BMC Complementary Medicine and Therapies

RESEARCH

Open Access

In vitro antioxidant and antibacterial activities of *Ajuga integrifolia* leaf extracts obtained with different solvents

Workineh Mengesha Fereja[1*], Wakuma Debessa Geleta[1], Engida Desalegn[2], Mitiku Moaneanda[3], Adugna Abdissa[4] and Firehiwot Belyneh Usamo[5]

Abstract

Background Many diseases are increasingly recognized as public health concerns worldwide because of the increasing incidence of multidrug-resistant bacteria. Recently, interest in the use of indigenous medicinal plants to treat infectious illnesses has increased, highlighting the need to find new bioactive phytochemicals. *Ajuga integrifolia* is a plant commonly utilized in traditional drugs to treat a wide range of diseases, although its effectiveness has not been scientifically validated. The present study aimed to evaluate the total phenolic and flavonoid contents and assess the biological activities of *A. integrifolia* leaf extracts produced via different solvent systems.

Methods Soxhlet extraction was employed to obtain crude extracts from different solvents (methanol, ethanol and water). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing power assays were used to measure the antioxidant activity, and the antibacterial activity of the extract was evaluated on the basis of its minimum inhibitory concentration (MIC) against two gram-negative bacteria (*Escherichia coli* (ATCC-25922) and *Pseudomonas aeruginosa* (ATCC-43495)) and two gram-positive bacteria (*Staphylococcus aureus* (ATCC-25923) and *Streptococcus pyogenes* (ATCC-19615)) via the agar disk-diffusion technique.

Results A significant amount of total phenolic content (TPC) and flavonoid content (TFC) were present in all the extracts. The extracts presented powerful antioxidant activity in all the assays. The disc diffusion and MIC results revealed the ability of the methanol and ethanol extracts of *A. integrifolia* leaves to inhibit *S. aureus* growth at a concentration of 3.125 mg/mL. However, the water extracts were ineffective against *E. coli* and *P. aeruginosa*.

Conclusions These findings indicate that *A. integrifolia* leaf extracts have reasonable biological activities. These findings underscore the importance of *A. integrifolia* leaves as a source of health benefits.

Keywords *Medicinal plants*, *Ajuga integrifolia*, Phenolic compounds, Flavonoid compounds, Antioxidant activity, Antibacterial activity, Minimum inhibition concentration (MIC)

*Correspondence:
Workineh Mengesha Fereja
workinehmengesha@gmail.com; workinehm@du.edu.et
[1]Department of Chemistry, and Energy and Environment Research Center, Dilla University, P. O. Box 419, Dilla, Ethiopia
[2]Department of Chemistry, Hawassa College of Teacher Education, P. O. Box 115, Hawassa, Ethiopia
[3]Department of Horticulture, Dilla University, P. O. Box 419, Dilla, Ethiopia
[4]Department of Biology, Dilla University, P. O. Box 419, Dilla, Ethiopia
[5]Department of Pharmacy, Dilla University, P. O. Box 419, Dilla, Ethiopia

Introduction

Secondary metabolites derived from various plants play critical roles in different biological (antioxidant and antibacterial) activities [1]. These compounds are distinct and specific to certain organisms or groups of organisms [2]. Natural products continue to have a significant impact on the development of drugs, as they make up nearly half of the new chemical entities discovered in drug research [3, 4]. According to the World Health Organization, approximately 80% of the world's population, particularly in developing countries, primarily uses traditional medicines for primary health care needs [5]. Additionally, there has been a rise in the international trade of traditional medicine and plant-based products, leading to widespread acceptance and expansion of knowledge in this field across different countries [6]. The majority of the antioxidant compounds in a regular diet are derived from plants and are classified into different groups with diverse physical and chemical characteristics [7]. Moreover, free radicals, their harmful effects, and bacterial infections continue to be the leading reasons for morbidity and death internationally despite the discovery of many antibiotics [8, 9]. The increasing number of bacterial strains resistant to many antibiotics is another key problem for human health and can lead to bacterial infections [10, 11].

Ajuga integrifolia Buch.-Ham. Ex D. Don (Lamiaceae), in the Ajuga genus, is locally known as akorarach in Amharic and harmegusa in Afaan Oromo in different parts of Ethiopia [12]. *A. integrifolia* is commonly used in traditional medicine to treat various ailments, such as diarrhea, stomach issues, the evil eye, retained placenta, ascariasis, malaria, swollen legs, hypertension, and jaundice. Additionally, it is also employed for veterinary purposes [13].

A. integrifolia is utilized as a medicinal herb by many societies to cure a variety of infectious diseases. However, their ability to act as biological agents and phytochemical entities in the treatment of drug-resistant bacteria has not been adequately examined. There is potential for greater utilization of plant parts in Ethiopia, but owing to the lack of scientific data, this potential is not fully realized. Little is known about *A. integrifolia*, and few studies have reported on the phytochemical content and biological activity of *A. integrifolia* extracts in various solvents. Furthermore, variations in bacterial strains, genotype, soil type, environmental conditions, harvesting techniques, extraction procedures, and extraction methods can influence the composition and efficacy of plant extracts [14–18]. The purpose of this work was to analyze the phytochemical constituents that are responsible for the biological (antioxidant and antibacterial) activities of *A. integrifolia* leaf extracts in various solvents with the goal of providing scientific knowledge that can contribute to drug development.

Materials and methods

Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, catechin, ascorbic acid, ethanol, methanol, sodium carbonate, potassium ferric cyanide, trichloroacetic acid, aluminum trichloride, ammonia, hydrochloric acid, sulfuric acid, ferric chloride, sodium nitrite, sodium hydroxide, chloroform, potassium hexacyanoferrate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, Tween 20, dimethylsulfoxide (DMSO), and all other reagents and solvents used in this study were of analytical grade and purchased from Sigma–Aldrich (St. Louis, USA). Bacterial media (Mueller Hinton agar) and Mayer's reagent (mercury chloride in potassium iodide) were used. Both solvents (Tween 20 and DMSO) were used as negative control and ciprofloxacin were used as positive controls.

Collection, identification and preparation of plant material

Fresh leaves of *A. integrifolia* were randomly collected to eliminate possible selection bias from branches during the month of November 2023 from Gelana Woreda in the West Guji Zone of Oromia regional state, Ethiopia. The sample was subsequently transported to the microbiology laboratory of Dilla University, Ethiopia. The taxonomical identification and authentication were performed by a botanist (Assistant Professor Tariku Berihun, Faculty of Biology). Further authentication was reconfirmed at the National Herbarium of Ethiopia, Addis Ababa University. The authenticated specimen was kept at the Ethiopian National Herbarium Center, Addis Ababa University, with voucher number WDG.01/2023. The harvested plant sample was subjected to washing with tap water to eliminate dust. The samples were subsequently dried in the dark for 15 days at room temperature (25±3 °C). The dried samples were powdered via an electric mill, sieved to obtain a uniform particle size (0.25 mm) and kept in a clean and dry plastic bag in a desiccator in the dark until use.

Extraction of secondary metabolites

The extraction process was carried out according to the protocol described by Ismael et al. [19], with minor modifications. Briefly, 10 g of the powdered sample was added to a beaker containing 100 mL of different solvents (methanol. ethanol, and water) to obtain the extracts. The obtained mixtures were shaken in an orbital shaker at room temperature for ten hours at 120 rpm. The extracts were filtered through Whatman No. 1 filter paper and then evaporated to dryness under vacuum at 40 °C via a rotary evaporator (Buchi, 3000 series, Switzerland). The

experiments were performed in triplicate for each solvent to obtain replicate results. The resulting dried crude extract was weighed to determine the percentage yield of the soluble constituents of the respective solvents via Eq. (1), as described in Goanar et al. [18] and Bandiola [20], and then the dried extract was kept in a sealed plastic continuer at -20 °C until use.

$$\text{Yield}\% = \frac{Weight\ of\ the\ dried\ extract}{Weight\ of\ the\ sample\ taken\ for\ extraction} \times 100 \quad (1)$$

Determination of bioactive constituents

Total phenolic content (TPC)

The amount of TPC in the extract was determined by using the method described by Ismael et al. [19], Banu et al. [21] and Shan et al. [22] with slight modifications, with Folin-Ciocalteu as a reagent (FCR) and gallic acid as the standard. Briefly, FCR was diluted ten times, 1 mL was added to 0.1 mL of extract (2.5 mg/mL), and after 5 min, 1 mL of Na_2CO_3 (75 g/L) was added to the mixture. The mixture was incubated for 90 min at room temperature, and then, the absorbance of the obtained blue color was measured via a UV-visible spectrophotometer (SPECTRONIC 20) at 765 nm. The TPC was determined from the linear equation of the calibration curve (y=0.024x - 0.014, R^2=0.996) drawn from the standard solution (10 to 100 g/mL) of gallic acid and was expressed as milligrams of gallic acid equivalent per gram of dried extract (mg GAE/g) Desalegn et al. [23].

Total flavonoid content (TFC)

The TFC was determined via the aluminum chloride colorimetric assay method as described by Esmael et al. [19], with slight modifications. This was accomplished on the basis of the appearance of a pink color due to the formation of the flavonoid-aluminium complex. Briefly, 1 mL of the extracts (2.5 mg/mL) was diluted with 1 mL of distilled water, and then 75 µL of 5% $NaNO_2$ was added to the mixture. After 6 min, 150 µL of 10% $AlCl_3$ was added, and then, 1 M NaOH was added after 5 min. The absorbance of the pink color solution was immediately measured via a UV-visible spectrophotometer at 510 nm and compared with that of the prepared water blank. The TFC was determined via a calibration curve (y=0.011x+0.132, R^2=0.97) from concentrations ranging from 10 to 100 µg/mL. The obtained result was expressed as milligrams of catechin equivalents per gram of dried extract (mg CE/g) via the method described by Gulcin et al. [24].

Antioxidant activities

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the extracts was evaluated via the methods described by Ismael et al. [19] and Desalegn et al. [23], with slight modifications. Briefly, test tubes with 1 mL of extract at concentrations ranging from 50 to 1000 µg/mL were made. Each test tube received a freshly prepared 2 mL solution of DPPH in methanol (0.06% w/v). The reaction mixtures, along with the reference standard (ascorbic acid), were vortexed and kept at room temperature in the dark for 30 min. The absorbance of the resulting solutions was measured at 520 nm, and water was used as a blank. The percentage reduction in DPPH radical activity was calculated via Eq. 2.

$$\text{DPPH scavenging percentage (R\%)} = \frac{(Ac - As)}{Ac} \times 100 \quad (2)$$

Ac: absorbance of DPPH without extract; As: absorbance of the test extract in the presence of DPPH.

The antioxidant activity of each extract was reported as an IC_{50}value. The IC_{50} value refers to the amount of extract in µg/mL required to scavenge 50% of the DPPH radical [25]. For those extracts, IC_{50} values less than 50 µg/mL were obtained by extrapolating the graph to zero via Origin 8 software."

Ferric ion reducing power

The reducing ability of the extracts to decrease ferric ions was evaluated to determine their antioxidant activity. This was performed via the methods described by Djamila et al. [1] and Ismael et al. [19], with slight modifications. The increased absorbance value indicates the reducing power ability of the extract. By monitoring the production of Perl's Prussian blue at 700 nm, one can see how the extracted antioxidants reduce the yellow ferric cyanide complex [$K_3Fe(CN)_6$] to the blue ferrous form. Briefly, 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferric cyanide (1%) were mixed with 1 mL of extract at a concentration of 1 mg/mL. The obtained mixture was incubated for 20 min at 50 °C. After adding 2.5 mL of (10%) trichloroacetic acid, the mixture was centrifuged for 5 min at 3000 rpm (Centurion, 1000 series, UK). An aliquot, 2.5 mL of the supernatant mixture, 2.5 mL of distilled water, and 0.5 mL of 0.1% $FeCl_3$ were combined to form a mixture. Finally, the absorbance was measured at 700 nm via a UV-visible spectrophotometer, and the value was expressed via an ascorbic acid calibration curve (y=0.004x+0.231, R^2=0.98) in milligrams of ascorbic acid equivalent per gram of dried extract (mg AAE/g).

Antibacterial activity

Culture and maintenance of bacterial strains

The antibacterial effects of the extracts were evaluated according to the method described by Goaner et al. [18] with minor modifications via the disc diffusion method against two Gram-negative bacterial strains (*E. coli* (ATCC-25922) and *P. aeruginosa* (ATCC-43495)) and two Gram-positive bacterial strains (*S. aureus* (ATCC-25923) and *S. pyogens* (ATCC-19615)). The pure cultures of bacterial strains were supplied by the Ethiopian Biodiversity Institute in Addis Ababa, Ethiopia, in March 2022. Before use, the bacterial strains were maintained on Mueller–Hinton agar media at 4 °C, and they were periodically subcultured on the same media to ensure that the bacterial population grew. The solvent used for extraction and Tween 20 were used as negative controls, whereas the ciprofloxacin disc was used as a positive control.

Disc diffusion assay

The antibacterial activity of *A. integrifolia* leaves extracted via different solvent systems (methanol, ethanol, and water) was examined via the disc diffusion assay method described by Bauer et al. [26] following the Clinical laboratory Standard Institute (LSI) Method for performance standards for antimicrobial susceptibility testing. To perform this experiment, a sterile cotton swab was used to spread 100 µL of each inoculum suspension (concentration 10^6 CFU mL^{-1} in each well) adjusted to that of the McFarland 0.5 turbidity standard evenly across the surface of 20 mL Mueller–Hinton agar set in 90 mm petri dishes. Discs with a diameter of 6 mm were sterilized at 121 °C for 15 min and impregnated with a positive control (ciprofloxacin 30 µg/mL) and *A. integrifolia* leaf extract solutions of 20 µL at various doses. After the disc was dried for 5 min, the impregnated discs were applied to the inoculated plates via flamed forceps. Each disc was pressed down firmly to ensure total contact with the Mueller–Hinton agar surface, and the plates were labeled and incubated at 37 °C for 24 h. The obtained result was observed, and the diameter (millimeter) that inhibits the growth of bacteria was measured by using a digital caliper.

Determination of the minimum inhibitory concentration (MIC)

The antibacterial activity potential of the extracts was evaluated by determining the MIC values of the extracts via the disc diffusion method reported by Goanar et al. [18] and Nasro et al. [27], with slight modifications. The extracts were first diluted to a concentration of 100 mg/mL. After cooling to 45 °C, the concentration of each plant extract in molten Mueller–Hinton agar was serially diluted twofold to achieve final concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 mg/mL in the agar plate. To do this, the agar and plant extracts were well mixed in a sterile container and dispensed into a Petri plate labeled with a specific concentration of diluted plant extract. The plates were incubated at 37 °C for 24 h before all the plates were examined for bacterial growth. The minimal concentration at which each solvent extract completely inhibited the growth of the tested bacterial strain was taken as the MIC.

Table 1 Mass and percentage yield of *A. Integrifolia* leaf powder (10 g) extracted with different solvents

Extraction solvent	Mass obtained	Percentage yield (%)
Methanol	2.756	27.56
Ethanol	1.6985	16.985
Water	0.9125	9.125

Statistical analysis

The data were analyzed via ANOVA, Origin 8 software, and Duncan's multiple range tests to differentiate means at $p<0.05$. The IC_{50} value was calculated via linear regression analysis. The results are presented as the means ± standard errors of triplicate measurements.

Results and discussion

Extraction yield and phytochemical screening

The yield of crude extracts from *A. integrifolia* leaf powder obtained via different solvents was determined to assess the effectiveness of different solvents in extracting specific components. Table 1 shows the mass and percentage yield of the samples dried with various solvents. The methanol leaf extract yielded the highest mass yield and percentage yield of secondary metabolites, whereas the water root extract had the lowest percentage yield in the following order: methanol > ethanol > water leaf extract. This trend is often observed in the extraction of bioactive substances and is believed to be due to the capacity of methanol to partially dissolve both polar and nonpolar compounds [28].

The yield of the methanol leaf extract was the highest (27.56%), followed by the ethanol extract at 16.985%. The ethanol and methanol extracts provided higher yields than did the water extract, probably because alcohol solvents contain both polar and nonpolar substances, enabling them to dissolve both polar and nonpolar compounds. Moreover, alcohol solvents are more effective at breaking down cell walls and extracting a greater number of polar molecules with intermediate and small polarities [29]. These results indicate that the secondary metabolite compounds found in *A. integrifolia* are mainly polar compounds and might be influenced by the nonpolar properties of the solvent [30].

Total phenolic content (TPC) and total flavonoid content (TFV)

The TPC and TFC of *A. integrifolia leaf* extracts varied in different solvent systems (Table 2). The results revealed that the extract was rich in phenolic and flavonoid compounds. A comparable TPC was reached at 18.74±0.38 and 17.45±0.38 mg GAE/g dried methanol and ethanol extracts, respectively. The water extract had the lowest value (6.67±0.15 mg GAE/g dry extract). Similarly, the methanol and ethanol extracts had the highest TFC (38.47±2.571 and 39.38±2.314 mg CE/g dry extract, respectively). However, the water extract had the lowest value (6.67±0.257 mg CE/g).

There were no significant differences ($p<0.05$) in the TPC or TFC between the ethanol, and methanol, extracts. However, a significant difference was observed for the *A. integrifolia* water extract compared with both the methanol and ethanol extracts. A phytochemical screening test of *A. remota* leaf extracts using aqueous and 80% methanol revealed the presence of phenolic compounds and flavonoids [31]. Another study reported that flavonoids and phenolic compounds were identified in aqueous and 70% ethanol extracts of *A. remota* leaves [32]. The results of the previous total phenolic and flavonoid contents and the results of the present study support similar findings, indicating that the phenolic and flavonoid contents were relatively high in the leaf extract of *A. integrifolia*.

Table 2 TPC and TFC of *A. integrifolia* leaves extracted with various solvents

Extraction solvent	TPC (mg GAE/g)	TFC (mg CE/g)
Ethanol	17.45 ± 0.60^{a}	39.38 ± 2.314^{c}
Methanol	18.74 ± 0.38^{a}	38.47 ± 2.571^{c}
Water	6.67 ± 0.15^{b}	6.67 ± 0.257^{b}

Note: The TPC and TFC values are expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g) and milligrams of catechin equivalents per gram of dried extract (mg CE/g), respectively. The results are presented as the means±SDs ($n=3$). Significant differences are indicated by different letters in the column after the mean ($p<0.05$)

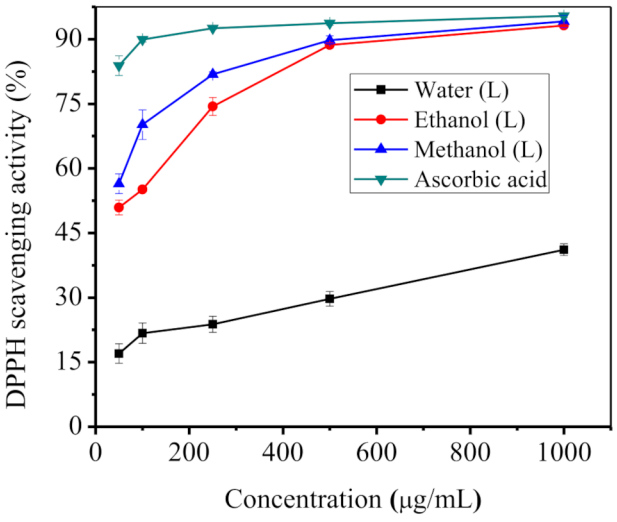

Fig. 1 DPPH radical scavenging activity (%) of methanol, ethanol, and water leaf extracts of *A. integrifolia* and the control (ascorbic acid) at different concentrations. The values are expressed as the means±standard deviations ($n=3$)

Antioxidant activity

DPPH scavenging activity

The antioxidant activity potential is the degree of inhibition of free radical activity and was evaluated on the basis of its ability to scavenge stable free radicals by donating an electron or hydrogen [1]. The disappearance of the purple color of DPPH suggested the presence of antioxidant activity in the crude extract capable of releasing hydrogen. The antioxidant potential was monitored by measuring the absorbance following the production of stable free radicals via a spectrophotometer. A reduction in the absorbance value indicates that the extract has stronger antioxidant activity because it donates hydrogen atoms [33]. The methanol and ethanol extracts demonstrated a greater percentage of inhibition than did the water extract, indicating that phytochemicals are soluble in these solvents (methanol and ethanol) (Fig. 1).

At all the tested concentrations, the leaf extracts of *A. integrifolia* presented DPPH scavenging activity in the following order: ascorbic acid>methanol>ethanol>water. At a concentration of 1000 μg/mL, *A. integrifolia* leaf extract presented greater DPPH scavenging activity than did the other extracts, and there was no significant difference in DPPH scavenging activity between the methanol and ethanol extracts (94.15±1.428% and 93.190±0.275%, respectively), and results comparable with those of commercially available standard antioxidants (ascorbic acid) were obtained. In contrast, the water-containing leaf extract had 41.124±1.363% activities and exhibited the lowest level of antioxidant activity (Fig. 1). These findings indicate that the dried leaf extract of *A. integrifolia* contains substances that are able to easily donate electrons/hydrogen and help neutralize unpaired electrons when extracted with methanol or ethanol. However, the utilization of water for extraction yields small electrons that can easily stabilize free radicals.

Moreover, the antioxidant capacity of the extracts was evaluated by determining the amount of sample capable of reducing the DPPH concentration to 50% (IC_{50}) of the initial amount [34]. The IC_{50} values of *A. integrifolia* leaf extracts were calculated from the graph of percentage scavenging activity versus extract concentrations tested. An extract that has a lower IC_{50} value has greater antioxidant activity than Ismael et al. [19]. According to the results presented in Table 3, the IC_{50} values for the extracts ranging from 10^3 μg/mL for the water leaf

Table 3 IC_{50} values of various solvents in *A. Integrifolia* leaf extracts

Solvent used	Water	Ethanol	Methanol	Ascorbic acid
IC_{50} value in µg/mL	$>10^3$	53.30 ± 3.39^b	48.01 ± 2.31^b	31.88 ± 0.20^a

Note: Values are averages of triplicate measurements (means ± SDs), and values with different superscript letters are significantly different at $p < 0.05$

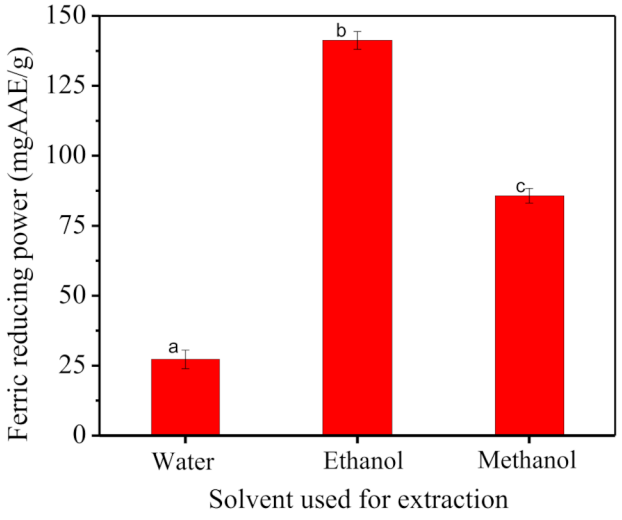

Fig. 2 Ferric reducing power (mg AAE/g) of water, ethanol, and methanol extracts from the air-dried leaves of *A. integrifolia*. The data are the average of triplicate measurements (mean ± SD). Values with different letters in the histogram bar are significantly different ($p < 0.05$)

extract presented the lowest DPPH scavenging activity to 48.01 ± 2.31 µg/mL for the methanol leaf extract, which presented the strongest DPPH scavenging activity.

The results revealed that there was no significant difference in the IC_{50} values of the methanol and ethanol extracts. Moreover, the DPPH scavenging activities of the ethanol and methanol extracts were not significantly different from the DPPH scavenging activity of the standard (ascorbic acid). However, these values are greater than the IC_{50} values and indicate the lowest DPPH scavenging activity, with an IC_{50} value of 31.88 ± 0.20 µg/mL for the standard (ascorbic acid). However, the IC_{50} value of the water extract was significantly lower than that of both the methanol and ethanol extracts ($p < 0.05$). The water leaf extract had the lowest DPPH scavenging activity ($IC_{50} > 10^3$) of all the extracts. As a result, the IC_{50} values of the extracts decreased in the following order: ascorbic acid > ethanol > methanol > water leaf extracts. This is because *A. integrifolia* has high contents of phytochemicals, such as phenolics and flavonoids, which are responsible for its antioxidant properties, which are known for its free radical quenching potential [35]. Overall, these findings suggest that the leaves of *A. integrifolia* have a strong ability to scavenge free radicals. Hence, they have the capacity to react with free oxygen species to generate phenoxyl radicals, which are stable and responsible for stopping the chain reaction [1].

Ferric reducing antioxidant power

The reducing power of the extract was determined by the ferric reducing antioxidant power (FRAP) of the bioactive compounds, which is associated with the antioxidant activity of the natural molecules [1]. The ability of the compound to transfer electrons and reduce iron (III) suggests electron-donating activity, which is an important mechanism in phenolic antioxidant reactions [36]. The process of converting ferric iron (Fe^{3+}) to its ferrous form (Fe^{2+}) is initiated by antioxidants present in the sample that donate electrons. Thus, this activity is proportional to the antioxidant ability of the extracts. At a concentration of 1 mg/mL, the reducing capacity of the leaf extracts decreased in the following order: ethanol (141.224 ± 3.174 mg AAE/g) > methanol (85.714 ± 2.597 mg AAE/g) > water leaf extracts (27.244 ± 3.3 mg AAE/g) (Fig. 2). Compared with the methanol leaf extract and water leaf extract, the ethanol leaf extract of *A. integrifolia* presented the strongest FRAP (the weakest FRAP), with a significant difference ($p < 0.05$).

At 1 mg/mL, the ethanol extract had considerably greater ferric reducing power ($p < 0.05$) than did the methanol and water extracts (Fig. 2). This finding is consistent with the results of the present study, where the ethanol extract and water extract had the strongest and weakest total antioxidant activities, respectively, as determined by the DPPH scavenging activity. There was a significant difference in the antioxidant activity of the ethanol, methanol and water extracts ($p < 0.05$).

This study confirmed that the leaf extracts of *A. integrifolia* presented strong antioxidant properties. Keshebo et al. [37] and Mashwani ZUR et al. [38]. reported that the methanol leaf extract of *A. integrifolia* had strong antioxidant properties. Moreover, other recent studies by Fagbemi et al. [39] and Keshebo et al. [37] on bioactive compounds revealed that the extract has antioxidant activity, and the researchers attributed the presence of secondary metabolites, including phenolics, flavonoids, terpenoids, β-sitosterol, cis-vaccenic acid and other compounds, such as alkaloids and steroids, to these properties. This study and the previous findings therefore confirm the evidence that suggests the involvement of *A. integrifolia* in drug development to combat oxidative stress. As a result, this study validates the traditional use of this plant and its application in the pharmaceutical industry.

Table 4 Antibacterial activity of *A. Integrifolia* leaf extracts in various solvents at various concentrations

Solvent and control used	Concentration (mg/mL)	Mean zone inhibition ± SD (mm)			
		Gram-positive bacteria		Gram-negative bacteria	
		S. aureus	S. pyogenes	E. coli	*P.* aeruginosa
Methanol	100	9.5 ± 0.5	7.66 ± 0.16	7.33 ± 0.16	7.16 ± 0.16
	50	8.33 ± 0.16	6.5 ± 0.5	6.33 ± 0.16	6.16 ± 0.16
	25	7.5 ± 0.33	-	-	-
	12.5	7.33 ± 0.16	-	-	-
	6.25	6.5 ± 0.33	-	-	-
	3.125	6.33 ± 0.16	-	-	-
Ethanol	100	9.00 ± 0.33	7.83 ± 0.66	7.5 ± 0.5	7.5 ± 0.33
	50	8.16 ± 0.16	7.00 ± 0.33	6.83 ± 0.16	7.00 ± 0.33
	25	7.33 ± 0.5	6.66 ± 0.33	6.5 ± 0.33	6.66 ± 0.16
	12.5	7.00 ± 0.33	6.16 ± 0.16	6.16 ± 0.16	6.33 ± 0.5
	6.25	6.5 ± 0.5	-	-	-
	3.125	6.16 ± 0.16	-	-	-
Water	100	6.83 ± 0.66	6.66 ± 0.33	6.33 ± 0.33	6.16 ± 0.5
	50	6.5 ± 0.5	6.33 ± 0.16	-	-
	25	-	-	-	-
	12.5	-	-	-	-
	6.25	-	-	-	-
	3.125	-	-	-	-
Ciprofloxacin 30 µg/mL		14.00 ± 0.5	15.5 ± 0.5	25.5 ± 0.33	26.5 ± 0.33
Tween 20 5%		-	-	-	-

Note: Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa), and Streptococcus pyogenes (S. pyogenes) indicate no antibacterial activity ($p < 0.05$)

Antibacterial activity

Table 4 shows the antibacterial activity of *A. integrifolia* leaf extracts in various solvents. The antibacterial effect of Tween 20 (considered a negative control) did not hinder the growth of the tested bacterial strains. All extracts at the tested concentrations presented lower antibacterial activity than did the antibiotics used (positive control). In this study, ethanol and methanol leaf extracts of *A. integrifolia* presented increased antibacterial activity against the gram-positive bacteria *S. aureus*, followed by antibacterial activity against *S. pyrogens*, *E. coli*, and *P. aeruginosa*. However, the results showed that the water extract is resistant to gram-positive bacteria (*S. pyrogens)*. According to the results of this study, among the bacteria tested, gram-negative bacteria were more resistant to *A. integrifolia* water leaf extract.

In this study, the Gram-positive bacterium *S. aureus* was *more* susceptible to ethanol and methanol leaf extracts of *A. integrifolia* at all the tested concentrations. This is due to the antimicrobial activity of polyphenols and flavonoid compounds [40, 41]. Table 4 shows that at a concentration of 100 mg/mL, the methanol extracts had the strongest bacterial inhibitory effect (9.5 ± 0.5 mm) on *S. aureus*, which was not significantly different from that of the ethanol extract. However, the results indicated that the extracts are resistant to *S. pyogenes*, *E. coli*, and *P. aeruginosa*. The water leaf extract of *A. integrifolia* had the lowest antibacterial efficacy against the gram-positive bacteria *S. pyogenes*, while the gram-negative bacteria *E. coli* and *P. aeruginosa* were more resistant to the water leaf extract of *A. integrifolia*. Many studies have suggested that the antibacterial effect of plant extracts is due to the interaction of the extract with the cell proteins of bacterial strains, leading to cell death [19, 42]. The synergetic effect of the high content of major flavonoids and their interaction due to their structure may lead to the high biological activities of the extracts.

Table 5 Minimum inhibitory concentrations (MICs) of *A. Integrifolia* leaf extracts in various solvents

Bacteria tested	MIC value of extracts using different solvent systems		
	Methanol	Ethanol	Water
S. aureus	3.125	3.125	50
S. pyogenes	50	12.5	50
E. coli	50	12.5	100
P. aeruginosa	50	12.5	100

Note: Staphylococcus aureus (S. aureus), Streptococcus pyogenes (S. pyogens), Escherichia coli (E. coli), and Pseudomonas aeruginosa (P. aeruginosa)

Minimum inhibitory concentration (MIC)

Table 5 summarizes the minimum inhibitory concentrations (MICs) of the *A. integrifolia* leaf extracts. The MIC values for the investigated bacterial strains ranged from 3.125 mg/mL to 100 mg/mL. The ethanol and methanol extracts displayed the highest activity against *S. aureus*,

with an MIC of 3.125 mg/mL, followed by moderate activity against *E. coli*, *S. pyogens*, and *P. aeruginosa*, with MIC values of 12.5 mg/mL for the ethanol extract and 50 mg/mL for the methanol extract. The MIC value for the water extract was 50 mg/mL against Gram-positive bacteria (*S. aureus* and *S. pyogens)* and 100 mg/mL against Gram-positive bacteria (*E. coli* and *P. aeruginosa*). *The* differences in the MICs obtained among the different solvent extracts were probably due to their ability to extract secondary metabolites [14]. Furthermore, *S. aureus* was the most susceptible bacterium to ethanol and methanol extracts, with an MIC value of 3.125 mg/mL. However, *S. pyogenes*, *E. coli*, and *P. aeruginosa* were more resistant to the water extract. Certainly, Gram-negative bacteria are more resistant to the extracts; this could be due to their impermeable external membrane, which limits the amount of antibiotics that may enter the cell [43].

A comparison of these findings with those of other studies confirmed that *A. integrifolia* has antibacterial activity. Keshebo et al. [37] reported that methanol extracts showed antibacterial activity against *Escherichia coli*, Shigella, *Staphylococcus aureus*, and *Bacillus subtilis*. Another study by Mashwani et al. [38] on *A. integrifolia* leaf extract revealed strong antibacterial activity. Moreover, Nagarkoti et al. [44] reported the antibacterial activity of *Ajuga* species (*Ajuga brachystemon*, *Ajuga integrifolia*, *Ajuga macrosperma and Ajuga parviflora*) belonging to the Lamiaceae family. Similarly, more recent studies by Fagbemi et al. [39] and Keshebo et al. [37] on bioactive compounds revealed that the extract had antibacterial activity on some tested bacterial strains, and the results were attributed to the presence of secondary metabolites, including phenolics, flavonoids, terpenoids, β-sitosterol, cis-vaccenic acid and other compounds, such as alkaloids and steroids, as reported by other researchers. The present study and the previous findings therefore confirm the evidence suggesting the involvement of *A. integrifolia* in drug development against pathogenic bacteria. Thus, the results of this study validate the traditional application of this plant and suggest its application in the pharmaceutical industry for the development of drugs from it.

The findings of this study have many important advantages, such as highlighting the potential of this plant for biological application. It can also be used for scientific communities by paving the way and promoting community awareness in conserving plants for medical assets.

Conclusion

In conclusion, the quantitative determination of *A. integrifolia* leaf extracts via methanol and ethanol yielded high total phenolic and flavonoid contents. This extract had stronger antioxidant activity and greater bacterial inhibition activity, which was proportional to its total phenolic and flavonoid contents. The ethanol and methanol extracts showed greater antibacterial efficacy against gram-positive bacteria. However, *P. aeruginosa*, *E. coli*, and *S. aureus* were resistant to water extracts. This study revealed that the leaves of *A. integrifolia* contain considerable amounts of compounds responsible for biological (antioxidant and antimicrobial) activities, which can be used as easily accessible sources for pharmaceutical applications. However, further studies are needed to isolate the pure compounds and evaluate their biological activities to better understand their antioxidant and antibacterial potential.

Abbreviations

ADDP	2,2-diphenyl-1-picrylhydrazyl
ANOVA	Analysis of variance
CE	Catechin equivalents
DMSO	Dimethyl sulfoxide
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
IC< Subscript>50</Subscript>	Half-maximal inhibitory concentration
TPC	Total phenolic content
TFC	Total flavonoid content
MIC	Minimum inhibition concentration
SD	Standard deviation
UV	Ultraviolet
WHO	World Health Organization
ZOI	Zone of inhibition

Acknowledgements

The authors would like to thank the Office of Vice President for Research and Technology Transfer, Dilla University, Ethiopia, for financial support as well as permission to use laboratory facilities. The authors would also like to thank the School of Nutrition, Food Science, and Technology, College of Agriculture, Hawassa University, Ethiopia, for permission to use laboratory facilities.

Author contributions

W.M.F., E.D. and W.D.G. contributed to the conception and methodology of the study. W.D.G., W.M.F. and E.D. carried out the experiments and statistical analysis and wrote the manuscript. W.M.F., E.D. and M.M. modified the experimental design, supervised the experimental process, and edited the final version of the manuscript. M.M. and F.B.U. edited the experimental process and performed the statistical analysis. All the authors read and approved the final version of the manuscript.

Funding

This study was financially supported by the Office of Vice President for Research and Technology Transfer, Dilla University, Ethiopia, with a funding code [DU/010/22].

Data availability

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

Declarations

Ethics approval and consent to participate

All the experimental methods were performed in accordance with the relevant guidelines and protocols. The Dilla University internal review board (DUIRB) issued an ethical clearance for this study. Data collection permission was obtained orally from the land owner.

Consent for publication

Not available.

Competing interests

The authors declare no competing interests.

Received: 3 May 2024 / Accepted: 27 September 2024
Published online: 12 October 2024

References

1. Djamila B, Ines B, Salah A, Chawki B, Douadi K. LC–MS/MS analysis, antioxidant and antibacterial activities of Algerian fir (Abies Numidica de LANNOY ex CARRIÈRE) ethyl acetate fraction extracted from needles. J King Saud Univ Sci. 2020;32(8):3321–7.
2. Werkneh AA. Isolation, structural elucidation, and bioactivity studies of leaf extract of Vernonia Amygdalina. Am J Appl Chem. 2015;3(1):14–20.
3. Tiruneh TA, Tiruneh GA, Abebe EC, Ayele TM. Phytochemical Investigation and determination of antibacterial activity of Solvent leave extracts of *Carissa Spinarum*. Infect Drug Resist. 2022;15:807–19.
4. Devanesan AA, Devanesan AA, Zipora T, Smilin G, Deviram BA, Thilagar G. Phytochemical and pharmacological status of indigenous medicinal plant *Pedalium murex L.-A review*. Biomed Pharmacother. 2018;103:1456–63.
5. World health organization (WHO). Traditional medicine strategy 2014–2023. 2013, Available online at: https://apps.who.int/iris/bitstream/handle/10665/92455/9789241506090_eng.pdf
6. Ekhtelat M, Mahmoodi MM, Fazlara A, Namjoyan F. Comparison of standard and impedance methods for the detection of Enterococci contamination in medicinal herbs in Iran. Jundishapur J Nat Pharm Prod 2019;14(2).
7. Sravani T, Paarakh PM. Antioxidant activity of Hedychium Spicatum Buch.-Ham. Rhizomes. IJNPR. 2012;3(3):354–8.
8. Madrid L, Seale AC, Kohli-Lynch M, et al. Infant group B streptococcal disease incidence and serotypes worldwide: systematic review and meta-analyses. Clin Infect Dis. 2017;65:160–72.
9. Schwarz S, Loeffler A, Kadlec K. Bacterial resistance to antimicrobial agents and its impact on veterinary and human medicine. Vet Dermatol. 2017;8:95–110.
10. Duval M, Cossart P. A new mechanism of antibiotic resistance: ribosome recycling. Med Sciences: Med Sci. 2019;35(8–9):613–5.
11. Blonk B, Cock IE. Interactive antimicrobial and toxicity profiles of Pittosporum Angustifolium L odd. Extracts with conventional antimicrobials. J Integr Med. 2019;17(4):261–72.
12. Seifu A. Bioprospecting Potential of Ajuga Integrifolia for Access and Benefit Sharing. *FAO, Rome, Italy*. 2017.
13. Vohra A, Kaur H. Chemical investigation of medicinal plant Ajuga bracteosa. J Nat Prod Plant Resour. 2011;1(1):37–45.
14. Naiyf SA, Sami AA, Govindan R, Chenthis KC, Shine K, Jamal MK, Khalid FA, Govindan R, Natesan M. Screening of antioxidant and antibacterial metabolites from brown algae Turbinaria ornata for inhibits the multidrug resistant *P. Aeruginosa*. J King Saud Univ Sci. 2020;32(8):3447–53.
15. Zakia K, Chew Shwu W, Irshad UI. Phytochemical screening and antimicrobial activity of Root and Stem extracts of Wild Eurycoma Longi Folia Jack (Tongkat Ali). J King Saud Univ Sci. 2015;27(1):23–30.
16. Brown SP, Grillo MA, Podowski JC, Heath KD. Soil origin and plant genotype structure distinct microbiome compartments in the model legume *Medicago truncatula*. Microbiome. 2020;8(1):1–17.
17. El-Shemy H, Aboul-Soud M, Nassr-Allah A, Aboul-Enein K, Kabash A, Yagi A. Antitumor properties and modulation of antioxidant enzymes' activity by Aloe vera leaf active principles isolated via supercritical carbon dioxide extraction. Curr Med Chem. 2010;17(2):129–38.
18. Goanar G, Tafesse G, Fereja WM. In vitro antibacterial activity of fruit pulp extracts of *Tamarindus indica* against *Staphylococcus aureus* and *Klebsiella pneumonia*. BMC Complement. Med. Ther. 2024;24:127.
19. Ismael J, Desalegn E, Fereja WM. In-Vitro antioxidant and antibacterial activity of leaf extracts of *Measa lanceolata*. Int J Food Prop. 2021;24(1):702–12.
20. Bandiola TMB. Extraction and qualitative phytochemical screening of Medicinal plants: a brief Summary. Int J Pharm. 2018;8(1):137–43.
21. Banu KS, Cathrine L. General techniques involved in phytochemical analysis. IJARCS. 2015;2(4):25–32.
22. Shan B, Cai YZ, Sun M, Corke H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J Agric Food Chem. 2005;53(20):7749–59.
23. Dessalegn E, Bultosa G, Haki GD, Rupasinghe HPV. Antioxidant and-amylase inhibition activities in vitro of various solvent extracts of Thymus schimperi Ronniger. J Med Plant Res. 2015;9(15):515–24.
24. Gülçin I. Antioxidants and antioxidant methods: an updated overview. Arch Toxicol. 2020;94(3):651–715.
25. Dessalegn E, Rupasinghe HPV. Evaluation of phenolic profiles and antioxidant activities of Moringa stenpetala from Ethiopia. Int J Food Prop. 2021;24(1):1581–891.
26. De Zoysa MHN, Rathnayake H, Hewawasam RP, Wijayaratne WMDGB. Determination of in vitro antimicrobial activity of five Sri Lankan medicinal plants against selected human pathogenic bacteria. Int J Microbiol. 2019.
27. Nasro H et al. Remifentanil vs. alfentanil in day-case microlaryngoscopy. Eur J Anesthesiol. 2000;17.
28. Dhawan D, Gupta J. Research article comparison of different solvents for phytochemical extraction potential from datura metel plant leaves. Int J Biol Chem. 2017;11(1):17–22.
29. Aghraz A, Gonçalves S, Rodríguez-Solana R, et al. Antioxidant activity and enzymes inhibitory properties of several extracts from two Moroccan Asteraceae species. S AFR J BOT. 2018;118:58–64.
30. Bekeri D, Adane L, Mamo F. Phytochemical investigation and isolation of compounds from ajuga Integrifolia root extract. WJ C. 2018;13(1):1–13.
31. Hailu W, Engidawork E. Evaluation of the diuretic activity of the aqueous and 80% methanol extracts of Ajuga remota Benth (Lamiaceae) leaves in mice. BMC Complement Altern Med. 2014;14(1):1–8.
32. Tafesse TB, Hymete A, Mekonnen Y, Tadesse M. Antidiabetic activity and phytochemical screening of extracts of the leaves of Ajuga remota Benth on alloxan-induced diabetic mice. BMC Complement Altern Med. 2017;17(1):1–9.
33. Padamanabhan V, Manimekalai G, Vasthi KE, Nirmala A, Jagajothi A. Phytochemical screening and antioxidant activity of extracts of the Leaf and Bark of *Albizzia Lebbeck (Benth)*. Acad J Med Plant. 2014;2(2):026–31.
34. Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol. 2004;26(2):211–9.
35. Onyebuchi AM, Uzor PF, Akazie-Nneji UN, Eze-Odurukwe CU, Ogbatue UB, Mbaoji EC. Antioxidant, total phenolic and flavonoid content of selected Nigerian medicinal plants. Daka Univ J Pharm Sci. 2015;14(1):35–41.
36. Thomas RH, Bernards MA, Drake EE, Guglielmo CG. Changes in the antioxidant activities of seven herb-and spice-based marinating sauces after cooking. J Food Compos Anal. 2010;23(3):244–52.
37. Keshebo DL, Washe AP, Alemu F. Determination of Antimicrobial and antioxidant activities of extracts from selected Medicinal plants. ASRJETS. 2016;16(1):212–22.
38. Mashwani ZUR, Wali R, Khan MF, Abasi F, Khalid N, Raja NI. Antibacterial activity of some selected medicinal plants of Pakistan. United Kingdom: Elsevier,: Medicinal Plants as Anti-Infectives, François Chassagne Ed; 2022. pp. 209–34.
39. Fagbemi KO, Aina DA, Adeoye-Isijola MO, Naidoo KK, Coopoosamy RM, Olajuyigbe OO. Bioactive compounds, antibacterial and antioxidant activities of methanol extract of. Tamarindus indica Linn Sci Rep. 2022;12(1):943240.
40. Shan B, Cai YZ, John D, Brooks B, Corke H. The in vitro antibacterial activity of dietary spice and medicinal herb extracts. Int J Food Microbiol. 2007;117:112–9.
41. Jayachithra RP, Adil FW, Amad MA-A, Rukhsana A, Hamed A, Imra A. Phytochemical analysis and antimicrobial activity of Enicostemma Littorale. J King Saud Univ Sci. 2020;32:3279–85.
42. Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi J BiolSci. 2018;25:361–6.
43. Janakat S, et al. Antimicrobial activity of amurca (olive oil lees) extract against selected foodborne pathogens. Food Sci Tech. 2015;35:259–65.
44. Kirti N, Jeewanti K, Om P, Ravendra K, Rawat DS, Pant AK, Ajuga L. A systematic review on Chemical composition, phytopharmacological and biological potential. Curr Bioact Compd. 2021;17(9):11–37.

Publisher's note