any toxic manifestation at doses as high as 5000 mg/kg. This could explain the safe use of the plant and encourage the local people to use *A. indica* in the treatment of different maladies.

Anti-malarial activity of *A. indica* leaf extract

The results of the suppressive activity of the leaf extracts of the study plant against

P. berghei in mice is shown below (Figure 1). The different solvent crude leaf extracts of *A. indica* showed anti-plasmodial effect at various doses. For instance, at dosages of 200 mg/kg, 400 mg/kg, 600 mg/kg, and 800 mg/kg, the 70% ethanol extract suppressed parasitemia by 43.65%, 76.76%, 83.89%, and 85.23%, respectively. At 200 mg/kg, water and acetone extracts suppressed parasitemia by 41.84% and 36.11%, respectively, whereas at 400 mg/kg, same extracts suppressed parasitemia by 67.31% and 58.98%, respectively.

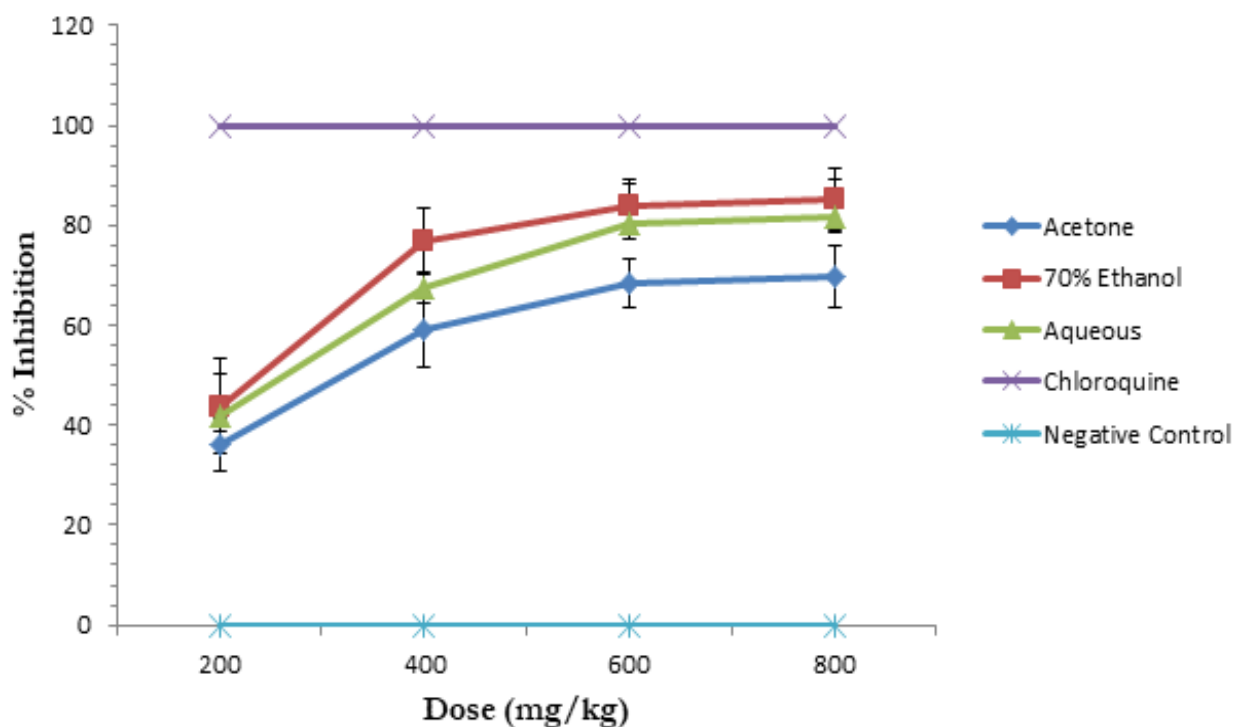


Figure 1: The antimalarial activity of *A. indica* leaf extracts at different doses against *P. berghei* infected mice. Data were expressed as mean $\pm$ SD (n= 5).

Furthermore, parasitaemia was suppressed by 80.21% and 81.54% for water and 68.31% and 69.01% for acetone extracts, respectively, at 600 and 800 mg/kg

dosages. As a result, mice treated with *A. indica* solvent extracts showed a lower parasite load than mice in the negative control groups. The results also implied that

the plant's leaf extracts had a dosage-dependent parasite suppressive effect, with the maximal suppressive effect (85.23%) at a dose of 800 mg/kg. Overall, the ethanolic extracts of *A. indica* showed the highest chemo-suppressive activity compared to water and acetone leaf extracts. After four days of treatments with the different doses of the plant solvent (70% of ethanol, water and acetone) extracts, the mean parasitemia

of the test groups ranged from 19.85 ± 0.53 to 3.99 ± 0.63 while the corresponding value of the negative control group was 28.6 ± 0.44 (Table 1). The treatment of mice with a daily dose of Chloroquine, 25 mg/kg, totally cleared the parasite on day four under identical condition. Nevertheless, the plant extracts did not clear the parasite completely and five animals were also euthanized from the negative control groups.

Table 1. The parasitemia (*P. berghei*) suppression data of the leaf extracts of *A. indica* in mice

Test extracts	Dose (mg/kg)	% mean parasitemia $\pm$ S.D.	% suppression	p-value
Acetone	200	19.85 ± 0.53	36.11	0.17
	400	12.75 ± 0.52	58.98	0.18
	600	9.85 ± 0.20	68.31	0.11
	800	9.11 ± 0.07	69.01	0.29
70% Ethanol	200	16.1 ± 0.42	43.65	0.10
	400	6.65 ± 0.49	76.76	0.16
	600	4.6 ± 0.10	83.89	0.09
	800	3.99 ± 0.63	85.23	0.12
Water	200	16.6 ± 0.33	41.84	0.12
	400	9.35 ± 0.41	67.31	0.10
	600	5.65 ± 0.1	80.21	0.08
	800	5.14 ± 0.19	81.54	0.19
Negative control	1ml	28.6 ± 0.44	0.00	
Chloroquine/positive control	1ml	0.00	100.00	

Key: S.D. = standard deviation

Anti-diabetic activity of *A. indica* leaf extract

Administrations of *A. indica* leaf extracts to alloxan induced diabetic mice

were periodically monitored for two weeks; the extracts reduced blood glucose levels appreciably (Figure 2). Among the test extracts, the 70% ethanol leaf extract

showed a considerable degree of blood sugar lowering. It was observed that the leaf extract at the dose levels tested was less active than the standard drug, glibenclamide, in lowering blood sugar levels. It was exhibited that a higher dosage of *A. indica*

leaf extracts brought about very good glycemic control compared to low dosage administration. This suggests that leaf extracts of *A. indica* have antidiabetic effects and could be of benefit in the management of diabetes mellitus.

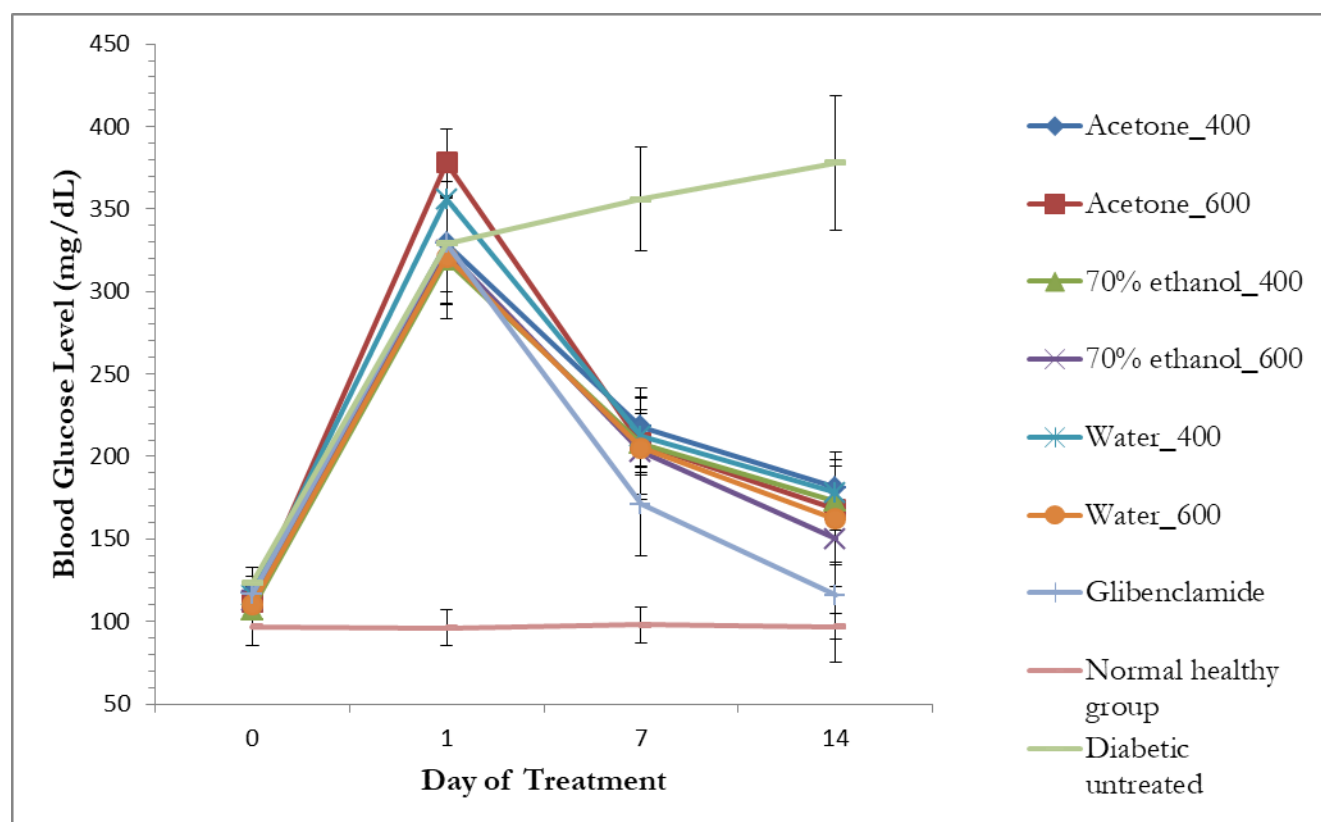


Figure 2: The effect of leaf extracts of *A. indica* on fasting blood glucose level in normal control and alloxan-induced diabetes mice. Acetone_600 and acetone_1000: Acetone Extract of *A. indica* (600 mg/kg and 1000 mg/kg per day, respectively); 70% ethanol_600 and 70% ethanol_1000: ethanol extract of *A. indica* (600 mg/kg and 1000 mg/kg per day respectively); water_600 and water_1000: water extract of *A. indica* (600 mg/kg and 1000 mg/kg per day, respectively); NC: Normal Control; DC: Diabetic Control; G10: Glibenclamide (10 mg/kg per day). Data was expressed as mean $\pm$ SD (n = 5)

Anti-microbial activity of *A. indica* leaf extracts

The antimicrobial activity test results showed that *A. indica* leaf extracts at concentrations higher than 1000 μ g/ml had

inhibitory action against the test microorganisms (Table 2). The activity was found to be dose-dependent. The data also revealed that among the six microorganisms against which the antimicrobial

activity was tested, *P. aeruginosa* was observed to be the most susceptible microbe to the three studied plant extracts. In the present study, the ethanolic extracts of *A. indica* showed a maximum zone of inhibition on *P. aeruginosa* (9.17 mm), *S. aureus* (8.33 mm), whereas the water extract

resulted in a moderate zone of inhibition for *P. aeruginosa* (8.00 mm) and *S. aureus* (7.17 mm) followed by the acetone extract, which also exhibited somehow moderate inhibition zone for *P. aeruginosa* (6.67 mm) and *S. aureus* (6.00 mm) (Table 2).

Table 2: The inhibition zones (in mm) of *A. indica* extracts at various concentrations on the test microorganisms

Test extracts	Conc. (µg/ml)	<i>E.c</i>	<i>S.p</i>	<i>P.a</i>	<i>S.a</i>	<i>A.n</i>	<i>C.a</i>
Acetone	4000	5.00±0.00	4.17±0.28	6.67±0.28	6.00±0.00	4.83±0.28	5.50±0.50
	2500	2.00±0.00	1.83±0.28	2.33±0.28	2.17±0.28	1.00±0.00	2.00±0.00
	1000	-	-	1.17±0.28	0.83±0.28	-	-
70% Ethanol	4000	7.33±0.57	6.83±0.28	9.17±0.28	8.33±0.28	6.33±0.28	6.83±0.28
	2500	4.67±0.28	4.00±0.00	6.00±0.00	5.00±0.00	4.33±0.28	4.33±0.28
	1000	-	-	1.83±0.28	1.83±0.28	1.17±0.28	-
Water	4000	6.83±0.28	6.17±0.28	8.00±0.28	7.17±0.28	6.33±0.28	5.00±0.28
	2500	3.50±0.50	3.67±0.28	4.33±0.28	4.00±0.00	3.00 ±0.00	3.50±0.50
	1000	-	-	1.50±0.50	1.33±0.28	0.83±0.28	-
Ciprofloxacin	0.3 %	29.83±0.28	25.50±0.00	33.16±0.28	29.17±0.28		
Ketoconazole	0.3 %					22.83±0.28	12.0±0.50
DMSO	3 %	0.00	0.00	0.00	0.00	0.00	0.00

Key: *E.c*=*E. coli*, *S.p*=*S. pyogene*, *P.a*=*P. aeruginosa*, *S.a*=*S. aureus*, *A.n*=*A. niger*, *C.a*=*C. albican*
(-) score was recorded for no activity; Conc. = concentration

Minimum Inhibitory Concentration (MIC) of the crude extracts

The MIC of *A. indica* inhibits the growth of microbial pathogens in the lowest concentration range of 875 to 1050 µg/mL.

The MIC for ethanol extracts of *A. indica* leaves for *E. coli*, *S. pyogenes* and *C. albicans* was shown to be 1050 µg/ml and for *S. aureus*, *P. aeruginosa* and *A. niger* was 875 µg/ml. However, the observed MIC

of the same extract for *A. niger* was altered from 875 µg/ml to 1050 µg/ml while the MIC for all the other species remained the same. On the other hand, aqueous extracts of *A. indica* leaf showed MIC at 875 µg/ml for

S. aureus, *P. aeruginosa* and *A. niger*. For *E. coli*, *S. pyogene* and *C. albicans* the water extract showed MIC at 1050 µg/ml (Table 3).

Table 3: The MICs of extracts of *A. indica* leaves

Test organisms	Acetone extract (µg/ml)	70% Ethanol extract (µg/ml)	Water extract (µg/ml)
<i>E. coli</i>	1050	1050	1050
<i>P. aeruginosa</i>	875	875	875
<i>S. aureus</i>	875	875	875
<i>S. pyogene</i>	1050	1050	1050
<i>A. niger</i>	1050	875	875
<i>C. albicans</i>	1050	1050	1050

Phytochemical studies of *A. indica* leaf extracts

The preliminary phytochemical screening of *A. indica* leaf extracts indicated

the presence of the most prominent bioactive compounds such as alkaloids, steroids, phenolics, flavonoids and tannins. Absence of saponins was also confirmed in this investigation (Table 4).

Table 4. The phytochemical screening test results of leaf extracts of *A. indica*

Phytochemical components	Reagents	Results
Alkaloids	Dragendroff's	++
	Mayer's	++
Steroidal compound	Chloroform and sulfuric acid	++
	Acetic anhydride and sulfuric acid	++
Polyphenolic compound	Ferric chloride and Potassium ferrocyanide	++

Flavonoids	Ethyl acetate	++
	10% Lead acetate	++
	Sodium hydroxide	++
Tannins	Ferric chloride	+
	Formaldehyde	+
	Hydrochloric acid	+
	Modified iron complex	+
Saponin	Froth test	—

Key: (+) positive, (++) strong positive, (-) negative (absence)

GC-MS analysis of the leaf oil of *A. indica*

The chemical constituents of the volatile oils of *A. indica* were determined by gas chromatography-mass spectrometry (GC-MS) technique. As presented in Table 5, a

total of 19 compounds comprising 99.99% of the total volatile oil composition were determined. The majority of the identified constituents were sesquiterpenes (75.45%) and monoterpenes and phenols comprised 1.1% and 23.44%, respectively.

Table 5: The constituents of *A. indica* leaf volatile oil identified by GC-MS analysis

No	KI	Name of compound	% yield	Compound Type
1	1212	Thymol	17.29	Phenol
2	1228	Carvacrol	6.15	Phenol
3	1402	α -Cubebene	1.10	Sesquiterpene hydrocarbon
4	1480	β -caryophyllene	8.35	Sesquiterpene hydrocarbon
5	1509	Elemene γ	2.84	Sesquiterpene hydrocarbon
6	1543	α -Caryophyllene	2.02	Sesquiterpene hydrocarbon
7	1557	Chamigrene β	0.59	Sesquiterpene hydrocarbon
8	1579	Ionone β	1.80	Sesquiterpene oxide
9	1588	Cedryl acetate	1.10	Monoterpene alcohol
10	1619	Cadinol< α >	0.41	Sesquiterpene alcohol
11	1639	Globulol	0.69	Sesquiterpene alcohol
12	1655	Himachalene< β >	2.00	Sesquiterpene hydrocarbon
13	1674	δ -Cadinene	0.98	Sesquiterpene hydrocarbon

14	1693	Valencene	0.66	Sesquiterpene hydrocarbon
15	1711	Aromadendrene	0.87	Sesquiterpene hydrocarbon
16	1792	Spathulenol	1.15	Sesquiterpene alcohol
17	1764	Nerolidol<trans>	19.06	Sesquiterpene alcohol
18	1996	Acetoxyelemol<8α>	5.50	Sesquiterpene alcohol
19	1561	Germacrene β	27.43	Sesquiterpene hydrocarbon

Key: KI: Kovat Index

Discussion

Medicinal plants are most often regarded as safe to use as they are “natural”. However, some active compounds derived from these economically important plants are still considered to have a potential to cause adverse effects. To this end, this study addressed the toxicity concern of *A. indica* and investigated that the leaf extracts of the plant tested on mice could not exhibit changes in physiological and general behavior and thus it is safe to use. This observation meets the criteria set by the center for drug evaluation and research (CDER) for the absence of acute toxicity (30). This toxicological test result is in agreement with a study that was carried out elsewhere i.e. orally administered aqueous extracts of *A. indica* leaves showed no toxicity effect, even at a concentration up to 5000 mg/kg in mice(31).

In this study, ethanol, water and acetone extract of *A. indica* leaves exhibited parasite suppressive activity on mice *P. berghei*. The

highest percentage of chemosuppression and reduction in the mean parasitemia level were observed at the maximum dosage (800 mg/kg) level of the plant extracts. This is because active compounds in natural products may not be detected at lower doses (32). Anti-plasmodial property demonstrated for water and acetone extracts were found to be weaker than that of ethanol leaf extracts. Thus, it is interesting to note that the active compound responsible for antimalarial activity has a polarity effect which might benefit the local practitioners who use commonly aqueous preparations for treatment. The anti-plasmodial activity observed in this study may be attributed to the presence of a range of classes of secondary plant metabolites, alkaloids, flavonoids, and phenolic compounds. Alkaloids have been the most important and have shown appreciable antimalarial activity, quinine is the first antimalarial drug that belongs to the class of alkaloids (21).

Comparison of the current findings with literature showed that Ethiopian neem tree has a better anti-plasmodial effect when compared with Indian neem leaf extracts. *In vivo* plasmodial suppressive test conducted in India depicted a mean percentage parasitemia of $22.4 \pm 4.7\%$ and percent suppression of parasitemia, 46%, at the dosage level of 1000 mg/kg. In a similar study, 200 mg/kg dosage of neem leaf extracts produce $21.6 \pm 3.8\%$ of mean parasitaemia (33). However, in the present study, the mean percentage parasitemia at the dosage level of 200 mg/kg was observed to be $16.1 \pm 0.42\%$. The dosages of 800 mg/kg produce $3.99 \pm 0.63\%$ mean parasitemia and 85.23 % suppression of parasitemia.

This significant suppression of parasitemia by the leaf extracts of *A. indica* coincides with the inhibition of parasitemia reported by earlier workers (34-36). *In vivo*, tested aqueous extracts of *A. indica* also exhibited antimalarial activity against *P. berghei* using 4-day suppressive test and about 70% parasitemia inhibition was recorded (32,37). The tablet suspension of the leaf extracts of *A. indica* showed anti-parasitic activity (79.6%, suppression of parasitaemia) against *P. yoelli nigeriensis* infected mice at a concentration of 800 mg/kg (34). Also, some other researchers

have reported the antiplasmodial activity of different parts of the neem tree (leaf, stem bark and seed) against *P. berghei* and *Plasmodium falciparum*, responsible for causing mammalian malaria (38,39). It was also indicated that extracts of neem leaf and bark reduced the level of parasitemia in *P. berghei* infected mice by about 51 - 80% and 56 - 87%, respectively (40). In addition, the *in vitro* anti-malarial activity of neem was also reported by Deshpande and colleagues in 2014, where they reported that an ethanolic extract of neem leaves showed LD₅₀ value of 5 µg/ml against *P. falciparum* (41). In 2007, similar results were obtained by the aqueous extracts of *A. indica*, Giemsa-stained microscopic slides showed an inhibition of (IC₅₀=2.0 µg/ml) developed schizonts in the ring stage of *P. falciparum* (42). These suggest that the active constituents in the extract of neem possess a significant anti-malaria potential and justify their usage in traditional medicine against malaria.

Furthermore, the present study also exhibited a promising antidiabetic activity of the neem extracts. The leaf extracts of *A. indica* applied for 14 days reduced the alloxan-induced diabetic mice glucose level remarkably, suggesting the plant antihyperglycemic properties. Upon increasing the dosage extracts of neem

leaves, an increased reduction in the blood glucose levels were recorded. Thus, experimental values indicate a dose-dependent reduction of blood glucose levels. The exact mechanism in reducing blood glucose level is not well understood. But the presumed mechanism of action for the antidiabetic effects of *A. indica* leaves might be due to an insulin mimetic effect of the plant on the peripheral tissues i.e. either stimulate or release of pancreatic secretion of insulin from existing β -cells.

A comparison of the results of this work with those previously reported for neem leaf extracts grown in India revealed considerable differences. The ethanolic neem leaf extracts, 250 mg/kg and 100 mg/kg, exhibited better blood glucose level reduction i.e. 64.2 ± 4.8 and 85 ± 1.8 , respectively, when compared to extracts of neem at the present study administered at 400 and 600 mg/kg doses (43). In accordance to the present findings, some other authors also reported noticeable fall in blood glucose level following administration of neem to streptozotocin-induced diabetic rats (44-47). On the other hand, Chattopadhyay, Mostofa and colleagues showed the therapeutic potential of *A. indica* leaf extracts as blood sugar lowering agent (48,49). All these prove the tendency of *A. indica* grown in Ethiopia in controlling hyperglycemia and

thus it opens an efficient alternative in management of diabetes mellitus.

In the present study, antimicrobial activity of *A. indica* leaves extracts were also tested against selected Gram-positive and Gram-negative bacterial species and fungal strains. The ethanol, aqueous and acetone extract of leaves of *A. indica* were found to limit the growth of bacteria and fungi species tested. Similar results were also observed by Autade *et al.* (2015) who presented growth inhibitory effect of acetone neem leaves extract (50). Mamman *et al.* (2013) found inhibitory effects of aqueous extracts of neem leaves against pathogenic microorganisms (51) and Tirumalasetty and colleagues did manage to measuring zone of inhibition for methanol extract of neem (52).

The bacteria and fungi inhibitory role of the leaf extracts of *A. indica* was found to be both organism and solvent dependent. *P. aeruginosa* and *S. aureus* are found to be sensitive to all tested solvent extracts of *A. indica*. Similar observations were also reported from Indian neem extracts (53,54). Ethanolic extracts exhibited pronounced activity against all tested organisms when compared to water and acetone extracts and thus ethanol as a solvent has more capacity to extract the secondary metabolites. This finding is consistent with the previous study

obtained from some other workers (55–58). In support of this finding, research work indicated that aqueous leaf extracts of *A. indica* was moderately active against the growth of many human pathogens including *S. aureus*, maximum inhibition of 8 mm (9, 26, 56). This demonstrated that the plant has retained its anti-microbial activity which showed somewhere else when grown out of its natural habitat such as in Ethiopia.

The MIC for the tested strains of microbes was ranged between 875 to 1050 µg/ml. Several other studies indicated that extracts of neem leaves are capable to inhibit the growth of *P. aeruginosa*, *E. coli* and *S. aureus* at low concentrations (from 0.25 to 4.0 mg/ml) (26,27). Thus, *A. indica* grown in Ethiopia showed somehow moderate antimicrobial activities against tested microbes. *A. indica* collected from Arsi Asela, 100 km South East of the capital Addis Ababa, Ethiopia, also demonstrated moderate to significant antibacterial activity against tested pathogens (59). This supports the finding of the present study; neem tree grown in Ethiopia has a moderate antimicrobial activity to human pathogens.

Phytochemical studies of *A. indica* extracts demonstrated the potential biological activity (such as antitumor, antiviral and antimicrobial) of the plant (60). Some other workers also documented the

antibacterial, antidiabetic, antihemorrhagic, antimalarial activity of *A. indica* plant extracts (53,61). Traditional medicinal plants containing various types of phytochemical constituents have a long history of serving people globally and phytochemical metabolites do play a variety of role in the pharmacological actions of individual plant source(62). The result of the present phytochemical investigation showed the presence of alkaloids, steroids, phenolics, flavonoids and tannins. However, none of the crude extracts showed color change for the saponins test. Thus, the significant medicinal properties of *A. indica* could be attributed to the resident of the above mentioned biochemical components in the leaf extracts of the plant (63–65). This finding is almost similar to phyto-components reported from the Indian indigenously harvested *A. indica* (66, 67).

The actual mechanism of action that brings up the biological activity of *A. indica* is yet to be understood but it is supposed to be brought up by the cumulative effect of secondary metabolites in the plant extracts. The presence of alkaloids from the crude extracts of *A. indica* represents the possibility of some biological activity such as antiplasmodial activity, anti-cholinergic, anti-tumor, vasodilating, anti-hypertensive, anti-protozoan, anti-hyperglycemic effect.

Furthermore, flavonoids and phenolic compounds have been reported to play a multitude of biological roles including modifying allergens and viruses, anti-inflammatory and antioxidant (59). The antimicrobial activity of the plant extract could be possibly attributed to presence of tannins (62).

Essential oil compositions of the leaves of *A. indica* growing in Ethiopia was obtained by hydrodistillation and analyzed by GC-MS. Nineteen components were identified accounting for 99.9 % of the total. Among all, Germacrene β , Nerolidol<trans>, Thymol and β -caryophyllene were identified as major components of the leaf oil of neem tree. The classification of identified compounds showed that the Sesquiterpene hydrocarbons (69.0%) constituted the major fraction of the oil, followed by phenols (23.44%) and monoterpenes, 1.1 %, (Table 5). Although the essential oil composition of *A. indica* has been studied elsewhere, GC-MS oil composition has been reported here in Ethiopia for the first time. The presence of monoterpenes may have a synergistic effect for the antimicrobial activity of neem tree against Gram-positive bacteria (68). Studies also indicated that essential oil possessing δ -cardinene and sesquiterpenes was found to

be responsible for antimicrobial activities (68).

A comparison of the results of this work with those of previously reported for essential oil composition of *A. indica* grown somewhere revealed similarity. The sesquiterpene hydrocarbons (69.0%) were constituted the major fraction of the oil and oxygenated sesquiterpens and some other compounds are the composition of the Iranian essential oil (69). However, the hydro distilled volatile oil obtained from India documented some differences; caryophyllene is the major identified component. The GC-MS results showed sesquiterpenes caryophyllene (56.03%), caryophyllene oxide (17.41%) and α -Caryophyllene (12.10%). α -Caryophyllene (monocyclic sesquiterpene) and caryophyllene (bicyclic sesquiterpene) are isomers (70). The difference in oil composition are perhaps correlated with differences in physical and chemical characteristics of soil, time of harvest, plant age etc (71).

Conclusion

The results obtained from the present study shows that the *A. indica* grown in Ethiopia had a pronounced anti-plasmodial activity, moderate beneficial effects on blood glucose levels in alloxan-induced diabetic mice and fair antimicrobial effects,

compared with the one grown indigenously. Moreover, the data on the phytochemical investigation and GC-MS analysis of the essential oil constituents of the leaf of *A. indica* was comparable to that of studied elsewhere. Thus, *A. indica* appears to be an attractive material for further studies, leading to possible drug development for malaria, diabetes and medication for microbial infection. In conclusion, the extracts of *A. indica* grown in Ethiopia retained the antimalarial, antidiabetic and antimicrobial activity that was shown elsewhere.

List of abbreviations

KI: Kovat Index; GC/MS: Gas Chromatography-Mass Spectrum; MIC: Minimum Inhibitory Concentration; *E.c*: *E. coli*; *S.p*: *S. pyogene*; *P.a*: *P. aeruginosa*; *S.a*: *S. aureus*; *A.n*: *A. niger*; *C.a*: *C. albican*; S.D: Standard Deviation; ANOVA: Analysis of Variance; SPSS: Statistical Package for the Social Sciences; CFU: Colony Forming Unit

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Authors' contributions

ESB: Participated in developing the original idea and protocol. FB: Participated in data analysis. MM: Participated in data analysis. MMA: Participated in data analysis and Manuscript preparation. All authors read and approved the final manuscript.

Ethical approval and consent to participate

An institutional ethics committee at Addis Ababa University in Addis Ababa, Ethiopia, approved the study's permission to conduct a scientific experiment on

laboratory mice. They were handled humanely in accordance to the recommendations of international guidelines for the care and use of laboratory animals, National Institutes of Health (NIH) Publication No. 86–23, Revised 1985, and the ARRIVE guidelines. Blood samples were collected from the tail under mild sodium pentobarbital anesthesia and all efforts were made to minimize the suffering of the mice.

Competing interests

We have no any competing interests to disclose.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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