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RESEARCH ARTICLE

Syzygium aromaticum L.: phytochemical investigation and comparison of the scavenging activity of essential oil, extracts and eugenol

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Abstract
Syzygium aromaticum L. is a popular flavor and fragrance spice used in cooking, traditional and modern therapy. No work has compared between the scavenging capacity of the essential oil, extracts and eugenol, or studied that of the hexane extract, as well as its phenolic and flavonoid contents. To reveal more details on phytochemical and scavenging characteristics of *Syzygium aromaticum* spice, we estimated the scavenging activity of the essential oil, each extract of water, ethanol, ethyl acetate and hexane obtained from clove buds, based on their effective concentration able to scavenge 50% (EC_{50}) of 2,2′-diphenyl-1-picrylhydrazyl, and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt free radicals. The essential oil showed high scavenging activity (EC_{50} of 0.40 ± 0.06 and 0.42 ± 0.02 mg/ml), and comprises 13 identified compounds. Eugenol, β-Caryophyllene, eugenyl acetate and α-Humulene are the major components. Comparing extracts, those of ethanol and hexane exhibited the greatest phenolic content (351.83 ± 17.90 and 348.04 ± 24.54 mg of gallic acid equivalent/$g_{extract}$ respectively) and the potent scavenging activities (EC_{50} of 0.41 ± 0.03 and 0.37 ± 0.00 mg/ml respectively). A moderate flavonoid content was observed, and aqueous extract was the best (21.90 ± 0.16 mg of quercetin equivalent/$g_{extract}$). According to thin layer chromatography screening, all extracts content eugenol except that of water, this phenol contributes mainly on the scavenging activity and seems to be more active in mixture with terpenes, like in essential oil.

Keywords Scavenging activity · *Syzygium aromaticum* · Essential oil · Polyphenols content · Eugenol · DPPH and ABTS radicals

Introduction

Free radicals appear as by-products in many of key biochemical reactions and play essential roles in living systems: reactive oxygen species include superoxide anion $O_2^{\bullet -}$ and hydroxyl radical $^{\bullet}OH$, function as signaling molecules and bactericides (Halliwell and Gutteridge 2015). Due to their potent chemical reactivity, when presented in abnormal concentration, free radicals act dramatically to cell structure through deteriorating indispensable macromolecules: lipids; proteins; nucleic acids and carbohydrates (Pacifici and Davies 1991). Among other deleterious processes, they initiate lipid peroxidation after snatching a hydrogen atom from an unsaturated fatty acid (Mead 1976). Consequently, free radical toxicity may contribute to ageing (Stadtman and Berlett 1997) and various diseases as cancer (Totter 1980).

To prevent biological systems from radical damage, the demand continues to grow on natural antioxidants, able to interact with free radicals and interrupt the degenerative reaction chains. Instead of synthetic antioxidants with side effects such as, butylated hydroxytoluene and butylated hydroxyanisole, natural antioxidants scavenge safely the free radicals and detoxify the human body. Many phytochemicals act differently as antioxidants via donating hydrogen atom or electron to reduce free radical, quenching singlet oxygen and inhibiting enzymes or chelating metals involved in free radical production. For example, phenolic acids are better free radical scavengers than metals chelating, while flavonoids

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are also good for both (Gramza-Michalowska et al. 2019; Brewer 2011).

Cloves (*Syzygium aromaticum* (L.) Merr. and L.M. Perry, Myrtaceae) are a popular flavor and fragrance spice used in traditional and modern therapy, and highly appreciated in cooking, represent one of the strong antioxidant and radical scavenger foods (Dudonné et al. 2009 ; Pérez-Rosés et al. 2016; Shan et al. 2005), with antibacterial (Bachmann 1916), preserving (Hoffmann and Evans 1911), antifungal and antiaflatoxigenic (Hitokoto et al. 1980; Mabrouk and El-Shayeb 1980) properties. Cloves are rich in phenols like eugenol—the major compound—and phenolic acids like gallic acid and its derivatives. Due to its phenolic structure, eugenol possesses a marked antioxidant activity (Kramer 1985). Several works have estimated the EC_{50} for essential oil of clove buds using the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and/or 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assays, but concerning the extracts they are scarce. Only the EC_{50} of the supercritical extract has been reported (Ivanovic et al. 2013). No work has compared between the scavenging capacity of the essential oil, extracts and eugenol on the DPPH and ABTS free radicals, or studied that of the hexane extract, as well as its phenolic and flavonoid contents.

Our aim was to reveal more details on phytochemical and scavenging characteristics of *Syzygium aromaticum* spice, and explore promising antioxidants sources, which can be benefit in food and pharmaceutical industry, through the investigation of chemical composition of the essential oil, phenolic and flavonoid contents of four extracts, and their scavenging activity together with pure eugenol, Trolox and ascorbic acid standards, using DPPH and ABTS free radicals scavenging assays.

Materials and methods

Chemicals and instruments

2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (Germany), 2,2′- azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (98%, ABTS) from Sigma-Aldrich (USA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%, Trolox) from Sigma-Aldrich (China). Folin-Ciocalteu reagent, sodium carbonate, aluminium chloride and ascorbic acid (99%) were obtained from Solvapur (Casablanca, Morocco). Eugenol (99%) was purchased from Alfa Aesar (Germany), and the polyphenol standards were from Sigma-Aldrich (Germany). All other chemicals and solvents were of analytical grade. The oil analysis was performed by GC–MS unit, consisted on a Shimadzu GC-2010 gas chromatograph with BP-5 capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm; SGE, Ltd.), and coupled to Shimadzu QP2010 Plus mass spectrometer (software version 2.50 SU1). Absorbance measurements were done using a UV-6300PC double beam spectrophotometer (VWR, China). Thin layer chromatography (TLC) was performed on Merck aluminium plate with silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany).

Clove material and extraction

The clove buds were purchased from local spice market (Casablanca; Morocco), and were stored in clear poly bags. Essential oil was obtained by hydrodistillation method. 150 g of dried clove materials were ground into fine powder and placed with 600 mL distilled water in 1 L round-bottom flask. The mixture was boiled for 4 h using Clevenger-type apparatus according to the European Pharmacopoeia (Council of Europe 2007). The extract was condensed in cooling vapor to form an azeotropic oil–water mixture. The essential oil was extracted by diethyl ether, dried over anhydrous sodium sulphate, and then kept at 4 °C, until used for the analysis. The yield (w/w) of the essential oil was expressed as a percentage of absolute dry weight of clove buds.

Extracts were prepared by maceration process using four solvents with varying polarity (water, absolute ethanol, ethyl acetate and n-hexane). 100 g of dried clove materials were ground into fine powder and were placed with 1L of each corresponding solvent in 2L flask. The mixture was stored in dark at room temperature, 1 day for water and 3 days for others, then the extract was filtered, concentrated in rotary evaporator and stored at 4 °C. The residue was treated twice in the same manner. The yield (w/w) of the collected extract was expressed as a percentage of absolute dry weight of clove buds.

GC–MS analysis of essential oil

The composition of essential oil was carried out by GC–MS. The oven temperature was programmed as described for GC analysis; transfer line temperature, 300 °C; ion source temperature, 200 °C; carrier gas, helium, adjusted to a linear velocity of 36.5 cm s^{-1}; split ratio, 1:40; ionization energy, 70 eV; scan range, 40,400 u; scan time, 1 s. Chemical constituents were performed on their retention time (RT) on the BP-5 capillary column, compared with those published in the literature (Adams 2007), and confirmed by comparing their mass spectra with a data bank (Shimadzu corporation library and NIST 05 database/ChemStation data system).

Total phenol content

The total phenol content (TPC) was determined according to the Folin-Ciocalteu colorimetric method (Singleton and Rossi 1965). To the mixture of 0.5 mL of sample (1 mg/mL),

and 2.5 mL of Folin-Ciocalteu's reagent (10%) was added 2 mL of sodium carbonate solution (7.5%). The mixture was well mixed, and incubated for 30 min at room temperature, the absorbance was measured at 765 nm. Gallic acid was used as standard for calibration curve. The TPC values were expressed in mg gallic acid equivalents per gram of dry weight of extract (mg GAE/$g_{extract}$).

Total flavonoid content

The total flavonoid content (TFC) was carried out as described by Ahn et al. (2007). To 1.5 mL of sample (1 mg/mL), was added 1.5 mL of $AlCl_3$-ethanol solution (2%). After vigorous stirring and incubation for 10 min at room temperature, the absorbance was measured at 420 nm. Quercetin was used as a standard for calibration curve. The TFC values were expressed in mg quercetin equivalents per gram of dry weight of extract (mg QuE/$g_{extract}$).

TLC chemical screening of extracts

The screening of polyphenols in the extracts was performed with the TLC technique. Different eluents were used to separate compounds: n-hexane 9/ethyl acetate 1 (v/v), chloroform 5/methanol 5 (v/v), and chloroform 9/methanol 1 (v/v), spots were detected under UV light and visualized by spraying the TLC plates with $FeCl_3$-methanol solution. The compounds in the extracts were identified by comparing their retention factor (R_f) with those of standards: eugenol, quercetin, catechin, caffeic acid, and syringic. Eugenol spot become visible after heating the plate at 100 °C for 5 min.

DPPH scavenging method

The DPPH free radical scavenging assay was estimated according to the method of Brand-Williams et al. (1995). 50 µL of sample at different concentrations was mixed with 1.95 mL of a fresh ethanolic solution of DPPH (60 mM) and incubated for 30 min at room temperature. The absorbance measurements (A_1) and (A_0) were read at 517 nm in the presence and absence of sample respectively. Ascorbic acid was the positive control. The EC_{50} was calculated from the plotted curve corresponding to the percentage scavenging of DPPH against the concentration of the sample.

ABTS scavenging method

The ABTS radical scavenging was determined using the procedure described by Dorman and Hiltunen (2004). 10 mL of ABTS (7 mM) was added to 10 mL of potassium persulphate aqueous solution (2.45 mM) and incubated for 16 h. The absorbance of stable ABTS solution was adjusted between 0.7 and 0.734 at 734 nm by diluting with ethanol. 20 µL of sample at different concentrations was mixed with 1.48 mL of freshly prepared solution of ABTS. The absorbance in the presence of sample (A_1) was read at 734 nm, after incubation for 30 min at room temperature, and that in the absence of sample (A_0) was read immediately. Trolox was the positive control. The EC_{50} was calculated from the plotted curve corresponding to the percentage scavenging of ABTS against the concentrations of sample.

Statistical analyses

All measurements were performed in triplicate and calculated as mean ± standard deviation (SD) using Microsoft Excel Software 2016. OriginPro 8 software was used to evaluate the significant difference and to draw the curves. One-way analysis of variance (ANOVA) followed by Tukey's test with a probability value of $P = 0.05$.

Results and discussion

Essential oil composition

The essential oil yield isolated from clove buds in a Clevenger-type apparatus was 8.58%. The identified compounds are summarized in Table 1, considering their order of elution on a BP-5 column. A total of 13 chemical ingredients recovers about 99.33% of the total essential oil composition. Eugenol, β-Caryophyllene, eugenyl acetate and α-Humulene are the most abundant.

Table 1 Chemical composition of clove bud essential oil

Component[a]	RT[b]	RT[c]	%
Eugenol	23.125	22.70	55.28
α-Copaene	23.806	23.49	0.58
Isocaryophyllene	25.157	24.95	tr
β-Caryophyllene	25.733	25.36	27.46
α-Humulene	27.165	26.82	3.64
γ-Muurolene	28.125	27.91	0.16
Germacrene D	28.324	28.15	0.05
β-Selinene	28.553	28.37	tr
Valencene	28.916	28.66	0.05
α-Farnesene	29.402	29.03	0.41
γ-cadinene	29.706	29.35	0.07
Eugenol acetate	30.253	29.70	11.48
Caryophyllene oxide	32.546	32.16	0.15

tr trace (< 0.05%)

[a]Order of elution on BP-5 capillary column

[b]Retention time on BP-5 capillary column

[c]etention time on DB-5 capillary column (Adams 2007)

Eugenol preponderates the essential oil composition of clove buds, with different proportions: Chaieb et al. (2007); Viuda-Martos et al. (2007); Srivastava et al. (2005); Lu et al. (2011); Sgorbini et al. (2015) exhibited high eugenol proportions of 88.58, 85.5, 82.6–70.0, 80.34, and 80.3% respectively. Whereas, some authors showed less high such as Omidbeygi et al. (2007); Hossain et al. (2014) with 63.37, and 63.64–57.17–54.97 respectively. Unlike previous studies, where acetate eugenyl was the second abundant compound (Chaieb et al. 2007; Fayemiwo et al. 2014; Sgorbini et al. 2015), in our case, it is β-Caryophyllene, which agrees with other ones (Lu et al. 2011; Omidbeygi et al. 2007; Srivastava et al. 2005; Viuda-Martos et al. 2007).

Many factors can be responsible to this variance in component ratios of clove essential oil, among others, genetic, distinct races, geographic origin, geology, climatic, rainfall, season, and extraction method chosen (Preedy 2016).

Phytochemical analysis of extracts

Interestingly, n-hexane solvent was efficient in term of phenols extraction; the lipid extract -seemed brown viscous oil, and smelled clove volatile oil- contains a great TPC amount, close to that of ethanol extract. The latter is the richest in phenols compared with published data; Gülçin et al. (2004) and El-Maati et al. (2016) found 264.9, and 293 mg GAE/$g_{extract}$ respectively. Ethyl acetate extract scores an important TPC value than that of reported study 58.8 mg GAE/$g_{extract}$ (El-Maati et al. 2016). A fewer value of aqueous extract against higher reported ones 179.8, and 230 mg GAE/$g_{extract}$ (El-Maati et al. 2016; Gülçin et al. 2004). Expect that of water, the other extracts have weak TFC values; water was the best extracting solvent for flavonoids, and characterizes higher TFC value compared with 17.5, 12, and 4.7 mg QuE/$g_{extract}$, extracted respectively by water, ethanol and ethyl acetate solvent (El-Maati et al. 2016) (Table 2).

Among little works on cloves polyphenolic content, none was interested by hexane extract. N-hexane is an excellent solvent-soluble oil (Wang and Weller 2006). The analysis of the lipidic extract on TLC plate revealed the presence of eugenol at $R_f = 0.42$, the same for extract from ethyl acetate and ethanol, except for aqueous extract. The spot color of eugenol was blue after spraying with $FeCl_3$. Spots of quercetin, catechin, caffeic acid, and syringic appeared brown to dark, and were not visualized in the developed extracts according to TLC screening. Probably the dominantly presence of the identified phenol can explain our finding on phenolic content, also increase the scavenging activity of the corresponding extracts.

Scavenging activity

All samples tested in radical scavenging reactions showed their capacity to neutralize DPPH and ABTS radicals resulting into decolorization and absorbance diminution of their solutions. Normally, the antioxidant activity depends widely to the phenolic and flavonoid compounds which, donate a hydrogen atom in the case of DPPH radical or transfer an electron in the case of ABTS radical (Mishra et al. 2012). The scavenging activity decreases in the following order for the DPPH assay; Ascorbic acid > eugenol–essential oil > ethanol extract > hexane extract > ethyl acetate extract > aqueous extract, and for the ABTS assay it decreases as follows; eugenol > Trolox > hexane extract > essential oil > ethanol extract > ethyl acetate extract > aqueous extract (Table 3).

This activity is inverse of EC_{50} value, the lower EC_{50} value means the stronger activity. Therefore, comparing extracts, that of hexane is the most powerful scavenger on ABTS assay, however on DPPH assay it is ethanol extract.

Table 3 Activity of essential oil, extracts and eugenol on the DPPH and ABTS assays

	DPPH-EC_{50} (mg/ml)	ABTS-EC_{50} (mg/ml)
Water extract	2.26 ± 0.04^{a}	3.10 ± 0.02^{a}
Ethanol extract	0.41 ± 0.03^{b}	0.43 ± 0.01^{b}
Ethyl acetate extract	0.68 ± 0.02^{c}	0.71 ± 0.08^{c}
N-hexane extract	0.57 ± 0.10^{bc}	0.37 ± 0.00^{be}
Essential oil	0.40 ± 0.06^{d}	0.42 ± 0.02^{b}
Eugenol	0.40 ± 0.01^{bd}	0.25 ± 0.02^{d}
Ascorbic acid	0.08 ± 0.00^{e}	nd
Trolox	nd	0.27 ± 0.00^{de}

Mean values marked with the same letter (a, b, c, d or e) within column are not significantly different (p = 0.05)

nd not determined

Table 2 Yield and total phenolic and flavonoid contents of clove bud extracts

Extracting solvent	Yield (%)	TPC (mg GAE/$g_{extract}$)	TFC (mg QuE/$g_{extract}$)
Water	4.0	45.57 ± 2.95^{a}	21.90 ± 0.16^{a}
Ethanol	14.7	351.83 ± 17.90^{b}	2.28 ± 0.01^{b}
Ethyl acetate	13.9	269.28 ± 13.27^{c}	0.88 ± 0.01^{c}
N-hexane	10.5	348.04 ± 24.54^{b}	0.24 ± 0.00^{d}

Mean values marked with the same letter (a, b, c or d) within column are not significantly different (p = 0.05)

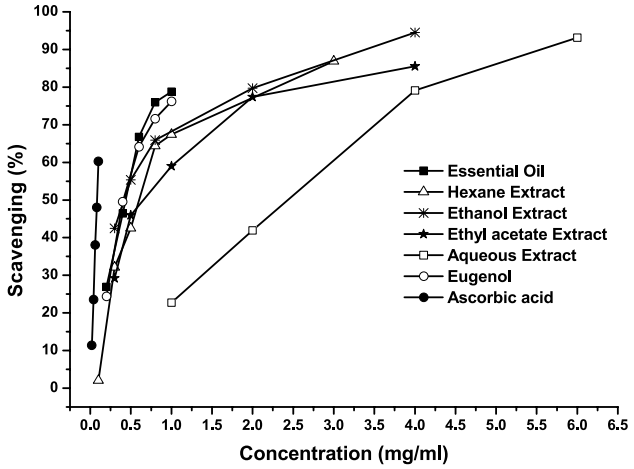

Fig. 1 Scavenging activity on the DPPH

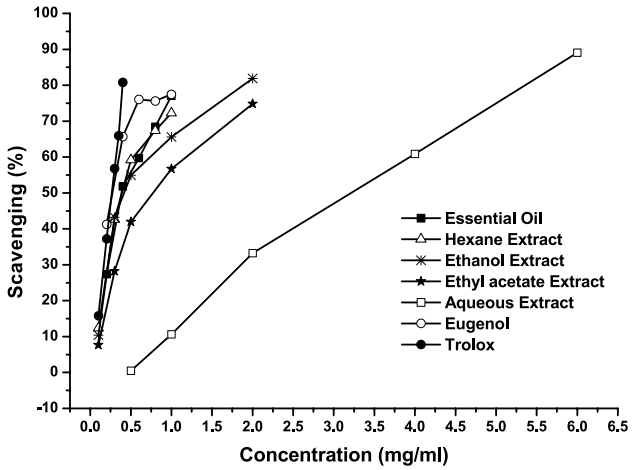

Fig. 2 Scavenging activity on the ABTS

Brito et al. (2017) recommended for food preparation and more antioxidant benefits, to use lipid and/or alcoholic fractions of clove, supporting our finding. While the aqueous extract is the least potent scavenger on the both assays despite its high TFC value. The EC_{50} of eugenol, the strongest scavenger sample, is slightly less than that of Trolox standard, but broadly higher than that of ascorbic acid standard. In the case of DPPH assay, to scavenge 50%, essential oil shows a strong activity; similar to the pure eugenol and higher than the extracts. But at an effective concentration of 0.8 mg/ml, essential oil advances pure eugenol, alcoholic and lipidic extracts to reach a scavenging of 75.98% against 71.62, 65.91 and 64.4% respectively (Fig. 1). In ABTS assay, at an effective concentration of 1 mg/ml, essential oil alongside pure eugenol with a scavenging power of 77.04% against 77.49%, and better than lipidic and alcoholic extracts with 72.23 and 65.61% respectively (Fig. 2). The essential oil activity may be attributed mainly to its one and major phenolic compound, eugenol, which seems to be more active in mixture with terpenes compared to pure eugenol. Extracts with high scavenging capacities are characterized by high levels of phenolic groups including eugenol, and poor concentration of flavonoids. The results for the extracts of hexane, ethanol and ethyl acetate, confirm these findings.

Conclusion

The present study demonstrates that ethanol, and hexane are the best solvents to extract phenols from clove buds. Their extracts, in addition to that from ethyl acetate and to the essential oil give important scavenging activities and seem to be good sources for food preparation and antioxidant benefits. The scavenging power of essential oil is remarkable in comparison with those of extracts and pure eugenol. Due to the poor concentration of flavonoids in the extracts, their activities may be attributed to the phenolic groups, including eugenol. The later contributes mainly to the essential oil activity, where is more effective than pure eugenol. This knowledge could enrich the scientific data on the chemical properties of spices, and provide a relevant information to food and pharmaceutical industry.

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Compliance with ethical standards

Ethical statements This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest Yassine El Ghallab has no conflict of interest. Abdellah Al Jahid has no conflict of interest. Jamal Jamal Eddine has no conflict of interest. Amal Ait Haj Said has no conflict of interest. Lhoussaine Zarayby has no conflict of interest. Sanae Derfoufi has no conflict of interest.

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