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Original article

Quantitative and Qualitative Analysis for Phenolic Compounds and Phytochemical Screening and Targeting of Leaves and Fruits of *Lotus tetragonolobus* L.

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ABSTRACT

Lotus tetragonolobus L. is a Mediterranean annual legume for which phytochemical information remains scarce. This paper made a qualitative comparative study on the phytochemical composition of aqueous and alcoholic extracts of leaves and fruits and investigated the distribution of 11 phenolic analytes of interest in both organs. Tannins and flavonoids always appeared in both tissues and both extraction media, but carbohydrates and/or glycosides appeared with lower qualitative strength. Saponins were observed in the alcoholic fruit extract only, sterols in the alcoholic leaf extract only, and triterpenoids in the aqueous leaf extract and the two fruit extracts. Under these conditions, alkaloids, anthraquinones, and cardiac glycosides were not detected. The cumulative response of the targeted phenolic panel was 8.029 in the leaves and 37.913 in the fruits, which means that the cumulative response in the fruits was about 4.72-fold higher. The highest values were 2-chlorophenol (8.219ppm), 4-nitrophenol(7.002ppm) and phenol (6.569ppm) in fruits; 2,4-dinitrophenol (3.495) and 4-nitrophenol (1.883) were the greatest in leaves. These results show that the chemical profile measured exhibits evident organ-dependent differences. Due to the presence of many reported analytes that are halogenated or nitrated phenols, as opposed to more conventional endogenous phenolics of plants, definitive biological interpretation requires confirmation of compound identity, quality-control performance, and possible environmental or procedural sources. The dataset is a useful preliminary basis for chromatogram validation and bioactivity-directed exploration of L. tetragonolobus.

Introduction

Plants generate chemically diverse primary and secondary metabolites which aid in growth, reproduction, defense and environmental adaptation. Of these constituents, phenolics and flavonoids are especially significant since their hydroxylated aromatic backbones play a role in redox activity, protein and membrane interactions and much of the biological activity of the plant extracts. Tannins, saponins, sterols, terpenoids and glycosides are also typical broad chemical classes commonly considered in the initial phase of pharmacognostic research. Qualitative phytochemical screening does not identify individual molecules, although it provides a rapid comparative overview of compound classes and guides further chromatographic and spectrometric studies [1,2]. Lotus (Fabaceae) is a genus of species that is found in temperate and subtropical areas. Current literature on the genus has reported flavonoids, phenolic acids, saponins, coumarins, sterols, and other metabolites, but metabolite composition and abundance vary by species, organ, developmental stage, environment, and extraction conditions [3]. High-performance liquid chromatography of L. arabicus and L. glaber provided a comparative analysis of each species, revealing several phenolic acids and flavonoids, with a significant interspecific variation, demonstrating the usefulness of combining broad screening with targeted chemical profiling [4].

Lotus tetragonolobus L. is a recognised species that is native to the Mediterranean region, including Libya and belongs to the family Fabaceae [5]. Although it has a regional distribution, the phytochemical composition of its leaves and fruits has received relatively little direct study. Assessment of these organs independently is significant since specialized metabolites are not uniformly distributed throughout a plant; metabolite accumulation reflects tissue activity, maturity, exposure, and

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transport. Another source of variation is the extraction medium, which extracts different metabolites in aqueous and alcoholic solvents based on their polarity and interaction with the matrix [2]. Various analyses were conducted on various environmental samples (plants, soils, waters, marine samples, and others) in some of the regions of Libya using different instruments that included HPLC, GC-Mass, atomic absorption, Spectrophotometer, XRD, and flame photometer [6 -111]. To this end, the current research paper sought to (i) draw comparisons between the major phytochemical classes identified in aqueous and alcoholic extracts of L. tetragonolobus leaves and fruits and (ii) characterize the distribution of the reported phenolic analytes in the two organs.

Methods

Plant material and sample preparation

Leaves and fruits of *Lotus tetragonolobus* L. were collected from the Al-Jabal Al-Akhdar region in northeastern Libya. The two organs were separated, washed with distilled water, and dried before analysis. For each extraction, 10 g of dried material was placed in a glass beaker, mixed with 100 mL of the designated solvent, and heated in a water bath at 75 °C for 4 h. The mixtures were allowed to cool, filtered, and stored in dark bottles until analysis. Figure 1 shows the studied plant.

Figure 1. The studied plant

Qualitative phytochemical screening

Conventional reagent-based assays were used to determine saponins, tannins, carbohydrates and/or glycosides, alkaloids, flavonoids, anthraquinones, sterols, triterpenoids, and cardiac glycosides in aqueous and alcoholic extracts of leaves and fruits following established phytochemical screening principles [9]. The intensity of the reactions was measured semi-quantitatively as absent (-), weak (+), moderate (++), or strong (+++).

GC-MS profiling

Extraction and GC–MS Determination of Phenolic Compounds from Plant Materials

Preparation of the plant material

To remove soil and foreign materials, fresh plant samples should be cleaned carefully. The samples are then dried by freeze-drying or heating in a well-ventilated oven at not more than 40–45 °C. Excessive drying temperatures should be avoided since this can lead to degradation or oxidation of some of the heat-sensitive phenolic compounds. The dry plant material is milled to a fine and homogeneous powder using a laboratory mill. Powder samples are kept in light-resistant containers at −20 °C before analysis. Long storage periods, moisture, direct light, and repeated freezing and thawing should be avoided.

Extraction of free phenolic compounds

Approximately 1.0 g of powdered plant material is accurately weighed into a centrifuge tube. Ten to twenty mL of 70–80% aqueous methanol or ethanol is then added. Organic solvents mixed with water tend to be more effective than pure water or pure alcohol since plant phenolics have a broad spectrum of polarities. The mixture is vortexed for about one minute and sonicated in an ultrasonic bath for about 20–30 minutes at room temperature. The extraction temperature ideally must not exceed 40 °C, but the sample can be shaken as well for 1–2 hours. The mixture is centrifuged at around 5,000–10,000 × *g* for 10–15 minutes after extraction. The supernatant will be collected and the residue of the plant will be extracted again with new solvent. The two supernatants are combined to enhance the extraction recovery. The combined extract is filtered with qualitative filter paper. It can then be filtered (using a 0.22- or 0.45-µm membrane) as required. The solvent is then removed under reduced pressure at a temperature not exceeding 40 °C or under a gentle stream of nitrogen. The use of acidified solvents must be carefully considered as acidification can enhance the stability or recovery of certain phenolic acids but also hydrolyse sensitive conjugated compounds.

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Fractionation using ethyl acetate

Fractionation can reduce the number of sugars, salts, pigments, and other interfering polar substances entering the GC–MS system. The aqueous extract is concentrated, and the pH of the extract is brought to around pH 2 by the addition of dilute hydrochloric acid. It is then extracted three times with an equal volume of ethyl acetate. Acidification involves changing a large fraction of the phenolic acids to their neutral forms, facilitating their passage into the organic phase. The layers of ethyl acetate are mixed and filtered through anhydrous sodium sulfate to eliminate any water. The solvent is then evaporated under nitrogen or reduced pressure. Until derivatization, the dried extract should be protected from light and moisture. This fractionation process is effective especially with free phenolic acids and relatively small molecules of phenolics. Glycosides with high polarities and polymeric tannins might not be effectively extracted in the ethyl acetate fraction. Plant phenolics can be free molecules or conjugates with sugars, organic acids, or cell wall structural components. To obtain total aglycone phenolics, an extra hydrolysis step can be carried out.

In the case of acid hydrolysis, a portion of the extract may be combined with dilute hydrochloric acid and heated under controlled conditions, usually at 70–90 °C, over 30–120 minutes. Ethyl acetate is used to extract the hydrolysed phenolic compounds after cooling. The conditions of hydrolysis should also be confirmed, as the presence of strong acid, high temperature, or long treatment may destroy sensitive compounds. Enzymatic or alkaline hydrolysis may be more suitable for particular types of conjugated or bound phenolics [1,2]. Findings upon hydrolysis must be provided separately of measurements of free phenolic compounds. Complete dryness is essential because residual water reacts with the silylation reagents and lowers the derivatization efficiency. The dried extract is reconstituted in about 50–100 µL of dry pyridine or acetonitrile. A suitable amount of BSTFA, typically 50–100 µL containing 1% TMCS is added. The vial is then closed tightly, mixed and heated at 60–70 °C within 30–60 minutes. Identification is then conducted by comparing the mass spectrum of each chromatographic peak with the spectra in a known mass spectral library, e.g. the NIST library. However, a library match by itself cannot be considered as definitive identification. The individual phenolic and substituted phenolic compounds were obtained by GC-MS at the Central Laboratory, Alexandria University, Egypt.

Results

Qualitative phytochemical screening

The phytochemical screening revealed a consistent core profile together with tissue- and solvent-specific differences (Table 1). Tannins showed the strongest reactions in the leaves (+++) and were also prominent in the fruits (++). Flavonoids were detected at moderate intensity (++) in every extract, while carbohydrates and/or glycosides were detected at lower intensity (+) throughout. In contrast, alkaloids, anthraquinones, and cardiac glycosides were not detected in either organ with either solvent. Saponins were confined to the alcoholic fruit extract. Sterols were detected only in the alcoholic leaf extract. Triterpenoids occurred in the aqueous leaf extract and in both aqueous and alcoholic fruit extracts, but not in the alcoholic leaf extract. Thus, the alcohol-dependent recovery of fruit saponins and leaf sterols contrasted with the broader distribution of tannins and flavonoids. Table 1

Table 1. Qualitative phytochemical screening of aqueous and alcoholic extracts of Lotus tetragonolobus leaves and fruits.

Chemical class	Leaves Aqueous	Leaves Alcoholic	Fruits Aqueous	Fruits Alcoholic
Saponins	–	–	–	+
Tannins	+++	+++	++	++
Carbohydrates and/or glycosides	+	+	+	+
Alkaloids	–	–	–	–
Flavonoids	++	++	++	++
Anthraquinones	–	–	–	–
Sterols	–	+	–	–
Triterpenoids	+	–	+	+
Cardiac glycosides	–	–	–	–

Phenolic compounds:

The numerical profile was substantially higher in fruits (Table 2). The cumulative value across the panel was 37.913 for fruits compared with 8.029 for leaves, a fruit-to-leaf ratio of approximately 4.72. This total is a descriptive sum of the reported values and is not a validated total-phenolic-content measurement. The fruit profile was dominated by 2-chlorophenol (8.219), 4-nitrophenol (7.002), and phenol (6.569), which together represented 57.5% of the summer fruit response. Further prominent fruit analytes were 2,4-dinitrophenol (3.818), 2,4-dichlorophenol (3.741), and 2,4,6-trichlorophenol (3.366). In

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leaves, 2,4-dinitrophenol was the largest value (3.495), followed by 4-nitrophenol (1.883); together these two analytes accounted for 67.0% of the summed leaf response. The smallest values were recorded for 2,4,6-trichlorophenol in leaves (0.035) and pentachlorophenol in both leaves (0.060) and fruits (0.112). Fruit values exceeded leaf values for every listed analyte. The largest proportional differences occurred for 2,4,6-trichlorophenol (approximately 96-fold), 2-chlorophenol (27.7-fold), and 2,4-dichlorophenol (27.3-fold). By contrast, the values for 2,4-dimethylphenol and 2,4-dinitrophenol were similar between organs, with fruit-to-leaf ratios of approximately 1.06 and 1.09, respectively.

Table 2. Phenolic Compounds in leaves and fruits of Lotus tetragonolobus.

Target analyte	Leaves	Fruits	Fruit/leaf ratio
Phenol	0.809	6.569	8.12
2-Chlorophenol	0.297	8.219	27.67
2-Nitrophenol	0.108	0.155	1.44
2,4-Dimethylphenol	0.852	0.907	1.06
2,4-Dichlorophenol	0.137	3.741	27.31
4-Chloro-3-methylphenol	0.134	1.315	9.81
2,4,6-Trichlorophenol	0.035	3.366	96.17
2,4-Dinitrophenol	3.495	3.818	1.09
4-Nitrophenol	1.883	7.002	3.72
2-Methyl-4,6-dinitrophenol	0.219	2.709	12.37
Pentachlorophenol	0.060	0.112	1.87
Sum of reported values	**8.029**	**37.913**	**4.72**

Discussion

The qualitative findings suggest that the leaves and fruits of L. tetragonolobus have a phytochemical basis characterized by tannins, flavonoids and carbohydrates and/or glycosides. This trend is widely consistent with the chemistry that has been reported on the genus Lotus, where flavonoids, phenolic acids, saponins, and sterol-related constituents are routinely reported [3]. It is also compatible with a comparative analysis [4] that identified several major phytochemical classes and a diverse phenolic/flavonoid profile in methanolic extracts of L. arabicus and L. glaber. The agreement, however, is to be understood at the level of the compound classes and not individual molecules since the species, organs, solvents, and methods of analysis vary.

The stronger tannin response in leaves than fruits could be due to the role of phenolic polymers in protecting photosynthetically active and externally exposed tissues. Leaf tissues often allocate resources to chemical defenses against herbivory, ultraviolet radiation, and oxidative stress. On the other hand, detection of saponins in fruits and the more widespread presence of triterpenoids in fruits indicate that reproductive tissues possess a unique combination of amphiphilic and terpenoid compounds. The observation of sterols exclusively in the alcoholic leaf extract is also chemically plausible as a solvent-selection effect: alcohol is able to extract moderately polar compounds, which may not be well represented in a simple aqueous extract. It has been demonstrated in studies with solvents of varying polarity that the apparent phytochemical profile is heavily dependent upon the extraction system, implying that a negative qualitative test means that the compound was not detected under the stated conditions, not necessarily that it is completely absent from the tissue [2].

The targeted-analyte data exhibit a pronounced organ effect, with the summed fruit response almost five times the leaf response. Tissue-dependent accumulation is biologically plausible and associated Lotus species show significant variation in the number and abundance of phenolic constituents [4]. However, the chemical identity of the dominant entries in the present panel requires unusual care. Chlorinated and nitrated phenols, such as 2-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol and dinitrophenols, are not the conventional phenolic acids and flavonoids commonly emphasized as endogenous Lotus metabolites. Hence, the numerical values cannot be uncritically characterized as the plant's natural phenolic content. A more precise definition at this point is a targeted phenolic-analyte profile. The reported signals might be explained by a variety of non-exclusive mechanisms: true uptake by soil or water, deposition by the local environment, transformation products, contamination during sampling or extraction, matrix interference, or false assignment due to a lack of chromatographic selectivity. The particularly high fruit values of 2-chlorophenol and 4-nitrophenol, and the approximately 96-fold fruit-to-leaf difference of 2,4,6-trichlorophenol, warrant confirmation rather than immediate biological attribution.

Confirmation should include retention-time matching with authentic standards, spectral or mass-spectrometric identity criteria, procedural and solvent blanks, matrix spikes, duplicate or triplicate extraction, recovery experiments, and reporting relative to dry or fresh mass. In the case of environmental exposure, matched soil, irrigation-water, and field-blank samples would help distinguish plant metabolism and external contamination. Lack of alkaloids, anthraquinones, and

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cardiac glycosides is informative and cannot be generalized beyond the sensitivity and specificity of the tests used. Similarly, the plus-sign scale is semi-quantitative and cannot be statistically compared unless there was a predefined, validated procedure to score reaction intensity. Future research should measure total phenolics and total flavonoids using proper standards, determine antioxidant activity using more than one mechanistically distinct assay, and profile characteristic Lotus metabolites using validated HPLC-DAD-MS or LC-MS/MS. This would enable the assessment of the relationship between broad phytochemical classes, individual compounds, and biological activity with more confidence. In general, both datasets are internally consistent to show chemical differentiation between leaves and fruits.

Conclusion

Lotus tetragonolobus leaves and fruits were found to contain detectable tannins, flavonoids and carbohydrates and/or glycosides, with saponins, sterols and triterpenoids showing tissue- and solvent-dependent distributions. The targeted analytical panel produced a cumulative fruit value of about 4.72 times that of leaves, with phenol, 2-chlorophenol and 4-nitrophenol contributing most strongly in the fruit profile. These observations are indicative of well-defined organ-level chemical differentiation. However, the prominence of substituted chlorophenols and nitrophenols requires confirmatory analysis and rigorous quality control before the compounds are assigned as endogenous phytochemicals or linked to biological activity. The article can therefore be regarded as a useful preliminary characterization that identifies promising phytochemical features and specific analytical questions for follow-up.

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