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Effect of Extraction Solvent Polarity on Antioxidant Activity of Various Fractions of Algerian *Galium tunetanum* Lam

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Abstract

Galium tunetanum Poiret (G. tunetanum) was extracted with different solvents (methanol, hexane, chloroform and ethyl acetate). Total phenolic and flavonoid contents of the extracts were estimated. The extracts were assayed for their antioxidant activities using six in vitro models (the 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS, reducing power, ferrous chelating test, ß- carotene/ linoleic acid assay system, ferric thiocyanate method and thiobarbituric acid method). Methanol extract (ME) showed the highest polyphenolic content: 131.29 ± 0.501 mg gallic acid equivalent/g of dry extract. The determination of flavonoid contents showed that ethyl acetate extract (EAE) had the highest content of flavonoids: 96.93 ± 1.2 mg quercetin equivalent / g of dry extract. ME extract exhibited the most significant antioxidant activity on DPPH radical and ABTS with IC50 value of 0.02 ± 0.00023 mg/mL and 0.012 ±0.0015 mg/mL, respectively. Using the reducing power test, ME extract showed the highest scavenging activity with an EC50 of 0.19±0.01 mg/mL. Similarly, ME extract exerted the highest chelating activity towards ferrous ions with an IC50 value of 21 ± 0.002 mg/mL. ME and CHE inhibited the oxidation of ß- carotene in important level compared to BHT as positive reference. In addition, all fractions inhibited strongly the lipid peroxidation. In conclusion, the results show that G. tunetanum Poiret represents a source of antioxidant compounds.

Keywords: Galium tunetanum, plant extracts, polyphenols, antioxidant activity.

1. Introduction

Active oxygen and free radicals exist in human body in the form of superoxide anion (O2.-) hydrogen peroxide (H_2O_2) and hydroxyl radical (OH.) and so on. Reactive Oxygen Species at low concentrations in the cell contribute to various metabolic pathways or regulate various physiological functions as a response to biotic and environmental stresses (Akgül et al., 2022). As normal metabolic action going on in human body, active oxygen and free radicals are constantly formed leading to oxidative stress (process that occurs at excessive production/generation of ROS in our cells and tissues), leading consequently to oxidation of the main cell macromolecules, abnormal gene expression, and cell death (Korkmaz et al., 2021; Derafa et al., 2022).

Antioxidants are compounds that can delay or inhibit the oxidation of lipids and other molecules and by doing so inhibit the initiation and propagation of oxidative chain reactions. They act by one or more of the following mechanisms: reducing activity, free radical scavenging, potential complexation of pro-oxidant metals and quenching of singlet oxygen (Mehlous et al. 2020). Plants develop different kind of antioxidants that aid in antioxidant defense system, protecting plants against damage caused by active O_2 formed due to exposure to ultraviolet radiation. In

the group of antioxidant and free radical scavenging agents, plants synthesize different compounds, principally phenolic derivatives, such as flavonoids, phenyl-propanoids, stilbenes and other (Amira *et al.*, 2012). Medicinal plants are a natural source of phytochemicals resulting from the secondary metabolism and having antioxidant activity which counteract oxidative stress (Benchikh *et al*., 2022b). The use of medicinal plants in health recovery has evolved over the times from the simplest forms of local treatment and up techno-logically sophisticated forms of industrial manufacturing currently used Clarice *et al*. 2018; Mehlous *et al*., 2022).

The Galium genus, belonging to the Rubiaceae family, is represented by 20 species in Algeria, including six endemic varieties (Quézel and Santa, 1963). Widely recognized in traditional medicine across various countries, Galium extracts are known for their strong antioxidant activity, attributed to their phenolic and flavonoid components, which neutralize free radicals. These extracts also demonstrate anti-inflammatory, antimicrobial, and antiviral effects (Vasilevna et al., 2016; Wanderley Sales, 2021), suggesting their potential in treating infections and inflammatory disorders. Additionally, Galium species are traditionally used to support urinary health and detoxification, with preliminary research indicating they may possess anti-cancer properties, along with liver and kidney-protective and anti-angiogenic effects (Goryacha et al., 2017; Mohamed Lotfy et al., 2022; Camero et al., 2018). Species such as Galium aparine and Galium verum contain bioactive compounds (Camero et al., 2018; Laanet et al., 2023; Ali Kanso et al., 2024) that reinforce their historical medicinal uses while underscoring the necessity for further clinical studies to validate these therapeutic benefits. This research, therefore, focuses on assessing the polyphenolic content and in vitro antioxidant activity of the methanolic extract and its fractions from *Galium tunetanum*, given its prominent role in traditional medicine.

2. Materials and Methods

2.1. Plant material

The fresh areal parts of *G. tunetanum* were collected by Pr. Smain Amira and Dr. Fatima Benchikh in Djimla, 45 km away from Jijel, Northeast Algeria. A voucher specimen has been deposited at the Herbarium Horti Botanici Pisani, Pisa, Italy (n. 8486 *Galium tunetanum*/1, Nuove Acquisizioni). The collected plant was dried under shade.

2.2. Extraction and fractionation

The extraction procedure was conducted as described by Benabdallah *et al*. (2020) with slight modification. The dried powder of *G. tunetanum* L. areal parts were extracted with methanol (85%) at room temperature for 3 days. The resulting suspension was then filtered and concentrated by evaporation at 50°C and fractioned by successive washing with different solvents of increasing polarity (hexane, chloroform and ethyl acetate). Each fraction was evaporated to dryness to obtain the following fractions: methanol extract (ME), hexane extract (HE), chloroform extract (CHE), ethyl acetate extract (EAE) and the remaining aqueous extract (AqE). The extracts were stored at 4°C until use.

2.3. Determination of total phenolic content

Total phenolic content was assessed by Folin Ciocalteu reagent as described by Mamache *et al* (2020). A volume of 100 µL of each extract was mixed with 500 µL of Folin Ciocalteu reagent (diluted 10 times). After 4 min, 400 µL of 7.5% of Na_2CO_3 solution was added. The final mixture was shaken and incubated in dark at room temperature for 1 hour and the absorbance of the reaction mixture was measured at 760 nm. The amount of total polyphenols in different extracts was determined from a standard curve of gallic acid. The results were expressed as mg of gallic acid equivalent (GAE) per gram of dried plant extract.

2.4. Determination of total flavonoid content

Total flavonoid content was determined using aluminium chloride assay (Kaoudoune *et al*., 2020). Briefly, 1 mL of each tested extract or standard (quercetin) were mixed with 1 mL of $AlCl_3$ (2%). After 10 min of incubation, the absorbance against a prepared blank was measured at 430 nm. The results were expressed as quercetin equivalent per gram of dry plant extract weight (mg QE/g DW) using a calibration curve of quercetin.

2.5. Determination of *in vitro* antioxidant activity

2.5.1. DPPH scavenging capacity

The 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of the extracts was determined spectrophotometrically in an MRXe tc (DYNEX Technologies GmbH, Denkendorf, Germany), by monitoring the disappearance of DPPH at 515 nm, according to the method described by Benchikh (2017). Briefly, 20 µL of *G. tunetanum* L. extracts or standard solution (ascorbic acid) in absolute methanol was added to 180 µL of DPPH reagent (0.004%) in 96 well plates. Absolute ethanol was used for reagent blank. All reagents were mixed and incubated

for 30 minutes at room temperature and protected from light. Experiments were done in triplicates. The percentages of the DPPH free radical scavenging activity were calculated as follows:

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100.

To determine the IC_{50} values, a dose response curve was plotted. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity.

2.5.2. ABTS radical scavenging activity assay

The radical scavenging assay against ABTS was measured using the method described by Benchikh *et al*. (2022a). The ABTS radical stock solution (7 mM in water) was mixed with 2.45 mM potassium persulfate and kept for 12-16 h in the dark at room temperature. The solution was then diluted with methanol to give an absorbance of ~0.7 at 734 nm. Then 50 µL of sample was mixed with 1 mL of ABTS mixture and kept for 30 min at room temperature in the dark. The absorbance of reaction mixture was measured at 734 nm. Trolox was used as positive control. All determinations were performed in replicates. Scavenging capability of test compounds was calculated from the following equation:

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] x100.

2.5.3. Ferrous ion chelating activity

The chelating effect of the extracts was determined according to the method of Decker and Welch *et al*. (1990) which is based on the inhibition of the formation of Fe^{2+}-ferrozine complex after treatment of samples with Fe^{2+} ions. Briefly, 250 µL of test material or EDTA at different concentration were added to 50 µL of $FeCl_2$ (0.6 mM in distilled water) and 450 µL of methanol. After 5 min of incubation, the reaction was initiated by the addition of 5 mM ferrozine (50 µL), the mixture was stirred and allowed to react at room temperature for 10 min. The control contained all the reaction reagents except the extract and EDTA. The absorbance of the Fe^{2+}-ferrozine complex was measured at 562 nm. The chelating activity was expressed as a percentage using the following equation:

Chelating activity (%) = [(Abs of control – Abs of test sample)/Abs of control] x100.

To determine the IC_{50} values, a dose response curve was plotted. IC_{50} is defined as the effective concentration of the test material that is required to chelate 50% of iron ions.

2.5.4. Reducing power

The reducing powers of the extracts from *G. tunetanum* L. and BHT were determined according to the method described by Chung *et al*. (2005). A 0.1 mL aliquot of each extract BHT were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide, and then incubated at 50°C for 20 min. 0.25 mL of 1% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 2790 g for 10 min. The supernatant (0.25 mL) was mixed with 0.25 mL distilled water and 0.1% $FeCl_3$ (0.5 mL) and then the absorbance was measured at 700 nm. The reducing powers of the tested samples increased with the absorbance values.

2.5.5. β-carotene/linoleic acid method

This assay is based on the capacity of antioxidant molecules to inhibit β-carotene oxidative degradation that is caused by oxidative compounds of linoleic acid according to the method of Kartal *et al.* (2007). β-carotene/linoleic acid emulsion was prepared by mixing 0.5 mg of ß-carotene in 1 mL of chloroform, 25 µL of linoleic acid and 200 mg of Tween 40. Chloroform was completely evaporated 40°C using a vacuum evaporator. Then 100 ml of oxygenated distilled water was added with vigorous shaking. To an aliquot of 2.5 mL of this emulsion, 350 µL of *G. tunetanum* L. or the reference antioxidant (BHT) were added and well mixed. The absorbance was recorded after 0, 1, 2, 4, 6 and 24 hours at 490 nm. A negative control consisted of 2.5 mL distilled water or solvent instead of extract or reference antioxidant. All samples were assayed in triplicate. The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation:

$$AA=[1-\frac{A_0-A_t}{A_0^0-A_t^0}]\times 100$$

Where, A_0 and A_0^0 were the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A_t^0 were the absorbance values measured in the test sample and control, respectively after incubation for 24 hours.

2.5.6. Ferric thiocyanate (FTC) assay

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation using the method described by Yen *et al.* (2003) with slight modifications. Linoleic acid emulsion (0.02 M) was prepared with linoleic acid (155 µL) and Tween 20 (155 µL) in phosphate buffer (50 mL, 0.02 M, pH 7.4). A reaction solution, containing extracts with different concentrations (0.5 mL), linoleic acid emulsion (2.5 mL), and phosphate buffer (2 mL, 0.02 M, pH 7.0) was placed in a glass vial with a screw cap and mixed with a vortex mixer. The reaction mixture was incubated at 40°C in the dark. To 0.1 mL of reaction mixture, 4.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added. Exactly, 3 min after the addition of 0.1 mL of 0.02 M $FeCl_2$ in 3.5% HCl, the peroxide value was determined by recording the absorbance at 500 nm every 24 hours until the absorbance of the control reached a maximum. The positive and negative controls were subjected to the same procedures as the sample, except for the negative control, in which only the solvent was added, and for the positive control in which the sample was replaced with BHT and vitamin C. The inhibition percentage of linoleic acid peroxidation was calculated as:

Inhibition% = (1 – Absorbance of sample at 500 nm/Absorbance of control at 500 nm) × 100.

2.5.7. Thiobarbituric acid (TBA) assay

The TBA test was conducted on the final day of FTC according to the method described by Kikuzaki and Nakatani (1993) to determine the malonaldehyde (MDA) formation from linoleic acid peroxidation. The same sample preparation method as described in the FTC method was used. To 1 mL of sample solution, 20% trichloroacetic acid (2 mL) and thiobarbituric acid solution (2 mL) were added. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was then centrifuged at 3000 rpm for 20 minutes. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was recorded based on the absorbance of the final day of the FTC assay using the following equation:

% inhibition = 100 – [(Abs sample /Abs control) × 100],

Where Abs control and Abs sample are the absorbances of the control (without sample) and the experimental (with sample) reactions, respectively.

2.6. Statistical data analysis

Results were expressed as means ± standard deviation (SD) and were analysed by one way analysis of variance (ANOVA) followed by Dunnet's test. The P Values of $P < 0.05$ were considered significantly different using Graph Pad Prism Version 6.0 (GraphPad Software, Inc, La Jolla, CA, USA).

3. Results and Discussion

3.1. Total phenolics and flavonoids contents

Methanol extract (ME) showed the highest polyphenolic content: 131.29 ± 0.50 mg gallic acid equivalent/g of dry extract (GAE/g). The determination of flavonoid contents showed that ethyl acetate extract (EAE) had the highest content of flavonoids: 96.93 ± 1.2 mg quercetin equivalent /g of dry extract (QE/g) (Table 1).

Table 1: Total phenolics and flavonoids contents of *G. tunetanum* L. extracts

Extracts	Total phenolics (mg GAE/g Dw)	Total flavonoids (mg QE/g DW)
ME	131.29 ± 0.5	80.45 ± 1.23
CHE	63.96 ± 0.93	15.42 ± 1.29
EAE	127.55 ± 1.86	96.93 ± 1.2
AqE	87.40. ± 1.03	84.80 ± 1.68

ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract, DW: Dry weight. Results are expressed as means ± SD (n=3).

3.2. *In vitro* antioxidant activities of *G. tunetanum* L. extracts

3.2.1. DPPH radical scavenging activity of *G. tunetanum* L. extracts

The results (Figure 2) show that ME extracts exhibited the highest antioxidant activity (IC_{50} = 0.02 mg/mL) compared to vit C as standard (IC_{50} = 0.0019 mg/mL), followed by CHE (IC_{50} = 0.28 mg/mL), AqE (IC_{50} = 0.034 mg/mL) then EAE (IC_{50} = 0.049 mg/mL).

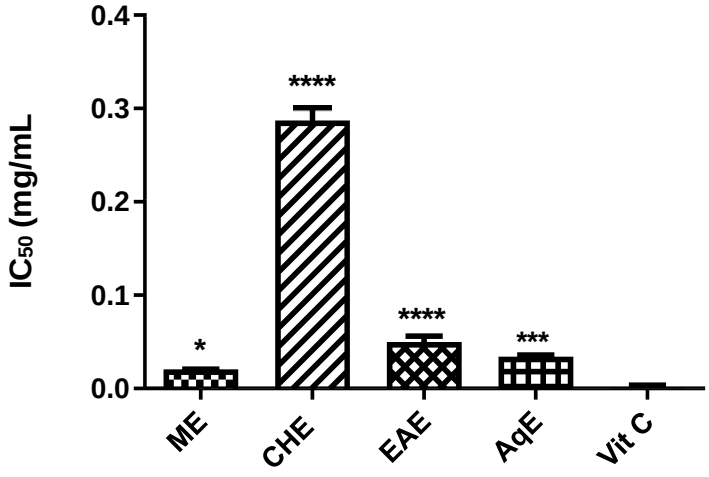

Figure 1: DPPH radical scavenging activity of *G. tunetanum* L. aerial parts extracts. ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as IC_{50} means ± SD (n=3). (****$P \leq 0.0001$; ***$P \leq 0.001$; *$P \leq 0.05$) *vs* Vit C as standard.

3.2.2. ABTS radical scavenging activity of *G. tunetanum* L. extracts

The ability of *G. tunetanum* extracts to scavenge the radical ABTS are shown in figure 1. All extracts exhibited high antioxidant activity and in the following order: ME (IC_{50} = 0.0118 mg/mL) > EAE (IC_{50} = 0.0119 mg/mL) > AqE (IC_{50} = 0.0129 mg/mL) > CHE (IC_{50} = 0.0268 mg/mL).

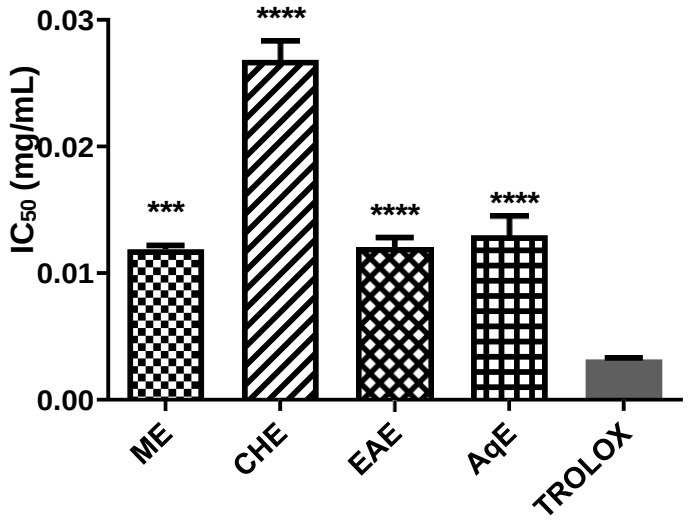

Figure 2: ABTS radical scavenging activity of *G. tunetanum* L. aerial parts extracts. ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as IC_{50} means ± SD (n=3) (****$P \leq 0.0001$; ***$P \leq 0.001$) *vs* Trolox as standard.

3.2.3. Reducing power capacity of *G. tunetanum* L. extracts

From the results (Figure 6), we can see that the best reducing power (the effective concentration at which the absorbance was 0.5) was for ME (EC_{50} = 0.19 ± 0.01 mg/mL) and AqE (EC_{50} = 0.27 ± 00 mg/mL). CHE showed the weakest activity (EC_{50} = 1.26 ± 0.06 mg/mL). None of the extracts reached the activity of BHT as reference drug (EC_{50} = 0.074 ± 00 mg/mL).

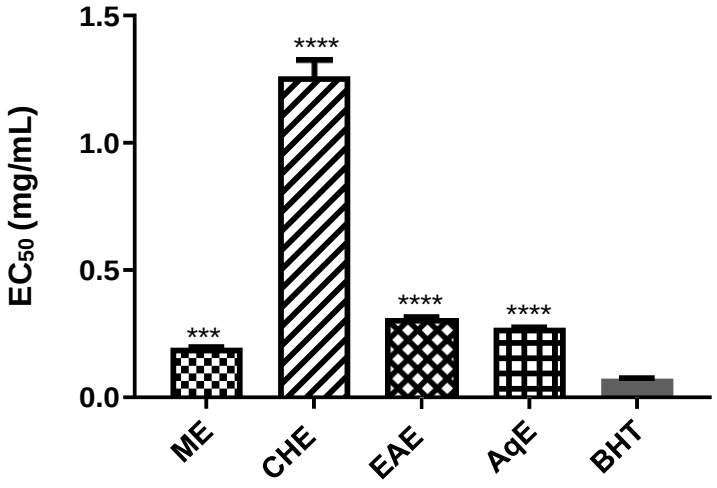

Figure 3: Reducing power activity of *G. tunetanum* L. aerial parts extracts. ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as EC_{50} means ± SD (n=3) (****$P \leq 0.0001$) *vs* BHT as standard.

3.2.4. Ferrous ion chelating activity of *G. tunetanum* L. extracts

All the extracts demonstrated an ability to chelate ferric iron (II) ions. The chelating abilities on ferrous ions were in descending order: ME (IC_{50} = 0.021 mg/mL) > AqE (IC_{50} = 0.09 mg/mL) > CHE (IC_{50} = 0.8 ± 0.089 mg/mL) > EAE (IC_{50} = 1.02 ± 0.043 mg/mL). ME showed close chelating activity as that of EDTA as reference drug (IC_{50} = 0.02 ± 00 mg/mL)

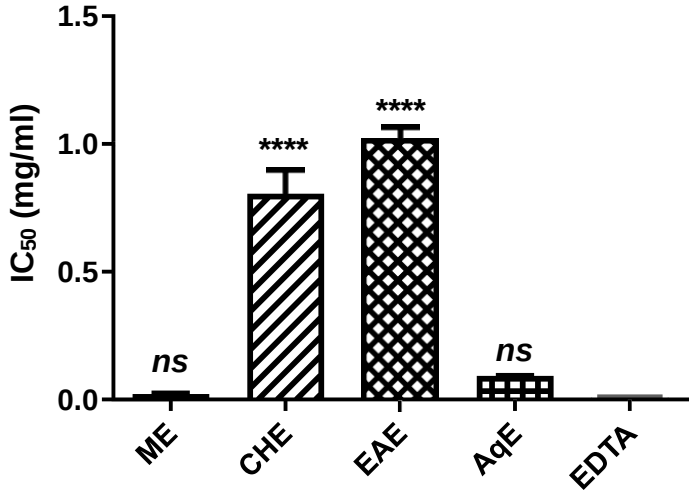

Figure 4: Ferrous ion chelating activity of *G. tunetanum* L. aerial parts extracts. ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as means ± SD (n=3). (****$P \leq 0.0001$; ns: not significant) *vs* EDTA as standard.

3.2.5. Antioxidant activity of *G. tunetanum* L. extracts determined by β-carotene /linoleic acid bleaching assay

Figure 5 shows the effect of *G. tunetanum* extracts on the changes in the percentage of the inhibition ratio of linoleic acid oxidation compared to BHT as positive control for 24 hours. The addition of the plant extracts and BHT at 2 mg/mL was markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β-carotene, in comparison with the negative control which contained no antioxidant component. ME showed the highest antioxidant activity (74.30 ± 0.58 %) compared to BHT (89.26 ± 1.52 %) followed by CHE (60.47 ± 0.82 %), AqE (57.97 ± 0.16 %) and EAE (52.64 ±0.18 %).

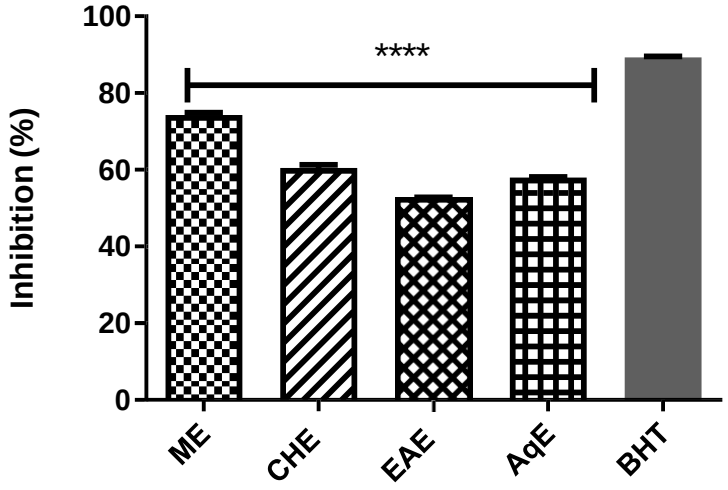

Figure 5: Antioxidant activity of *G. tunetanum* L. aerial parts extracts (2 mg/ml) using β-carotene /linoleic acid bleaching assay after 24 h. ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as means ± SD (n=3). (****$P \leq 0.0001$) *vs* BHT as standard.

3.2.6. Antioxidant activity of *G. tunetanum* L. extracts determined by FTC assay

As shown in figure 6, the different extracts showed good antioxidant potential with percent inhibition ranging from (77.12 ± 4.83 %) to (97 ± 1.21 %) as compared with BHT as positive control. The best activity was observed in EAE and CHE (97.87 ± 1.21 and 97 ± 0.5%, respectively), which were as strong as that of BHT as positive control (98.16 ± 0.6 %) ($P \geq 0.05$).

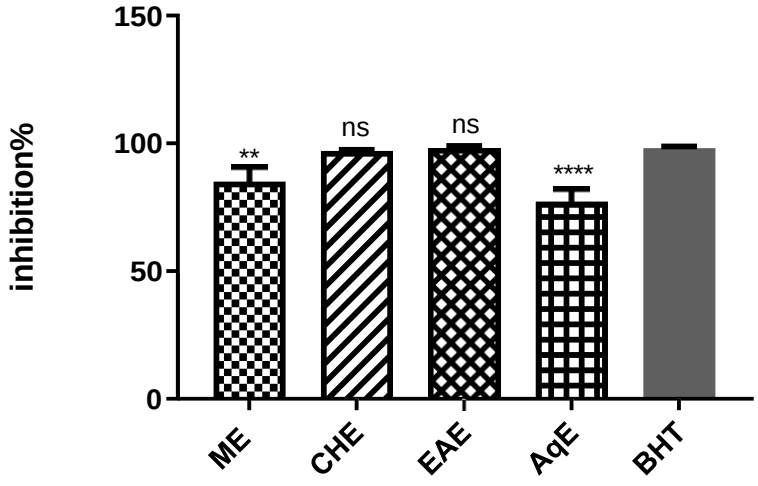

Figure 6: Antioxidant activity of *G. tunetanum* L. aerial parts extracts (2 mg/ml at 96 h of incubation) measured by FTC method. ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as means ± SD (n=3). (****$P \leq 0.0001$; **$P \leq 0.01$; ns: not significant) *vs* BHT as standard.

3.2.7. Antioxidant activity of *G. tunetanum* L. extracts determined by thiobarbutiric acid assay (TBA)

As shown in figure 7, the plant extracts inhibited MDA formation in the following order: BHT (98.16 ± 0.6%) > EAE (97.87 ± 1.21%) > CHE (96.34 ± 1.15%) > ME (75.08 ± 5.62%) > AqE (73.12 ± 11.04 %). The percentage of inhibition exhibited by EAE and CHE was comparable to the positive control (BHT).

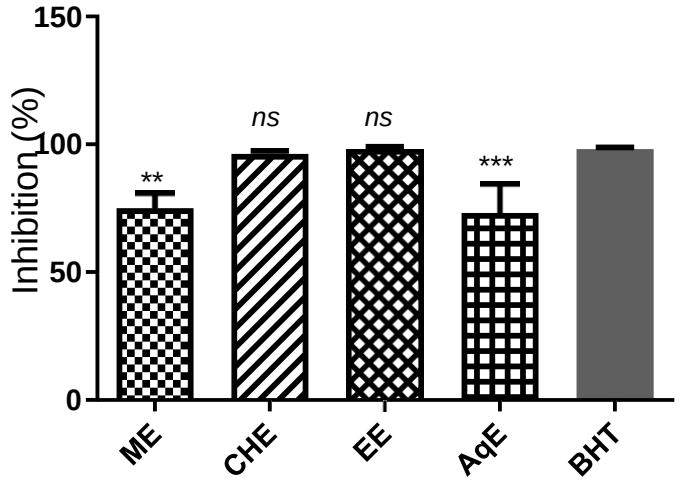

Figure 7: Antioxidant activity of *G. tunetanum* L. aerial parts extracts (2 mg/mL at 96 h of incubation) measured by TBA assay. ME: methanol extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as means ± SD (n=3). (***$P \leq 0.001$; (**$P \leq 0.01$); ns: not significant) *vs* BHT as standard.

Discussion

G. tunetanum L. extracts were examined for their total polyphenols and flavonoids; the results of the present study revealed that they have high amounts of polyphenols. highest levels of polyphenols were identified in methanol extract. Whereas EAE contained the highest amount of flavonoids.). These findings align with the results of Gaamoune et al. (2014). It has been suggested that polar solvents serve as the most effective extracting media for polyphenols and flavonoids. This is likely due to the increased polarity of flavonoids upon conjugation with glycosides and hydroxyl groups, which enhances their solubility in polar solvents (Spingo et al., 2006).

The choice of extraction solvent plays a crucial role in accurately isolating botanical compounds from plant materials (Ounis et al., 2023). Therefore, the antioxidant activities of the plant samples were assessed using five complementary techniques: DPPH radical scavenging, ABTS radical cation decolorization, metal ion chelating activity, reducing power, and lipid peroxidation assays. This comprehensive approach aims to provide more conclusive results regarding the antioxidant properties of the extracts. The IC_{50} parameter is utilized to express the antioxidant activity of a molecule, where a lower IC_{50} value indicates a higher antioxidant activity of the compound.

The DPPH free radical scavenging assay is used to evaluate the antioxidant potential of a test sample by measuring its capacity to neutralize free radicals. This reflects the sample's ability to effectively prevent, intercept, and repair damage within a biological system (Loucif et al., 2020a; Amira et al., 2020). In this study, the DPPH scavenging activity varied between 0.02 mg/mL and 0.049 mg/mL across the four plant fractions. Among these, the methanol extract (ME) demonstrated the highest scavenging capacity, followed by the chloroform extract (CHE). The results for the ethyl acetate fraction were similar to those reported by Gaamoune et al. (2014).

At 734 nm, the ABTS blue/green chromophore loses color as it is scavenged by antioxidant molecules. The decrease in ABTS absorbance with the tested extracts reflects the degree of ABTS inhibition, demonstrating the ability of antioxidant substances to form stable radicals, which is often linked to their scavenging capacity (Amira et al., 2023). BHA and α-tocopherol are known for their powerful antioxidant effects, particularly for their strong antiradical activity. In this study, *G. tunetanum* extracts exhibited notable activity comparable to that of trolox, a standard reference antioxidant. This high radical-scavenging potential could be attributed to the monoterpenes and sesquiterpenes that are prevalent in Galium species (Al-Snafi et al., 2018).

The chelating activity of ferrous iron was assessed by measuring the inhibition of the Fe^{2+}-ferrozine complex formation after incubating *G. tunetanum* extract with Fe^{2+}, following the method of Decker and Welch (1990). Ferrozine quantitatively forms a complex with Fe^{2+}, producing a distinctive color. However, in the presence of chelating agents, this complex formation is disrupted, resulting in diminished color intensity. This reduction is used to estimate the chelation activity of the coexisting agent. Divalent ferrous ions act as catalysts in oxidative processes, promoting superoxide and hydroxyl radical formation via Fenton reactions. Consequently, chelating Fe^{2+} can exert significant antioxidant effects by delaying metal-catalyzed oxidation (Benchikh et al., 2022a; Benabdallah et al., 2022).

Polyphenols and flavonoids are considered excellent chelators for iron and copper ions. Notably, flavonoids can chelate metals through various mechanisms, depending on their structure, metal ion type, and reaction pH—such as acidic conditions in the stomach and alkaline conditions in the intestine (Derafa et al., 2022). The EAE extract demonstrated weak chelating activity, despite its high flavonoid content. This outcome suggests that flavonoids may not be highly effective metal chelators. Additionally, none of the extracts exhibited superior iron (II) ion chelation compared to the positive control, EDTA, used in the assay

In the reducing power assay, antioxidants present in the samples lead to the reduction of the ferric cyanide complex to its ferrous form, which can be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. An increase in absorbance at this wavelength indicates a greater reducing power of the samples (Benchikh et al., 2022b). The results from this study demonstrated that the methanol extract (ME) exhibited the strongest reducing power, while the ethyl acetate extract (EAE) and chloroform extract (CHE) showed the weakest activity. The unique structures and functional groups of phytochemicals in plants influence their polarity and solubility in the various extraction solvents utilized. It is believed that the contents of polyphenols and flavonoids are closely linked to the reducing power of the extracts (Mehlous et al., 2020).

Lipid peroxidation occurs when reactive oxygen species (ROS) attack lipids, forming carbon radicals that react with oxygen to produce peroxyl radicals, leading to lipid peroxides (Benabdallah et al., 2014). This process causes oxidative degradation of unsaturated fatty acids, compromising the structural integrity and permeability of cell membranes. To investigate the effects of *G. tunetanum* extracts on lipid peroxidation, three assays were employed. The β-

carotene bleaching method (involving the coupled oxidation of β-carotene and linoleic acid) evaluates the antioxidant capacity of plant extracts by measuring their ability to scavenge linoleic acid peroxide radicals, which oxidize β-carotene in an emulsion phase. In the absence of antioxidants, β-carotene rapidly decolorizes as it reacts with linoleic acid radicals, losing double bonds and its characteristic orange hue (Loucif et al., 2020b; Kaoudoune et al., 2024).

The ferric thiocyanate (FTC) method evaluates lipid peroxidation by monitoring the oxidation of linoleic acid. During this process, peroxides are generated, which subsequently oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} ions then form a complex with thiocyanate, producing a colour with maximum absorbance at 500 nm. Thus, a higher absorbance signifies a greater extent of linoleic acid oxidation. Additionally, the thiobarbituric acid (TBA) assay measures malondialdehyde (MDA) a byproduct of lipid hydroperoxide decomposition. MDA reacts with TBA in an acidic medium, forming a pink chromophore that absorbs at 532 nm, reflecting the degree of lipid peroxidation (Miguel et al., 2010).

G. tunetanum extracts demonstrated inhibition of lipid peroxidation across three assays. The methanol extract showed the most significant activity in the β-carotene bleaching assay, while the ethyl acetate (EAE) extract exhibited the strongest inhibition in both the FTC and TBA assays. The conversion of thiobarbituric Acid Reactive Substances (TBARS) to malondialdehyde (MDA) equivalents is a commonly used as indicator for measuring the extent of lipid peroxidation. Compounds such as flavonoids, phenolic acids, and tannins play a role in inhibiting both enzymatic and non-enzymatic pathways involved in lipid peroxidation (Benabdallah et al., 2014).

G. tunetanum extracts effectively inhibited lipid peroxidation in all three tests, with the methanol extract (ME) and ethyl acetate extract (EAE) showing the highest activity. These findings align with those of Gaamoune et al. (2014), who also demonstrated the strong inhibitory effects of *G. tunetanum* extracts on lipid peroxidation. Phenolic compounds and other chemical components present in the extract may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination (Benchikh et al., 2018).

Conclusion

To conclude, *G. tunetanum* L. methanol extract and its fractions are rich in polyphenols and flavonoids. They have significant antioxidant activities in different in vitro assay systems. These results suggest that *G. tunetanum* L. can be used as sources of natural antioxidants in the pharmaceutical and food industry. However, further investigations on *in vivo* antioxidant activities are highly recommended.

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Competing Interests

Authors have declared that no competing interests exist

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