

Evaluation of Antibacterial and Acute Oral Toxicity of *Impatiens Tinctoria* A. Rich Root

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Keywords: Antibacterial, acute toxicity, plant extract, *Impatiens tinctoria* A. Rich

Posted Date: September 2nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-64882/v1>

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Abstract

Background: Infections due to a variety of bacterial etiologic agents become common and are taking the big share of morbidity and mortality. On the other way, development of antibacterial drug resistance has been commonly reported from all over the world. As a solution of stated problems scientific studies have to be conducted on the traditional medicinal plants to develop new, effective and safe antimicrobial drugs since plants are important sources. Traditionally, the study plant (*I. tinctoria* A. Rich) used to treat fungal infections like ring worms that cause tinea pedis and it has also different medicinal values. These were some of the provoking information to undertake the evaluation of antibacterial activities with its oral acute toxicity study.

Objectives: To evaluate the antibacterial activities and acute oral toxicity of aqueous, ethanol and ethyl acetate root extracts of *Impatiens tinctoria* A. Rich.

Methods: The roots of *Impatiens tinctoria* A. Rich were extracted using solvents of water, ethanol and ethyl acetate. Agar well diffusion for preliminary antibacterial screening and agar dilution methods for determination of minimal inhibitory concentration were used. The minimum bactericidal concentration of the extracts was determined. by taking inoculums from all concentrations of the plant extract plates exhibiting invisible growth (from inhibition zone of minimal inhibitory concentration plates) and subcultures onto appropriate media plate. Finally, the plant extracts were subjected to oral acute toxicity study according to the organization of economic co-operation and development test Guidelines 420.

Result: Gram positive bacteria were more susceptible to the extracts compared to gram negative bacteria especially against *S. aureus* and *S. epidermis* which are commonly found in the skin even though the traditional application is to control fungal infections and to toughen the skin. Ethyl acetate extract was more potent than ethanol and aqueous extracts. The LD50 was above 9600 mg/kg.

Conclusion: This study provides scientific basis as the root of *I. tinctoria* A. Rich had a promising antibacterial activity in extract dependent manner in which ethyl acetate extract showed better potency. Therefore, the antibacterial potential and practically non toxicness of the study plant could take the attention of scientific communities for the development of new, effective and safe antimicrobial drugs by further studying the plant in different directions.

1. Introduction

Infectious diseases are the world leading cause of premature deaths, killing almost 13.4 million people per year. The World Health Organization (WHO) forecasts 13 million deaths attributed to this cause in 2050 (1, 2). Infections due to a variety of bacterial etiologic agents become common and are taking the big share of the burden (1). Severe infections, including sepsis, meningitis, and pneumonia, are estimated to cause about a third of the 2.6 million neonatal deaths globally in which most of them are in less affluent regions of our planet (1). In Ethiopia, the top five leading causes of premature mortality in 2015 were lower respiratory infections, tuberculosis, diarrheal disease, ischemic heart disease, and Human

Immunodeficiency Virus/Acquired Immune Deficiency Syndrome that indicates the dominant fatality of the infectious diseases (3).

There is also an alarming increase in the incidence of new and reemerging infectious diseases of which some of them don't have drugs that act against them (4, 5). For instance, over the past 40 years a minimum of 50 emerging infectious agents have been identified across the globe; approximately 10% of them are bacterial agents (5). Additionally, drug resistance has been commonly reported from all over the world (6). For example, the development of resistance to methicillin has decreased the usefulness of this antibiotic in treating serious staphylococcal infections within the community and hospitalized patients (6). Presently, approximately 60,000 people in Europe and United States die each year due to serious infections caused by antimicrobial resistant bacteria (7). The problem is also high in Ethiopia as indicated by few studies (8–11).

In spite of such problems, medicinal plants have been used since ancient time to treat various diseases. They are bases for most of traditional healing practices in which around 4.3 billion people of the world's population use herbal medicines for some aspect of primary healthcare (12). Surveys carried out in developed countries like Germany and Canada tend to show that no less than 70% of their population have used herbal remedies at least once that reaches 80% when we come to the emerging world (13). Traditional remedies are the most important and sometimes the only source of therapeutics for nearly 80% of the Ethiopian population and 95% of the preparations are of plant origin (14).

As a solution of stated problems scientific studies have to be conducted on the traditional medicinal plants to develop new, effective and safe antimicrobial drugs. Locally, Ethiopian women chop or mash the inside of the roots of *I. tinctoria* A. Rich in to a paste to dye the palms and nails of the hands and feet as a beauty treatment, to control fungal infections and to toughen the skin (15, 16). The root decoction is also drunk against abdominal pains and as a purgative. The stem is chewed to treat mouth and throat diseases (17). In view of this, this study initiated to scientifically justify the antimicrobial potential of this medicinal plant root extracts against selected bacteria. The acute oral toxicity evaluation was necessary to identify the range and concentration of dose that could be used and the possible clinical signs elicited by this medicinal plant.

2. Materials And Methods

2.1. Herbal material collection and preparation

The whole plant material of *Impatiens tinctoria* A. Rich was collected from Butajira, Southern Nations and Nationalities Region, Ethiopia and identified by a botanist. The collected root part washed with clean water, cut, and dried at room temperature, by using milling machine, the plant cutlets was milled to powder. The powder was weighted using electronic weighting balance and packed in polyethylene bags to avoid entrance of air and any other contaminant and stored in closed container with proper labeling for further extraction processes.

2.2. Extraction

The extraction was by mixing the root powder and extraction solvents (distilled water, ethanol and ethyl acetate) with a proportion of 1 gram of powder and 20 ml solvents. After thoroughly mixed macerated in rotary-shaker at 100 rpm for 24 hours and filtered through Whatman filter paper followed by concentration of it under reduced pressure (vacuum) by rotary evaporator at 40 °C to obtain the extract. These concentrated extracts were kept in water bath set at 40 °C to avoid the remaining organic solvent and water (18, 19).

2.3. Microorganisms

The antibacterial activity of the root extracts of the study plant was evaluated on 13 standard strains (American Type Culture Collection (ATCC)) or clinical isolated bacteria. The tested bacterial strains were *S. aureus* (ATCC 25923), MRSA (clinical isolate), *S. epidermidis* (ATCC 12228), *S. pyogenes* (ATCC 19615), *Streptococcus agalactiae* (*S. agalactiae* (ATCC 12386)), *E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *S. typhimurium* (ATCC 13311), *Shigella flexneri* (*S. flexneri* (ATCC 12022)), *Shigella sonnei* (*S. sonnei* (ATCC 25931)), *P. aeruginosa* (ATCC 27853), *K. pneumoniae* (ATCC 700603) and *proteus mirabilis* (*P. mirabilis* (ATCC 35659)). These microorganisms were maintained in the laboratory of microbiology in the Traditional and Modern Medicine Research Directorate (TMMRD) of Ethiopian Public Health Institute (EPHI) on Triptosoya + 20% glycerol broth at -78 °C.

2.4. Inoculums preparation

All strains were refreshed for the actual test with in Petri dishes containing nutrient agar, except *S. pyogenes* (ATCC 19615) and *S. agalactiae* (ATCC 12386) which were grown on 5% sheep blood nutrient agar, by incubation for 18-24 hours at 37 °C. The grown bacteria few inoculums were harvested using 5 ml of nutrient broth, its absorbance was adjusted at 625 nm and diluted to attain viable cell count of 10^7 CFU/ml using spectrophotometer (20, 21).

2.5. Screening antimicrobial activity of the extract

Agar well diffusion method was used to evaluate the antimicrobial activity of the extract. The agar plate surface was inoculated by spreading a volume of the microbial inoculums, taken from adjusted suspensions, over the entire agar surface. Then, a hole with a diameter of 8 mm was punched aseptically with a sterile cork borer or a tip, and a volume (100 µl) of the antimicrobial agent or extract solution at desired concentration (100 mg/ml, 200 mg/ml and 400 mg/ml) was introduced into the well. Then, the plates were incubated under suitable conditions (at 37 °C for 18-24 hours). The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. The presence of inhibition zones were measured by ruler and considered as indication for antimicrobial activity (22).

2.6. Minimum Inhibitory Concentration

All tested extracts were manipulated to determine their minimum inhibitory concentration (MIC) using agar dilution method by preparing different concentrations of extracts (from 64 mg/ml to 0.0625 mg/ml)

through two-fold serial dilution. Then, incorporation of the prepared concentrations of the extracts into an agar medium (molten agar medium) followed by the inoculation of defined microbial inoculums on to the agar plate surface. The incubation time is similar to the screening method. The MIC was considered as the lowest concentration which inhibits the growth of the respective bacteria under suitable incubation conditions was expressed in mg/ml (23).

2.7. Determination of Minimum Bactericidal Concentration

Streaks were taken from all concentrations of the plant extract plates exhibiting invisible growth (from inhibition zone of MIC plates) and subcultures onto appropriate media plate. The plates were incubated under suitable conditions depending upon the test microorganism (at 37 °C for 18-24hours). Then, examined for bacterial growth in corresponding to extract concentrations. Minimum Bactericidal Concentration (MBC) was taken as the concentration of plant extract that did not exhibiting any bacterial growth on the freshly inoculated agar plates. All assays were performed in triplicate (24).

2.8. Oral acute toxicity

Acute toxicity study was performed according to the organization of economic co-operation and development (OECD) test Guidelines 420 (Acute Oral toxicity–Fixed dose procedure) with slight modification. Healthy young adult, nulliparous and non pregnant female albino mice was used. The testing animals were randomly selected from 8–12 weeks old mice, marked to permit individual identification, and kept in their cages for 5 days prior to dosing to allow for acclimatization to the laboratory conditions. The animals were fasted 3-4hours (food withdrawn but not water) prior to dosing after which the animals weighed to determine the fasted body weight. Each animal, at the commencement of its dosing, was 25-33gm weight (25).

The starting dose was 300 mg/kg which was increased by bi-fold till 9600 mg/kg. Five animals were used for each dose. Treatment of animals at the next dose was delayed until assuring confident of survival of the previously dosed animals. The extract was calculated according to the body weight and dissolved in a consideration of the administered volume not exceed 1 ml/100 g of mice body weight. Then, the solvent alone for control groups and diluted extract for treated groups were administered with oral gavage. After administration each mouse was closely observed for the first 30 minutes, hourly during the first six hours, two hourly during the first 24 hours, and daily for a total of 14 days. All observations like changes in breathing, alertness, restlessness, diarrhea, behavioral pattern, mortality and consumption of food and water were systematically recorded. Moreover, the change in body weight was measured at initial day and at 7 and 14 post treatment days (25).

2.9. Data analysis and interpretation

The extracted data was examined for its completeness and checked for consistency. Then, entered into excel spreadsheet, exported to Minitab 16 software and analyzed. The statistical differences of the antibacterial activity of crude extracts on each bacteria and the effect of each extracts on body weight of the albino mice were carried out by employing one way analysis of variance (ANOVA) followed by Tukey's

multiple comparison tests. The experimental data was expressed as Mean \pm Standard Deviation (SD). The result considered statistically significant at $P < 0.05$.

3. Results

3.1. Antibacterial activity screening by well method

The antibacterial activity of aqueous, ethanol and ethyl acetate extracts of the roots of *I.tinctoria* A. Rich were screened against selected bacteria. A total of 13 bacteria by agar well diffusion assay were assessed at a concentration of 100, 200, 400 mg/ml for each extract in triplicates.

3.1.1. Antibacterial activity against gram positive bacteria

The average zone of inhibition formed by all tested concentrations of ethanol and ethyl acetate extracts against *S. aureus* was better compared to the aqueous extract, significantly different at $P \leq 0.05$. Each extract has produced notable inhibition zone against MRSA at all concentrations. *S. epidermidis* produced the largest average zone of inhibition when compared to the other tested gram positive bacteria in which the inhibition zone against this bacterium ranges from 38.0 ± 1.0 mm by 100 mg/ml aqueous extract to 43.7 ± 1.5 mm by 400 mg/ml ethyl acetate extract. Comparisons of the mean growth inhibition zones for *S. aureus* at 100, 200 and 400 mg/ml concentrations of the three tested extracts showed no significant differences ($P < 0.05$). It indicates absence of concentration dependent inhibition difference which was also observed on MRSA and *S. epidermidis*.

All extracts showed less inhibition zone against the two Streptococci bacteria (*S.pyogen* and *S.agalactiae*) when compared with other tested gram positive bacteria with each corresponding concentration. Erythromycin (15 μ g) showed better inhibition diameter against the two tested streptococci than the three extracts at all concentrations that were significantly different at $p \leq 0.05$. In extract type dependent manner, better inhibition against *S. pyogen* and *S. agalactiae* were obtained by ethyl acetate extract followed with ethanol extract. The least inhibition diameter 17 ± 0.0 mm at 100 mg/ml aqueous extract against *S.agalactiae* was recorded when compared with all tested gram positive bacteria.

The three extracts showed better inhibition on 400 mg/ml than 100 mg/ml and 200 mg/ml concentrations to *E. faecalis* with a significant difference at $P \leq 0.05$. The effect of vancomycin (30 μ g) on this bacterium revealed less inhibition to all extracts and doses, with a significant difference at $P \leq 0.05$, except the aqueous extract at 100 mg/ml concentration. Similarly, erythromycin (15 μ g) that produced 21.3 ± 2.3 mm inhibition zone was greater than the inhibition of aqueous extract at 100 mg/ml concentration and lesser than or equal to other extracts at all concentrations. The potency of almost all extracts on this bacterium was better than *S. pyogenes* and *S. agalactiae*, but lesser than *S. epidermidis*, *S. aureus* and MRSA (Table 1).

Table 1

Inhibition zone diameter measurement (mm) of crude extracts of *I. tinctoria* A. Rich roots against gram positive bacteria.

Different solvent extracts (mg/ml)		Inhibition Zone Diameter (mm), including well diameter (8 mm)					
		<i>S. aureus</i>	MRSA	<i>S. epid</i>	<i>S. pyogen</i>	<i>S.agal</i>	<i>E. faecalis</i>
Aqueous	100	25.7 ± 0.6 ^d	27.0 ± 1.0 ^d	38.0 ± 1.0 ^b	18.0 ± 0.0 ^f	17.3 ± 0.6 ^h	19.0 ± 1.0 ^{de}
	200	27.3 ± 0.6 ^{cd}	28.3 ± 0.7 ^{cd}	41.0 ± 1.0 ^{ab}	21.7 ± 0.6 ^e	18.0 ± 0.0 ^{gh}	22.3 ± 0.6 ^{cd}
	400	29.3 ± 1.2 ^{bc}	28.7 ± 0.6 ^{cd}	41.7 ± 1.5 ^{ab}	24.0 ± 1.0 ^{cd}	21.3 ± 0.6 ^{cde}	27.7 ± 1.5 ^{ab}
Ethanol	100	35.0 ± 1.0 ^a	29.7 ± 0.7 ^{bcd}	41.0 ± 1.7 ^{ab}	22.3 ± 0.6 ^{de}	19.0 ± 0.0 ^{fgh}	21.3 ± 1.5 ^{cde}
	200	35.7 ± 0.6 ^a	30.3 ± 0.6 ^{bcd}	42.0 ± 1.0 ^a	23.3 ± 0.6 ^{de}	19.7 ± 1.5 ^{efg}	27.7 ± 0.6 ^{ab}
	400	36.7 ± 0.6 ^a	31.7 ± 1.5 ^{bc}	43.0 ± 1.0 ^a	24.0 ± 1.0 ^{cd}	21.7 ± 1.2 ^{cde}	28.7 ± 1.2 ^a
Ethyl acetate	100	36.3 ± 0.6 ^a	30.0 ± 1.7 ^{bcd}	41.3 ± 0.6 ^{ab}	25.7 ± 0.6 ^b	22.3 ± 0.6 ^{bcd}	24.3 ± 0.6 ^{bc}
	200	36.0 ± 1.0 ^a	33.0 ± 2.7 ^{ab}	42.0 ± 1.0 ^a	26.7 ± 0.6 ^b	23.0 ± 1.0 ^{bc}	27.7 ± 0.6 ^{ab}
	400	37.0 ± 1.0 ^a	35.7 ± 1.2 ^a	43.7 ± 1.5 ^a	26.0 ± 1.0 ^b	24.0 ± 1.0 ^b	29.7 ± 1.5 ^a
Distilled water	-	8.0 ± 0.0 ^f	8.0 ± 0.0 ^f	8.0 ± 0.0 ^e	8.0 ± 0.0 ^g	8.0 ± 0.0 ⁱ	8.0 ± 0.0 ^f
5% tween 80	-	8.0 ± 0.0 ^f	8.0 ± 0.0 ^f	8.0 ± 0.0 ^e	8.0 ± 0.0 ^g	8.0 ± 0.0 ⁱ	8.0 ± 0.0 ^f
Vancomycin	30 µg	21.3 ± 1.5 ^e	21.3 ± 1.5 ^e	21.7 ± 2.1 ^d	22.0 ± 1.0 ^{de}	20.7 ± 0.6 ^{def}	18.7 ± 1.2 ^e
Erythromycin	15 µg	31.7 ± 2.1 ^b	31.7 ± 2.1 ^{bc}	32.3 ± 2.1 ^c	30.3 ± 1.5 ^a	31.0 ± 1.0 ^a	21.3 ± 2.3 ^{cde}

Key notes

Values are expressed as Mean ± SD (n = 3), 8.0 ± 0.0 = no inhibition (well diameter), "Means" that do not share a superscript letter are significantly different (only column wise) at P ≤ 0.05. *S. epid* - *S. epidermidis*,

3.1.2. Antibacterial activity against gram negative bacteria

S. flexneri, *S. soni* and *P. mirabilis* showed the highest inhibition zone among all gram negative bacteria at 400 mg/ml concentration of ethyl acetate extract with inhibition zone of 22.7 ± 0.6 mm compared to other gram negative bacteria. *K. pneumoniae* measured the smallest inhibition zone of 17.3 ± 1.2 mm at this concentration and extract type. However, the growth of this bacterium did not show any inhibition by 100 mg/ml aqueous extract.

The positive control, ciprofloxacin (5 µg/ml), showed significantly higher inhibition of the growth of all gram-negative bacteria compared to all tested extracts at all concentration ($P < 0.05$). On the other hand, among the extract type, aqueous extract has showed significantly lower inhibition activity against the tested gram-negative bacteria ($P < 0.05$). Moreover, no statistically significant different inhibitions were observed against *E. coli*, *S. typhimurium*, *P. aeruginosa*, *K. pneumoniae* and *P. mirabilis* at a concentration of 100 mg/ml of aqueous extract compared to the treatment of negative control (distilled water and 5% tween 80) ($P < 0.05$). Table 2 showed the inhibition zone diameter and association of the total seven tested gram negative bacteria were used to assess the antibacterial activity of the extracts.

Table 2

Inhibition zone diameter measurement (mm) of crude extracts of *I.tinctoria* A. Rich roots against gram negative bacteria.

Extract Type	Conc.	Inhibition Zone Diameter (mm), including well diameter (8 mm)						
		<i>E.coli</i>	<i>S.typhim</i>	<i>S.flexneri</i>	<i>S.sonnei</i>	<i>Paerog</i>	<i>K.pneum</i>	<i>P.mirabi</i>
Aqueous	100	8.7 ± 0.6 ^e	9.0 ± 0.0 ^{ef}	11.3 ± 0.6 ^h	11.3 ± 0.6 ^g	11.0 ± 1.0 ^f	8.0 ± 0.0 ^f	9.3 ± 0.6 ^g
	200	11.7 ± 0.6 ^d	11.7 ± 0.6 ^e	13.3 ± 0.6 ^g	13.0 ± 1.0 ^{fg}	13.7 ± 0.6 ^{ef}	9.0 ± 0.0 ^f	11.7 ± 0.6 ^f
	400	15.0 ± 0.0 ^c	15.3 ± 0.6 ^d	16.7 ± 0.6 ^f	15.0 ± 0.0 ^{ef}	16.0 ± 1.0 ^{de}	12.0 ± 1.0 ^e	14.7 ± 0.6 ^e
Ethanol	100	16.3 ± 0.6 ^c	16.7 ± 0.6 ^{cd}	16.0 ± 1.0 ^f	14.7 ± 0.6 ^{ef}	17.7 ± 1.2 ^{cd}	13.7 ± 0.6 ^{de}	19.3 ± 0.6 ^{cd}
	200	19.3 ± 0.6 ^b	19.0 ± 1.0 ^{bc}	17.3 ± 0.6 ^{ef}	15.3 ± 0.6 ^{ef}	19.3 ± 1.5 ^{bc}	15.0 ± 0.0 ^{cd}	21.3 ± 0.6 ^{bc}
	400	20.7 ± 0.6 ^b	20.7 ± 1.5 ^b	18.7 ± 0.6 ^{de}	17.3 ± 1.2 ^{df}	19.7 ± 1.2 ^{bc}	16.7 ± 0.6 ^{bc}	23.3 ± 0.6 ^b
Ethyl acetate	100	15.7 ± 0.6 ^c	17.3 ± 0.6 ^{cd}	20.0 ± 0.0 ^{cd}	19.0 ± 1.0 ^{cd}	17.3 ± 1.2 ^{cd}	14.7 ± 0.6 ^{cd}	18.7 ± 0.6 ^d
	200	19.3 ± 1.5 ^b	19.3 ± 1.5 ^{bc}	21.3 ± 0.6 ^{bc}	21.3 ± 1.5 ^{bc}	19.3 ± 1.5 ^{bc}	15.7 ± 1.5 ^{bcd}	19.7 ± 1.5 ^{cd}
	400	21.0 ± 1.0 ^b	21.0 ± 1.7 ^b	22.7 ± 0.6 ^b	22.7 ± 2.3 ^b	21.7 ± 0.6 ^b	17.3 ± 1.2 ^b	22.7 ± 1.5 ^b
DW	-	8.0 ± 0.0 ^e	8.0 ± 0.0 ^f	8.0 ± 0.0 ⁱ	8.0 ± 0.0 ^h	8.0 ± 0.0 ^g	8.0 ± 0.0 ^f	8.0 ± 0.0 ^g
5% T80	-	8.0 ± 0.0 ^e	8.0 ± 0.0 ^f	8.0 ± 0.0 ⁱ	8.0 ± 0.0 ^h	8.0 ± 0.0 ^g	8.0 ± 0.0 ^f	8.0 ± 0.0 ^g
Cipro	5 µg	30.7 ± 0.6 ^a	33.7 ± 0.6 ^a	32.3 ± 0.6 ^a	31.0 ± 0.0 ^a	28.7 ± 0.6 ^a	23.3 ± 0.6 ^a	32.3 ± 0.6 ^a

Key notes

Values are expressed as Mean ± SD (n = 3), 8.0 ± 0.0 = no inhibition (well diameter), Means that do not share a superscript letter are significantly different (only column wise) at $P \leq 0.05$. Conc.-Concentration, Cipro- Ciprofloxacin as positive control, DW- Distilled Water as negative control 1, *Paero*-*P. aeruginosa*, *S.typhim*-*S. typhimurium*, T80-Tween 80 as negative control 2

3.2. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the extracts

Each tested microorganism was examined, starting from a high concentration of 64 mg/ml by descending with serial bi-fold dilution till 0.0625 mg/ml, to determine the MIC value. Based on the study the MIC value of the extracts was in agreement with its preliminary antimicrobial activities screening (on well method) against most of the microorganisms. The ethyl acetate extract of the plant was more potent against all organisms than ethanol and aqueous extracts which supported by MBC value, too. *S. aureus* and *S. epidermidis* inhibited at lower concentration by ethanol extract than aqueous extract. For other microorganisms the two extracts showed similar MIC value.

S. epidermidis was the most susceptible bacteria with MIC value of 0.7 ± 0.3 mg/ml, 1.0 ± 0.0 mg/ml, 2.0 ± 0.3 mg/ml for ethyl acetate, ethanol and aqueous extracts, respectively. The low antimicrobial activity was recorded by *E. faecalis* with the highest MIC value of 8.0 ± 0.0 mg/ml by ethyl acetate extract and 16.0 ± 0.0 mg/ml by both ethanol and aqueous extracts compared to other gram positive bacteria. From gram negative bacteria *S. typhimurium*, *S. sonnei* and *P. mirabilis* were most susceptible bacteria which have showed similar MIC value as *E. faecalis* at the three extracts. The lowest antimicrobial activities were recorded by *P. aeruginosa* and *K. pneumoniae* with MIC value of above 64 mg/ml for aqueous and ethanol extracts and 16 mg/ml for ethyl acetate extracts. These values were in agreement with MBC values in which *E. coli*, *K. pneumoniae* and *P. aeruginosa* MBC value were above 64 mg/ml by ethanol and aqueous extracts where as ≤ 64 mg/ml in case of ethyl acetate extract. The minimum MBC value among all bacteria was 4.0 ± 0.0 mg/ml that was by ethyl acetate extract against *S. epidermidis*(Table 3).

Table 3
MIC and MBC value of tested microorganisms

Microorganisms	MIC and MBC	Water extract	Ethanol extract	Ethyl acetate extract	Positive control
<i>S.aureus</i>	MIC	8.0 ± 0.0	2 ± 0.0	0.8 ± 0.3	0.5 ± 0.0
	MBC	32.0 ± 0.0	16 ± 0.0	8 ± 0.0	1.0 ± 0.0
MRSA	MIC	8.0 ± 0.0	8 ± 0.0	4 ± 0.0	0.5 ± 0.0
	MBC	32.0 ± 0.0	32 ± 0.0	8 ± 0.0	4.0 ± 0.0
<i>S.epidermidis</i>	MIC	2.0 ± 0.0	1 ± 0.0	0.7 ± 0.3	0.25 ± 0.0
	MBC	16.0 ± 0.0	16 ± 0.0	4 ± 0.0	0.25 ± 0.0
<i>S.pyogenes</i>	MIC	4.0 ± 0.0	4 ± 0.0	2 ± 0.0	0.5 ± 0.0
	MBC	16.0 ± 0.0	16 ± 0.0	8 ± 0.0	4 ± 0.0
<i>S.agalactiae</i>	MIC	4.0 ± 0.0	4 ± 0.0	2 ± 0.0	0.5 ± 0.0
	MBC	16.0 ± 0.0	16 ± 0.0	8 ± 0.0	1 ± 0.0
<i>E.faecalis</i>	MIC	16.0 ± 0.0	16.0 ± 0.0	8.0 ± 0.0	0.5 ± 0.0
	MBC	32.0 ± 0.0	32 ± 0.0	16 ± 0.0	4 ± 0.0
<i>E.coli</i>	MIC	32 ± 0.0	32 ± 0.0	8 ± 0.0	0.5 ± 0.0
	MBC	> 64 ± 0.0	> 64 ± 0.0	16 ± 0.0	0.5 ± 0.0
<i>S.typhimurium</i>	MIC	16 ± 0.0	16 ± 0.0	8 ± 0.0	0.08 ± 0.04
	MBC	32 ± 0.0	32 ± 0.0	16 ± 0.0	0.5 ± 0.0
<i>S.flexneri</i>	MIC	16 ± 0.0	16 ± 0.0	8 ± 0.0	0.08 ± 0.04
	MBC	32 ± 0.0	32 ± 0.0	16 ± 0.0	0.5 ± 0.0
<i>S.sonnei</i>	MIC	16 ± 0.0	16 ± 0.0	8 ± 0.0	0.5 ± 0.0
	MBC	32 ± 0.0	32 ± 0.0	16 ± 0.0	0.5 ± 0.0
<i>P.aeruginosa</i>	MIC	> 64 ± 0.0	> 64 ± 0.0	16 ± 0.0	1.0 ± 0.0
	MBC	> 64 ± 0.0	> 64 ± 0.0	32 ± 0.0	2 ± 0.0
<i>K.pneumonia</i>	MIC	> 64 ± 0.0	> 64 ± 0.0	16 ± 0.0	1.0 ± 0.0
	MBC	> 64 ± 0.0	> 64 ± 0.0	32 ± 0.0	4 ± 0.0

Key notes: Erythromycin and ciprofloxacin are positive control drugs for gram positive bacteria and gram negative bacteria, respectively. The MIC and MBC values are expressed in mg/ml for extracts and in µg/ml for positive controls.

Microorganisms	MIC and MBC	Water extract	Ethanol extract	Ethyl acetate extract	Positive control
<i>P.mirabilis</i>	MIC	16 ± 0.0	16 ± 0.0	8 ± 0.0	0.5 ± 0.0
	MBC	32 ± 0.0	32 ± 0.0	16 ± 0.0	0.5 ± 0.0

Key notes: Erythromycin and ciprofloxacin are positive control drugs for gram positive bacteria and gram negative bacteria, respectively. The MIC and MBC values are expressed in mg/ml for extracts and in µg/ml for positive controls.

3.3. Acute oral toxicity study

3.3.1. Behavioral pattern and LD50

The results of an acute oral toxicity study showed that the aqueous extracts of the plant were appeared to be safe up to the dose of 9600 mg/kg. Testing parameters of restlessness, touch response, pain response, urination, skin color, fur erection, and food and water intake were assessed (Table 4).

Drowsiness and erection of fur were observed at a dose of 4800 and 9600 mg/kg. Nevertheless, other groups did not show any sign of toxicity. Generally, the study revealed absence of signs of toxicity for most of the setted parameters and absence of mice death records up to the 14th day. Therefore, LD50 of the extract might be considered to be greater than 9600 mg/kg.

Table 4

General appearance and behavioral observations of acute toxicity study for control and treated groups.

Observation	Dose of extracts in mg/kg						
	Control	300	600	1200	2400	4800	9600
Food intake	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Diarrhea	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen
Urination	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Breathing	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Skin color	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Drowsiness	Not seen	Not seen	Not seen	Not seen	Not seen	present	Present
Hypersensitivity	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen
Erection of fur	Not seen	Not seen	Not seen	Not seen	Not seen	present	Present
Sedation	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen	Not seen
Death	Alive	Alive	Alive	Alive	Alive	Alive	Alive

Key notes: n = 5 ,“present” means at least 1 out of the 5 mice showed the symptom, “not seen/normal” means no mice showed the symptom .

3.3.2. Body Weight

A weekly body weight has weighed on initial day, 7th and 14th days of the six groups as displayed on Table 5. At these days all treated groups didn't show any statistically significant difference changes in the body weight compared with control groups ($p < 0.05$).

Table 5

The effect of aqueous extract of *I. tinctoria* A. Rich roots on body weight of mice at different days.

Group	Doses(mg/kg)	Weight(gm)		
		Initial day	7th day	14th day
I	Distilled water	29.4 ± 3.4 ^a	31.0 ± 4.2 ^a	31.2 ± 2.1 ^a
II	300	27.0 ± 2.3 ^a	30.1 ± 2.6 ^a	31.7 ± 3.2 ^a
III	600	27.4 ± 2.2 ^a	30.2 ± 1.9 ^a	30.1 ± 1.5 ^a
IV	1200	30.2 ± 2.3 ^a	30.2 ± 3.7 ^a	31.6 ± 2.5 ^a
V	2400	27.1 ± 1.4 ^a	31.8 ± 3.4 ^a	33.1 ± 3.8 ^a
VI	4800	27.0 ± 1.4 ^a	29.1 ± 1.8 ^a	32.3 ± 2.2 ^a
VII	9600	28.4 ± 2.1 ^a	29.5 ± 1.0 ^a	32.2 ± 1.7 ^a

Key notes: Values are expressed as Mean ± SD (n = 5) one-way ANOVA followed by Tukey's multiple comparison tests ($P \leq 0.05$), “Means” that do not share a superscript letter are significantly different (only column wise).

4. Discussion

4.1. Antibacterial activity

The inhibition zone of the most susceptible bacteria (*S. aureus*, *S. epidermis* and MRSA) in the well method assay did not show any significant difference ($p < 0.05$) at the tested concentration of 100 mg/ml, 200 mg/ml and 400 mg/ml for most tested extracts. Therefore, these bacteria have resulted with similar susceptibility at both the lowest and highest tested extract concentration in this method. These observations could possibly be explained as the effect of these concentrations might be the maximal efficacy portion on the dose-response curve where as the steepest portion might be below 100 mg/ml concentration with the assumption of the dose-response curve is sigmoidal curve. From this the minimum effective dose might be ≤ 100 mg/ml. The remaining tested gram positive bacteria *S. pyogenes*, *S. agalactiae* and *E. faecalis* also did not show any significance difference inhibition at

200 mg/ml and 400 mg/ml concentration for ethanol and ethyl acetate extracts which was a similar scenario with most of gram negative bacteria.

The antibacterial activity of the extracts against MRSA resulted with a highest inhibition zone, lowest MIC and MBC value of 35.7 ± 1.2 mm, 4 mg/ml and 8 mg/ml, respectively. All extracts at all concentrations (100 mg/ml, 200 mg/ml and 400 mg/ml) showed a better antibacterial activity than vancomycin (30 µg) on the well method with a statistically significant difference at ($p < 0.05$). This might be due to the ability of the extracts to inhibit penicillin-binding proteins of the bacteria that are involved in the synthesis of peptidoglycan which is impossible by the antibiotic methicillin. Therefore, it could be a good alternative as a natural product, as we are now in a situation where, in some cases, the glycopeptides antibiotic vancomycin, is the only option for antimicrobial therapy even its non susceptibility in *S. aureus* is on the increase (6, 26).

The well method zone of inhibition was in line with the MBC and MIC value concentration for most of the tested microorganisms except *S. pyogenes* and *S. agalactiae* that might suggest the consistency of the testing methods. The inconsistency of the two organisms might be due to the usage of 5% sheep blood muller-hinton agar. The sheep blood might in some extent decrease the looseness of the media that lead a weak diffusion of extracts than the pure muller-hinton agar that used for other bacteria. On the other way, these two bacteria might be susceptible for large molecules or hydrophobic molecules of the extracts constituents which did not diffuse easily as other studies support it (27). These might be the reasons that the two organism record better MIC and MBC value than those bacteria that had longer inhibition zone than them. For instance *S. agalactiae* and *E. faecalis* on 400 mg/ml ethyl acetate extract showed inhibition zone of 24.0 ± 1.0 mm and 29.7 ± 1.5 mm, respectively (significantly different at $p < 0.05$). This value was inversed as *S. agalactiae* records 2 mg/ml and 8 mg/ml where as *E. faecalis* records 8 mg/ml and 16 mg/ml of MIC and MBC value, respectively.

As observed from the inhibition zone, MIC and MBC value of the extracts the study plant also showed antibacterial activity against gram negative bacteria in extraction solvent dependent manner. Of the extracts ethyl acetate extract showed better antibacterial activity against all gram negative bacteria. For example, *K. pneumoniae* and *Paeroginosa* had > 64 mg/ml of both MIC and MBC on water and ethanol extracts where as ethyl acetate extract had 16 mg/ml MIC and 32 mg/ml MBC which was a great difference in between. This notable better efficacy of ethyl acetate extract supported by other previous studies on plant extracts (28–30). Thus, of the extracts ethyl acetate extracts might has a better penetration ability of the outer membrane of gram negative bacteria and disturbing cellular function, metabolism, and loss of cellular constituents, leading their inhibition and death of the bacteria.

It has been found that the gram positive bacteria were more susceptible to the extracts compared to gram negative bacteria. Many other studies on different medicinal plants also revealed as gram positive bacteria tend to be more sensitive to the antimicrobial properties of plant extracts than gram negative bacteria (31–34). These could be due to gram negative bacteria have an outer membrane that is

composed of high density lipopolysaccharides that serves as a barrier to many environmental exposures including antibiotics (35).

In addition, this study confirms as the roots of *I. tinctoria* A. Rich had also a promising antibacterial activity especially against *S. aureus* and *S. epidermis* which are commonly found in the skin even though the traditional application is to control fungal infections and to toughen the skin (15, 16). Hence, locally dyeing of skin, applying on cloths and different materials might prevent infection transmission of Staphylococci (*S. aureus*, MRSA and *S. epidermidis*), the most abundant skin-colonizing (biofilm forming) bacteria and the most important causes of community associated and hospital acquired skin infections (36–38).

4.2. Acute toxicity

The evaluation of the toxic characteristics is usually a preliminary step in screening medicinal plants for pharmacological activity. But, there is a lack of scientific validation on the toxicity and adverse effects of medicinal plants. Therefore, scientific knowledge towards acute oral toxicity study is much needed since it helps to identify the dose that could be used subsequently and to reveal the possible clinical signs elicited by these medicinal plants under investigation. In addition, in order to increase the confidence on medicinal plants or preparations safety to human being the data of toxicity studies should be obtained (39).

The oral acute toxicity study of the tested plant extracts was carried out on albino mice at a single dose of 300, 600, 1200, 2400, 4800 and 9600 mg/kg body weight and was continuously monitored for first 4 hours, followed for a period of 14 days daily for any toxic effect after the treatment period. Major changes in behavior and mortality were not observed in all groups. However, drowsiness and erection of fur were observed in each mouse of treated groups of 4800 and 9600 mg/kg body weight. These signs were disappeared after the 4th hours almost among all of the mice that showed the symptom. The extract seems to be safe at a dose level of 9600 mg/kg, and the LD₅₀ is considered be > 9600 mg/kg. According to Hodge and sterner toxicity classification the root extract of *I. tinctoria* A. Rich is classified at least as practically non toxic herbal medicine as LD₅₀ between 5000 to 15000 mg/kg is practically non toxic according to this classification (40).

The body weight of each mouse was carefully weighed at first day, 7th day and on the day of sacrifice. The body weights of tested animals of both control and treated groups were increased progressively throughout the study period though it was not statistically significant changes ($p < 0.05$). The body weight changes serve as a sensitive indication of general health status of animals (41). Therefore, the normal increment in body weight and the zero death report could give confidence to state roots of *I. tinctoria* A. Rich did not interfere with the normal metabolism of animals.

5. Conclusion

This study provides scientific basis as the root of *I.tinctoria* A.Rich had a promising antibacterial activity in extract dependent manner in which ethyl acetate extract showed better potency. Gram positive bacteria especially *S.aureus* and *S.epidermidis* were more susceptible to the extracts compared to gram negative bacteria. On the other way, the acute oral toxicity study of the aqueous extracts of the plant were appeared to be safe up to the maximum tested dose that classify *I.tinctoria* A.Rich at least with in practically non toxic category. Therefore, the antibacterial potential and practically non toxicness of the study plant can take the attention of scientific communities for the development of new, effective and safe antimicrobial drugs by further studying the plant in different directions.

Declarations

Ethics approval and consent to participate

The Departmental Research and Ethics Review Committee of Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University approved the study protocol by giving a protocol number of DRERC/392/19/MLS. Laboratory animals were managed scientifically according to the international guideline of the care and use of laboratory animals.

Consent for publication

Not applicable

Availability of data and materials

The corresponding author has all fabricated data that support the findings of this study and majority of them are presented in the main manuscript.

Funding

There is no funding for this research.

Competing interests

No competing interests

Author Contributions

Designed the experiments: Sileshi Degu, Adane Bitew, Negero Gemedo, Abiy Abebe

Extraction: Sileshi Degu

Performed the bioassay and toxicity experiments: Sileshi Degu, Abiy Abebe

Analyzed the data: Sileshi Degu, Negero Gemed, Abiy Abebe

Wrote the paper: Sileshi Degu

Over all advising and technical support: Adane Bitew, Negero Gemed, Abiy Abebe

Acknowledgement

First, we would like to acknowledge Traditional and Modern Medicine Research Directorate of Ethiopian Public Health Institute that helped us by providing microorganisms, reagents, supplies, free space laboratories and equipments for this study. Besides these, we would like to thank the National Bacteriology and Mycology Reference Laboratory of Ethiopian Public Health Institute for giving of some of the tested microorganisms.

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