Identification of Major Constituents of Hypericum perforatum L. Extracts in Syria by Development of a Rapid, Simple, and Reproducible HPLC-ESI-Q-TOF MS Analysis and Their Antioxidant Activities

Abdalrahim Alahmad, * Ibrahim Alghoraibi, Raghad Zein, Sergej Kraft, Gerald Dräger, Johanna-Gabriela Walter, and Thomas Scheper

ABSTRACT: Hypericum perforatum Linn (St. John’s wort) is a popular and widespread medicine in Syria, which is used for a wide range of conditions, including gastrointestinal diseases, heart disease, skin diseases, and psychological disorders. This widespread use prompted us to identify the main compounds of this plant from Syria that are responsible for its medicinal properties, especially since its components differ between countries according to the nature of the soil, climate, and altitude. This is, to the best of our knowledge, the first report in which St. John’s wort, a plant native to Syria, is extracted using different solvents and its most important compounds are identified. In this study, the dried above-ground parts, i.e., leaves, stem, petals, and flowers, were extracted using different solvents (water, ethanol, methanol, and acetone) and extraction protocols. By increasing the polarity of the solvent, higher yields were obtained, indicating that mainly hydrophobic compounds were extracted. Therefore, we conclude that extraction using the tea method or using a mixture of water and organic solvents resulted in higher yields compared with pure organic solvents or continuous boiling with water for long periods. The obtained extracts were analyzed using high-performance liquid chromatography equipped with a diode array detector (HPLC-DAD), coupled with UV–visible spectrophotometry at a full spectrum (200–800 nm). The HPLC spectra of the extracts were almost identical at three wavelengths (260 nm for phloroglucinols (hyperforin and derivates), 590 nm for naphthodianthrones (hypericins), and 350 nm for other flavonols, flavones, and caffeoylquinic acids), with differences observed only in the intensity of the peaks. This indicates that the same compounds were obtained using different solvents, but in different amounts. Five standards (chlorogenic acid, quercetin, quercetin hydrate, hyperoside, and hypericin) were used, and a comparison with retention times and ultraviolet (UV) spectra reported in the literature was performed to identify 10 compounds in these extracts: hyperforin, adhyperforin, hypericin, rutin, quercetin, quercitrin, quercitrin hydrate, hyperoside, biapigenin, and chlorogenic acid. Although the European Pharmacopoeia still describes ultraviolet spectroscopy as a method for determining the quantity of Hyperici herba, interference from other metabolites can occur. Combined HPLC–DAD and electrospray ionization–mass spectrometry (LC-ESI-MS) in the positive mode have therefore also been used to confirm the presence of these compounds in the extracts by correlating known masses with the identified masses or through characteristic fragmentation patterns. Total phenolic contents of the extracts were determined by the Folin–Ciocalteu assay, and antioxidant activity was evaluated as free radical scavenging capacity using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. The results indicate that the aqueous extracts prepared by the tea method gave the highest total phenols, while the pure organic solvents gave very low phenols. Also, the extracts that contain the largest amount of phenols gave lower IC50 values or higher antioxidant activity than that of others.

INTRODUCTION

Hypericum perforatum Linn, generally recognized as St. John’s wort, is a flowering plant native to Asia and Europe. It belongs to the Hypericaceae family and contains over 1000 species and about 55 genera. The Hypericum genus comprises over 450 species distributed worldwide in tropical and subtropical...
regions.\textsuperscript{1–3} This species can grow under various environmental conditions and has a very extensive ecological capacity. It is found in pastures, thickets, forest clearings, varied types of oak forest, meadows, and burnt areas.\textsuperscript{3} The height of the stem, which is red in color and branched in its upper section, is about 40–100 cm (Figure 1 a). Stems might appear articular from leaf scars although it is wooden close to its base.\textsuperscript{4,5} Branches are grouped as opposite pairs and rotated at 90\textdegree angles (intersecting), at the base of every leaf.\textsuperscript{4,6} The leaves are narrow-oblong, nonserrated, yellowish green in color, with less stalk, 2–4 cm long on the major stalk, 1–2 cm long on branches, and covered with scattered translucent glands in the form of dots (Figure 1 b). The dots are translucent when seen against light; this gives the leaves a pierced appearance as indicated by the plant’s Latin name.\textsuperscript{4,7} The multiflowered flowers are very branched and compact to round and bright yellow in color with black dots with a length of up to 2.5 cm. Each flower has five petals and sepals, which are 4–6 mm long, in the shape of a narrow shaft with a pointed tip, and sometimes have some black glands.\textsuperscript{4,8,9} A total of 50–80 stamens are grouped into three or five fascicles; these patterns are separated, and the stigmas are in dense head-like groups (Figure 1 c). The fruit is a three-chamber capsule that contains many raw seeds that are rough and netted with coarse grooves, with length from 1 to 1.3 mm (Figure 1 d).\textsuperscript{4,9}

Different extracts (alcoholic or aqueous extracts) of the aerial parts of \textit{H. perforatum} revealed that its bioactive natural components (phytochemicals) consist of seven groups:\textsuperscript{4,10–25} (1) naphthodianthrones—anthraquinone derivatives (e.g., hypericin, isohypericin, protopypericin, pseudohypericin, protopseudohypericin, etc.), (2) phloroglucinols (e.g., hyperforin, adhyperforin, hydroperoxycadiforin, etc.), (3) flavonol glycosides: flavonoids—flavonols (e.g., quercetin, kaempferol, etc.), glycosides (e.g., rutin, hyperoside, quercitrin, isorquercitrin, etc.), and flavones (e.g., apigenin, luteolin, etc.), (4) bilavones (e.g., flavone (13,118-biapigenin), amentoflavone (13′,118-biapigenin), and catechins (flavonoids are often associated with condensed tannins)), (5) phenylpropanes (e.g., p-coumaric, chlorogenic, caffeic, vanillic, p-hydroxybenzoic, and ferulic acids), (6) proanthocyanidins and tannins (e.g., dimeric procyanidin B2, dimeric, trimeric, and tetrameric procyanidins), and (7) xanthones (e.g., 1,3,6,7-tetrahydroxyxanthone and kielcorin C).\textsuperscript{4,10–25} Moreover, minor amounts of other common components include acids (palmitic, isovalerianic, myristic, stearic, nicotinic, citric, and malic), pectin, choline, carotenoids, B-sitosterol, amino acid derivatives (tryptophan, \textgammavaminobutyric acid, and melatonin), nicotinamide, vitamin C, sugars (glucose, fructose, saccharose, and lactose), fatty acids, bisanthraquinone, glycosides, and hydroperoxycadiforin. Hydroalcoholic extracts also contain essential oils. Roth (1990) published a list of 29 ingredients that make up about 65\% of the steam distillates: \textalpha-pinene and 2-methylcine were the major ingredients. Essential oils from \textit{St. John’s wort} also contain typical terpenes such as monoterpenes \textalpha-pinene and \textbeta-
pinacol, limonene, B-caryophyllene, myrcene, geraniol, germacrene D, B-farnesene, humulene, and larger amounts of long-chain alkanols, hydrocarbons, and alkanols such as undecane, n-undecane, n-nonane, n-tetradecanol, 2-methylpentane and -decane, 2-methyl-dodecane, C16 and C29 alkanes and C24, C26, and C28 alkanes and 2-methylbutenol. Although the specific chemical differences between plants grown in different regions of the world appear to be hereditary, the composition of extracts is, according to several studies, also largely influenced by environmental factors. The plant’s location, including the height above sea level, the time of harvest (in which month of the year; before flowering, full flowering, or fruit group stages), the harvested parts (flowers, leaves, bells, root, or stalk), the polarity of the solvent (methanol, ethanol, acetone, water, etc.) used in the extraction, pH of medium, temperature, pressure, and exposure to light all play an important role in the type, nature, and concentration of the chemical compounds obtained from the extraction.\(^1,14,17,19,22,23,25-28\)

**H. perforatum L. (St. John’s wort)** is used as a traditional medicinal plant all over the world and was accepted in the European Pharmacopoeia 6. It is utilized in many countries of the world for the production of plant-based pharmaceutical products\(^1,10,22\) due to its broad diversity of ingredients, such as hypericins and hyperforins, which harbor considerable pharmaceutical effects.\(^30\) Traditional uses have included topical or oral application for the treatment of diabetes, bruises, rheumatism, burns, malaria, skin wounds, biliary disorders, migraines, eczema, common cold, gastric ulcer, menorrhagia, diarrhea, bedwetting, sprains, bronchitis and urogenital diseases, indigestion, hemorrhoids, snake bite, hysteria, neuralgia, and psychiatric disorders, especially depression.\(^3,4,11,13,14,16,37-39\)

As detailed above, *St. John’s wort* extracts contain various polyphenols, which are considered to be the main source of antioxidant activity and, consequently, a potential cancer prevention agent.\(^11,36\) Consumers prefer natural antioxidants because of their assumed lower potential toxicity compared to that of synthetic antioxidants.\(^36\) Both phytochemicals and essential oils of *St. John’s wort* exhibit pharmacological effects, such as anti-inflammatory, antiviral, antibacterial, anti-diabetic, antifungal, chologenic and choleretic, analgesic, anticholinesterase, antioxidant, antidepressive, anti-nociceptive, anxiolytic, and cytotoxic activities.\(^2,10,11,32,37-42\)

Hypericum as a major photosensitizing agent has been used in *vitro* and *in vivo* in photodynamic cancer therapy, including squamous cell carcinoma, human leukemia, and nasopharyngial carcinoma, or viral infections such as sindbis virus, herpes simplex virus types II and I, vesicular stomatitis, influenza virus, HIV-I, and murine cytomegalovirus.\(^14,15,17,45-46\) Hyperforin suppresses the proliferation of allogeneic T cells, inhibits the proliferation of peripheral blood mononuclear cells, inhibits the growth of MT-450 breast carcinoma in immunocompetent Wistar rats, and activates mitochondria-mediated apoptosis when added to MT-450 cells.\(^14,15,47,48\) Flavonoids have exhibited activity against cancer and influenza virus.\(^14,15,17\)

High-performance liquid chromatography (HPLC), linked with UV detectors, is a routine technique in most laboratories for chemical separation and detection. HPLC is also the preferred separation technology for the isolation of nonvolatile, often polar, and unstable components present in natural products. In the literature, many HPLC methods have been reported in the identification of the main components such as chlorogluccinols, naphthodianthrones, flavonoids, phenolic acids, biflavones, aurones, and xanthones from *St. John’s wort*.\(^1,12,18,20,21,26,49-53\) (see Table S1 in the Supporting Information).

In this study, the aerial parts (leaves, stems, petals, and flowers) of *St. John’s wort* harvested from Syria were cleaned, dried, and extracted using eight different extraction procedures: water (boiling with water for almost 3 h (water I) or using the tea method (water II)), ethanol, methanol, and acetone (100 and 70%). A new, simple, effective, accurate, and repeatable HPLC method was developed for the isolation and identification of the major chemical ingredients. HPLC diagrams for all eight extracts contained the same peaks and only varied in the intensity of these peaks. HPLC–DAD-ESI-MS analysis was performed in the positive ionization mode to obtain the exact mass data in the MS full scan experiment and to identify the compounds. The compounds that were identified in these extracts, either by UV, HPLC, or by HPLC-MS analysis, were chlorogenic acid, hypericin, hyperforin, hyperoside, quercetin, quercitrin hydrate, rutin, bipigenin, quercitrin, and adhyperforin. The antioxidant effect and free radical scavenging activity of extracts of *St. John’s wort* were determined in two ways: first is based on the compounds’ capability to transform the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (purple-colored) to its reduced form DPPH-H (yellow-colored) and second is by their capability to act as an electron-donating radical scavenger inhibiting the formation of the green-colored ABTS** radicals. Total phenols in the extracts (water I, water II, EtOH 100%, and MeOH 100%) were quantified, and it was found that the antioxidant activity increased with the increase of total phenols.

### MATERIALS AND METHODS

**Chemicals.** Aerial parts (leaves, stem, petals, and flowers) of *H. perforatum L. (St. John’s wort)* were collected in July–August 2018 from the Ghab Plain in Syria (Google maps: 35.586856, 36.355724 and 180–200 m above sea level) and harvested during the flowering season. Hypericin and quercetin were purchased from Cayman Pharma (Neratovice, Czech Republic); hyperforin was purchased from Roth (Karlsruhe, Germany); quercitrin hydrate, chlorogenic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium persulfate, and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (Darmstadt, Germany); Whatman 90 mm filter paper was purchased from GE Healthcare Life Sciences (Freiburg Germany); a 0.22 μm nylon syringe filter, Sartolab Vakuumfilter 180C5, 0.22 μm polyethersulfon, 500 mL, 25 mm syringe filter, 0.45 μm RC with GF prefiltre, and 0.45 μm PTFE filter were purchased from Sartorius (Goettingen, Germany); and 0.45 μm prefiltre was purchased from Wicom (Heppenheim, Germany). Ethanol, methanol, and acetone were HPLC grade from Roth (Karlsruhe, Germany); acetonitrile was obtained from VWR (Hannover, Germany), and water was purified using a QM system from Sartorius (Goettingen, Germany).

**Instrumentation.** The HPLC system consisted of a VWR HITACHI liquid chromatograph equipped with a S160 pump, a S260 autosampler, a S430 diode array detector, an organizer (eluent stand), and temperature controller for the column and sampler (S310 column oven) (VWR, HITACHI, MA, JAPAN). The absorption was measured in the range of 200–800 nm, with the UV–visible detector working at 260 nm for hyperforins and 350 and 590 nm for hypericins. The chromatographic data were recorded and processed with...
Agilent Open LAB Control Panel software. For UPLC-ESI-QTOF-MS analysis, a Waters Acquity high-performance liquid chromatograph (UPLC, Waters) coupled with a time-of-flight Q-TOF micro-mass spectrometer (Waters) and equipped with electrospray ionization (ESI) was used. Mass spectra were recorded in the positive ion mode at 3 kV capillary voltage and 230 °C desolvation temperature.

Raw Plant Preparation and Extraction. The aerial parts (leaves, stem, petals, and flowers) of St. John’s wort were washed in cold water to clear them of mud and soil, dried, cut into small pieces, and then ground using a ball mill until a fine powder was obtained. A total of 0.360 g of this powder was placed in a 1000 mL beaker; then, 800 mL of distilled water was added. The beaker was placed on a heater at boiling temperature and stirred. Concentration was performed for about 4 h until the volume of the solution was reduced to about 200 mL (water I). A total of 0.360 g of this powder was placed in a 1000 mL beaker; then, 800 mL of distilled water was added. The beaker was placed on a heater at boiling temperature and stirred. Concentration was performed for about 4 h until the volume of the solution was reduced to about 200 mL (water I). The same amount of powder was placed in a 400 mL beaker, and 150 mL of boiling water was added; the beaker was placed on the heater, stirred, and left at room temperature for about 20 min (water II). Subsequently, the aqueous extracts were filtered through a filter paper (Whatman, no. 589/2). The resulting filtrate was centrifuged at 24630 g for 30 min to remove residual particles. The supernatant was filtered through a 0.22 μm nylon syringe filter. The flow-through was collected (see Figure 2), and the solvent was evaporated under nitrogen gas at 30 °C to prevent oxidation.

For ethanol, methanol, and acetone extraction, the process was modified as follows: 2 g of powder was placed in a beaker and 150 mL of solvent (100 and 70% for each solvent) was added. The solution turned dark red for EtOH 70% and acetone 70%, light red for EtOH 100%, black red for MeOH 70%, and red for MeOH 100% and acetone 100%. The beaker was covered with paraffilm and stirred for 5 h at room temperature (RT). The supernatant was decanted and stored at −80 °C. A total of 200 mL of solvent was added to the sediment; the beaker was covered with paraffilm and then stirred at RT for 17 h. The supernatant was decanted, added to the previous supernatant, and then stored at −80 °C. A total of 100 mL of solvent was added to the sediment. The beaker was then covered with paraffilm and stirred for 3.5 h at RT. The solution turned slightly reddish with a light-brown haze. The supernatant was decanted and added to the previous one. The solutions were filtered first with a filter paper (Whatman, no. 589/2) and then with a Sartoban vacuum filter 180C5; 0.22 μm polyethersulfon (PES) was used for ethanolic and methanolic extracts. For acetonic extracts, a 0.45 μm PTFE filter was used (see Figure 2). The solvent was concentrated by means of a rotary evaporator (water bath temperature = 37 °C) as far as possible, and the residual solvent was removed by freeze drying.

Standard and Reference Compound Solutions. Five reference compounds (standards) were used in this study: chlorogenic acid, quercetin, hyperoside, quercitrin hydrate, and hypericin. These substances and different extracts were dissolved in HPLC-grade ethanol in different amounts, as shown in Table 1, and filtered through a 0.45 μm prefilter (Wicom, Germany) before undertaking HPLC analysis.

**Table 1. Overview of the Measured Samples, Standards, and Spiked Samples**

<table>
<thead>
<tr>
<th>St. John’s wort extracts</th>
<th>amount [mg] dissolved per mL EtOH</th>
<th>standards</th>
<th>amount [mg] per mL EtOH</th>
<th>ethanol extract with standards together</th>
<th>spiking ratio Vextract [μL] + Vstandard [μL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>water I</td>
<td>20</td>
<td>quercetin</td>
<td>0.5</td>
<td>extract + quercetin</td>
<td>100 + 50</td>
</tr>
<tr>
<td>ethanol 100%</td>
<td>30</td>
<td>chlorogenic acid</td>
<td>1.4</td>
<td>extract + chlorogenic acid</td>
<td>13 + 5</td>
</tr>
<tr>
<td>ethanol 70%</td>
<td>30</td>
<td>quercitrin hydrate</td>
<td>0.9</td>
<td>extract + quercitrin hydrate</td>
<td>120 + 20</td>
</tr>
<tr>
<td>methanol 100%</td>
<td>33.8</td>
<td>hyperoside</td>
<td>1.1</td>
<td>extract + hyperoside</td>
<td>50 + 50</td>
</tr>
<tr>
<td>methanol 70%</td>
<td>25</td>
<td>hypericin</td>
<td>1.7</td>
<td>extract + hypericin</td>
<td>50 + 50</td>
</tr>
<tr>
<td>acetone 100%</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acetone 70%</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Colors of extracts obtained using different solvents in this study: (A) 100% acetone, (B) 70% acetone, (C) 100% methanol, (D) 100% ethanol, and (E) water (water I and water II exhibited the same color). Photograph courtesy of the corresponding author Abdalrahim Alahmad. Copyright 2020.
Chromatography. High-Performance Liquid Chromatography (HPLC). For HPLC analysis, a Chromator HPLC-diode array detection (DAD) system supplied with an autosampler (Shimadzu, VWR HITACHI, Germany) was utilized. The extract compounds were identified using a Kinetics 5 C18 100 A column (Phenomenex, Germany, dimensions: 100 mm × 4.6 mm, 5 μm particle size) and C18 4 × 3.0 mm guard column (Phenomenex, Germany) at room temperature (25 ± 2 °C). Solvent A was 0.1% formic acid, and solvent B was 0.1% formic acid, 95% MeOH, and 5% H2O. The mobile phase was freshly prepared daily, filtered through a 0.45 μm nylon filter, and degassed after preparation for 15 min in an ultrasonic bath. The gradient mode shown in Figure S1 (0 min 95% A, 5% B; 82 min 0% A, 100% B; 92 min 0% A, 100% B; 93 min 95% A, 5% B; 100 min 95% A, 5% B) was used with a flow rate of 1 mL/min. Specific amounts of the dried extract (Table 1) were dissolved in HPLC-grade ethanol. All samples were filtered through a 0.45 μm prefilt (Wicom, Germany) before undertaking HPLC analysis. The injection volume was 10 μL, and every sample solution was injected in triplicate at different time periods and with different concentrations taken for all experiments, indicating that the method is accurate and repeatable. For the identification of the compounds, standards such as chlorogenic acid, hyperoside, quercitrin hydrate, quercetin, and hypericin were run under the same conditions, using dilutions in HPLC-grade ethanol (Table 1). The detection was performed at 260, 350, and 590 nm for all samples and standards. St. John’s wort compounds of different extracts (water I and II, EtOH%100, EtOH%70, MeOH%100, MeOH%70, acetone%100, acetone%70) were identified by comparing their retention times with each other and with the retention times of standards, in addition to comparing their UV spectra and literature data.

UV–vis spectra. The UV spectra of pure hypericin and St. John’s wort extracts were run with a Chromator S430 diode array detector, and spectra were acquired over the absorption range of 200–800 nm. Identification of hyperoside, quercitrin hydrate, quercetin, and hypericin in the extracts (EtOH, MeOH, and acetone; 100 and 70%, respectively) was performed by comparing curves from the maxima of absorptions of extracts at 260 nm at 19.9, 24.36, 29.4, and 81.49 min, respectively, with the UV–vis spectra of the standards at the same wavelength and time.

HPLC-MS Spectra. For the HPLC-MS analysis, the column used in HPLC analysis was installed in another HPLC device to which an MS device was directly connected. For high-performance liquid chromatography with mass detection (HPLC-MS) analysis, a Waters Acuity UPLC with a Kinetex 5 μm C18 column (Phenomenex, 100 × 4.6 mm) was used with a linear gradient of (A) water with 0.1% formic acid and (B) 0.1% formic acid, 95% MeOH, and 5% H2O at a flow rate of 500 μL/min (initial: 95% A, 5% B; 82 min: 0% A, 100% B; 92 min: 0% A, 100% B; 93 min: 95% A, 5% B; 100 min: 95% A, 5% B; runtime: 100 min). The injection volume was 5 μL. A chromatogram was recorded in parallel to the mass spectrum. Therefore, the UV measurement was only carried out at two wavelengths, namely 260 and 350 nm. MS analysis was carried out on a Q-ToF Premier (Waters) using electrospray ionization (positive ions, 3 kV capillary voltage; 250 °C desolvation temperature; 650 L/h desolvation gas flow (nitrogen)).

Determination of the Total Phenolic Content of Crude Extracts of H. perforatum L. The total phenolic content of plant extracts was determined as shown in the references54–56 with some modifications. Gallic acid represents one phenolic compound of H. perforatum L. and was therefore used as a standard. A total of 10 mg of the standard (gallic acid) was dissolved in 100 mL of distilled water to give a concentration of solution of 100 μg/mL.

Preparation of the Standard Calibration Curve of Gallic Acid. Aliquots of 0.2S, 0.5, 1.0, 1.5, and 2.0 mL of the 0.1 mg/mL gallic acid solution were placed in five different 15 mL glass test tubes. A total of 2.5 mL of a 10-fold dilution of Folin–Ciocalteau phenol reagent (1:10 v/v with distilled water) and 2.0 mL of a 7.5% w/v sodium carbonate (Na2CO3) solution were added to each tube. The volume in the tubes was increased up to 10 mL with distilled water, resulting in gallic acid concentrations ranging from 2.5 to 20 μg/mL. A mixture of reagents and water was used as a blank. The resulting solutions were put in a water bath at 45 °C for 30 min. Thereafter, the absorbance at 765 nm was read using a UV–vis spectrophotometer.

Preparation of Sample Extract Solutions. A total of 10 mg of each of the extracts was dissolved in 10 mL of an appropriate solvent (water, ethanol, or ethanol) to get 1 mg/mL solution. Also, 1 mL of this solution was then put in a 15 mL glass test tube, and color development was undertaken in the same manner as for the standard. The absorbance of the test solutions at 765 nm against a reagent blank (appropriate solvent) was measured. All the tests were performed in triplicate; results were averaged and expressed as mean ± standard error of the mean. The concentration of phenols in the test samples was determined by extrapolation from the gallic acid standard calibration curve and calculated as mg of gallic acid equivalents (GAE) per g of dried extract (de) (mg GAE g⁻¹) using the formula described by56,57

\[ P = C \times V / M \]

where \( P \) = total phenolic content in milligrams per gram of the dry plant material (mg/g), \( C \) = concentration of gallic acid established from the calibration curve in milligram per milliliter (mg/mL), \( V \) = volume of the extract solution in milliliters (mL), and \( M \) = weight of the extract in grams (g).

Determination of Antioxidant Activity. - DPPH. The antioxidant activity of water, ethanol, and methanol extracts was measured depending on their scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. DPPH is a recognized radical and a scavenger (snare) for other radicals. Because of a powerful absorption band at around 517 nm, the DPPH radicals have a profound violet color in solution, and when neutralized, they turn pale yellow or colorless. Therefore, the change in absorption at 517 nm allows the calculation of the number of primary radicals. The DPPH assay was performed as previously described58,59 with some modifications. Different concentrations (1–500 μg/mL) of the extracts and standard were prepared; then, 1 mL of each solution was added to 3 mL of 0.004% ethanolic DPPH free radical solution. The absorbance of the preparations was measured after about 30 min of exposure to normal light using a UV–vis spectrophotometer at 517 nm. The results were compared with the corresponding absorption of standard ascorbic acid concentrations (1–500 μg/mL). Finally, the free radical scavenging ability (RSC) expressed as a percentage was calculated by the following equation

\[ \text{RSC} \% = 100 \times \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \]
The inhibitory concentrations (IC$_{50}$) were determined. The IC$_{50}$ value indicates the concentration of sample required to scavenge 50% of the DPPH free radicals.$^{59,60}$

- **ABTS.** We use the spectrophotometric method to assess the loss of color when adding antioxidants to the blue-green chromophore ABTS$^{•+}$ (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). ABTS$^{•+}$ is reduced by antioxidants to ABTS and loses its color. ABTS antioxidant activity was measured as described elsewhere$^{61}$ with some minor modifications. ABTS was prepared with 7 mM concentration using water as a solvent. The ABTS solution was mixed with 2.45 mM potassium persulfate at a ratio of 1:1 (v/v). The mixture was placed in the dark at room temperature for 18 h. The ABTS$^{•+}$ solution was diluted about 20 times with water (or ethanol or methanol based on the nature of the solvent employed in the extraction) to reach an absorbance of 0.850 ± 0.05 at 734 nm. A total of 150 $\mu$L of this ABTS$^{•+}$ solution was added to 50 $\mu$L of different concentrations of extracts and incubated for 6 min at room temperature. For the control, 50 $\mu$L of solvents (water, ethanol, and methanol) used in the respective extraction were used in place of the extract. Ascorbic acid was used as a positive control. Absorbance at 734 nm was measured spectrophotometrically in a 96-well microplate. The percentage of inhibition was calculated utilizing the same equation as in the DPPH assay, and the radical scavenging activity was expressed as the IC$_{50}$ value.

The results of all experiments (samples, standards, and blank) were reported as mean ± standard error of three separate determinations. Statistical analysis was performed by analysis of variance using Origin statistical software.

### RESULTS AND DISCUSSION

**Extraction.** The water I and water II extraction processes resulted in a yellow-brown solid with a yield of 21% for water I and 16% for water II. The organic solvents resulted in a black-gray solid with a yield of 25.5% for ethanol 70% (light gray), 7% for ethanol 100% (black gray), 22.9% for methanol 70% (light gray), 18% for methanol 100% (black gray), 22.3% for acetone 70% (light gray), and 7% for acetone 100% (black gray). The yields resulting from the mixture of water with organic solvents were much higher when compared with the yields of the pure organic solvents. Also, extracting with water using the tea method resulted in higher yields than boiling with water for a long time. By increasing the polarity of the solvent, higher yields were obtained, indicating that mainly hydrophobic compounds were extracted. Therefore, we conclude that extracting the plant with water using the tea method or using a mixture of water and organic solvents produces higher...
yields of the resulting extract when compared with pure organic solvents or continuous boiling with water for long periods.

Characterization of Extracts Using HPLC. Reversed-phase chromatography has analytical and preparatory applications in the field of phytochemical separation and purification, and the active ingredients of a plant extract can be isolated using an appropriate isolation procedure. During the protocol development process, many factors were changed and tested, such as column type (nature of the stationary phase and its particle size), mobile phase or gradient elution (the exact composition of the solvents and its polarity and gradients of different solvents), flow rate, temperature of the column, pressure used (maintaining linear velocity), and wavelength. Subsequently, we were able to develop the protocol used, through which well-separated peaks were obtained without any obvious overlap between them. The different extracts were measured using HPLC under the same conditions and can thus be directly compared with each other. The chromatograms of the measurement series at the three most important wavelengths (260, 350, and 590 nm) are shown in Figure 3. The respective graphs show no general differences between ethanolic, methanolic, and acetonic extracts with regard to the position of the obtained peaks; only differences in the intensity of the peaks were observed as some solvents extract certain compounds more effectively than others. The ethanolic, methanolic, and acetonic extracts (whether 100 or 70%) are similar concerning the ingredients present but differ in the concentration of these ingredients. In contrast, the aqueous extract is completely different to the ethanolic, methanolic, or acetonic extracts. Aqueous extracts obtained by water I and water II extraction procedures were identical, especially at 260 and 350 nm, where especially, phloroglucinols, flavonoids, and phenolic acids could be expected (active constituents, hyperforin and its derivatives). While the aqueous extract has fewer peaks or very low peaks (the peaks are present, but when drawn in the same scale as the chromatograms of organic extracts, they are not visible due to their low intensity) at 590 nm of the chromatogram, where, e.g., naphthodianthrones (hypericin exhibits a very low level of solubility in pure water because of its hydrophobicity could be expected. Also, it can be concluded that at 100 °C, in particular, the strongly
Figure 5. continued
polar compounds are virtually all decomposed. Plant extracts are usually composed of a mixture of different types of phytochemicals or specialized metabolites with different polarities, in the reversed-phase chromatography; the bioactive polar compounds eluted prior to the less or nonpolar one. This explains why in the water extract fewer peaks appear at high retention times because the high retention times correspond to the less or nonpolar specialized metabolites. It is clear that less or nonpolar substances are more difficult to extract from water. It should also be noted that some ingredients of St. John’s wort are not stable at high temperatures and should therefore always be extracted at low temperatures. It is also noteworthy that the main constituents in the St. John’s wort extracts, especially hypericins, may be retained by some cartridges in the filter during the filtration process.17,20 Hypericin and pseudohypericin are substantially insoluble in water at ambient temperature and can only be obtained with less than 40% yield at higher temperatures.4,17−19 When prepared by the tea method, the extract contains very little amounts of hyperforin.19,25 The concentration of chlorogenic acid, which has been detected in St. John’s wort, is below 1%.17

Characterization of Extracts with Standards Using HPLC. In this series of measurements (Figures 4 and 5), the extracts, standards, and extracts spiked with standards were measured under the same conditions. Standards were used to analyze the constituents of St. John’s wort extracts and were chosen to cover the complete chromatogram and the most important wavelengths and, based on their stability and solubility, in the solvents used for extraction. Five standards were selected for this, which represent the most important and best-known compounds of St. John’s wort. These include quercetin and quercitrin, which have been used as a hydrate, and hyperoside, chlorogenic acid, and hypericin. The first four mentioned standards cover the two important wavelengths 260 and 350 nm and are distributed in the middle of the obtained chromatograms of the St. John’s wort extract. Hypericin is represented at a high retention time (approximately 81.5 min) of the St. John’s wort chromatograms and also covers a significant absorbance at 590 nm (Figure 4).

Extracts were analyzed by addition of different standards (Figure 5). If the compound to be examined were already present in the extract, the corresponding peak would increase in the chromatogram by the added standard amount. This increase in the peak would thus provide a good indication that it is the same compound. By adding the standards to be confirmed to the sample, the effect of the column drifts can be prevented. Figure 5 shows four chromatograms, each with a different standard added. Due to the addition of the respective standard, a peak increased in each chromatogram (indicated by an arrow) when compared with chromatograms of the pure extract. Thus, within the scope of its analytical accuracy, this method indicates the presence of the four compounds, hyperoside, quercetin, quercitrin hydrate, and hypericin, in the ethanolic extract. This also applies for methanolic, acetonic, and aqueous extracts and also is in agreement with the literature [7].

However, a final identification of substances is not possible using this method as other components could have the same retention time. This method of substance analysis by adding a standard thus serves only as an indication of the presence of certain compounds and simplifies the comparison of the chromatograms with the literature information. Therefore, further and more precise analysis methods have been used for the final substance analysis.

UV-vis Analysis. Each substance has a characteristic UV spectrum. The various substances separated via HPLC can
thus be identified by comparing the measured UV spectra with the corresponding spectra from the literature or measured for a standard. When analyzing St. John’s wort extracts, it must be borne in mind that ingredients such as quercetin and other likely ingredients are sensitive to changes in pH, which leads to the formation of new substances.65 For this reason, UV spectra

Figure 6. Overlay UV spectrum of the peaks detected by HPLC−DAD. (a) Retention time of 19.907 min and the standard hyperoside at the same time; (b) retention time of 24.364 min and the standard quercitrin hydrate at the same time; (c) retention time of 29.404 min and the standard quercetin at the same time, and (d) retention time of 81.547 min and the standard hypericin at the same time.

Figure 7. Chromatogram of the ethanolic extract (ethanol 100%) at 260 nm.
of some peaks with suspected constituents of the measured samples (EtOH 100 or 70%) were compared with the UV spectra of the standards, which were recorded under the same conditions. Figure 6 shows the UV spectra of the measured standards hyperoside, quercitrin hydrate, quercetin, and hypericin each with the UV spectra of the selected corresponding peaks of the ethanolic extract. The peaks selected are those that experienced a peak increase when the ethanolic sample was spiked with the respective standards. In Figure 6, it can be clearly seen that the UV−vis spectra for the peaks at retention times of 19.907, 24.364, and 29.404 min are completely identical with the UV−vis spectra obtained for the standards hyperoside, quercitrin hydrate, and quercetin, respectively. Figure 6d shows that the UV spectrum of the hypericin standard differs slightly in its intensity from the selected peak of the ethanol sample at the same time, although the peak of the sample is well isolated, possibly due to the difference in concentration. The results of these comparisons show that this method is only of limited use for the identification of analytes. UV−vis analysis merely indicates the presence of these compounds. The presence of other analytes at the same peak in the chromatogram can change the UV spectrum significantly. Furthermore, other analytes can have the same UV spectrum at the same retention time. For this reason, this method is only suitable for a quick and basic orientation when interpreting chromatograms.

Identification of the Main Compounds Using HPLC-MS Analysis. The HPLC-MS method exhibits unique selectivity and sensitivity because it combines high-performance liquid chromatography, which has strong separation ability, with detection via mass spectrometry, which has an unparalleled structural analysis ability. This technique is a fast, specific, and delicate analytical method and is one of the most efficient processes for the determination of metabolites and has thus become a key tool in the metabolic exploration of plant extracts. 

Figure 8. Chromatogram of the ethanolic extract (ethanol 100%) at 350 nm.

Figure 9. Representation of the base peaks after HPLC separation of the sample (ethanol 100%).
The measurement of the masses for the eluted components is preceded by the detection of UV absorption at 260 and 350 nm for the ethanolic extract. The chromatograms of the sample (Figures 7 and 8) correspond to the previous measurement (Figures 4 and 5). The mass spectrum (Figure 9) of the ethanolic extract has many peaks over the entire spectrum, in particular in the range between 50 and 70 min, in which there is a very strong superimposition of the peaks. To provide a better overview, the peaks of the mass spectrum are marked with a number and shown in Table S2 with the corresponding retention times and ion masses. For a clear comparison of the chromatogram with the respective mass spectrum, the retention times of the chromatograms and the mass spectra are compared in Tables 2 and 3. It should be noted that not all separation. In view of the low concentrations of compounds in the fractions, HPLC devices would have to be used for particularly small sample quantities. When evaluating the mass spectrum (Figure 9), all peaks are more or less superimposed. Separate groups were observed only up to 45 min. From 45 to about 72 min, so many peaks were recorded that only the outstanding peaks can be viewed more closely.

Table 4 shows the substances identified from the mass spectrum of the ethanolic extract. With the exception of hypericin, the most important and best-known compounds can be identified. In the positive ESI mode, the prominent protonated molecular ion \([M + H]^+\) at \(m/z\) 355.17 refers to chlorogenic acid. \(m/z\) 303.03 was characterized from the losses of the sugar residue from rutin and hyperoside. \(m/z\) 303.04 indicates quercitrin without the sugar residue, while \(m/z\) 303.05 matches quercetin, and \(m/z\) 359.09 was determined to be a characteristic fragment ion of biapigenin. \(m/z\) 551.42 indicates adhyperforin, and \(m/z\) 537.43 refers to hyperforin. However, most of the compounds (Table S3) cannot be identified despite the huge number of peaks. Possible reasons for this could be that not all peaks of the mass spectrum are taken into account and many peaks are superimposed. This means that most of the peaks are still unknown compounds because (i) some of the compounds cannot be detected using a UV detector (ii) because of interference, where some peaks may merge from several peaks; (iii) the molecular weights may not be present in the device library. To be able to analyze these aspects, additional HPLC separation steps of individual fractions must be carried out, as already mentioned. Additional methods are also required in which molecular fragments arise during the mass spectrometry measurement to enable an even more precise analysis.

### Table 4. Peak Identification from Table 7 with Already Known Substances

<table>
<thead>
<tr>
<th>compound</th>
<th>(m/z)</th>
<th>time [min]</th>
<th>peak-Nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>chlorogenic acid</td>
<td>355.17</td>
<td>26.97</td>
<td>15</td>
</tr>
<tr>
<td>rutin and hyperoside each</td>
<td>303.03</td>
<td>29.32</td>
<td>16</td>
</tr>
<tr>
<td>quercitrin without the</td>
<td>303.04</td>
<td>32.00</td>
<td>18</td>
</tr>
<tr>
<td>sugar residue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>quercetin</td>
<td>303.03</td>
<td>35.37</td>
<td>20</td>
</tr>
<tr>
<td>quercetin</td>
<td>303.04</td>
<td>35.49</td>
<td>21</td>
</tr>
<tr>
<td>quercetin</td>
<td>303.05</td>
<td>35.59</td>
<td>22</td>
</tr>
<tr>
<td>biapigenin</td>
<td>539.09</td>
<td>40.10; 40.23</td>
<td>24, 25</td>
</tr>
<tr>
<td>adhyperforin</td>
<td>551.42</td>
<td>71.28</td>
<td>43</td>
</tr>
<tr>
<td>hyperforin</td>
<td>537.43</td>
<td>80.19</td>
<td>47</td>
</tr>
</tbody>
</table>

The sample-sparing electrospray method was used for the ionization of the sample, in which positively charged ions are formed, and there is negligible fragmentation of the sample molecules. The comparison of the peaks from the chromatogram with the peaks from the mass spectrum shows that the chromatogram peaks correspond to the mass spectrum peaks. In the respective tables (Tables 2 and 3), however, a mass spectra peak cannot be given for each chromatogram peak. This can be demonstrated with a visual comparison, however. In the two chromatograms of the ethanolic extract (Figures 7 and 8), it can be seen that almost all peaks are quite narrow. As a result, the method used to separate the ingredients of the extract can be considered successful. The peaks that cannot be separated completely are too similar in retention behavior to be separated in one step with the chosen conditions. This requires fractionation of the eluate and an additional HPLC.
the Folin–Ciocalteau assay and expressed as gallic acid equivalents (GAE). There was no significant difference in the resulting phenolic compounds for the different water-based methods (Table 5). Although the difference is minimal, we note that extracting the plant after the addition of boiling water gives a better result than heating the water with the plant gradually. A significant difference was observed between the methanolic extract (93.2 mg GAE/g) and ethanolic extract (64.4 mg GAE/g). The value for aqueous extracts is much higher than for methanol and ethanol; this may be due to the fact that methanolic and ethanolic extracts do not dissolve fully in water. We note that the standard calibration curve of gallic acid ($Y = 0.06063X + 0.06734$) is linear in the range of $0–20 \mu g/mL$ with a correlation coefficient ($r^2$) of 0.997 (Figure S2).

Table 5. Total Phenolic Contents for the Studied Extracts of St. John’s Wort

<table>
<thead>
<tr>
<th>St. John’s wort</th>
<th>total phenolics (mg GAE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>water I</td>
<td>170.6 ± 1.7</td>
</tr>
<tr>
<td>water II</td>
<td>174.8 ± 0.9</td>
</tr>
<tr>
<td>ethanol extract</td>
<td>64.4 ± 1.5</td>
</tr>
<tr>
<td>methanol extract</td>
<td>93.2 ± 1.3</td>
</tr>
</tbody>
</table>

The appropriate selection of the extracting solvent is not as straightforward as it may seem. Effective extraction of phenolic compounds relies on suitable solvent selection, elevated temperatures, and mechanical agitation. The solubility relies on the polarity of phenolic compounds that exist in the plant. Phenolic compounds have the ability to form hydrogen bonding with water molecules, so they are soluble in water, a polar solvent.

Antioxidant Activity—DPPH. In the DPPH test, we examined the capacity of the tested $H. \text{perforatum} \ L.$ extracts to act as donors of electrons or hydrogen atoms in the conversion of DPPH radicals into their reduced form DPPH-$\cdot$$H$. In the present study, aqueous, ethanolic, and methanolic extracts were investigated. All extracts showed free radical scavenging activity (Figure 10), meaning that all the assessed extracts were able to reduce the stable, purple-colored radical DPPH into the yellow-colored DPPH-$\cdot$$H$ (Figure 11). Antioxidant activities of the phytochemicals existing in these extracts perhaps rely on structural factors, such as the number of keto groups, free carboxylic groups, methoxyl groups, phenolic hydroxyl, flavone hydroxyl, and other structural advantages. For the calculation of the IC$_{50}$ values, the following theoretical function was fit to the measurements using Microsoft Excel solver (Figure 10).

![Figure 10](https://doi.org/10.1021/acsomega.1c06335)
Figure 11. Change in the structure of the 1,1-diphenyl-2-picrylhydrazyl compound from the radical form to the reduced form with the change of its color.

Table 6. Percentage of Neutralization of the DPPH Radical by St. John’s Wort Extracts in the DPPH Assay

<table>
<thead>
<tr>
<th>St. John’s Wort extract</th>
<th>concentration (μg/mL)</th>
<th>IC50 (μg/mL) ± STD error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500</td>
<td>300</td>
</tr>
<tr>
<td>water II</td>
<td>83.46</td>
<td>80.60</td>
</tr>
<tr>
<td>water I</td>
<td>87.26</td>
<td>86.10</td>
</tr>
<tr>
<td>ethanol</td>
<td>84.84</td>
<td>84.12</td>
</tr>
<tr>
<td>methanol</td>
<td>82.12</td>
<td>80.28</td>
</tr>
</tbody>
</table>

\[ f(x) = \frac{A}{1 + e^{-B(\ln(x) - \ln(C))}} \]

A, B, and C are the parameters of the function, x is the concentration, and \( f(x) \) is the corresponding activity. Extracts marked by * the A value were set to the A value of the corresponding ascorbic acid value because otherwise no reasonable fit is obtained. The parameter errors are determined by the inverse of the Fisher information matrix. Using the error propagation calculation, the standard error of the IC50 values and the confidential interval with \( \alpha = 0.05 \) are calculated. We found that IC50 of the extracts of H. perforatum L. (St. John’s wort) is 51 μg/mL for the extract prepared using 150 mL of boiling water, 60 μg/mL for the extract prepared using 750 mL of cold water and then boiling until the volume of the solution is reduced to approx. 100 mL, 75 μg/mL for the ethanolic extract, and 67 μg/mL for the methanolic extract, which indicates the remarkable antioxidant activity of the extracts.

The most powerful extracts in terms of antioxidant activity were those obtained from the water I method (Table 6). This can be explained by the difference in the free radical scavenging activity of extracts based on their chemical composition and content of total phenols and flavonoids. Generally, a positive correlation between phenols and antioxidant activity was found, but the relationship is not obvious as it depends on the complex composition of the sample. The pool of the phenolic compounds can be low, but due to a high content of, e.g., ascorbate, the total antioxidant status can be very high. The type of phenols and the amount of individual phenolic compounds present in the extract affect the antioxidant properties, and different classes of compounds with low and/or high antioxidant activity could be present in the extract. It is important to analyze each class of compound and relate it to the antioxidant activity.

Data previously published indicate that there are significant differences in the results of scavenging activities of H. perforatum L. (St. John’s wort) extracts, which are partly related to extraction medium (MeOH, EtOH, water, or other solvents) and thus to the content of the various phenolic compounds. Ethanolic samples, which contained the least amount of phenols, showed a lower activity (IC50 75 μg/mL) in agreement with several studies that have reported the relationships between the phenolic content and the antioxidant activity.70 The correlation is shown in Figure 12.

Antioxidant Activity—ABTS. The solvents used in the DPPH assay, methanol or ethanol, yield incorrect results for polar antioxidants (DPPH is insoluble in water), so this method does not provide useful information on the actual reaction of the antioxidant when viewed alone. In contrast, in the ABTS assay, the radical is produced in water just before the test by the reaction of ABTS with an oxidizing agent, such as potassium persulfate. The antioxidant activity is specified as the amount of ABTS** that is quenched after a specified period of time (ABTS** is a radical cation, so antioxidants react with it by an electron transfer mechanism) and compared to the activity produced by ascorbic acid. No distinction is made between radical trapping kinetics and stoichiometry; the result relies on the time chosen before the absorption reading.
ABTS is often employed in plant medicine research to measure the antioxidant characteristics of hydrogen-donating and chain-breaking antioxidant agents. This method is usable for both hydrophilic and lipophilic antioxidants. For the calculation of the IC50 values, the same previous theoretical function used in the DPPH method was fit to the measurements. The scavenging activities of the ABTS radical cation of various extracts (water, ethanol, and methanol) of *H. perforatum* L. are illustrated in Figure 13. The synergistic effects of these extracts with various solvents on the ABTS cation scavenging activity were calculated (Table 7). The results clearly imply that the aqueous, ethanolic, and methanolic extracts of *H. perforatum* L. inhibit the ABTS radical or scavenge the radical in a dose-dependent manner. The radical scavenging activity of extracts of *H. perforatum* L. was estimated by comparing the percentage inhibition of formation of ABTS** radicals with that of ascorbic acid. The activity was concentration-dependent, and the maximum scavenging activity was found in water extract II (IC50 = 42 μg/mL), followed by water extract I (IC50 = 51 μg/mL), and then the methanolic extract (IC50 = 71 μg/mL) and ethanolic extract (IC50 = 97 μg/mL) (Figure 13). All the extracts obtained with different solvents showed scavenging effects on ABTS** in the μg/mL range. This result is extremely promising because it indicates that lower cost extraction processes can be achieved as no expensive solvents are needed to obtain the same amount of phenols and active substances from the plant (Figure 14).

**Statistical Analysis.** For the experimental technical harvesting methods, statistical analysis showed that the correlation difference between the water I extract method and the ethanol 70% method is of high significance at a p-value of 0.007, while the water II extract method leads to harvest with no significant

<table>
<thead>
<tr>
<th>St. John’s wort extract</th>
<th>500</th>
<th>300</th>
<th>100</th>
<th>50</th>
<th>10</th>
<th>1.5</th>
<th>IC50 (μg/mL) ± STD error</th>
</tr>
</thead>
<tbody>
<tr>
<td>water II</td>
<td>94.42</td>
<td>94.03</td>
<td>74.45</td>
<td>44.62</td>
<td>13.23</td>
<td>3.63</td>
<td>42 ± 9.3</td>
</tr>
<tr>
<td>water I</td>
<td>94.40</td>
<td>93.63</td>
<td>72.74</td>
<td>44.20</td>
<td>24.84</td>
<td>5.48</td>
<td>51 ± 4.1</td>
</tr>
<tr>
<td>methanol</td>
<td>94.57</td>
<td>93.11</td>
<td>51.66</td>
<td>33.38</td>
<td>24.77</td>
<td>9.93</td>
<td>71 ± 14</td>
</tr>
<tr>
<td>ethanol</td>
<td>93.07</td>
<td>92.30</td>
<td>45.67</td>
<td>26.32</td>
<td>11.17</td>
<td>7.57</td>
<td>97 ± 29</td>
</tr>
</tbody>
</table>
difference with the ethanol 70% method. Therefore, the water II method is recommended for extraction of a higher amount of harvest nearly to the level of the ethanol 70% method rather than the water I method. The statistical correlation showed no significance in the harvest when using 70 and 100% of the methanol, acetone, and ethanol solvents, but our recommendation is to use 70% of these solvents, which give a high amount of harvest.

As for the HPLC diagrams, as shown in Figures S3–S10, the iterations of the eight extracts have shifted to the right or to the left, although all the iterations of all extracts have the same spectrum, that is, all of the peaks in the spectra are shifted by about the same time interval. Also, the repetitions of the samples were using different concentrations, so the intensity of the peaks varies from one plot to another according to the concentration taken. The main reasons that affect the Rt in our case are that the pump was old and did not always work with the same efficiency and the column was used to a great extent, for other experiments of other students also during the process of repeating our experiments. However, it is clear from the sampling frequency plots (Figures S3–S10) that the protocol is repeatable and we will get the same spectrum and the same peaks with the same time difference for each extract when the scan is repeated.

**Paired Sample T Test.** To compare the DPPH scavenging activity of various extracts of *St. John’s Wort* with Ascorbic acid as a standard, the paired T-test showed that *St. John’s Wort* extracts, i.e., water extracts I and II and ethanol have a low significance difference with ascorbic acid under the same conditions, while the methanol extract has a higher significant difference with ascorbic acid at a p-value of 0.134. This is probably due to the preparation of ascorbic acid during this experiment, clearly appearing with the lowest recorded number of DPPH activities in comparison to the other tested groups; see Tables S24 and 26 in the Supporting Information.

There is a strong correlation between the DPPH scavenging activity of ascorbic acid and *St. John’s Wort* extracts as represented in Table S25 in the Supporting Information. These correlations can be classified into very strong correlations for both water extracts I and II at values of more than 0.85, while the correlation is moderate between 0.5 and 0.85 for ethanol and methanol extracts. This means that for the highest DPPH scavenging activity, it is a preference to use water as an extraction solvent rather than ethanol and methanol with the *St. John’s Wort* plant.

The same comparison has been done by the ABTS method using the paired sample T-test; the difference between *St. John’s Wort* extracts, i.e., water extracts I and II, ethanol, methanol, and ascorbic acid, was low at a p-value ranging from 0.001 to 0.007, as seen in Tables S27 and 29 in the Supporting Information. This means that the previously mentioned probability concerned with the preparation of ascorbic acid for the methanol extract would be correct. For testing the correlation between *St. John’s Wort* extracts, i.e., water extracts I and II, ethanol, methanol, and ascorbic acid standard by ABTS, the same result shown in Table S28 in the Supporting Information was confirmed as previously mentioned for DPPH.

**Conclusions.** In the present study, the aerial parts (leaves, stem, petals, and flowers) of *St. John’s Wort* from Al-Ghab Plain in Syria were harvested, cleaned with cold water, dried, ground, and extracted using different solvents such as water (either boiling with water for about 3 h (water I) or adding to boiling water for 20 min (water II)), ethanol, methanol, and acetone, either pure or mixed with water at 70%. The results indicate that preparing the extract using the tea method (water II) and using organic solvents mixed with water gave a high yield compared to the water I method and pure organic solvents. A rapid, simple, and reproducible high-performance liquid chromatography diode array (HPLC–DAD) and UV absorbance detection protocol was developed to determine the phytochemical profiles present in the various extracts of *St. John’s Wort* from Syria. Concerning the presence of compounds, no general differences were observed in the HPLC patterns of the eight extracts, with the exception of the patterns of aqueous extracts at the wavelength of 590 nm. This is the region of the appearance of hypericins, which do not easily dissolve in water except at high temperatures and in very small proportions. The other difference was in the intensity of the existing peaks, referring to the respective concentrations of the compounds. This can be explained by the fact that different solvents extract the compounds in the plant in different proportions. High-performance liquid chromatography–mass spectrometry (UPLC-MS) in the positive ionization mode was conducted to separate the bioactive molecules ions [M + H]+ of the compounds in the extracts by their mass-to-charge ratio (m/z) and to detect them qualitatively and quantitatively by their respective m/z and abundance. Various techniques were used to identify the compounds: (i) comparison with five reference substances (chlorogenic acid, quercetin, hyperoside, quercitrin hydrate, and hypericin), (ii) comparison of the retention times of peaks in HPLC diagrams with those of standards and those reported in the literature, (iii) comparison of the UV absorption spectra of peaks with the UV spectra of reference substances and spectra from the literature, and (iv) matching the prominent protonated molecular ions [M + H]+ of compounds with the molecular weights of compounds known to be present in this plant. Ten compounds, hypericin, rutin, hyperoside, quercitin, quercetin, biapigenin, hyperforin, quercitin hydrate, adhyperforin, and chlorogenic acid, were identified, proven, and confirmed. All *St. John’s Wort* extracts (water I or water II, ethanolic, methanolic, and aceticonic) were effective scavengers of the free stable ABTS− and DPPH• radicals, and particularly, extracts containing more phenolic compounds were more effective scavengers. This antioxidant
activity may be explained by the presence of phytochemicals previously identified using HPLC, UV−vis, and HPLC-MS analyses, as they have all been shown to have antioxidant activity.

**Future Studies.** The aqueous extract of this plant has been used in our previous study as a reducing agent for silver ions into metallic silver and a protective agent (capping agent or stabilizer) to protect the silver nanoparticles and prevent their aggregation. This synthesis of AgNPs using the aqueous St. John’s wort extract from Syria represents a green, simple, one-pot method, which is cost-effective and environmentally friendly and provides natural capping agents for the stabilization of AgNPs, without necessitating high temperature, pressure, energy, and toxic chemicals. Phenolic compounds originating from the aqueous St. John’s wort extract on the surface of AgNPs played an important role in their antioxidant and anticancer activity, as shown in the above-mentioned study. The current work examines the conjugation of AgNPs with aptamers selective toward specific cancer cells. Thus, the phenolic compounds from St. John’s wort on the surfaces of silver nanoparticles could play an important role in targeted therapy.

**REFERENCES**


**ACKNOWLEDGMENTS**

The authors are thankful to all the lab members for their help. They are very thankful to Dr. Bernd Hitzmann, Head of the Department of Process Analysis and Grain Science at the University of Hohenheim, for his support in statistical analysis. They also thank Dr. Nina McGuinness from EU University Office Hanover for reviewing and correcting the grammar of the article. The authors express their gratitude to Dr. Ulrich Krings (Institut für Lebensmittelchemie, Leibniz Universität Hannover), Dr. Abdalla A. Elshereef, Dr. Osama Al-Madanat, Dr. Yamen AlSalka, and Martina Weiss for support during this work and for access to technical facilities. The authors are very thankful to Dr. Ayham Abazid, Bremen University, for his valuable suggestions during this work. Abdalrahim Alahmad is supported by PhD grants from Avicenna Studienwerk e.V. The publication of this article was funded by the Open Access Publishing Fund of Leibniz Universität Hannover.

**AUTHOR INFORMATION**

**Corresponding Author**

Abdalrahim Alahmad − Institute of Technical Chemistry, Leibniz University of Hannover, 30167 Hannover, Germany; orcid.org/0000-0002-9843-5883; Email: alahmad@iftc.uni-hannover.de, rahimclsp84@gmail.com

**Authors**

Ibrahim Alghoraib – Department of Basic and Supporting Sciences, Faculty of Pharmacy, Arab International University, 20872 Damascus, Syria; Physics Department, Faculty of Science, Damascus University, 20872 Damascus, Syria

Raghad Zein – Physics Department, Faculty of Science, Damascus University, 20872 Damascus, Syria

Sergej Kraft – Institute of Technical Chemistry, Leibniz University of Hannover, 30167 Hannover, Germany

Gerald Dräger – Institute of Organic Chemistry, Leibniz University of Hannover, 30167 Hannover, Germany

Johanna-Gabriela Walter – Institute of Technical Chemistry, Leibniz University of Hannover, 30167 Hannover, Germany

Thomas Scheper – Institute of Technical Chemistry, Leibniz University of Hannover, 30167 Hannover, Germany

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsomega.1c06335

**Notes**

The authors declare no competing financial interest.


(22) Avato, P.; Gaspare, G. Determination of major constituents in St. John’s Wort under different extraction conditions. *Pharm. Biol.* 2004, 42, 83–89.


