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Bioactive compounds (phytoestrogens) recovery from *Larrea tridentata* leaves by solvents extraction

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ABSTRACT

Methanol, ethanol, and acetone at four different concentrations (90%, 70%, 50%, and 30% v/v) were used for extraction of phytoestrogens (nordihydroguaiaretic acid (NDGA), kaempferol, and quercetin) from *Larrea tridentata* leaves. Besides the phytoestrogens extraction, the antioxidant potential, and the contents of total phenols, flavonoids, and protein in the produced extracts were also determined. The solvent and concentration used for extraction strongly affected the phytoestrogens recovery. The highest NDGA, quercetin, and kaempferol contents (46.96 ± 3.39, 10.46 ± 1.01, and 87.00 ± 6.43 mg/g DW plant, respectively) were recovered using 90% (v/v) methanol. All the produced extracts showed antioxidant capacity, but those obtained using 70% and 90% (v/v) methanol had significantly higher ($p < 0.05$) FRAP (ferric reducing antioxidant power) values (2.55 ± 0.09 and 2.73 ± 0.11 mM FE(II)/g DW plant, respectively) than the remaining ones. Extract produced by using 90% (v/v) methanol contained also the highest contents of total flavonoids (19.29 ± 0.79 mg QE/g DW plant) and protein (131.84 ± 6.23 mg/g DW plant), and elevated total phenols concentration (263.60 ± 25.78 mg GAE/g DW plant).

1. Introduction

Phytoestrogens including flavonoids (comprising isoflavonoids and flavonols derivatives), lignans, and coumestanes, are secondary plant metabolites that have attracted great attention due to their protective action against several health disorders such as cardiovascular diseases, cancer, brain function disorders, menopausal symptoms, and osteoporosis [1]. Such compounds have the ability to imitate or modulate the effectiveness of endogenous estrogens. This biological response is based on their structural and/or functional similarity to estradiol and their capacity to bind to the human estrogen receptors (ER). Some studies have shown that selective ER modulators, including phytoestrogens, inhibit cell proliferation *in vitro* [2] and *in vivo* [3].

Larrea tridentata (*Zygophyllaceae*), commonly known as creosote bush, is a plant that grows in semidesert areas of Southwestern United States and Northern Mexico [4]. This plant was traditionally used for centuries by North American Indians as a medicine for several illnesses including infections, kidney problems, gallstones, rheumatism and arthritis, diabetes, and to treat tumors [5]. *L. tridentata* is an outstanding source of natural compounds with approximately 50% of the leaves (dry weight) being extractable matter [6]. Among several bioactive compounds present in this plant, nordihydroguaiaretic acid (NDGA), kaempferol, and quercetin can be found at considerable high concentrations [7].

NDGA (Fig. 1A) is a phenolic lignan with biological activities of large interest in the health area, such as antiviral, antifungic, antimicrobial, and antitumorgenic [8]. The therapeutic potential of this compound for the treatment of tumors and cancer has been demonstrated, being related to an inhibition on cancer cells growth via an apoptotic mechanism [9]. Kaempferol and quercetin are flavonols that exist as a variety of glycosides or in aglycone form. The aglycone forms of kaempferol and quercetin are structurally similar, differing only by one hydroxyl group in the B-ring (Fig. 1B and C). Research on cell culture models has shown important biochemical effects of both compounds, which are relevant to carcinogenesis, including increase of differentiation and gap junction function [10], metal chelation [11], antioxidant properties [12], the inhibition of hepatic enzymes involved in carcinogen activation [13], the induction of Phase II (conjugating) enzymes [14], and the influence of ER-transcriptional activity of ERE-reporter systems [15]. Despite the anticarcinogenic capacity of kaempferol and quercetin, these compounds are also known for their anti-inflammatory and antinociceptive capacities [16].

Nowadays, bioactive compounds with potential health benefits have attracted great interest for use in several industrial areas, and researches on this topic have been strongly encouraged. Extraction is the first step in the isolation of compounds from natural sources.

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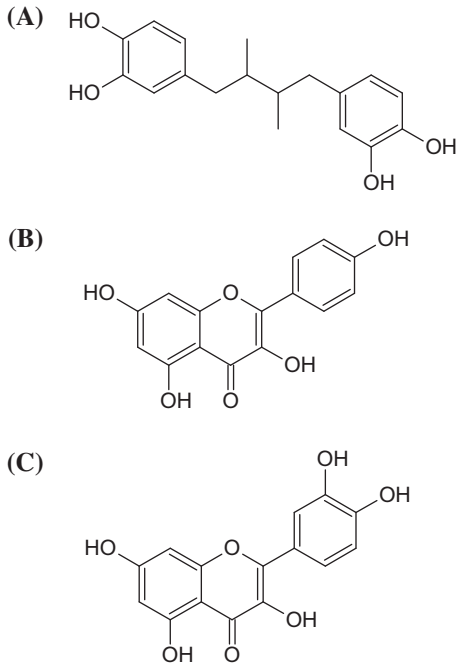

Fig. 1. Chemical structure of NDGA (A), kaempferol (B), and quercetin (C).

Among the variety of techniques that can be used for this purpose, solid–liquid extraction has been widely employed to extract bioactive compounds from plant materials and agro-industrial residues [17]. However, the efficiency of this extraction process is greatly affected by the type of solvent and its concentration [17,18], and therefore, studies to define the best conditions for these variables are necessary to maximize the extraction yields to each different plant material. Despite several studies evaluating the best solvents to extract the maximum content of phenolic compounds from plant matrices and the antioxidant potential of the produced extracts are reported in the literature, to the best of our knowledge, no detailed study has been developed with *L. tridentata*. Thus, the purpose of this study was to evaluate the effect of different organic solvents on the extraction of phytoestrogens, in particular, NDGA, kaempferol, and quercetin, from *L. tridentata* leaves. The antioxidant potential of the produced extracts, as well as the contents of total phenols, flavonoids, and proteins were also determined and are discussed.

2. Materials and methods

2.1. Plant material and chemicals

Plant material (*L. tridentata*) was collected from the Chihuahuan semidesert (North Coahuila, Mexico) during Spring season (April, 2009). Nordihydroguaiaretic acid (NDGA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin, kaempferol, aluminum chloride, 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ), sodium acetate, ferrous sulfate, and iron (III) chloride were purchased from Sigma–Aldrich (Saint Louis, MO, USA). Reagent-grade methanol, ethanol, acetone, acetic acid, and Folin–Ciocalteau were from Panreac (Barcelona, Spain). Potassium acetate was purchased from AppliChem (Darmstadt, Germany). HPLC-grade acetonitrile was obtained from Fisher Scientific (Leicestershire, UK). Ultrapure water from a Milli-Q System (Millipore Inc., USA) was used.

2.2. Extraction methodology

Air-dried leaves of *L. tridentata* were ground to fine powder and stored in dark bottles at room temperature for further use. Extractions were performed by mixing 1 g of plant material with 20 ml of organic solvent (methanol, ethanol or acetone, in a concentration of 90%, 70%, 50%, or 30% v/v) or distilled water. The mixtures were heated during 30 min in a water-bath at 70 °C when using methanol, ethanol, or water, and at 60 °C when using acetone, due to its lower boiling point. After this time, the produced extracts were filtered through qualitative filter paper and stored at −20 °C until further analysis.

2.3. Bioactive compounds quantification

NDGA, kaempferol, and quercetin concentrations were determined by high performance liquid chromatography (HPLC) on an equipment LC-10 A (Jasco, Japan) with a C_{18} 5 μm (3.9 × 300 mm) column at room temperature, and a UV detector at 280 nm. The response of the detector was recorded and integrated using the Star Chromatography Workstation software (Varian). The mobile phase consisted of acetonitrile (solvent A) and 0.3% acetic acid in water (v/v) (solvent B) under the following gradient profile: 30% A/70% B (0–2 min), 50% A/50% B (2–11 min), 70% A/30% B (11–17 min), 100% A (17–22 min), and 30% A/70% B (22–40 min). The mobile phase was eluted in a flow rate of 1.0 ml/min, and samples of 10 μl were injected. Previous the analysis, all the extracts were filtered through 0.2 μm membrane filters. NDGA, kaempferol, and quercetin were expressed as the ratio between mass of the compound in the extracts and mass of plant material (dry weight).

2.4. Determination of total phenols content

Total phenols content was determined by the Folin–Ciocalteu method with modifications. Briefly, 5 μl of the filtered extracts duly diluted were mixed with 60 μl of sodium carbonate solution (7.5% w/v) and 15 μl of Folin–Ciocalteu reagent in a 96-well microplate. Then 200 μl of distilled water were added and solutions were mixed. After standing for 5 min at 60 °C samples were allowed to cool down at room temperature. The absorbance was measured using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria) set at 700 nm. A calibration curve was prepared using a standard solution of gallic acid (200, 400, 600, 800, 1000, 2000, 3000 mg/l, $r^2 = 0.9987$). The total phenols content determined according to the Folin–Ciocalteau method are not absolute measurements of the phenolic compounds amounts, but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid. Thus, total phenols content was expressed as milligram gallic acid equivalent (mg GAE)/g DW plant material (dry weight).

2.5. Determination of total flavonoids content

Total flavonoids content was quantified by colorimetric assay. Briefly, 30 μl of the diluted and filtered extracts was added to 90 μl of methanol in a 96-well microplate. Subsequently, 6 μl of aluminum chloride (10% w/v), 6 μl of potassium acetate (1 mol/l) and 170 μl of distilled water were added to the mixture. The absorbance of the mixture was measured after 30 min at 415 nm against a blank prepared with distilled water, using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). A calibration curve was prepared using a standard solution of quercetin (25, 50, 100, 150, 200 mg/l, $r^2 = 0.9994$). Total flavonoids content was expressed as milligram quercetin equivalent (mg of QE)/gDW plant material (dry weight).

2.6. Determination of protein content

Total protein content was estimated using the Bradford assay.