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Enhancement of Phytochemical Analysis and Drying Efficiency of Annual Herbaceous harvested in Tunisia Flora: Used as Efficiency Remedy in Ayurveda Therapy

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Abstract

The aim of the present study was to detect, quantify and identify the phenolic compounds in *Tribulus terrstris* (used as remedy in infertility disorder by Ayurveda therapy) extract hydrolyzedby HCL, using High-Performance Liquid Chromatography (HPLC) to detect and quantify non-volatiles compounds. Gas-Chromatography Coupled to Mass Spectrometry (GC-MS) was performed to detect and quantify volatile-compounds. A total of sixteen, eighteen[1], and twenty[2] compounds from Tribulus extract, respectively in seeds, leaves and roots were identified as belonging to various structural classes, mainly phenolic compounds. Analysis by HPLC finger print allowed the identification of procatechic-acid in leaves part detected with high peakarea (8.2 mg / g MS).

Keywords: Dietary-supplement; Nutraceutical; Phenolic-compounds; Procathechic-acid; Separation-process; RP-HPLC

Introduction

Polyphenolics, which are widely distributed in plants, currently are among the most studied phyto chemicals because of their perceptible chemical properties[1]. Polyphenols, a large group of compound sub iquitously expressed in plants constitute one of the most numerous and large groups of substances in the plant kingdom, currently with over 8000 known phenolic structures published[3]. These compounds can be sub divided into various classes according to the number of their phenol rings and the structural elements linked to the basic units[4]. A classification of the twenty one[5] principal structures based on the number of carbons in the molecule has been established[4]. These secondary molecules accumulated in plants and participated in defense mechanisms against different environmental stress conditions such as wounding, infection, excessive light or UV irradiation[6] and constitute a wide and complex array of phyto chemicals that exhibit antioxidant action and consequently a beneficial physiological effect[2]. Generally, polyphenols keletons are derived from two different active precursors (i.e. 4-coumaroyl-CoA and malonyl-CoA), and they arise biogenetically from acetate and shikimate pathways. Poly phenols are present in foods and beverages of plant origin (fruits, vegetables, cereals, herbs, spices, legumes, nuts, olives, chocolate, tea, coffee, and wine) and are the most abundant antioxidants in the human diet[7]. Epidemiological studies have shown that a diet rich in poly phenols can prevent a wide variety of human diseases. Poly phenols show many beneficial effects on human health including antimicrobial, anti-inflammatory, antiviral, anticancer, and immunomodulatory activities[1,8-10]. Their ability to delay lipid oxidation in food stuffs and biological membranes, in addition to their propensity to act as a prophyl actic agent has motivated research in food science and biomedicine[11,12]. The determination of phytoconstituents is largely performed by the relatively expensive and often laborious techniques such as Gas (GC) and Liquid Chro-

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matography (LC) combined with specific detection schemes. In the last few years, GCe-MS has become firmly established as a key technological metabolic profiling in plant species. One of them Tribulus terrestris. L (TT) (US dietary supplement marketed product), which has been carefully investigated by Farombi, E.O .,et al[13] and further developed in our last publication and communication to evaluate its anti-cancer activity by invitro assay[13]. The plant is used individually as a single therapeutic agent or as a prime or sub ordinate component of many compound formulations and food supplements. It's consumed by sports men since 1980 in Bulgaria as muscular enhancer, tonic, energetic, strengthening. To the best of our knowledge, no data are present in the extant literature about the use of GC-MS and LC-ESI-MS/MS applied to the characterization of phenolic compounds of Tribulus. Therefore, the aim of the present study is to report for the first time the phyto chemical investigation of TT parts harvested in Tunisia flora. Sophisticated methods of detection and quantification (liquid and gas chromatography) were performed to identify the volatile and non-volatile compounds. As a result these methods have succeeded in identifying many compounds; while liquid chromatography has showed a high peak area of procatechic-acid: major non-volatile compound.

Material and Methods

Reagents

Folin-Ciocalteu reagent, β-carotene, cis, cis-9, 12-Octa decadienoic acid (linoleic acid), di (phenyl)-(2, 4, 6-tri nitro phenyl) iminoazanium (DPPH), 2, 6-bis (1, 1-di methyl ethyl)-4- methylphenol (BHT), aluminium chloride (AlCl3), Hydrochloric Acid (HCl)

Tri hydroxyl benzoic acid (gallicacid), quercetin, catechin were procured from Sigma-Aldrich Chemise. Analytical grade ethanol, chloroform and Tween 40 were obtained from Merck.

Collection of Plant Material

***Tribulus terrestris* L:** Samples were collected from plants grown in the region of Boukrim: Hawaria (northe as to fnorthern Tunisia). Herbaceous was collected between June-Septembers 2015-2016, when the fruits were ripening. The harvested plants were identified as mentioned in our last publication[13]. Voucher specimens were deposited in the herbarium four laboratories for future reference. Leaves, seeds and roots were separated and dried at room temperature under dark conditions prior to use.

Extraction of phenolic compounds

The dried separated organs were then powdered, different extraction methods were adopted for these powders:
(1: one step extraction), using different solvents with increasing polarity: hexane, chloroform, 1-butanol, acetone, ethanol, ethanol 70 % and water. Technique: vegetable matter is socked with solvent under magnetic stirring for 60min, and then the solutions are stored at4 °C for 48hours, and then filtered under vacuum through a Whatman No.4 filter paper (porosity of 25 µm). All organic extracts were concentrated by rotator evaporation under vacuum at 40°Ca 60°C to determine the different yields. (Ref)

Sample preparation by sequential and reflux extraction

The dried and powdered plant material (powder: 30 g) was extracted in a succession by chloroform at room temperature (3x270x1h) and 70% v / v ethanol (reflux at 80º C, 3x450 mLx2h).The combined ethanol solutions were concentrated under vacuum at70º C to a small volume ~150 mL and extracted in the separator funnel with n-butanol(3 times 60, 45 mL). The butanol layers were concentrated to dryness giving the crude organic fraction[13].

Total poly phenolic content

Total poly phenol content was determined with the Folin–Ciocalteu reagent using the method described in our study[13]. 100 µL of the diluted sample were dissolved in 500 µL (1 / 10 dilution) of the Folin–Ciocalteu reagent and 1 mL of distilled water. The solutions were mixed and incubated at room temperature. After 1 min, 1.5 mL of 20% sodium carbon at solution was added. The final mixture was shaken thoroughly and then incubated for 2 hrs in the dark at room temperature. The absorbance of all samples was measured at 760 nm and results were expressed in mg of gallic acid equivalents per gram (mg GAE.g-1).

Total flavonoid content

The $AlCl_3$ method was adapted[13] for the purpose of determining the total flavonoid content of the ethanol extracts. 1.5 mL of extracts was added to equal volumes of a solution of 2% $AlCl_3$ $6H_2O$. The mixture was thoroughly mixed and incubated for 10 min at room temperature the absorbance was read at 367.5 nm. Data were expressed in mg quercetin equivalents per gram (mgQE.g-1).

Total proanthocyanidins content

The HCl / butan-1-ol assay was used[13] to quantify total proanthocyanidins. 0.25 mL of extract was added to 3 mL of a 95% solution of n-Butanol / HCl (95:5 v / v) and 0.1 mL of a solution of $NH_4Fe(SO_4)_2$ $12H_2O$ in 2Ml HCl in stoppered test tubes. The tubes were incubated for 40 min at 95°C. The absorbance of the red color was read at 550 nm, data expressed as mg catechin equivalents per gram (mgCE.g-1).

Hydrolysis of phenolic extract

Dried samples from Tribulus parts were hydrolyzed according to the method of[14] which was slightly modified. The acidic hydrolysis was used to release the aglycones in order to simplify the identification process since the free forms of phenolic compounds are rarely present in plants and they occur as esters, glycosides or bound to the cell wall[15]. Twenty milliliter of methanol containing BHT (1g / L) was added to 0.5 g of a dried sample. Then 10 mL of 1MHCl was added. The mixture was stirred carefully and sonicated for 15 min and refluxed in a water bath at 90°C for b 2h. The obtained mixture dried under vacuum was further injected to HPLC.

Identification of phenolic compounds using RP-HPLC

The actives compound analysis was carried out using an Agilent Technologies 1100 series liquid chromatography (RP-HPLC) coupled with a UV-v is multi wavelength detector. The separation was carried out on a 250×4.6 mm, 4 µm HypersilODSC18 reversed phase column at ambient temperature. The mobile phase consisted of aceto nitrile (solvent B) and water with 0.2%

formic acid (solvent C). The flow rate was kept at 0.7 mL / min. The gradient programme was as follows: At 6 min: 35% of Band 65% of C, at 9 min:60% of B and 40% of C, at 14 min 80% of B and 20% of C, at 25 min 100% B and 0% C, at 30 min 35% of B and 65% of C[16,17]. The injection volume was 20 µL, and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before injection. Actives compounds were detected according to literature dates as well as the same experimental conditions. Analyses were performed in triplicate.

Identification of phenolic compounds using GC-MS

Gas Chromatography-Mass Spectrometry (GC-MS) analyses were carried out on a gas chromatograph; an HP 5890 series (II) coupled to an HP 5972 mass spectrometer (Agilent Technologies, PaloAlto, CA, USA) with electron impact ionization (70eV). AnHP-5MS (5% Phenyl Methyl Silox) capillary column (30 m × 0.25 mm, 0.25 m film thickness Agilent Technologies, Hewlett-Packard, CA, USA) was used. Column temperature was programmed as follows 40°C for 1 min, 8°C / min to 100°C for 5 min, 10°C / min to 200°C for 3 min, 12°C / min to 300°C for 20 min. The carrier gas was helium with a flow rate of 0.9 mL / min and a split ratio of 100:1.Scantime and mass range were 1 sand 50–550 m / z, respectively[17].

Theory of calculation

The content to feach peak area (PA) was calculated from a calibration curve that was generated using the 1, 2, Buthylhydroxy-Toluene (BHT) as an internal standard.

Statistical analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P < 0.05$. Correlations were carried out using the correlation and regression in the EXEL program.

Results

Phyto chemical investigation

Extraction Yields: Solvent extraction is the most frequently used technique for the isolation of bioactive compounds from plants. Therefore, the recovery of bioactive compounds has been typically accomplished using various solvents, such as methanol and ethanol, as well as hot water and buffers[18]. Nevertheless, the majority of the studies focused on solvent extracts because the efficacy of solvent extraction is higher than simple water extraction. Moreover, solvents can dissolve the many useful organic molecules found in plants, such as phenolics, and terpenoids compounds.

Two methods were adapted to extract TT: In the first extraction method (#1: one step extraction), different solvents with increasing polarity (hexane, chloroform, 1-butannol, acetone, ethanol, ethanol 70% and water) were used. Yields of different extracts obtained from leaves, seeds and roots. The highest yield (14.12%) was recorded from ethanol 70% by the one-step method. There is no study in the literature which mentions the extraction yields using our methods or other methods. Ethanolic extracts yields varied significantly between Tribulus organs with for leaves 9.28%, 8.6% for seeds and 1.21% for roots. In the literature adding water to solvent increase the polarity of solvent and improves the efficiency of extraction[17,5], so we note that leaves exhibited the highest yield (ethanol 70%:14.12%) is more better one and half than ethanol yield for the same organ (9.28%). In general the extraction yields are influenced by the nature of solvent, the mixture of solvent and the part of the plant studied. The leaves chloroformed fraction yield (9%), obtained by mechanical agitation for 3 hours are three times better than extract yields given from magnetic agitation for 30 min (3.32%), so the extension of extraction time improves the efficiency of extraction process.

Total polyphenol, total flavonoidand total condensed tannin contents: Results from the quantitative determination of total poly phenols, total condensed tannins and total flavonoids of various extracts of Tribulus parts. Total flavonoids and total condensed tannin contents were determined as catech in equivalents in milligrams per gram of dry weight (mgCE / gDW), while total polyphenol contents were calculated as gallicacid equivalents in milligrams pergram of dry weight (mgGAE / gDW). The total polyphenol contents varied significantly ($P < 0.05$) between the three part studied, leave extract had higher total polyphenol content (45.2 ± 0.01mg GAE / g) than seed (35.62 ± 0.01 mg GAE / g) and root (13.88 ± 0.02 mg GAE / g) ones.

Identification and quantification of non-volatile compounds by RP-HPLC

In order to quantify and separate phenolic compounds of the three studied parts of Tribulus, RP-HPLC coupled with a UV–vis multi wave length detector was used and the obtained results. To the best of our knowledge, there are no previous reports dealing with the characterization of phenolic compounds in Tribulus methanolic extracts (after acidic hydrolysis). So[8] compounds were identified in Tribulus parts by HPLC including[6] phenolic acids (procatechic-acid, 3,4-dihydroxybenzoic-acid, genestic-acid, cafeic-acid, phloretic-acidand 2, hydroxyphenyl acetic-acid). At observation 3, 4- di hydroxyl benzoicacid is respectively detected by RP-HPLC in leaves, seed sand roots as follows: 2229.11mau; 514.61mau and 500.41mau, the variability of amount of chemical composition is clearly influenced by the part of plant used. Furthermore variability in nature of chemical composition according to the part was also confirmed, mainly procatechic acid is widely detected and quantified in leaves part (8124.82mau), however this compound is not detected in seeds and roots samples.

Identification and quantification of volatile-compounds by GC-MS: For the first time qualitative and quantitative analysis of hydrolysis acid extract was carried out using a Gas Chromatography–Mass Spectrometer (GC–MS) system.

From the GC–MS profile it can be noted that enormous numbers of compounds have been identified tentatively. For instance, many aliphatic ester and ketones, heterocyclic, alkaloids, alkanes, terpene, phenolics, flavonoids, silane compounds have been identified from seeds, leaves and roots. The retention time at10.26 min, 38.22 min, 42.18 min, 42.28 min, 31.82 min, 42.97 and 42.27 min, correspond to molecules with percentages higher than 3% from seeds samples. At showing in chromatogram profile (x) the major compounds identified in seeds is methyl-linol

(42.18 min; 58.02 ± 0.01%), while an ethole (22.61 min; 14.87 ± 0.01%) is major molecule in leaves part, and octa decanoic acid (42.05 min; 12.77 ± 0.01%) identified in roots.

Discussion

Development of current approaches and methods for characterization of metabolites in complex samples mainly plant remains a challenging task, particularly if they are present at varied and minor concentration levels[17]. The ability to detect a large number of metabolomics in low concentrations in a single analysis by current chromatography being important benefits to scientific and researchers compared to other analytical methods.

The range of phenolic compounds extracted from plant materials is influenced by the chemical nature of the compounds, the sample particle size, the part of the plant used, the extraction method performed, the extraction time and conditions, and the presence of interfering substances[19]. Phenolic extracts of plant materials are composed of a mixture of different classes of phenolics (metabolomics) determined by their solubility in the solvent system used[20,21]. Acetone, ethyl acetate, methanol, ethanol, and water, and their combinations were frequently used to extract poly phenolics[22]. Non-polar organic solvents (i.e., petroleum ether, n-hexane, chloroform, and dichloromethane) have relatively low extraction abilities for poly phenols, and were usually used in sample treatment to remove chlorophyll and lipids or to prevent enzymatic reactions[23]. Moreover, given that some extracted phenols were present as glycosides, same caution was exercised in extracting the m to avoid hydrolysis, Precautionary techniques, including isolation in the dark and under low temperature was performed. Soluble phenolic molecules can be easily isolated from plant tissues by extraction[24,25] with methanol or methanol acidified with 0.1 % (v / v) HCl. Methanol / water(1:1) for example has been widely used to extract low molecular weight phenolic compounds (hydroxyl benzoic, and hydroxyl cinnamic acids).

In the present study, acidic hydrolysis of phenolic compound has been performed, further, it's analyzed by HPLC, so we think that acidic hydrolysis extract need to be more investigated, and measured by Folin-Ciocalteau method. This latter method was predictable due to the weak selectivity of the Folin–Ciocalteu reagent, as it reacts positively with different natural compounds (phenolic and non-phenolic substances[26]. Besides the traditional solvent reflux extraction, ultrasound extraction, supercritical fluid extraction, and micro wave extraction were favored alternative extraction methods compared with the first method because of their high efficiency and low solvent consumption[27]. In some studies, the authors described an increase into total polyphenols by ultrasound[28,29]. This observed increase was attributed to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave, which accelerated the transfer of organic substances from plant materials. SFME, a relatively new extraction method, was another alternative to the traditional and conventional techniques because it was easy, environmental, rapid, Inexpensive and efficiency[30]. This technique does not require solvents, thereby reducing sample manipulation, further more has a positive impact on human health[31].

It is well known that an important function of flavonoid sand phenolic acids offers their action in plant defense mechanisms[32]. Effectively, flavonoid shave many biological activities such as cyclo oxygenase activity[33], the inhibition of plasma platelet aggregation and, the suppression of histamine release, potent nitric oxide radical scavenging activity and exhibiting antiviral, antibacterial, anti-inflammatory and anti allergenic effects[34-36].

Conclusion

In the present study, RP-HPLC has been confirmed as a powerful analytical technique for separating and detecting phenolic and other polar compounds in concentrated extracts. For the first time with this method, eight[37] compounds were tentatively identified in performed extract and based on their chromatographic retention time. Further most representative groups of compounds tentatively identified by GC-MS were flavan-3-ols (oligomeric forms). Of these compounds, (epi) fisetinidol-(epi) catechin isomers and other. Our results clearly showed that the presence of secondary metabolites (polyphenols, flavonoids, tannins) in high appreciable amounts in the plant parts could contribute to their nutritional and medicinal value.

Conflict of Interest: The author's declare that there are no conflicts of interest.

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