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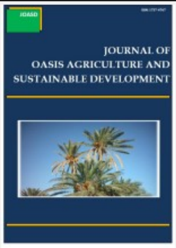

JOURNAL OF OASIS AGRICULTURE AND SUSTAINABLE DEVELOPMENT

www.joasdjournal.org

JOASD

p-ISSN: 2724 – 699X
e-ISSN: 2724-7007

Hydrosol extract from aerial parts of *Rosmarinus officinalis* cultivated in Metouia oasis - Biochemical composition and enzymes content

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Article info

Article history:
Received: 06 July 2023
Accepted: 22 August 2023

Keywords: Antioxidant Potential, Enzyme Activities, Hydrosol, *Rosmarinus Officinalis*, Total Phenolic Content.

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Conflict of Interest: The authors declare no conflict of interest.

Abstract

Hydrosols, are by-products of the hydrodistillation of plants. They consist of the distillation water in which very small amounts of essential oils remain dispersed. The aim of this study is the valorization of the hydrosol of a Tunisian aromatic and medicinal plant, *Rosmarinus officinalis*, by evaluating its phenolic content, antioxidant properties and enzyme activities. Through pH measurements, it has been determined that the hydrolat possesses an acidic character, with pH value falling within the range of 4.9. Total phenolic content assay (55.76 µg GAE/ml) and antioxidant assays DPPH radical scavenging activity (36.08%) unequivocally demonstrated the presence of antioxidants in the hydrosol and highlighted its potential to combat free radicals. The results showed that *Rosmarinus officinalis* presented an interested tyrosinase (2.16 U/mg protein), polyphenol oxidase (8.37 U/mg protein) and lipoxygenase (2.24 U/mg protein) activities. Detected enzymes are identified for the first time in plant hydrosol and are searched for health care and manufacturing industries.

1. INTRODUCTION

Hydrosols, referred to hydrolats or aromatic waters, are the water-based solutions obtained through hydrodistillation of various components of aromatic plants. After the distillation process is complete, these solutions separate from the essential oil phase. Although there is a wealth of scientific literature on essential oils, the understanding of hydrosols is comparatively limited (D'Amato et al., 2018). In Mediterranean countries, they have been employed for various purposes. Aromatic waters are used in invigorating beverages and as flavoring agents to enhance the savory dishes. Furthermore, they have been recognized as traditional remedies for ailments such as chronic pain, depression and dysmenorrheal (Hamedi et al., 2017; Aćimović et al., 2020).

Research has demonstrated that certain key components, predominantly oxygenated compounds, can exhibit higher concentrations in hydrosols compared to their corresponding essential oils (Khan et al., 2018; Vuko et al., 2021; Moukhles et al., 2022). Moreover, studies have indicated that those extracts are suitable for sanitizing tools involved in food preparation processes (Chorianopoulos et al., 2008). It was revealed also that they exhibited antioxidative, antimicrobial and anti-inflammatory properties (D'Amato et al., 2018). Hydrosols have a long-standing tradition of application in skincare, valued for their antiseptic and antispasmodic properties (Saxena et al., 2012; Mahboubi, 2016). The reason behind their frequent inclusion as one of several ingredients in formulations is primarily due to their tendency to possess an acidic to neutral pH (Brand-Williams et al., 1995).

The well-known therapeutic benefits of medicinal plants have been gathered as significant data in the field of ethnomedicine. However, this data can serve as a foundational starting point for the utilization of medicinal plant species. However, it is crucial that their application as alternative medicine be grounded in scientific assessment through chemical, biological, and pharmacological assessments (Andritoiu et al., 2020; Salmeron-Manzano et al., 2020). Tunisia boasts a rich variety of approximately 2,000 medicinal and aromatic plants species, offering vast potential as natural ingredients, hydrosols, essential oils and aromas. Notably, 90 percent of the overall production comes from wild species. Among these, key wild plants in terms of surface area include rosemary, lentisque, thyme and myrtle (Weber et al., 2019). Rosemary, scientifically known as *Rosmarinus officinalis* L., is widely growing in several areas in Tunisia and mainly in Seliana and Kef occupying around 39000 hectars (Technical sheet, 2016). The plant is a shrub that belongs to the Lamiaceae family. The array of biological properties exhibited by rosemary has positioned it as a promising candidate for therapeutic applications in the treatment of various diseases. It is renowned for its remarkable properties, including antimicrobial, antidiabetic, anticarcinogenic, antithrombotic, diuretic and antinociceptive effects (Haloui et al., 2000; Yamamoto et al., 2005; Rafie et al., 2017). Therefore, the aim of this paper was to assess the phenolic composition and to analyze the antioxidant potential of hydrosol of *Rosmarinus officinalis* growing in Metouia oasis. Furthermore, the enzymatic activities including polyphenol oxidase, tyrosinase and lipoxygenase were investigated.

2. MATERIALS AND METHODS

2.1. Plant material and extraction

Fresh *Rosmarinus officinalis* was collected in March from Metouia oasis. Located in the southeastern part of Tunisia, near the coast of Gabes City, the Metouia oasis is characterized by an arid climate with infrequent and unpredictable precipitation occurrences. The region was identified as plant agro-biodiversity where the plants are arranged in tiers, corresponding respectively to palm trees and pomegranate trees on the first and second levels, and vegetables and medicinal plants on the third level.

Aerial parts of rosemary were harvested from flowering and vigorous plants. The species underwent water-steam distillation utilizing a semi-industrial processing line of a Clevenger-type apparatus. The distillation process parameters were set as follows: the raw material consisted of 2 kg, the hydro module ratio was 1:6, and the duration of the distillation process was 60 minutes. Throughout the distillation, the distillate temperature was carefully maintained within the range of 25-30°C (Georgieva et al., 2019). The plant material was subjected to three replicates (3 × 2 kg) during the extraction process. The essential oil obtained during the distillation process was carefully extracted from the top of the hydrosol using a glass syringe. Hydrosol was collected and it was then stored at 4°C until analysis during the same week.

2.2. Determination of pH

The pH of the hydrosol at room temperature (25°C) was measured using a precisely calibrated pH meter (SCHOTT Instruments; SI Analytics Mainz, Mainz, Germany).

2.3. Total phenolic and flavonoids content

The total phenolic content of hydrosols was determined using the Folin–Ciocalteu method (Singleton and Rossi, 1995). The spectral absorbance reading was conducted at 765 nm. The results are expressed as micrograms of gallic acid equivalent per milliliter of hydrosol. To quantify the total flavonoids content in the extract, a spectrophotometric method was employed (Lamaison and Carnat, 1991). It was measured, at 430 nm, in micrograms per milliliter of hydrosol and expressed as quercetin equivalents.

2.4. Antioxidant activity

The antioxidant activity of the sample was assessed using a spectrophotometric method that involved the utilization of the synthetic radical DPPH (2,2-diphenyl-1-picrylhydrazyl). The spectral absorbance at 518 nm was promptly recorded (Brand-Williams et al., 1995). Results are presented as the percentage of DPPH radical inhibition using the following formula:

$$\% \text{ inhibition} = ((A0-As)/A0)*100$$

with:

A0 corresponded to the absorbance of DPPH solution without tested sample

As corresponded to the absorbance of DPPH solution with tested sample

2.5. Polyphenol oxidase assay

Polyphenol oxidase was assessed at a wavelength of 475 nm, utilizing L-tyrosine as a monophenolic substrate. The steady-state rate of enzyme activity was determined by analyzing the linear range of the product accumulation curve after the lag period (Pérez-Gilabert et al., 2001). The protein content of the *Rosmarinus* hydrosol was quantified using the Lowry method (Lowry et al., 1951).

2.6. Tyrosinase assay

The activity of tyrosinase was assessed at a temperature of 30°C using a colorimetric assay at a wavelength of 457 nm. A substrate solution was freshly daily by dissolving 10 mM L-DOPA in phosphate buffer (0.1 M, pH 7.0). Absorbance was measured every 30 seconds over a 5-minute duration (Lerch and Ettlinger, 1972).

2.7. Lipoxygenase assay

The lipoxygenase activity was assessed using a borate buffer (0.2 M, pH 9.00) and employing linoleic acid (21.53 x 10-4 M) as a substrate. Peroxides formation was monitored over a period of 4 minutes by measuring the increase in absorbance at 234 nm. The lipoxygenase (Lox) activity was quantified as the absorbance, measured at 234 nm, per minute with 1 ml of protein present in the *Rosmarinus* hydrosol (Axelrod et al., 1981).

2.8. Statistical analysis

Independent samples of each item were analyzed in triplicate. The data is represented as mean ± standard deviation (S.D.). Statistical analyses of the data were performed using the SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Phytochemical composition and antioxidant analysis

The examined *Rosmarinus officinalis* aromatic water showed an acid pH of 4.9 (Table 1). According to previous reports, the pH value of hydrosols was found to be influenced by both the species from which they were extracted and the chemical composition of the volatile fraction present in the hydrolat. Hydrosols containing functional groups capable of releasing protons, such as acids and phenols, exhibited lower pH levels (Rao, 2012; El Bouny et al., 2021). However, the optimal pH range for normal facial skin is typically between 4.7 and 5.6. Consequently, it is crucial for hydrosols to maintain a pH level within this range to avoid potential negative effects and skin problems that may arise from excessively high or low pH levels. The acidic properties of aromatic waters can be harnessed to create natural skin care products. They are searched for natural skincare routines due to their ability to replenish and restore the acid mantle of the skin after cleansing with soap (Tarun et al., 2014).

The total phenolic contents (TPC) of the hydrosol amounted to 55.76 µg GAE/ml (Table 1) when measured against the gallic acid standard curve. It was higher than values obtained with aromatic waters of chamomile (9.33 mg/L) and Damascus rose (32.54 mg/L) (Jakubczyk et al., 2021). The TPC of ultrasound-assisted extracted *Rosmarinus officinalis* exhibited ranged from 55 to 85 mg GAE/g (Dhouibi et al., 2023). A low level of phenols is attributed due to their low water solubility (Georgieva et al., 2019). Total flavonoid contents (TFC) ranged 14.24 µg QE /ml. It was recorded that the flowers exhibited the greatest concentrations of flavonoids followed by leaves (Jakubczyk et al., 2021). These parameters may be affected by the plant origin, the extracted part and the conservation method of the hydrosol. Indeed, abiotic and biotic parameters, along with genetic factors, exert significant influence on the biosynthesis and accumulation of polyphenols compounds (Kumar et al., 2018).

Phenolic compounds possess numerous biological properties, including their notable ability to enhance antioxidant activity. This is attributed to the presence of hydroxyl groups, which can act as hydrogen donors (Pourreza, 2013; Djabou et al., 2014). Experimental evidence revealed variations in the antioxidant properties of plant hydrosols. *Rosmarinus officinalis* showed a DPPH activity of 36.08%. The correlation between the total phenolic content and antioxidant capacity suggests the involvement of additional mechanisms or the presence of specific concealed bioactive compounds (Lahmar et al., 2022; Lahmar et al., 2023). Phenolic compounds possess a chemical

Table 1. Biochemical composition and antioxidant potential (DPPH) of *Rosmarinus officinalis* hydrosol.

	pH	TPC (µg/ml)	TFC (µg/ml)	DPPH (%)
Plant hydrosol	4.9±0.86^{a}	55.76±0.57^{ab}	14.24±0.91^{a}	36.08±1.3^{b}

TPC: Total phenolic content; TFC: Total flavonoids content.

structure that enables them to neutralize free radicals, break down peroxides and scavenge singlet and triplet oxygen (Skotti el al., 2014). It is possible that the primary mechanism revolved around the interaction between highly reactive peroxyl radicals and antioxidants, leading to the formation of cross-reactions among radicals (Rossetto et al., 2002).

3.2. Enzymes activities

The profiles of the measured optical densities varied across different enzymes (Fig. 1). Moreover, activities expressed in unit per mg of protein revealed on the hydrosol are different (Fig. 2).

In order to safeguard cells against the detrimental effects of excessive reactive oxygen species, plants have developed enzymatic antioxidant defense systems, including polyphenol oxidase (Hasanuzzaman et al., 2017).

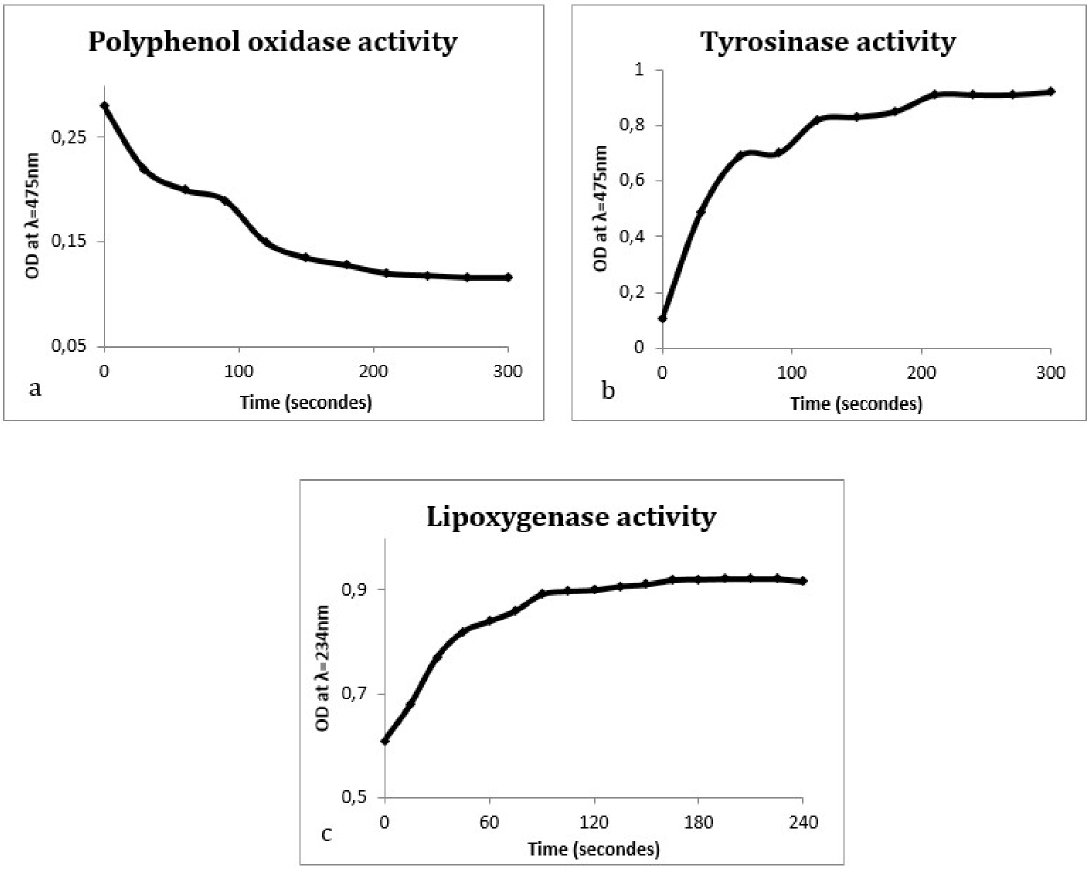

Fig. 1. Profiles of the optical densities measured for (a) polyphenol oxidase, (b) tyrosinase and (c) lipoxygenase on the hydrosol of *Rosmarinus officinalis*.

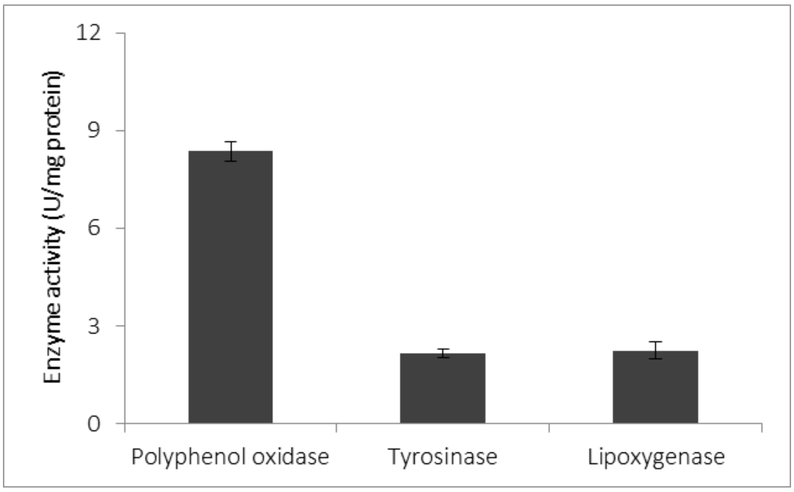

Fig. 2. Enzymes activities measured for polyphenol oxidase, tyrosinase and lipoxygenase on the hydrosol of *Rosmarinus officinalis.* The values are the mean of three determinations ± standard error.

Polyphenol oxidase is an enzyme that facilitates the hydroxylation of monophenols, converting them into ortho-diphenols. Additionally, the enzyme catalyzes the oxidation of o-diphenols, resulting in the formation of quinones. These quinones spontaneously polymerize, giving rise to dark-colored phytomelanins. The activation of the enzyme exclusively takes place when the plastids are disrupted following tissue wounding (Tomás-Barberán and Espín, 2001). The screening of polyphenol oxidase in the studied hydrolat showed an enzyme activity of 8.37 U/mg protein. This result was higher than what is was found with the African Mango extracted for 3 min in the presence of ascorbic acid showing a specific activity of 4.1 U/mg protein (Sanni, 2016). The field been presented an enzyme activity accounted of 2.01 U/mg protein (Paul and Gowda, 2000). The activities are influenced by numerous factors, which encompass the pH of the extraction mixture, extraction temperature, buffer type, mass to solvent ratio, extraction time and the additives employed. They depended also from the extracted species, plant organ, geographical habitat and period of collect. Polyphenol oxidase was implied as phenol removal in extracts of rejected white *Pleurotus ostreatus*. It can be also utilized for phenolic compound removal in bioremediation efforts and in applications within the food and drug industries (Murniati et al., 2018).

Tyrosinase is considered as a pivotal enzyme involved in melanogenesis. It facilitates the initial two stages of melanin biosynthesis. These phases encompass the ortho-hydroxylation of L-tyrosine and the oxidation of L-DOPA (Fan et al., 2021). The hydrosol obtained from the water-steam distillation of *Rosmarinus offcinalis* presented a tyrosinase activity of 2.16 U/mg protein. Tyrosinase is categorized as a polyphenol oxidase and plays a role in regulating the redox potential within plants (Walker and Ferrar, 1998). This enzyme possesses numerous biotechnological and industrial applications. It is used for treating wastewater that is contaminated with dyes and phenols (Girelli et al., 2006). It facilitated also the protein cross-linking in food technology as well the synthesis and bioconversion of o-diphenol drugs (Halaouli et al., 2006). Manufacturing L-DOPA using tyrosinase in batch reactors was proved (Pialis et al., 1998). In addition, the enzyme was applied in vitro for the conjugation of the protein gelatin to the polysaccharide chitosan (Chen el al., 2002).

Within plant tissues, the primary substrates for lipoxygenase enzyme are linoleic and linolenic acids, both of which contain methylene-interrupted cis and cis-pentadiene structures (Baysal and Demirdöven, 2007). Indeed, the studied aromatic water showed an enzyme activity amounted to 2.24 U/mg protein. A previous study reported an activity of 1.01 U/mg protein of a crude extract of *Ecballium elaterium* (Lahmar and Lubov, 2016). Lipoxygenase is responsible for catalyzing oxygenation at various positions along the carbon chain, a phenomenon known as "positional" specificity. This specificity holds great significance in the metabolic pathways leading to the formation of several vital secondary metabolites from the resulting hydroperoxides (Veldink et al., 1998). Extensive documentation supports the utilization of lipoxygenase active soy flour for fortifying wheat flour as strengthening and bleaching agent (Xu et al., 2014; Hayward et al., 2016). In the food and beverage industries, this enzyme has gained widespread usage as a biocatalyst for producing aroma compounds in abundance and at a cost-effective rate (Gigot et al., 2010). Leading to the production of diverse arachidonic acid-like substances, lipoxygenase holds significant importance in cancer prevention and treatment (Lee et al., 2011).

4. CONCLUSION

The findings derived from this study reveal the captivating phytochemical and antioxidant capacity exhibited by *Rosmarinus officinalis* hydrosol. These results serve as a compelling impetus to delve deeper into the realm of the examined hydrosol, with the aim of developing secure and eco-friendly bioproducts. Additionally, this research introduces a novel natural biological material that warrants exploration as a potential source of polyphenol oxidase, tyrosinase, and lipoxygenase.

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