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Effects of extraction solvents on polyphenols content and biological activity of *Ajuga iva* extracts

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ABSTRACT: Different solvent systems have been used for the extraction of polyphenols from plant material, however, the appropriate solvent system is more effective for extracting the total phenolic of any plant extract and evaluating the antibacterial activity is not determined yet. Thus, the objective of this research was to determine the most effective solvent for extraction and characterization of polyphenols as well as antibacterial activity of the aerial parts *Ajuga iva* extracts. The Soxhlet method was devised to extract polyphenols from aerials parts of *Ajuga iva* powders, for this matter, three different solvents were used In order to analyze and quantify the result an in vitro evaluation of the antibacterial activity of the various plant extracts was carried out. The preliminary evaluation of the chemical composition made it possible to highlight the presence of some chemical groups. The quantitative determination of polyphenols is twofold, first the dichloromethanic extract contains the highest levels of polyphenols (3.38 mg GAE/g), second the ethanolic extract contains the highest levels of flavonoids (6.59 mg CE/g DW) and tannins (14.58 mg CE/g DW). On other hand, a remarkable antibacterial activity of some tested extracts was detected. The results showed that solvents with different polarities significantly affected polyphenol content and antibacterial activity.

Keywords: Antioxidant activity; Polyphenols; Composition; *Ajuga iva*.

1. INTRODUCTION

Antibiotic resistance is a phenomenon as old as the advent of antibiotics [1], so the search for antibacterials from natural sources has received much attention, and efforts have been put into identifying compounds that can act as suitable antibacterials to replace synthetic ones. In addition, these naturally-occurring antibacterials can be formulated to give nutraceuticals that can help prevent the increasing prevalence of drug-resistant pathogenic bacteria, which represents an alarming global threat to public health [2]. Among these natural sources, we find *Ajuga iva*, one of the most commonly prescribed drugs in Algerian pharmacopeia thanks to their compounds, such as secondary metabolites that exert a broad spectrum of biological and pharmacological actions [3].

Nevertheless, solvents used during the extraction process are reported to influence the nature and the extract of secondary metabolites extracted from medicinal plants [4]. Thus, the choice of proper extraction

solvent is necessary for the desired pharmacological activity of these extracts. This study endeavours to appraise the effect of three solvents extraction on the qualitative and quantitative profile of *Ajuga iva* polyphenols and their antibacterial activities.

2. MATERIALS AND METHODS

2.1. Plant materials

Arial parts of *Ajuga iva* were collected from" KEURTE" in Mascara city in the Northwest of Algeria in April 2018 and were oven-dried and milled into uniform dry powder and packed in paper bags, and stored at +4°C before experiments.

2.2. Chemicals

All chemicals were purchased from Sigma (USA) and Merck (Germany).

2.3. Preparation of extracts

Polyphenols were extracted using solvents of increasing polarity method (dichloromethan and ethanol/soxhlet) [5]. The powder of the previously prepared plant is first put in contact with the dichloromethane at the rate of 300 ml of solvent per 25 g of drug-using the Soxhlet device. After several siphoning (5 siphonings), the heterogeneous mixture is filtered with filter paper, and the residue is extracted two times every 24 hours again under the same conditions. The filters are joined together, the solvent evaporated using a rotating evaporator (Büchi-Rotavapor; 56°C), and the dry residue is dried and weighed: this is called raw dichloromethanic extract (DCM). The residue not extractable with dichloromethane is treated using the same method with ethanol. After combining the filters, evaporating the solvent and freeze-drying, the ethanolic crude extract (EtOH) is obtained

2.4. Screening of phytochemicals

Phytochemical components of aerial parts from *A. iva* were screened using the methods of [6-10]. The components identified were: flavonoids, tannins, alkaloids, anthraquinones, free quinones, cardiac glycosides, irridoides, senoides and mucilage.

2.5. Determination of total phenolic content (TPC)

Folin-Ciocalteu colorimetric method was used to determine the total phenolic content of plant extracts [11]. Each extract was mixed with Folin-Ciocalteu reagent (0.2 N), and after 5 min, aqueous sodium carbonate (75 g/L) was added, and the mixture was incubated for 90 min at room temperature. Absorbance was measured at 760 nm using a UV/Vis spectrophotometer (Beckman Coulter DU530). Results were expressed in milligram per g dry weight.

2.6. Determination of total flavonoids (TFC)

The aluminum chloride colorimetric method was used to quantify the total amount of flavonoids [12]. Leaf extracts were mixed with 1.5 ml of alcohol, 0.1 ml of aluminum chloride (10%), 0.1 ml of potassium acetate (1 M) and 2.8 ml of deionized water. The absorbance of the reaction mixture was recorded at 415 nm after 40 min of incubation at room temperature. A calibration curve was prepared using quercetin, and results were calculated as milligram per gram dry weight.

2.7. Determination of total tannin content (TTC)

The reaction with vanillin transformed condensed tannins into anthocyanidins [13]. Reduced tannin contents of each organ (three replicates per treatment) were expressed as mg catechin equivalents per gram (mg CE/g) through the calibration curve with catechin [13].

2.8. Antibacterial activity assays

2.8.1. Bacterial strains and antibiotic susceptibility test

Several bacterial strains were isolated from Ghriss hospital laboratory (Mascara, Algeria) and subjected to disk diffusion method using different antibiotics [15]. In the end, seven strains were selected for their antibiotic resistance. According to the standardization of susceptibility in human medicine at the national level, and the recommendations of the Committee on Antimicrobial the French Society for Microbiology (2008): *Enterococcus feacalis*, *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Staphylococcus aureus.*

2.8.2. Disc diffusion test

Antimicrobial activity was determined by the agar disc diffusion assay [16]. Inoculum for the assays was prepared by diluting scraped cell mass in 0.85% NaCl sterile solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometric reading at 580 nm. Cell suspensions were finally diluted to 10^6 CFU/ml. The extracts were dissolved in dimethyl sulfoxide (DMSO) or distilled water. Petri plates were prepared with 20 ml of sterile Mueller Hinton agar (Sigma, Paris, France) surface inoculated by cell suspension (200 µl).

The test cultures were swabbed on the top of the solidified media and dry for 10 min. The tests were conducted at a concentration of the sterile phenolic extract (100 mg/ml) and two dilutions (50 and 25 ml) of *A. iva* in sterile filter paper discs (6 mm). The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. The plates were incubated at 37°C for 24 h. Pristinamycin, nitroxolin, spiramycin were used as positive controls. Negative controls were performed using paper discs loaded with 20 µl of the aqueous DMSO.

The antimicrobial activity was evaluated by measuring the growth inhibition zone surrounding the discs. After that, the inhibition zones were measured in millimeters by Vernier calipers. All tests were repeated two times to minimize test error. An inhibition zone of 14 mm or greater (Including diameter of the disc) was considered as high antibacterial activity [16].

2.8.3. Determination of MIC by microdilution method

MIC of the compounds under study was determined by the microdilution method as described by [17]. All wells were filled with 50 µl of Muller Hinton broth (MHB). Extracts were dissolved in DMSO and added to the first well (50 µl). Serial two-fold dilutions were made then. An overnight culture of bacteria suspended in MHB was adjusted to turbidity equal to 0.5 McFarland standards, so 10^4 CFU/ml of bacterial inoculums size. Each test included two growth controls: the medium with the solvent (DMSO) and the medium with bacterial suspension. Each plant extract was run in duplicate. The test plates were incubated at 37°C for 18 h. Then the turbidity was measured every two hours using a microplate reader (TECAN brand) at 620 nm wavelength. The MIC was taken as the minimum concentration of the dilutions that inhibited the growth of the test microorganism.

2.9. Statistical analysis

The analysis results were performed in triplicate: The results obtained were presented with their standard deviations (mean±SD). All statistical comparisons were made by ANOVA test, and statistical significance was defined as $P<0.05$. Statistical analysis was carried out with Graph PadPrism version 7.00 for Windows, GraphPad Software, San Diego California USA.

3. RESULTS

3.1. Phytochemical screening and extraction yields

The results displayed in Table 1 show the presence of alkaloids, tannins, flavonoids, anthocyanins, irroides and quinones with varying intensities, with an absence of coumarins and senoides.

Table 1. Phytochemical constituents of the *Ajuga iva.*

Components		Results
Alkaloids		+
Tannins	Catechic T	++
	Gallic T	+
Flavonoids		+++
Coumarins		–
Anthocyanins		+++
Free-Quinones		++
Cardiac-glycosides		+
Irroides		+++
Senoides		-
Mucilages		+

–: Absent, +: Low in abundance, ++: Moderate in abundance, +++: High in abundance.

Additionally, the presence of tannins is revealed by the appearance of a dark blue color for gallic tannins. A greenish-blue coloration is a sign of catechins that were weakly present in the powder tested; however, alkaloids were found to be higher in the present sample.

The extraction stage from the plant generated two types of extracts: a dichloromethanic extract (DCM) and an ethanolic extract (EtOH). Yields were determined against 10 g of powder. The results were expressed as a mass percentage and are presented in Figure 1.

According to the experimental findings, the dichloromethanic extract has a brown color with a pasty consistency. On the other hand, the ethanolic extract that retained the initial color of the sample was viscous. It should be noted that both extracts have a less intense smell than that of the plant. The extraction yields demonstrated a slight difference between the two extracts.

The highest rate was noted for E. DCM, with an average percentage of $2.54 \pm 1.68\%$ and ethanol extraction yield was remarkably low compared to DCM extraction with a value of $1.20 \pm 1.2\%$

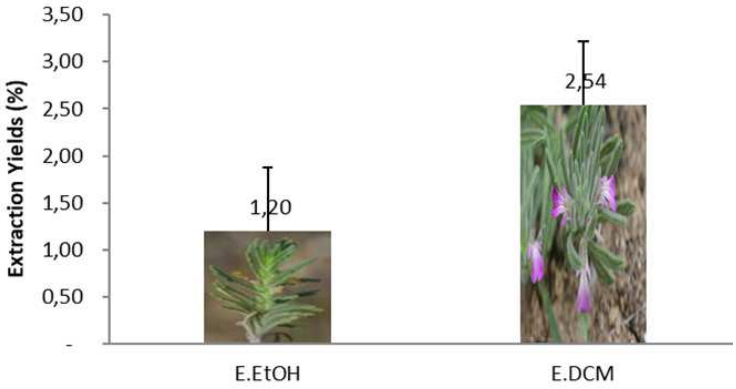


Figure 1. *Ajuga iva* extracts yields.

3.2. Phenolic compound's content

Table 2 details the phenolic compound's content. The dichloromethanic extract represents the richest polyphenol-rich extract with: 3.38 ± 0.01 mg GAE/g PS compared to ethanol extract 0.91 ± 0.05 mg GAE/g PS. However, the latter is the richest in flavonoids (6.59 ± 0.21 mg EC/g) compared to the plant's dichloromethanic extract (1.82 ± 0.01 mg EC/g). The same result was observed in the content of condensed tannins which is higher in ethanolic extract (Table 2).

Table 2. The effect of different solvents on polyphenol content in *A. iva* extracts obtained by both solvents.

	TPC (mg GAE/g)	**TFC (mg QE/g)**	**TTC (mgCE/g)**
DCM.E	3.38 ± 0.01	1.825 ± 0.01	14.58 ± 0.22
EtOH.E	0.911 ± 0.05	6.59 ± 0.21	20.7 ± 0.0066

Values are expressed as mg GAE/g dry weight (means ± standard deviation of three measurements). TPC: Total Polyphenols Content, TFC: Total Flavonoids Content, TCT: Total Condensed Tannins.

3.3. Antibacterial activity results

3.3.1. Disc diffusion test

Plant extracts and pure phenolic acids were determined as an evaluation of their antimicrobial activity against selected pathogenic bacteria. Using the disk diffusion and agar dilution methods, we tested the ability of bacteria to produce visible growth when a given amount of plant extract or pure phenolic acid was added.

According to the results obtained, Table 3 shows that the antibacterial effect is more or less important depending on the nature of the strain and the active substance's concentration (100, 50 and 25 mg/ml). Subsequently, we notice the diameter of the varied inhibition zone (8-13 mm) so we can say that our extract has a moderate effect on the four strains tested (Table 3).

On the one hand, the most effective extract is dichloromethane extract which gives a zone of inhibitions (13 mm) on *Klebsiella pneumoniae* and exerting a significant effect on the four strains. On the other hand, for the ethanolic extract, no effect was observed on the *Pseudomonas aeruginosa* strain so there is a potential resistance (6 mm). As a result, the studied plant has an average antimicrobial effect on both strains *Klebsiella pneumoniae* and *Enterococcus faecalis* and a weak effect on the strain *Staphylococcus aureus*.

Table 3. Antimicrobial activity caused by phytochemicals through agar diffusion method (inhibition zone in mm).

		Staphylococcus aureus	*Klebsiella pneumoniae*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*
Control	Sp	12	14	6	10
	PTK	6	6	6	6
	NTX	6	25	6	6
DCM extract (mg/ml)	100	10	13	10	8
	50	6	6	6	6
	25	6	6	6	6
EtOH extract (mg/ml)	100	8	11	11	6
	50	6	6	6	6
	25	6	6	6	6

SP: Spiramycine; PTK: Pristinamycine; NTX30: Nitroxoline.

The qualitative and quantitative results of polyphenolic extracts analysis reveal that the DCM extract contains a high level of polyphenols [18]. Polyphenols can cause inhibition of intracellular enzymes.

3.3.2. Microdilution test

It is noted that both extracts have an inhibitory effect on the four bacterial strains tested. For the DCM extract, The concentration of 75 mg/ml is sufficient to inhibit the growth of *S. aureus*. On the other hand, *Klebsiella sp*., *E. faecalis* and *P. aeruginosa* are inhibited at the concentration of 150 mg/ml. On the other hand, the ethanolic extract is shown to have an inhibitory effect on *K. pneumonia,* S. *aureus*, *P. aeruginosa* strains at a concentration of 150 mg/ml, on the other hand, no effect on *E. faecalis*. Both extracts have moderate antibacterial activity, but if we compare the ethanolic extract to a weak antimicrobial activity when added to the DCM extract.

Aligiannis et al. [19] proposed a classification of plant material based on MIC results as follows:

• Strong inhibition: MIC less than 500 µg/ml

• Moderate inhibition: MIC varies from 600 µg/ml to 1500 µg/ml.

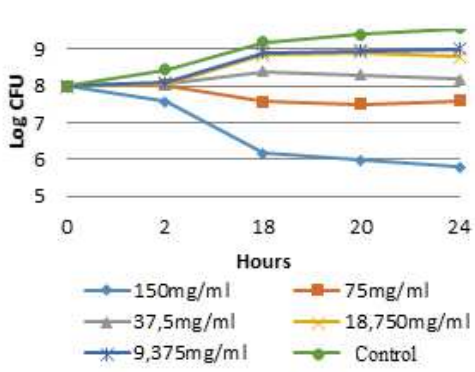
Relying on the research results at hand, the antibacterial properties of the extracts are due to their chemical composition and to the nature of the germ itself. The inhibitory activity of the extracts has been identified in a wide range of concentrations ranging from 25 to 50 mg/ml for the polyphenolic extracts (Table 4 and 5). According to the graphs, it is noteworthy which MIC blocks bacterial growth, this means that after 18 h the microbial load becomes stable at the starting point (10^6 germs/ml), the germ does not multiply.

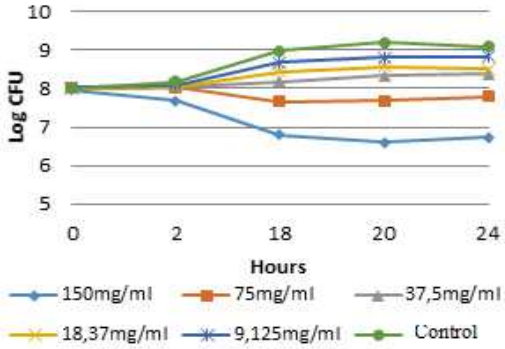
Table 4. Minimal inhibitory and bactericidal concentration of *A. iva* extracts.

	MIC (mg/ml)		**MBC (mg/ml)**	
	DCM	**EtOH**	**DCM**	**EtOH**
K. pneumoniae	150	150	>150	>150
S. aureus	75	150	75	>150
E. faecalis	150	>150	150	>150
P. aeruginosa	150	150	>150	>150

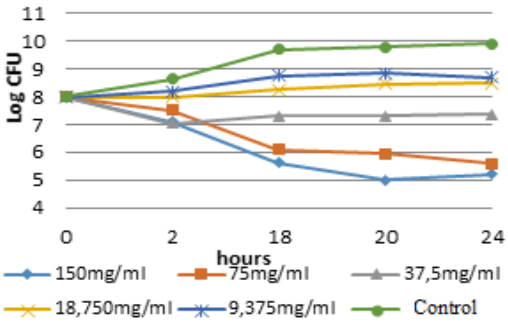
Table 5. Minimal Inhibitory Concentration (MIC) of plant extracts and phytochemicals against antibiotic-resistant bacteria.

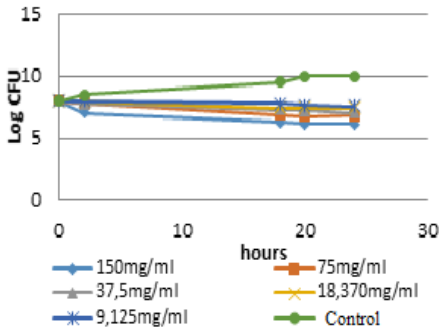
K. pneumoniae



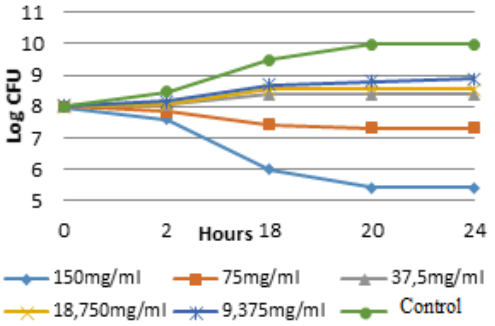


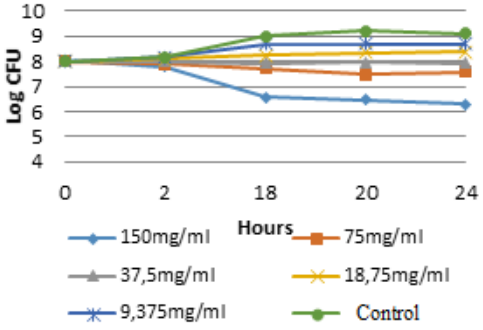
S. aureus



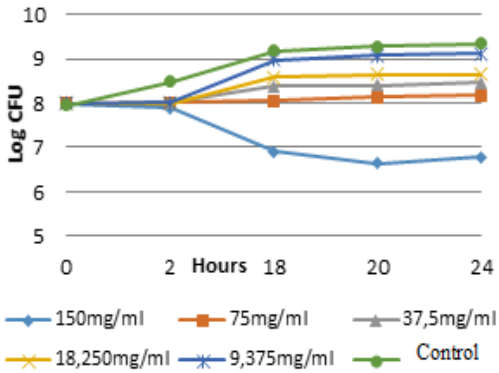


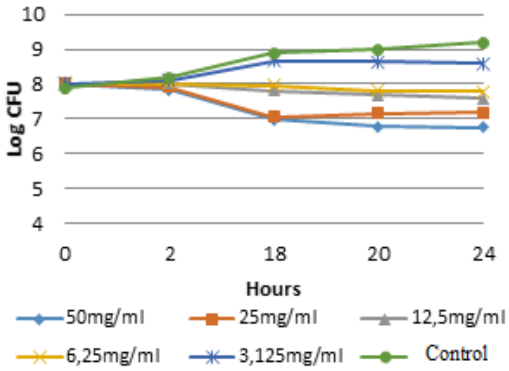
E. faecalis





P. aeruginosa





4. DISCUSSION

The presence of alkaloids, tannins, flavonoids, anthocyanins, irroides and quinones with varying intensities and an absence of coumarins and senoides was noted in our sample. In effect, the other works [3,20,21] reported the presence of the same chemical groups at the aerial parts of *A. iva*, namely: tannins, flavonoids, sterols, steroids, volatile oils and saponosides, which is comparable to the obtained results.

The presence of tannins is revealed in our sample, contrasting the results of Hariri and Ouis [23], who noted their absence. The presence of irrioides and anthocyanins is typical for the Ivette species, whose presence was confirmed in our experiment and proven previously by the work of Amarowicz et al. [24], especially for ajugarin. However, The absence of coumarins and senoides is confirmed by Bendif et al. [25]. However, alkaloids were found to be higher in our sample using Mayer's reagent [26] which found similar results. Alkaloids and flavonoids have been reported to be responsible for plant antibacterial activity [27].

For extraction yields, our results are consistent with Bendif et al. [25] for the ethanolic extract of *A. iva* (2.56-0.05). This is close to Bendif's value (2.56%) [25]. On the other hand, Arrar et al. [28] found higher yields with percentages between 4.07 and 2% for ethanolic and methanol extracts, respectively. It is worthwhile to note that the effect of solvents on extraction yield has been reported in numerous studies [29, 30]. Qasim et al. [31] have also shown that solvent polarity is of great importance, and therefore, variation in yields of various extracts can be attributed to the polarities of different compounds.

The dichloromethanic extract represents the most TPC extract with: 3.38-0.333 mg GAE/g PS compared to ethanol extract 0.91-0.333 mg GAE/g PS; these results are comparable to those found by Rouibi et al. [21], which showed that the total phenol levels of the gross extract of *Ajuga iva* is 3.49 mg EGA/mg MS. but also those of Saad et al. [32], which found content of 1.32 mg GAE/g PS of TPC in the polar methanolic extract of *A. Iva.* On the other hand, Mohamed et al. [33] have shown that the phenolic content of the methanolic extract of some plants belonging to different *Ajuga iva and Punica granatum* families, *Retama raetam*, *Thymus capitatus, Rosmarinus officinalis, Ruta chalepensis, Lawsonia inermis* and *Agave americana* range from 1.68 to 11.07 mg/g of dry matter expressed in gallic acid equivalent.

Concerning the flavonoids content (FC), the ethanolic extract is the richest (6.59-1.662 mg EC/g) compared to the plant's dichloromethanic extract (1.82-0.660 mg EC/g). Our values have been compared to previous work on species of the same family that are superior to *Teucrium polium* [34] for ethanol extract, whereas it is almost similar to that of *Teucrium polium* for dichloromethanic extract [35]. We also noted that the content of condensed tannins in the ethanolic extract is higher than that of dichloromethanic extract. Indeed, a study by Taleb-Senouci et al. [36] shows that tannin levels the methanolic extract of A. 26.86 mg EGA/mg MS respectively. This content remains higher than the results found in this research which are lower in the different extracts. Nevertheless, these values are exciting, proven by the qualitative study on tannins discussed previously, are of great significance.

Indeed, the presence of secondary metabolites in an extract can be influenced qualitatively and quantitatively by several factors such as the mode and time of extraction, temperature, the nature of the solvent and its polarity that allows solubilize and extract similarly polarized compounds [37,38] have shown that the type of solvent significantly influences the total phenol and flavonoid amounts of plants.

With regard to the antibacterial activity and according to the literature, there is a close relationship between phenolic compounds and antimicrobial activities for the antibacterial activity. Therefore, in general, the phenolic compounds in our extract seem effective against the strains tested [46]. Indeed, an ethnopharmacological study revealed that the *Ajuga* plant has important antibacterial activities linked to the content of active compounds, such as ajugapyrin A, bracteonin A, lupulin C and iridoids which have a wide range of biological and pharmacological activity [47]. However, most of the work investigating the mechanisms of action of phenolic compounds suggests that their main site of action is the bacterial plasma membrane [48].

The growth of different strains of bacteria has been dramatically influenced, and a very significant reduction is proportional to the dose of the natural extract. These antibacterial activities are not due to the presence of a particular substance only but to the synergistic or antagonistic effect of each of the extract constituents; they can disintegrate the cell membrane of bacteria [46]. The cell wall and membrane destroy its permeability and release its intracellular constituents. Still, they are likely to interfere with different cellular functions: electron transport, synthesis of proteins and nucleic acids, and enzymatic reaction [49].

5. CONCLUSION

Based on the results, it can be concluded that the qualitative and quantitative phytochemical study demonstrated a richness of the aerial part of the plant *Ajuga iva* in bioactive compounds; nevertheless, the quantitative findings reveal that dichloromethanic extract contains a high polyphenol content, unlike ethanol extract which is rich in flavonoids and tannins.

Moreover, the chemical composition of plant extracts depends mainly depends on the solvent used. Ethanol was particularly efficient in extracting polyphenolic compounds from *Ajuga iva* areal parts. The investigation also confirms that the plant material extraction efficiency proportionately increases with increasing solvent polarity. Likewise, *A. iva* extracts showed significant antibacterial activity marked by the strongest effect of the dichlorometanic extract. These results open new avenues of research on these board-spectrum plants in the field of herbal medicine.

Authors' Contributions: BA, BS, BS and TTA: Designed the study wrote the protocols. BA: Gathered the initial data and performed preliminary data analysis and interpretation. BA and SLK: Managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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