Original Article

Antibacterial Activity of *Ritchiea albersii* Gilg and *Cynoglossum amplifolium* Leaves Extracts against Selected Bacteria

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Abstract

Background: The increase in antimicrobial resistance worldwide has necessitated the search for alternative therapeutic agents. The leaf extracts of *Ritchiea albersii* and *Cynoglossum amplifolium* have been used as traditional medicine for the management of eye, ear and wound infections in Ethiopia.

Objective: The objective of the study was to evaluate the antibacterial activity of *R. albersii* and *C. amplifolium* against three common bacteria.

Materials and Methods: In this experimental study, the antimicrobial properties of 80% methanol, chloroform and acetone extracts of *R. albersii* and *C. amplifolium* were evaluated against two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumoniae* ATCC 49619) and one Gram-negative bacterium (*Escherichia coli* ATCC 25922) using the agar-well diffusion method. Ciprofloxacin 0.05 mg/disc was used as a positive control. Furthermore, a preliminary phytochemical study was carried out.

Results: The zones of inhibition shown by all extracts of the two plants against the tested bacteria were significantly lesser ($P < 0.05$) than the standard drug. *E. coli* and *S. aureus* were the most susceptible strains for most extracts studied. The acetone extract of *R. albersii* exhibited a higher inhibitory effect ($P < 0.05$) against *S. pneumoniae* (16 mm) and *E. coli* (19 mm) compared with its methanol extract. The chloroform extract of *R. albersii* was more effective than its methanol extract ($P < 0.05$) against all tested bacteria. The acetone extract of *C. amplifolium* displayed a higher inhibitory effect (20 mm) against *E. coli* than its methanol and chloroform extracts.

Conclusions: The leaf extracts of *R. albersii* and *C. amplifolium* exhibited broad-spectrum antimicrobial activity, highlighting their potential as phytotherapeutic drugs in preventing and treating infections caused by *S. aureus*, *S. pneumoniae* and *E. coli*. Further investigations for isolating specific compounds and elucidating mechanisms are required to address the need for novel antibacterial drugs.

Keywords: Antibacterial activity, *Cynoglossum amplifolium*, *Escherichia coli*, *Ritchiea albersii*, *Staphylococcus aureus*, *Streptococcus pneumoniae*

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INTRODUCTION

Infectious diseases are a leading cause of mortality worldwide, particularly in low-income countries.[1] According to the World Health Organization, in 2016, lower respiratory infections, diarrheal diseases and tuberculosis accounted for 3 million, 1.4 million and 1.3 million deaths, respectively, making them three of the top ten causes of deaths worldwide.[2]

Antimicrobials remain valuable resources for treating and preventing infectious disease despite the global increase in antimicrobial resistance (AMR).[3] However, AMR is a key issue in public health and has increased the rates of morbidity, mortality and socioeconomic costs.[4] The increase in multi-drug resistant (MDR) pathogenic bacteria is limiting the choices of effective antibacterial treatment, as this phenomenon has not been paralleled by the development of new antibiotics.[5] Consequently, by the year 2050, an increase in AMR is estimated to annually put 10,000,000 lives at risk.[6] Hence, there is an urgent need for newer antibacterial agents with novel mechanisms of action.[7,8] Some areas for discovering such antibacterials are natural products of plant origin and antimicrobial peptides.[9-11]

In Ethiopia, about 90% of the population is reliant on traditional remedies for the management of diseases.[12] Studies conducted on numerous traditionally used ethnomedical plants of Ethiopia have shown antibacterial activities including *Nuxia congesta,*[13] *Zehneria scabra*, *Ricinus communis*,[14] *Rhamnus prinoides*,[15] *Justicia schimpriana*,[16] *Jasminium abyssinicum*, *Myrsine africana*, *Foeniculum vulgare*,[17] *Verbasicum sinaticum, Calpurnia aurea*, *Salvia schimperi*, *Hypericum revolutum*, *Pterolobium stellatum*,[18] *Datura stramonium*, *Croton macrostachyus* and *Acokanthera schimperi*.[19]

Ritchiea albersii Gilg (*Capparidaceae*) is a small tree with a short thick trunk (11 m high). In Ethiopia, its various parts are used in traditional medicine for the treatment of meningitis, wound, cataract, respiratory tract problems and tonsillitis. *Cynoglossum amplifolium* (Boraginaceae) is a perennial herb or subshrub that is 0.3–1.8 m tall and has a thick tuberous root of up to 45 cm and large leaves and tall stems. In Ethiopia, *C. amplifolium* is prescribed by traditional medicine practitioners to treat ear, eye and wound infections.[20-22] Despite their use as traditional medicines, no antibacterial studies of *R. albersii* and *C. amplifolium* have been conducted to date. Therefore, the aim of this study was to screen the antibacterial activities of 80% methanol, chloroform and acetone crude extracts of *R. albersii* and *C. amplifolium* leaves against *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli*, which are MDR and common causes of ear and wound infections.

MATERIALS AND METHODS

Collection of plant material

For this experimental study, fresh leaves of *R. albersii* and *C. amplifolium* were collected in December 2017 from its natural habitat in Bench district, namely Temenja Yaxi and Andekel Kebele, Southwest Ethiopia, about 574 km from Addis Ababa. The leaves were covered in plastic sheet during transportation. The collected plants were then identified and authenticated as *R. albersii* and *C. amplifolium* by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where voucher specimens (no. HE 001 and HE 002, respectively) were deposited for future reference.

Preparation of plant extract

Fresh leaves of the plants were thoroughly washed by tap water and cleaned with gauze to remove dirt and soil. These samples were then air-dried under the shade and crushed into coarse powder using sterile pestle and mortar. Subsequently, 231 g powder of *R. albersii* and 210 g powder of *C. amplifolium* were divided into three portions and extracted by cold maceration technique with 80% methanol (800 mL), acetone (1000 mL) and chloroform (1000 mL) solution in an Erlenmeyer flask for 3 consecutive days at room temperature to get the crude hydroalcoholic, acetone and chloroform extract, respectively. The same volume of solvent was used for the successive extraction of the residues. The extraction process was facilitated using a mechanical shaker (Bibby Scientific Limited, Stone Staffordshire, UK) at 120 revolutions per minute. The resulting crude extracts were separated from the marc with gauze and then filtered by Whatman filter paper Grade-1 using suction twice by the addition of fresh solvent to acquire the maximum yield. The filtrates were combined and concentrated by Rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland) under reduced pressure. All extracts were then dried and further concentrated using a dry oven (Leaders Engineering, Hastings, UK). Finally, the extracts were transferred into an amber glass bottle and kept at −20°C until use. The respective percentage yield of 80% methanol, acetone and chloroform of *R. albersii* was 15.9%, 16.3% and 14.8% and of *C. amplifolium* was 16%, 14.9% and 14.6%, respectively. All the extracts were reconstituted with dimethylsulfoxide (DMSO) to obtain 100 mg/mL concentrations.

Phytochemical screening

Each crude extract obtained by different solvent extractions was separately tested using standard procedures for the presence of various phytoconstituents, namely alkaloids, flavonoids, saponins, terpenoids, tannins and phenolic compounds.[23-25]

Test organisms

Standard bacterial strains of two Gram-positive bacteria (*S. pneumoniae* ATCC 49619 and *S. aureus* ATCC 25923) and one Gram-negative bacterium (*E. coli* ATCC 25922) were obtained from Ethiopian Public Health Institute and used in this study. They were preserved at −20°C until the preparation of inoculums. Each bacterial strain was activated by streaking on culture media aseptically. For *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, nutrient agar was used, and for *S. pneumoniae* ATCC 49619, 5% sheep blood agar was used. The culture media inoculated with *S. pneumoniae* was enclosed in a candle jar to supply 5%–10% of carbon dioxide. All the inoculated strains were incubated for 24 h at 37°C in an incubator. Then, the inoculum of each bacterium was prepared by taking 3–5 colonies and transferring them to tubes containing 5 ml of normal saline. The immersed colonies of bacteria were mixed gently to form a homogeneous suspension until the turbidity of the suspension became attuned to 0.5 McFarland standards (1.5×10^8 CFU/mL).[26]

Antibacterial activity assay

A sterile cotton swab was implemented to remove surplus suspension by gentle rotation of the swab against the surface of the tube. It was then used to dispense the bacteria evenly over the whole surface of the Mueller–Hinton Agar (MHA). For *S. pneumoniae*, MHA supplemented with sheep blood (5%) was used. The agar well diffusion method, which is equivalent to Kirby–Bauer disc-diffusion method, was used to assess the antibacterial effects of all extracts extracted from the study plants, as described previously.[27] Wells of 6-mm diameter were formed on the inoculated agar media with a sterile cork borer. Around 100 μL of each extract solution (100 mg/ml) was added into each well. Ciprofloxacin 0.05 mg/disc was used as a positive control. The solvent (DMSO) used for the reconstitution of each extract was used as a negative control. The plates were then incubated at 37°C for 18 h. Antibacterial activity was interpreted by measuring the diameter of clear inhibition zones surrounding the wells according to the standards of Clinical and Laboratory Standards Institute, 2015.[28] Each extract was examined in triplicate to ensure the quality and the mean value was calculated.

Statistical analysis

Data were organized, edited and analyzed using SPSS version 22 for Windows (IBM Corp., Armonk, NY, USA). The results of the antibacterial activity were expressed as mean ± standard error of mean. Statistical significance was determined by a one-way analysis of variance followed by the Tukey *post hoc* test to compare the inhibition zone against the selected bacteria between control and treatment groups. $P < 0.05$ was considered statistically significant at a 95% confidence interval.

RESULTS

Phytochemical screening

The qualitative phytochemical screening of the crude methanol and chloroform leave extracts of *R. albersii* revealed the presence of all the tested phytoconstituents except saponins [Table 1]. However, only alkaloids and phenols were detected in the acetone extract. Similarly, the methanol extract of *C. amplifolium* comprised all of the tested constituents except terpenoids, while its chloroform extract contained flavonoids, tannins, phenols and terpenoids and the acetone extract comprised flavonoids, tannins and saponins.

Screening of antibacterial activity

The antibacterial activities of three solvent extracts from *R. albersii* and *C. amplifolium* against each bacterium are tabulated in Table 2. The inhibition zones shown by all extracts of both the plants against each bacterial species were significantly lesser ($P \leq 0.001$) than the positive control [Table 2]. From *R. albersii* test groups, the chloroform extract demonstrated a greater inhibition zone against *E. coli* (21.67 mm) and *S. aureus* (19.67 mm). For the acetone extract, *E. coli* was the most susceptible strain with a growth inhibition of 19 mm followed by *S. aureus* (18 mm). There were no significant differences between the inhibition sizes by chloroform and acetone extracts. Overall, the methanol extract of *R. albersii* exhibited a significantly lower inhibitory effect than acetone against *S. pneumoniae* and *E. coli* ($P \leq 0.001$) and than chloroform against *S. aureus* ($P = 0.002$), *S. pneumoniae* ($P = 0.014$) and *E. coli* ($P \leq 0.001$).

From *C. amplifolium* extract groups, the largest area of inhibition was attained by the acetone extract against *E. coli* (20 mm) followed by methanol extract against *S. aureus* (19.33 mm). All of its extracts showed an inhibitory effect against *S. aureus* and *S. pneumoniae,* with no significant differences between them. However, the methanol and chloroform extracts had a significantly smaller zone of growth inhibition ($P \leq 0.001$) against *E. coli* as compared with the acetone extract [Table 2].

Table 1: Phytochemical constituent of *Ritchiea albersii* and *Cynoglossum amplifolium*

Constituents	*Ritchiea albersii*			*Cynoglossum amplifolium*		
	Methanol extract	Chloroform extract	Acetone extract	Methanol extract	Chloroform extract	Acetone extract
Alkaloids	+	+	++	+	–	–
Flavonoids	+	+	–	+	+	+
Tannins	+	++	–	+	+	+
Saponins	–	–	–	+	–	+
Phenols	+	+	+	+	+	–
Terpenoids	–	+	–	–	+	–

+: Trace amount; –: Absent; ++: High amount

Table 2: Inhibition zone diameter of extracts from leaves of *Ritchiea albersii* and *Cynoglossum amplifolium* against three pathogenic bacteria

Test groups	*Staphylococcus aureus* (ATCC 25923)	*Staphylococcus pneumoniae* (ATCC 49619)	*Escherichia coli* (ATCC 25922)
MRA	16.33±1.86[a,c]	11.33±1.67[a,c,d]	15.00±0.58[a,c,d]
CRA	19.67±0.88[a]	14.00±1.00[a]	21.67±1.76[a]
ARA	18.00±1.16[a]	16.00±0.00[a]	19.00±0.58[a]
MCA	19.33±0.88[a]	14.00±1.00[a]	15.00±0.00[a,g]
CCA	17.33±0.88[a]	16.67±1.67[a]	14.00±0.58[a,g]
ACA	18.67±1.33[a]	16.33±0.88[a]	20.00±1.00[a]
PC	30.00±0.00	26.00±0.00	32.00±0.00
NC	–	–	–

Data are expressed as mean±SEM (n=3). [a]As compared to PC; [b]As compared to MRA; [c]As compared to CRA; [d]As compared to ARA; [e]As compared to MCA; [f]As compared to CCA; [g]As compared to ACA; $P<0.05$. The NC has shown no antibacterial activity. MRA – Methanol extract of *Ritichea albersii*; CRA – Chloroform extract of *Ritichea albersii*; ARA – Acetone extract of *Ritichea Albersii*; MCA – Methanolic extract of *Cynoglossum amplifolium*; CCA – Chloroform extract of *Cynoglossum amplifolium*; ACA – Acetone extract of *Cynoglossum amplifolium*; PC – Positive control; NC – Negative control; SEM – Standard error of mean

In terms of comparison across the various extracts, it was found that all the tested extracts were lesser effective against *S. pneumoniae* than *S. aureus* and *E. coli*, except the chloroform extract of *C. amplifolium*. The methanol extracts of both the plants exhibited the same inhibition diameter (15 mm) against *E. coli*, but the inhibition diameter of *C. amplifolium* methanol extract was greater against *S. aureus* compared with that of *R. albersii* (19.33 mm vs. 16.33 mm, respectively). Against *S. pneumoniae*, the chloroform extract of *R. albersii* and the methanol extract of *C. amplifolium* had the same inhibition diameter (14 mm). Of all the extracts, the chloroform extract of *R. albersii* had the highest inhibition diameter (21.67 mm against *E. coli*, followed by 19.67 mm against *S. aureus*). In terms of the acetone extracts, the zones of inhibitions of both the extracts against the Gram-positive bacteria were almost identical but differed by about 1 mm against the Gram-negative bacteria.

DISCUSSION

An increase in AMR has resulted in medicinal plants gaining importance for their therapeutic potential in producing bioactive substances that inhibit the growth of microbes. The preliminary results of this study, therefore, justify the use of such plants in the complementary and alternative medicine system against some common microbes of public health importance as well as highlight potential sources for developing effective antimicrobial agents in the future.[29]

Organic solvents were used in this study for extraction, as these have been reported to result in higher antibacterial activity compared with aqueous extract.[30] Acetone has been reported to be highly effective for extraction, as it dissolves a wide range of active compounds from plants including both hydrophilic and hydrophobic components.[31,32] In addition, the use of organic solvent as an extractant does not confer any negative effect on the bioactivity against the bacteria tested.[33]

To the best of the authors' knowledge, this is the first study on the antibacterial activity and phytoconstituents of *R. albersii* and *C. amplifolium* extracts. Here, the qualitative phytochemical analysis of the extracts of both plants verified the existence of different secondary metabolites [Table 1], which are well known to produce antimicrobial effects in other plants.[34,35] Thus, the antibacterial activity of both the plants in this study may be associated with the availability of these chemicals that act synergistically or individually.

Despite showing a zone of inhibition, the extracts of these plants did not produce a significant growth inhibition as compared with the standard control. The use of crude extracts of plants can limit their antibacterial potency.[36] In the current study, a single dose of the crude extract was used, which may have resulted in a lower concentration of the active components. Therefore, future studies should be conducted with multiple doses of the extracts with

increasing concentrations to determine its effectiveness compared with the standard control.

Among the extracts of *R. albersii* leaves, a maximum zone of inhibition against *E. coli* and *S. aureus* was observed with the chloroform extract [Table 2]. This may be because the chloroform extract had a greater amount of the active component(s) such as flavonoids and, particularly, tannins than that in the 80% methanol extract; these components were absent or present in an undetectable amount within acetone extract [Table 1]. Tannins enhance the therapeutic efficacy, as they are able to (a) bind proteins and thus inhibit cell protein synthesis, (b) form a complex with the microorganism membrane because of its astringent properties and (c) deprive iron through precipitation and/or its effect on bacterial metabolism through inhibition of oxidative phosphorylation.[37,38] Moreover, the presence of terpenoids in chloroform extract, which was absent in the methanol and acetone extracts, may have directly/indirectly enhanced its growth inhibitory effects against the studied bacteria.

The acetone extract of *R. albersii* had only slightly lower inhibition against *E. coli* and *S. aureus* strains as compared with the chloroform extract. In the qualitative test, this acetone extract was found to have high alkaloid contents and phenols, which have antimicrobial properties[39] and thus may have contributed to the effectiveness against these strains. The comparable antibacterial effects of the acetone and chloroform extracts against all tested bacteria suggest that medium polar and nonpolar compounds of *R. albersii* are likely responsible for its bioactivity, which is similar to the findings of Teka *et al*.[40] The methanol extract had lesser activity on most tested bacteria compared with that of acetone and chloroform extracts, indicating that the active components that inhibit the growth of the studied bacteria might be dissolved better in acetone and chloroform than in 80% methanol. Nonetheless, the zone of inhibition by the 80% methanol extracts of both plants against *E. coli* is similar to that of the same solvent extract of *P. stellatum*,[18], *Ceterach officinarum* DC and *Echinophora tenuifolia* L. subsp. sibthorpiana (Guss) Tutin.[41]

In terms of the extracts of *C. amplifolium*, the acetone extract had significantly higher efficacy against *E. coli* than the 80% methanol and chloroform extracts [Table 2]. The acetone extract consisted of saponins, which was absent in the chloroform extract and may be the differentiating factor for this higher efficacy. This also justifies the localization of active compounds in acetone extract, as 80% methanol and chloroform extract exhibit lesser effect on this microorganism. Against *S. aureus* and *S. pneumoniae*, all crude extracts of *C. amplifolium* showed comparable effects. This may describe the relativity of active phytoconstituent composition among those extracts and/or may be because these are Gram-positive bacteria.

All the extracts of this study were found to inhibit growth in all three studied bacteria. This indicates that these extracts contain compounds with broad-spectrum antibacterial activity, highlighting their potential as alternatives to antibiotics. In terms of the mechanism, the active ingredients in the extracts may affect the overall impermeability and integrity of the bacterial cell wall.[35,42] Flavonoids, which are a diverse group of secondary metabolites that often present in relatively high concentrations in plants,[43,44] have been shown to have effective antimicrobial phytochemicals against various disease-causing organisms (i.e., have a wide range of bioactivities). This biological activity is because of their ability to form a complex with the bacterial cell wall and with extracellular and soluble proteins. Similarly, alkaloids and phenols, which occurred in most extracts in this study, have been recognized to have a growth suppression tendency against various Gram-positive and Gram-negative bacteria.[45,46]

The bioactivity of all extracts against all studied bacteria varied except for the 80% methanol extracts of both the plants against *E. coli* and the chloroform extract of *R. albersii* and 80% methanol extract of *C. amplifolium* against *S. pneumoniae* [Table 2]. These dissimilarities in activity could be linked to the disparity in solvent used for extraction purpose,[13] in addition to phytochemical variations in composition and/or concentration in the respective plant extracts. The sensitivity of microorganisms to chemotherapeutic compounds can change even in different strains of a single bacterial species. Like this study, the extract of various plants inhibited the growth of selected microorganisms at different ratios in another study. The phytochemicals and their concentrations differ across plants, which explains the difference in antimicrobial effect.[41] On the other hand, the extract from the same plant species has shown variable activity against different bacterial species, presumably because of the difference in sensitivity of the microorganisms to specific active ingredients in a plant.[27]

It is interesting to note that *S. pneumoniae* is less susceptible than *S. aureus* to different extracts of the respective plants despite both microbes being Gram-positive, thereby suggesting that genetic variations between the two bacteria[47] could be a factor making *S. pneumoniae* more resistant to the extracts. Furthermore, this might be because of the more complex nature of cell wall of *S. pneumoniae* compared with that of *S. aureus*.

Although the *in vitro* finding of this study suggests that the extracts from *R. albersii* and *C. amplifolium* are effective against the selected bacterial species, this may not necessarily be the same in *in vivo* studies, as seen in previous studies.[27,36] Therefore, more detailed *in vitro* studies, including determination of minimum inhibitory concentration and minimum bactericidal concentration, and *in vivo* investigation of these medicinal plants should be carried out.

CONCLUSIONS

The preliminary findings of this study revealed that because the extracts of *R. albersii* and *C. amplifolium* have a wide spectrum of activity against selected bacteria, they may have potential beyond their current use in ethnomedicine. However, further detailed investigation and isolation of compounds from the extracts should be done so enable more precise testing for the development of newer and safer antibacterial agents. This work can be a basis for elucidation of the actual mechanism of action of these plants.

Peer review

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Conflicts of interest

There are no conflicts of interest.

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