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HPLC-DAD profile of phenolic compounds and antioxidant activity of leaves extract of *Rhamnus alaternus* L.

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ABSTRACT

This research investigated the polyphenols profiling and antioxidative properties of *Rhamnus alaternus* L. A comparative analysis of the defatted and eluted fractions of methanol extract from leaves of *R. alaternus* L. was performed on high-performance liquid chromatography coupled to diode array detection. The total phenolic, flavonoid, monomeric anthocyanin contents, and antioxidant activity of all the fractions of the leaves extract were quantified. All fractions showed the presence of phenolic compounds and exhibited different levels of free radical scavenging activity. Seven individual phenolic compounds and anthraquinones as a group of phenolic compounds in the samples were identified. Luteolin, quercetin-3-rhamnoside, *p*-coumaric acid, ferulic acid, gallic acid, and rutin were the newly identified compounds in the extract, confirmed by the presence of kaempferol and anthraquinones. Knowledge of these compounds in the leaves extract will help in formulating pharmaceutical products for various diseases.

1. Introduction

Ethnomedicine has been practiced since prehistory by virtually all human cultures around the globe, including Europe, Asia, Americas, and Africa (Slikkerveer, 2006). North Africa has one of the oldest and richest traditions associated with the use of medicinal plants (Batanouny et al., 2005), where Herodotus (430 B.C) wrote that medicine in Egypt is practiced among them on a plan of separation, by using an infinite number of drugs from medicinal plants (Rawlinson and Blakeney, 1910). Farther, North Africa is known for its illustrious savant king Juba II of Libya (Mauritania), who died about the year 20 AD, leaving at least a dozen scientific works. One of his most esteemed treaties is that which Pliny and Galen attributed the title "Deherba euphorbia.„ it is the variety of medicinal plant, to which he discovered the medicinal potential (Eugène-Humbert, 1952).

In North Africa, there are about 10,000 vascular plant species and about 70% found in the wild have medicinal, aromatic, and other uses. In Algeria, an estimated number of 3164 identified species are qualified as traditional medicinal plants (Vasisht and Kumar, 2004). Among the identified plant species on the earth, only a small percentage has been phytochemically investigated and the fraction submitted to biological or pharmacological screening is even smaller. Moreover, a plant extract may contain several thousand different natural products and any phytochemical investigation of a given plant will reveal only a narrow spectrum of its constituents (Hostettmann et al., 2000).

Plants represent a rich source of natural products, with almost infinite molecular diversity (Hostettmann and Marston, 2002), of which active ingredients of medicinal plants are mostly secondary metabolites (Zeng et al., 2013). The phenolic compounds are large and heterogeneous groups of these secondary metabolites, that are distributed throughout the plant kingdom (Maestri et al., 2006). Phenolic compounds are amongst the most desirable phytochemicals due to antimicrobial, antiviral, anti-inflammatory properties, and high antioxidant capacities (Ignat et al., 2011). Antioxidants are defined as compounds that can delay, inhibit, or prevent the

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oxidation of oxidizable materials by scavenging free radicals. Phenolics have been considered powerful antioxidants in vitro (Dai and Mumper, 2010). Many efforts have been made to provide a highly sensitive and selective analytical method for the determination and characterization of phenolic compounds. It is very important to understand the profile of phenolic compounds in medicinal plants by using highly effective extractions and analytical methods (Ignat et al., 2011).

Rhamnus alaternus L. is a fleshy-fruited, shrub of the Mediterranean region (Ben Ammar et al., 2005). One of the species commonly used in reforestation programs, due to its fruit characteristics and ability to survive in xeric environments, which represents an important water and nutrient source for birds and small mammals (Gulias et al., 2004). It has traditionally been used as laxative; purgative; hypotensive; and it is also used for treatment of the dermatological, ocular, burning, odontological, hepatic (Ben Ammar et al., 2008; Ben Ammar et al., 2005), psychological, depression, and goiter problems (Mati and de Boer, 2011). The wood has been also used as cosmetic for split hair (Lardos, 2006) and as a dyeing agent for wool (Guarrera, 2006). In addition, it has been reported that the leaves extracts of *R. alaternus* showed the highest antimutagenic level in a bacterial assay system, high xanthine oxidase inhibition (Ben Ammar et al., 2008) and inhibition of both rat intestine and purified porcine liver carboxylesterase (Stocker et al., 2004).

As a prominent local medicinal plant, *R. alaternus* L. has been widely distributed in the Northern Algeria. The leaves are used locally to in Kabylia by decoction as purgative and laxative, and also used fresh for treatment of jaundice. The purpose of this study was (i) defatting and fractionation of methanol extract from leaves of *R. alaternus* L. (ii) to investigate profiling of the phenolic compounds including phenolic acids, flavonoids, and anthocyanins by HPLC-DAD, and (iii) assessing total phenolic compounds, flavonoids, monomeric anthocyanins using colorimetric methods, and antioxidant activity using a radical scavenging assay of all fractions of defatting and fractionation steps of methanol leaves extract.

2. Materials and methods

2.1. Chemicals

Acetonitrile, hexane, acetic acid, sodium carbonate, Folin–Ciocalteau reagents, aluminium trichloride ($AlCl_3$), potassium chloride, and sodium acetate trihydrate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethyl acetate and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The phenolic standards, i.e., catechin, caffeic acid, quercetin-3-rhamnoside, *p*-coumaric acid, gallic acid, ferrulic acid, luteolin, apegenin, vanillic acid, and (−) epigallocatechin were purchased from Sigma–Aldrich (St, Louis, MO, USA). Chlorogenic acid and rutin were purchased from Acros organics (Thermo Fisher Scientific, Fair Lawn, NJ, USA) and naringenin and kaempferol from Sigma–Aldrich (Gillingham, Dorset, United Kingdom). All solvents used for this work were of HPLC grade.

2.2. Preparation of methanol extract

R. alaternus L. leaves were collected in March 2013, from Hengued, Adekar, North-West part of Bejaia in Kabylia (Algeria) (latitude: 36°43′15.46″N and longitude: 4°34′54.15″E) and kept for drying under a forced air oven at 60 °C up to moisture content of about 3.25% (determined by dry weight in oven at 105 °C until constant weight) (Spigno et al., 2007). The dried material was crushed to prepare powder, which was milled through a 1 mm sieve (final powder size <1 mm). Fifty grams of powder were macerated with 500 mL of methanol for 72 h at room temperature. After filtration through a filter paper, methanol was entirely removed using a rotary evaporator (Buchi, Flawii, Switzerland) at 45 °C (at 337 mbar). The dry extract was weighed and then re-dissolved in methanol to obtain a solution, with known concentration which was designated as crude extract (CE). The extractive value was calculated on dry weight basis from the formula given below:

$$\%\text{extractivevalue(yield\%)} = \frac{\text{Weightofdryextract}}{\text{Weighttakenforextraction}} \times 100$$

2.3. Defatting and fractionation of extract

All the steps of extraction, defatting, and fractionation are summarized in Fig. 1. Fifty milliliters of crude extract were subjected to entire evaporation of the methanol on a rotary evaporator (Buchi, Flawii, Switzerland) at 45 °C (at 337 mbar). The resulting dry extract was dissolved in 90 mL of Ultrapure water (Milli-Q) to obtain aqueous solution and was defatted several times, with the same volume of hexane, until a clear solution of hexane resulted. The hexane in the solution was evaporated using a rotary evaporator to get dry extract at 45 °C (at 335 mbar). The dry extract was re-dissolved in 40 mL of methanol and designated as hexane fraction (HF). The insoluble fraction formed between hexane and aqueous solution was washed several time with ultrapure water (Milli-Q), dried, dissolved in 25 mL of methanol and is designated as intermediate fraction (INT).

C-18 Sep-Pak Vac 35-cc (Waters, Milford, MA, USA) column was sequentially rinsed with 50 mL of ethyl acetate, acidified methanol (0.01% v/v HCl), and acidified water (0.01% v/v HCl), and charged separately with 20 mL of defatted aqueous solution of extract. The column was then washed with 60 mL of acidified Ultrapure water (Milli-Q) (AW), to remove any organic sugars and acids remaining in the solution (fraction AW), followed by elution with 60 mL of ethyl acetate and was designated as fraction EA. The final elution was made with 60 mL of acidified methanol and was designated as fraction AM (Lacombe et al., 2012). The ethyl acetate fraction EA contained a small amount of water, resulting from the previous elution with acidified water, which led to the separation of ethyl acetate solution designated as EA1 and water solution designed as EA2.

The solvents of the fractions HF, EA1, AM were removed completely on a rotary evaporator (Buchi, Flawii, Switzerland) at 45 °C (at 240 mbar). For the aqueous solutions AW and EA2, the solvents were removed by lyophilization (VirTis, New York, USA). All dry extracts HF, EA1, AM, AW, EA2, and INT fractions were dissolved in HPLC grade methanol and were subjected to HPLC analysis, phytochemical dosages, and antioxidant activity.

2.4. Determination of total phenolic compounds

The total phenolic content of the standards, crude extract (2 mg/mL) and all fractions (INT, HF, AW, EA1, EA2, and AM) without dilution were determined by Folin–Ciocalteu's phenol method following the procedures of Velioglu et al. (1998). Briefly, 20 μL of standard or samples were mixed separately with 150 μL of Folin–Ciocalteu reagents (previously diluted 1:10 with distilled water) and allowed to stand at 22 °C for 5 min. After incubation, 150 μL of sodium bicarbonate (6 g/100 mL) were added and after 90 min at 22 °C, the absorbance was read on the BMG LABTECH FLUOstar Omega plate reader (Germany) at 725 nm against a blank. The experiment was carried out in triplicate and the concentration of total phenolic compounds in the extract were expressed in mg gallic acid equivalent (GAE) per gram plant powder (PPW) i.e., (mg GAE/g PPW).