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Phytochemical Screening, and *In vitro* Evaluation of Antioxidant Activity of *Lolium perenne* L.

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Abstract

The current study aimed to investigate the preliminary phytochemical screening, quantify the total phenolic (TPC), flavonoidal (TFC) contents and evaluate the antioxidant activities of the total methanol extract (ME) of the aerial parts and its derived fractions of *Lolium perenne* L. (*L. perenne*). The preliminary phytochemical screening of ME displayed the presence of carbohydrates and/or glycosides, steroids and/or triterpenes, phenolic compounds, proteins and/or amino acids, flavonoids, and quinones. Dichloromethane (DFr) and ethyl acetate (EFr) fractions showed the highest TPC (82.42±2.70 and 70.85±2.27 mg gallic acid equivalent (GAE)/g dried sample), respectively. Also, DFr and EFr displayed the highest TFC (115.65±6.43 and 146.00±1.06 mg rutin equivalent (RE)/g dried sample), respectively. All samples showed variable antioxidant properties. While EFr, followed by DFr, demonstrated the least IC_{50} value of 120.04±0.43 and 166.81±0.53 µg/mL, respectively, indicating their high antioxidant potential.

Keywords
Lolium perenne; Antioxidant activities; Flavonoidal content; Phenolic content; Phytochemical screening.

Introduction

Lolium, a genus belonging to family Gramineae (Poaceae), includes eight species, according to recent studies [1-3]. The species are native to Europe, the North Atlantic Islands, temperate Asia, and North Africa, but they are widely distributed in other parts of the world [1, 4]. Perennial ryegrass called English ryegrass (*Lolium perenne* L.), and annual ryegrass, named Italian ryegrass (*Lolium multiflorum* Lam.), are considered as forage grasses [1]. The use of *L.* perenne as a fodder plant is crucial to the rural economy [5, 6]. It is also used on winter games pitches, tennis courts, heavy-duty lawns, landscaping, cricket fields, golf tees, and fairways due to its fine texture, and bright green colour, and its ability to withstand heavy foot traffic, and recover quickly from damage [7]. Overall, *L. perenne* has several biological activities that make it a valuable plant for forage, turf, and soil improvement [8]. Moreover, *L. perenne* and *L. multiflorum* have been traditionally used in herbal medicine for their anti-inflammatory, anti-septic, antioxidant activity, and analgesic properties [9-14]. *Lolium*, has reportedly been informally employed in traditional cures for cancer, diarrhoea, haemorrhage and malaria, particularly *Lolium temlentum* L. seeds [15, 16]. *Lolium perenne* has been utilised as an anti-diarrheic, and anti-periodic agent, in addition to having detergent, and anti-gangrene effects. *Lolium temulentum* is occasionally used in folk medicine to treat headache, and rheumatism [17]. The Moroccans use a decoction of *L. temulentum* as a treatment for bleeding, and urine incontinence. Moreover, the powdered seeds of the latter species were taken internally as a meal to serve as a psychological suppressant, and used externally in the form of a poultice in cases of skin diseases [17, 18]. Research on humans has demonstrated that incorporating antioxidants from plant sources into one's diet can enhance the overall antioxidant levels in the bloodstream and potentially mitigate or prevent diseases linked to oxidative stress, such as cancer, inflammatory conditions, and cardiovascular ailments [19, 20]. However, more research is needed to determine its efficacy for medicinal use. *Lolium perenne* contains several active principles, including loline alkaloids (these are a group of alkaloids that are unique to ryegrass species), flavonoids, phenolic acids, and essential oils [21-25]. So, it is challenging to determine which constituents of *Lolium* may be crucial for animal performance, and human medicine, because their profiles have not yet been clearly illustrated. In this context, the methanol extract of the whole aerial part of *L. perenne* plant was undergone phytochemical screening to look for the presence of secondary metabolites. Also, determining the total phenolic content (TPC), total flavonoidal content (TFC), and antioxidant activity (*in vitro*) of the methanol extract of the whole aerial parts of *L. perenne*, and its derived fractions was the goal of the current investigation.

2. Materials, Apparatus and Techniques

2.1. Plant material

The aerial parts of *L. perenne* L. were collected from a wheat crop field 30 km south of Sohag in March 2020. It was identified by Prof. Ahmed Alkordy, Botany, and Microbiology Dept., Faculty

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of Science, Sohag University, Egypt. A voucher specimen was kept in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Minia University, Minia, Egypt. Voucher specimen number (Mn-ph-Cog-067).

2.2. Solvents and chemicals

n-Hexane, dichloromethane, ethyl acetate, *n*-butanol, and methanol were purchased from EL-Nasr Pharmaceutical, and Chemical Co., Egypt (ADWIC). DPPH: (2,2-diphenyl-1 picryl hydrazyl), rutin, gallic acid, aluminium chloride, and Folin-Ciocalteu were purchased from Sigma Co., St. Louis, MO, USA. Sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), and sodium nitrite ($NaNO_2$) were purchased from Wako Co., Osaka, Japan.

2.3. Instruments

Spectrophotometric results were obtained by using a UV-visible spectrophotometer (instruments, Leicestershire, UK). The measurements were taken in one-centimeter quartz cells.

2.4. Preparations of samples

The fresh whole aerial parts of *L. perenne* were air-dried in the shade, and then reduced to a fine powder. The powdered plant material (2.5 kg) was then extracted by maceration in 80% methanol (10 L, 3x, a week intervals), and the methanol extract (ME) was concentrated under vacuum to a syrupy consistency (300 g). Methanol extract was suspended in the least amount of distilled water (100 mL), then transferred to a one-litre separating funnel, and partitioned by liquid/liquid extraction, successively, and exhaustively with *n*-hexane, dichloromethane, ethyl acetate, and finally with *n*-butanol. The fractions were concentrated under reduced pressure at 40 °C to afford *n*-hexane (HFr, 40 g), dichloromethane (DFr, 17 g), ethyl acetate (EFr, 15 g), and *n*-butanol (BFr, 26 g) fractions. While, the remaining mother liquor was the aqueous fraction (AFr, 202 g).

2.5. Preliminary phytochemical screening

A phytochemical screening of ME of *L. perenne* for the presence of carbohydrates, and/or glycosides, steroids and/or triterpenes, alkaloids, saponins, terpenoids, anthraquinones, cardiac glycosides, coumarins, phenolic compounds, proteins, and amino acids, flavonoids, and quinones was carried out.

Test for carbohydrates, and/or glycosides was done according to the method reported by Parimelazhagan et al [26], while that for steroids and/or triterpenes, terpenoids, anthraquinones, and flavonoids was undertaken according to the methods reported by Gul et al [27]. Moreover, screening for presence of cardiac glycosides, coumarins, alkaloids, nitrogenous base, and quinones was exerted by methods reported by Shaikh et al [28], while that for saponins, and phenolic compounds was carried out according to Singh et al [29].

2.6. Estimation of total phenolic content

The content of total phenolic compounds for ME, and its derived fractions was determined by the Folin-Ciocalteu method [30]. For preparation of the calibration curve, 50 µL aliquots of 0.25, 0.50, 1, and 2 mg/mL methanolic gallic acid solutions were mixed with 50 µL Folin-Ciocalteu reagent (2N), 300 µL of 20 % anhydrous sodium carbonate, and 3.5 mL deionized water. The absorption was measured after 30 min at λ_{max} 728 nm compared to a blank of absolute methanol using a UV-visible spectrophotometer. The ME, and its derived fractions 50 µL (10 mg/mL) were separately treated as mentioned above, and after 30 min, the absorption was measured at λ_{max} 728 nm. All determinations were performed in triplicate, and the total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g of dried sample.

2.7. Estimation of total flavonoidal content

The content of total flavonoidal compounds in ME, and its derived fractions was determined using a modified colorimetric method [31]. For the preparation of a calibration curve, two mL aliquots of 0.2, 0.3, 0.4, and 0.5 mg/mL methanolic rutin solutions were each mixed with 300 µL $NaNO_2$ (5%), left for 6 min, then 300 µL of 10 % $AlCl_3$ was added, and allowed to stand for another 6 min, followed by 1 mL of NaOH (4%), followed by distilled water, which was added immediately to make the final volume 6 mL, where the mixture was thoroughly mixed, and allowed to stand for 15 min. The absorption was measured at λ_{max} 510 nm. For each concentration, three determinations were carried out, then the absorbance for each was plotted versus its concentration, and the calibration curve was drawn. Twenty-five mg of ME, and its derived fractions were dissolved in 10 mL of methanol to obtain the working samples for this study. The same reagents as previously mentioned were added to 2 mL of each fraction (2.5 mg/mL), and after 15 min, the absorbance was measured at λ_{max} 510 nm. All analyses were carried out 3 replicates, and the total flavonoidal content was expressed as mg of rutin equivalents (RTE)/g of dried sample.

2.8. *In vitro* antioxidant activity

Using DPPH, the free radical scavenging activity of ME, and its derived different fractions were measured spectrophotometrically utilising the earlier mentioned technique [32-34]. This technique was commonly used due to its simplicity, speed, and good reproducibility [35]. In essence, methanol was used to dilute sample stock solutions (1.0 mg/mL) to final concentrations of 250, 125, 50, 25, 10, and 5 µg/mL, in ethanol. 2.5 mL of sample solutions in various concentrations were combined with 1 mL of a 0.3 mM DPPH methanol solution, and the mixture was left to react at room temperature. The absorbance readings were recorded at λ_{max} 518 nm after 30 min. As stated previously, the control was created without any sample. The following equation was used to evaluate the scavenging activity based on the percentage of DPPH radicals scavenged.

Scavenging effect (%)
= [(control absorbance - sample absorbance) / (control absorbance)] × 100

IC_{50} values were calculated by linear regression of the plots, with the horizontal axis representing the concentration of the tested sample, and the vertical axis representing the average percentage of antioxidant activity from three separate replicates. Antioxidant activity is highest, where the IC_{50} values are low.

2.9. Statistical analysis

Results are given as the mean ± standard deviation (SD). Three or more means were compared using one-way analysis of variance (ANOVA). Excel 2010 was used for statistical calculations. Results were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Phytochemical screening

Phytochemical screening of ME demonstrated the presence of carbohydrates, and/or glycosides, steroids and/or triterpenes, phenolic compounds, proteins, and/or amino acids, flavonoids, and quinones. On the other hand, alkaloids, saponins, terpenoids, anthraquinones, cardiac glycosides, and coumarins haven't been detected, and the results are listed in **Table 1**. The presence of phenols and flavonoids in consistence with the research of Kagan [25].

Table 1: Phytochemical screening of ME of *L. perenne*

No.	Phytochemical Constituents	Methanolic Extract
1	Carbohydrates and/or glycosides	+
2	Steroids and/or triterpenes	+
3	Alkaloids	-
4	Saponins	-
5	Terpenoids	-
6	Anthraquinones	-
7	Cardiac glycosides	-
8	Coumarins	-
9	Phenolic compounds	+
10	Proteins and/or amino acids	+
11	Flavonoids	+
12	Quinones	+

(+) Present / (-) Absent

The various pharmacological and traditional benefits of plants may be explained by the existence of such phytochemical substances. Observations of phytochemical screening serve as a good guide for further research into how phytochemical groups affect a plant's biological activity.

3.2. Estimation of total phenolic and total flavonoidal contents

Table 2 and **Figure (2A)** demonstrate that total phenolic compounds in different samples ranges considerably between 11.65±0.65 and 82.42±2.70 mg (GAE)/g dried sample. Dichloromethane and ethyl acetate fractions exhibited high of total phenolic content. Rutin equivalents, a measure of flavonoid concentration, ranged from 12.48±2.99 to 146.00±1.10 mg rutin equivalent/g dried sample (**Table 2**, and **Figure (2B)**. Also, the high flavonoidal levels are found in the DFr and EFr, followed by the BFr and AFr.

3.3. In vitro antioxidant activity

The scavenging effects of ME and its derived fractions on the DPPH radical are shown in **Figure (3C)** to be in the following order: EFr > DFr > BFr > ME > AFr > HFr.

Table 2: Total phenolic and flavonoidal contents of ME, and its derived fractions.

Sample	TPC (mg GAE/g dried sample)	TFC (mg RE/g dried sample)
Methanol extract (ME)	26.46±2.21	25.37±0.35
n-Hexane Fr. (HFr)	24.99±0.97	12.48±2.99*
DCM Fr. (DFr)	82.42±2.70*	115.65±6.43*
Ethyl acetate Fr. (EFr)	70.85±2.27*	146.00±1.10*
n-Butanol Fr. (BFr)	50.80±3.21*	100.69±7.17*
Aqueous Fr. (AFr)	11.65±0.65*	36.49±1.46*

GAE: Gallic acid equivalent; RE: Rutin equivalent. Each value is represented as mean±SD (n = 3) with statistically significant differences *($p < 0.05$) relative to ME according to ANOVA.

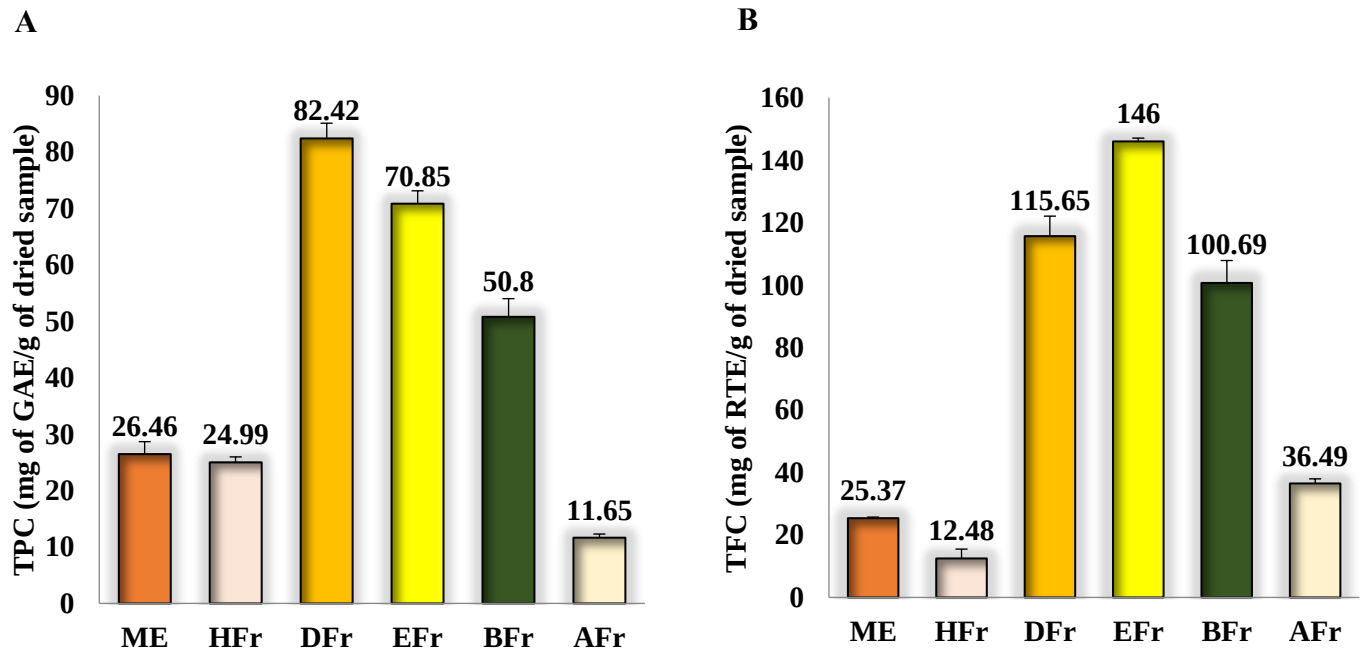

Figure 2: (**A**) Total phenolic content, and (**B**) Total flavonoidal content in ME and its derived fractions (HFr, DFr, EFr, BFr, and AFr).

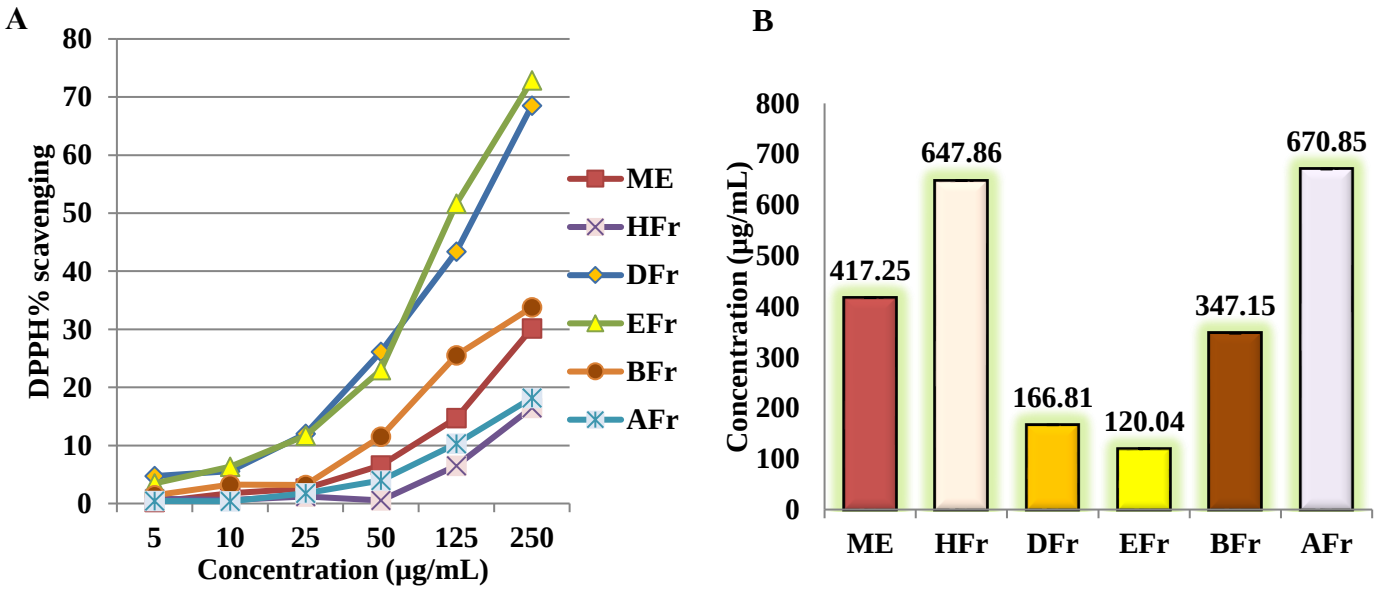

Figure 3: (A) Antioxidant activities and (**B**) IC_{50} for DPPH radical scavenging assay for ME and its derived fractions (HFr, DFr, EFr, BFr, and AFr) at various concentrations (each value represents a mean ± SD (n = 3).

Table 3: The Coefficient of Determination (R^2), and IC_{50} values of the DPPH radical scavenging activities of ME and its derived fractions.

Samples	R^2	IC_{50} (µg/mL)
Methanol extract (ME)	0.9985	417.25±0.664
n-Hexane Fr. (HFr)	0.9977	647.86±0.513
DCM.Fr. (DFr)	0.9701	166.81±0.535
Ethyl acetate Fr. (EFr)	0.9990	120.04±0.432
n-Butanol Fr. (BFr)	0.9265	347.15±0.250
Aqueous Fr. (AFr)	0.9913	670.85±0.954

Each value is represented as a mean±SD (n = 3). Regression Analysis was applied to determine the IC_{50} values of the DPPH radical scavenging activities and the value of R^2.

The amount of medication needed to scavenge 50% of DPPH free radicals is known as the IC_{50}. According to **Table 3** and **Figure (3D)**, IC_{50} values for the EFr and DFr were 120.04±0.432 µg/mL, and 166.81±0.535 µg/mL, respectively. These values represent the lowest values, followed by BFr with 347.15±0.25 µg/mL, then ME with 417.25 ±0.66 µg/mL and, finally HFr with 647.86 ±0.51 µg/mL, and AFr with 670.85±0.95 µg/mL, respectively.

The results of antioxidant potential discovered that EFr and DFr have prominent antioxidant activity; indicating the strong relationship between the presence of phenolics and the free radical scavenging activity, these fractions displayed high phenolics and flavonoidal contents, and also had potent radical scavenging activity. This explanation is in consistent with the research by López, Kagan and Rice et al, respectively [13, 36, 37]. This knowledge can be used to develop new drugs and therapies for various diseases. The antioxidant properties of plants can be used to develop new plant-based products that can be used in the food and cosmetic industries. For example, plant extracts can be used in the formulation of anti-aging creams and lotions. Overall, the antioxidant study of plants contributes to the field of phytochemistry by providing new insights into the properties of plant compounds and their potential applications in various industries.

4. Conclusion

The abovementioned findings revealed the plant's chemical profile and showed that the dichloromethane and ethyl acetate fractions of *L. perenne* exhibited the highest values of total phenolic and flavonoidal contents. Also, they demonstrated the directional relationship between the antioxidant potential and the amount of phenolic and flavonoidal content. Accordingly, *L. perenne* could be considered a major source of natural antioxidants. This inspired us to investigate their antibacterial and anti-inflammatory properties in more detail, which may lead to the identification of novel antibiotics derived from natural sources and may prevent diseases linked to oxidative stress, such as cancer, inflammatory conditions, and cardiovascular ailments. However, further in-depth studies of chemical constituents are needed to isolate and confirm antioxidant compounds in DFr and EFr.

Conflict of Interests

The authors declare that there is no conflict of interest regarding this study.

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