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Impact of Geographical Conditions on Phenolic and Flavonoid Contents and Antioxidant Activity of Different Extracts of *Ajuga iva*

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ABSTRACT

In the present investigation, various cultivars of *Ajuga iva* were collected from different locations in Morocco in 2022. These cultivars were subjected to extraction using different solvents, namely water, ethanol and methanol, to obtain extracts from their aerial parts. The extracts were then analyzed to determine their total phenolic content, total flavonoid content, total sugar content, hydrolysable tannin content, condensed tannin content and antioxidant activity. The results obtained from the analysis revealed the following quantities for the different parameters: The total phenolic content ranged from 226.04±8.47 to 22.59±2.43 mg GAE/g dw, the total flavonoid content ranged from 22.27±0.11 to 3.35±0.006 mg QE/g dw, the total sugar content ranged from 38.78±2.56 to 2.88±0.18 mg/g dw, the reducing sugar content ranged from 7.17±0.45 to 0.41±0.007 mg/g dw, the hydrolysable tannin content ranged from 12.25±0.017 to 0.75±0.15 mg TAE/g dw, the condensed tannin content ranged from 25.49±0.53 to 3.35±1.85 mg/g dw and the total antioxidant capacity ranged from 0.18±0.012 to 0.010±0.004 mg AAE/g dw. Furthermore, a principal component analysis was conducted to assess the relationship between the different parameters. The analysis revealed a strong correlation between the total phenolic content, total flavonoid content and hydrolysable tannin content with the total antioxidant capacity. This suggests that these compounds contribute significantly to the antioxidant capacity of *Ajuga iva*. Overall, the findings of this study demonstrate that *Ajuga iva* contains substantial amounts of bioactive compounds and possesses a noteworthy antioxidant capacity. These results contribute to the understanding of the chemical composition and potential health benefits of *Ajuga iva.*

Key words: *Ajuga iva*, Antioxidant potency, Bioactive compounds, Oxidative stress.

INTRODUCTION

Plants have been a vital part of human diet since ancient times, providing essential nutrient and protective substances against various diseases (Crozier *et al*., 2008; Li *et al*., 2023). However, determining the appropriate time to use medicinal plants as a medication can be a daunting task (Phillips, 2023). With the discovery of the beneficial properties of phytochemicals, the study of medicinal plants has become a promising field for drug discovery (Rasouli *et al*., 2017).

Ajuga iva (Al) is a member of the Lamiaceae family with numerous vernacular names, including “Chendgora” and “Touftelba”, this plant is grown in different parts of the world (Lahrizi *et al*., 2022). Al has medicinal importance and is traditionally used as a natural medication for different ailments such as diabetes, obesity, inflammation and cancer (Bouyahya *et al*., 2020). The ethnopharmacological studies were considered a keystone of the start of the experimental investigation of different biological functionalities of natural products. In this context, several studies were conducted to document different usages of Al in folkloric medicine. Furthermore, Lyoussi *et al*. (2023) conducted a study documenting the ethnopharmacological properties of different medicinal plants used in the Sefrou region (Middle-North of Morocco). They found that the Al was used in different forms to treat psychic diseases, diabetes, cancer, asthma, rheumatism, digestive disorders and hypertension (Lyoussi *et al*., 2023). Nowadays, the determination of the phytochemical composition for the beneficial properties of medicinal plants is coming to the forefront in the search for safe and efficacious medication against human diseases (Khatteli *et al*., 2020; Makni *et al*., 2013; Senhaji *et al*., 2020). The pharmacological properties of Al seem to be mediated by the bioactive compounds found in Al extracts, including phenolic acids, flavonoids, tannins, proteins, minerals and vitamins (Khatteli *et al*., 2020). In fact, different techniques used to determine chemical composition, accounting high-performance liquid chromatography (HPLC), gas chromatography- mass spectroscopy (GC-MS), nuclear magnetic resonance spectroscopy (NMR), detected several

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bioactive compounds in different amounts such as, ajugasterone, apigenin, carvacrol, cyasterone, 20-hydroxyecdysone, ecdysterone and palmitic acid (Bouyahya *et al*., 2020). The biological properties of AI were ascribed to its bioactive compounds which positively react with different biological attributes.

The impact of various factors on the phytochemistry of medicinal plants if significant, with environmental factors being the most prominent (Ncube *et al*., 2012; Ousaaid *et al*., 2019). The diversity in phytochemical content of medicinal plants collected from different geo-biological sources is well-established (Agrawal *et al*., 2021; Kumar *et al*., 2017; Senhaji *et al*., 2020; Touati *et al*., 2022) and this variability has a direct impact on their *in vitro* and *in vivo* biological properties. In light of this, our study aims to assess the antioxidant activity, phenolic and flavonoid contents of *Ajuga iva* samples collected from different geographical locations in the Fez Meknes regions.

MATERIALS AND METHODS

Sampling sites

Different samples of AI were collected in July 2022, from five distinct altitude locations in Fez-Meknes region, Morocco, including Fez (34°01′26″N 5°00′06″W), Emmouzzer Kandar (33°50′21″N 5°00′35″W), Moujou (33°48′36″N 4°45′0″W), Jbel Zerhoune (34°01′56″N 5°31′09″W) and Azzaba (33°49′40″N 4°42′29″W). to Different samples of AI were collected in July 2022, from five distinct altitude locations in Fez-Meknes region, Morocco, including Fez (Variety 1: V1) (34°01′26″N 5°00′06″W), Emmouzzer Kandar (Variety 2: V2) (33°50′21″N 5°00′35″W), Moujou (Variety 3: V3) (33°48′36″N 4°45′0″W), Jbel Zerhoune (Variety 4: V4) (34°01′56″N 5°31′09″W) and Azzaba (Variety 5: V5) (33°49′40″N 4°42′29″W). The areal parts of plant were cleaned, air-dried and powdered (Fig 1). The prepared powder was kept under suitable conditions until *in vitro* experimentation. The experimentations were carried out in the Laboratory of Functional Ecology and Environmental Engineering, Faculty of sciences and technology, Sidi Mohamed Ben Abdellah University, Fez, Morocco.

Extracts preparation

The preparation of extracts involved mixing five grams of powder from each plant sample with 50 mL of three extractor solvents with varying polarities, namely water, ethanol and methanol. The maceration process was sustained 24 hours at ambient temperature, after which the mixtures were filtered and the resulting filtrates were stored at a suitable temperature of 4°C until experimentation.

Determination of total sugars (TS)

TS were assessed according to (DuBois *et al*., 1956), slightly modified method. Briefly, 1 mL of each extract was blended with 1 mL of phenol solution (5%), then 600 µL of distilled water and 5 mL of sulfuric acid (96%) were added. The incubation sustained 10 min and then the mixture was transferred to a water bath at 30°C for 30 min. The optic density was read at 488 nm. The results are expressed as milligram of glucose per gram of dried weight.

Dosage of bioactive content

Total phenolic content (TPC)

The measurement of TPC quantity was realized using the colorimetric method designed previously by (Singleton *et al*., 1999), using Folin-Ciocalteu reagent. Briefly, an aliquot of 100 µL of each sample extract was blended with 450 µL of Folin reagent solution (0.2N) and 450 µL sodium carbonate (75 g/L) was added after 5 min. The mixtures prepared were incubated for two hours in darkness. The optical density was read using a UV-spectrophotometer at wave length of 760 nm. The TPC outcomes were presented as milligram of gallic acid equivalent per g of dry weight (mg GAE/g dw).

Total flavonoid content (TFC)

The TFC quantity measurement was assessed adopting the protocol designed by (Ordonez *et al*., 2006), using aluminum chloride. The reaction mixture consisted of blending 250 µL of each extract with 150 µL of $AlCl_3$ (10%), 75 µL of sodium carbonate (Na_2CO_3, 1M) and 500 µL of sodium hydroxide. The volume was adjusted to 2.5 mL with distilled water. The incubation was sustained for one hour in the dark conditions. The optical density was measured at 510 nm using a UV spectrophotometer. The TFC quantities were expressed as

Fig 1: Powder of dried areal plant parts of *Ajuga iva*.

milligram of quercetin equivalent per gram of dry weight (mg QE/g dw).

Dosage of total tannins (TT)

Spectrophotometric determination of total tannins was carried out as per (Prieto *et al*., 1999). The procedure included blending 1mL of each extract with 1.5 mL of37% HCl solution and 0.5 ml of distilled water. The mixture was then divided into two tubes, Tube A and B. Tube A was kept at room temperature, while tube B was incubated in a water bath at 95°C for 30 minutes. The optical density was measured at 550 nm using UV-spectrophotometer. The total tannins were calculated using the following formula:

TT (g/L)= (optic density TB - optic density TA) *19.33

Dosage of hydrolysable tannins

The HT determination was conducted using the method outlined by (Willis, 1998), with slight adjustments. In summary, the procedure involved mixing one milliliter of each extract with five milliliters of potassium iodate. The optic density was then measured at 550 nm after 4-minute incubation period under dark conditions and at room temperature. The results are expressed as milligrams of tannic acid equivalent per gram of dry weight (mg TAE/g dw).

Antioxidant activity

Total antioxidant capacity (TAC)

The TAC determination was evaluated according to the protocol outlined by (Prieto *et al*., 1999) utilizing the phosphomolybdenum method. In summary, the assay involved combining various concentrations of distinct extracts with one milliliter of molybdate solution (Comprising 0.6 M sulfuric acid, 28 Mm of sodium and 4Mm of ammonium molybdate). Subsequently, the mixture was incubated in a water bath at 95°C for a duration of 90 minutes. The obtained results are expressed as milligram of ascorbic acid per gram of plant after measuring the optical density at 695 nm.

Statistical analysis

The mean±SD values were utilized to express the results and the statistical analyses were conducted through ANOVA-two way using Graph Pad Prism 5 software. Additionally, the significance of the difference was determined at <0.05 by calculating Pearson correlation coefficients using Past 3.

RESULTS AND DISCUSSION

Bioactive content of different samples of *Ajuga iva*

Table 1 displays the obtained results of quantification of bioactive content of different samples of AI collected from different locations. The TPC results ranging from 22.59 to 226.04 mg GAE/g of dry weight. It is clearly seen that the extracts (Aqueous and ethanol extracts) of variety 4 present the highest values of phenolics and flavonoids (226.04 mg GAE/g and 22.27 mg QE/g). Therefore, from the obtained results for all extracts under study, the most suitable extractor solvents were water and ethanol. The obtained results are in accordance with those found by several studies (Bendif *et al*., 2017; El-lamey, 2022; Fettach *et al*., 2019; Makni *et al*., 2013; Salem *et al*., 2016; Senhaji *et al*., 2020). Bioactive compounds are synthesized in different structures and chemical natures which affect their extraction (Joana Gil-Chávez *et al*., 2013). The above-presented outcomes unequivocally demonstrate that the chemical nature of solvent can affect the extraction yield of bioactive content. Makni *et al*. reported that methanol and water were the most suitable extractor solvents to maximize the extraction of polyphenols with values ranging between 16.52 and 25.69 mg GAE/g (Makni *et al*., 2013). Chloroform and hexane solvents showed the weakest ability to extract polyphenolic and flavonoids contents (Makni *et al*., 2013). In fact, the

Table 1: Total phenolic and flavonoids contents.

Extracts	Variety	TPC	TFC	Total sugar	Reducing sugar	Hydrolysable tannins	Total tannins
Aqueous extract	V1	70.83±1.10	6.17±0.01	13.84±0.46	3.81±0.07	6.61±0.015	6.37±0,080
	V2	46.66±0.66	4.76±0.005	15.99±0.49	7.17±0.45	5.4±0.012	14.4±0,36
	V3	60.62±0.07	5.48±0.007	9.67±0.60	3.27±0.11	12.25±0.017	25.49±0,53
	V4	226.04±8.47	22.27±0.11	19.69±2.71	4.43±0.07	6.36±0.015	21.18±0,68
	V5	46.66±0.66	3.35±0.006	38.78±2.56	2.43±0.02	3.91±0.013	11.05±0,54
Ethanolic extract	V1	103.64±13.92	9.80±0.21	12.38±0.57	2.97±0.05	1.31±0.48	18.55±1.30
	V2	95.26±3.53	6.81±0.23	15.20±1.31	0.57±0.13	0.98±0.23	3.35±1.85
	V3	75.88±3.38	7.92±0.22	8.53±0.69	0.41±0.007	0.75±0.15	1.67±0.7
	V4	204.46±1.38	9.75±0.48	24.30±5.38	0.84±0.42	1.33±0.14	14.75±0.77
	V5	49.53±5.91	9.71±0.12	8.25±0.63	0.75±0.09	0.83±0.33	31.44±1.66
Methanolic extract	V1	26.09±0.66	10.62±0.21	2.88±0.18	4.97±0.49	4.40±0.49	6.08±0.06
	V2	22.59±2.43	9.58±0.13	11.49±3.32	1.40±0.10	1.40±0.10	4.63±0.08
	V3	34.26±4.13	12.43±0.52	11.96±1.17	1.82±0.07	1.82±0.07	10.28±0.89
	V4	62.38±7.34	15.23±0.18	12.45±1.08	1.01±0.05	1.02±0.05	8.60±0.97
	V5	36.32±1.91	14.73±0.28	14.57±2.20	2.13±0.22	2.13±0.22	10.05±0.9

impact of organic solvents on bioactive compounds extraction is widely studied and found that the solvents with different polarities significantly affected the extraction yield and consequently the beneficial properties *in vitro* and *in vivo* (Belmimoun *et al*., 2022).

Medicinal herbs constitute raw matter to extract a pool of biogenic molecules produced under different conditions for many purposes, including resistance to unfavorable conditions and infections (Jamieson *et al*., 2017). Robust evidence confirmed the phytochemical functionalities against numerous human diseases such as diabetes obesity, cardiovascular diseases, cancer and pathogenic bacteria and fungi (Jhang *et al*., 2018; Patra, 2012; Rochfort and Panozzo, 2007). Furthermore, seasonal variations considerably affect the phytochemical composition of *Ajuga iva* (El-lamey, 2022), which can explain the high variability of phenolic contents of different samples under study and consequently their biological properties.

Concerning hydrolysable tannins, the highest amount was registered in the aqueous extract of variety 3 (12.25 mg TAE/g dw), while the lowest value was found in the ethanol extract of the same variety (0.75 mg TAE/g dw). The total tannins ranged between 25.49 and 1.67 g/L.

The findings agree with those evoked by Salem *et al*. (Salem *et al*., 2016). The leaves of AI contain considerable amounts of tannins with values varying between 2.69 and 14.93 µg ECAT/mg of dry weight (Salem *et al*., 2016). The accumulation of tannins in the areal parts of plants is closely related to herb defense mechanisms against animal pests (Fuller-Thomson, 2019; Hassanpour *et al*., 2011).

For total sugar, the analysis of obtained results showed that the ethanol extract of sample 5 registered the highest amount of total sugar (38.78 mg GE/g dw), while the methanol extract of the sample 1 showed the lowest value (2.88 mg GE/g dw). The aqueous extract of sample 2 showed the highest content of reducing sugar with value of 7.17 mg/g dw, while the ethanol extract of sample 3 registered the lowest amount with value of 0.41 mg/g dw.

Antioxidant activity

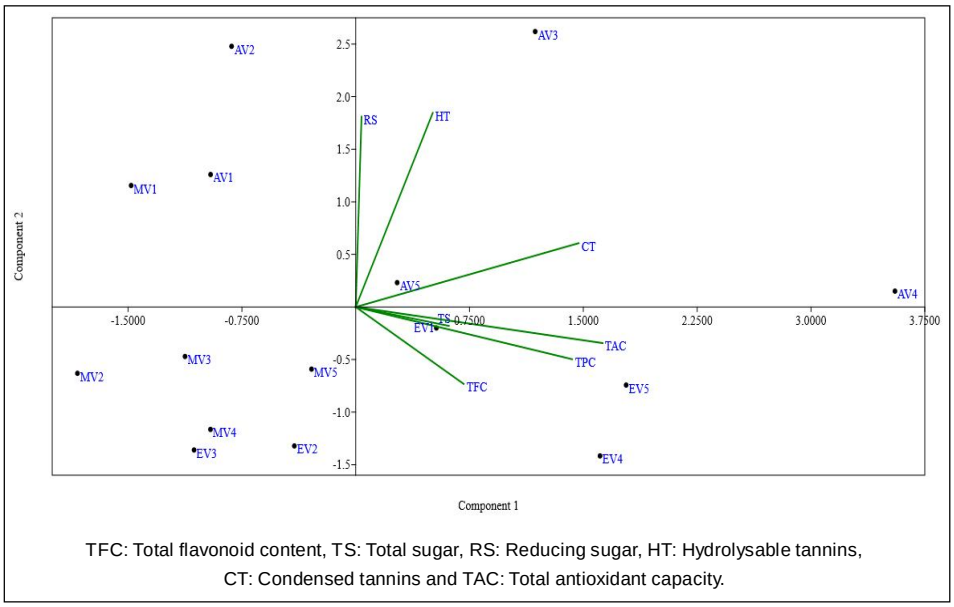
The ability of extracts to scavenge free radicals is associated with their phytochemical composition. The antioxidant potential of extracts under study was assessed by phosphomolybdenum assay. Table 2 displays the obtained results from three tests adopted to examine the antioxidant potency of different extracts prepared. The analysis of results showed significant variability of antioxidant potential between extracts, which was related to the type of the extractor solvent. All extracts exerted excellent antioxidant abilities. The variability of values found of the same extract using different antioxidant tests could be explained by the fact that the same bioactive compounds present in the extract may react differently against different radicals used (El Mannoubi, 2023; Venkatesan *et al*., 2019). The obtained results from this study agreed with the outcomes of Fettach *et al*. who proclaimed that methanol extract was the strongest DPPH radical, ABTS and FRAP scavengers compared to aqueous extract (Fettach *et al*., 2019). The same findings are evoked by Senhaji *et al*, citing that the methanol extract of AI was the most active extract against DPPH radical with an IC_{50} of 78.40 µg/mL (Senhaji *et al*., 2020).

Multivariate analysis

The statistical analysis serves as a robust tool for comprehending the distribution and differentiation among all samples under investigation based on the attributes being studied. Principal component analysis (PCA) is one of the most commonly employed statistical tools to reveal the relationship between various parameters and samples. Fig 2 illustrates the two principal components derived from the analyzed samples. The cumulative variance of the first two principal components amounts to 56.502%. PC1 accounts for 31.48% of the variance and encompasses positive contributions from EV1, EV4, EV5, AV3, AV4 and AV5. Conversely, the negative part of the first component contains AV1, AV2, EV2, EV3, MV1, MV2, MV3, MV4 and MV5. On the other hand, PC2 explains 25.022% of the variance and distinctly divides the studied extracts into two groups. The positive part of PC2 comprises all aqueous extracts and the methanol extract of the first sample (MV1), while the negative part includes all ethanol extracts and four methanol extracts (MV2, MV3, MV4 and MV5). In terms of homogeneity, EV1, EV4 and EV5 exhibit uniformity in TPC, TFC and TS, which are positively correlated with TAC. Conversely, AV3, AV4 and AV5 demonstrate high homogeneity in HT and CT. It is worth noting that the distribution of the studied samples correlates with their geographical origin, as indicated by the obtained results. Fig 2 showcases the principal component analysis (PCA) of the different extracts from the studied samples, utilizing the determined attributes as input, namely TPC (total phenolic content), TFC (total flavonoid content), TS (total sugar), RS (reducing sugar), HT (hydrolysable tannins), CT (condensed tannins) and TAC (total antioxidant capacity). The analysis of the Pearson correlation coefficients between the various studied parameters reveals a strong positive

Table 2: Antioxidant effect of different extracts under study.

Extract	Variety	TAC mg AAE/g dw
Aqueous extract	V1	0.03±0.001
	V2	0.02±0.001
	V3	0.13±0.023
	V4	0.18±0.012
	V5	0.12±0.011
Ethanolic extract	V1	0.10±0.007
	V2	0.12±0.019
	V3	0.09±0.002
	V4	0.12±0.016
	V5	0.22±0.017
Methanolic extract	V1	0.034±0.001
	V2	0.010±0.004
	V3	0.019±0.003
	V4	0.011±0.001
	V5	0.089±0.006



TFC: Total flavonoid content, TS: Total sugar, RS: Reducing sugar, HT: Hydrolysable tannins, CT: Condensed tannins and TAC: Total antioxidant capacity.

Fig 2: Principal component analysis (PCA) of the extracts of different samples under study using the determined attributes as an input: TPC: Total phenolic content.

Table 3: Pearson correlation coefficients between the determined attributes of different extracts under study.

	TPC	TFC	TS	RS	HT	CT	TAC
TPC	-		0.098689	0.2276	0.90266	0.87425	0.34965
0.064494							
TFC	0.44239	-	0.55535	0.8572	0.55805	0.63426	0.63761
TS	0.33139	-0.16558	-	0.93458	0.9738	0.95814	0.43002
RS	-0.034566	-0.050842	-0.023204	-	0.015645	0.58902	0.39272
HT	0.044723	-0.16447	0.0092842	0.61047	-	0.20139	0.79157
CT	0.25984	0.1339	0.014838	0.15185	0.34967	-	0.0050609
TAC	0.48875	0.13259	0.22035	-0.23814	0.074613	0.68243	-

correlation between TPC, TFC and HT with TAC (r=0.064494, r=0.63761, r=0.7915) (Table 3).

Phytochemicals play a pivotal role in plant protection and pollinator attraction (Wani *et al*., 2022). They gained huge attention from the scientific community thanks to their biological functionalities, especially antioxidant abilities (Shoaib *et al*., 2023). Several studies have unveiled a remarkable contribution of phytochemicals to antioxidant abilities, which could be explained by the high correlation between polyphenolic compounds and total antioxidant capacity (Ferreyra *et al*., 2020; Mukhtar *et al*., 2023; Stefaniak *et al*., 2020).

CONCLUSION

This study aimed to determine the phytochemical contents and antioxidant capacity of various samples of *Ajuga iva* collected from different geographical locations. The results showed a significant variation in the amounts of different attributes, which were found to be correlated with the extractor solvent and geographical location. The most effective solvents for extracting phenolic and flavonoid compounds with high antioxidant capacity were water and ethanol. *Ajuga iva* was found to contain a substantial amount of bioactive compounds, making it an abundant source of active compounds with diverse effects.

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Data availability

The manuscript has no associated data.

Declarations conflict of interest

The author declares that they have no relevant financial or non-financial interests to disclose.

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