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ONE STEP PURIFICATION OF 6-SHOGAOL FROM *ZINGIBER OFFICINALE ROSCO*, A PHENOLIC COMPOUND HAVING A HIGH EFFECTIVENESS AGAINST BACTERIAL STRAINS

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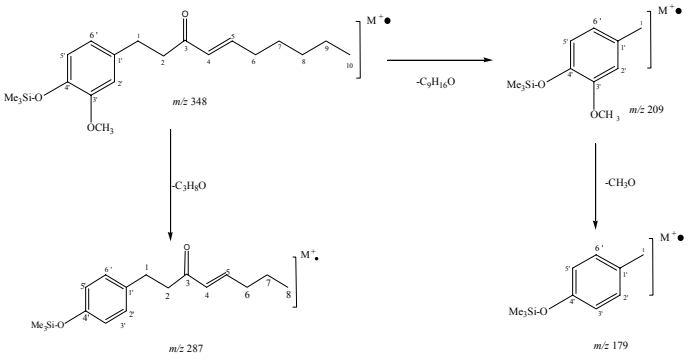
For the first time, one step purification of 6-shogaol (N) from rhizome of *Zingiber officinale Rosco* methanol (20 %) extract was carried out. In the present study, a solid phase extraction procedure was optimized for the extraction of 6-shogaol which was eluted from a silica cartridge with 150 mL of *n*-hexane-diethyl ether (70/30, v/v). The structure elucidation of compound N was based on the spectral evidence UV, MS, ^{1}H-NMR and ^{13}C-NMR. The antioxidant as well as antimicrobial properties of the pure compound and root extracts were investigated by different methods. It exhibited an antimicrobial activity against both tested Gram-positive and Gram-negative bacteria with MICs values ranging from 0.312 to 2.5 mg/mL. The results of the present study can be proved useful in the purification of 6-shogaol to produce a potentially bioactive compound.



INTRODUCTION

Ginger derived from the rhizome of *Zingiber officinale Rosco* is a common condiment for a variety of compounded foods and beverages. It is cultivated in many tropical and subtropical countries including China, India, Nigeria, Australia, Jamaica and Haiti.[1-3] Many reports have mentioned that Ginger rhizome contains rich source of pungent bioactive compounds. These substances, which are phenolic ketones include the gingerols as well as the shogaols with a range of unbranched alkyl chain lengths.[2.4.5] As it has been reported, *Zingiber officinale* has been proved to have several pharmacologic activities and anti-inflammatory activities.[6] Besides, Ali and co-workers[7] proved that ginger has anti-microbial activities against various bacteria, fungi and nematodes. In addition, Ginger has been proved to be effective on various viruses.

The purpose of this study was to fractionate the phenolic extracts by solid-phase extraction (SPE), in order to obtain pure compound with a high yield and to evaluate its antioxidant activities with regard to the free radical 2,2-diphenyl-1-picryl hydrazyl (DPPH), as well as Free Reducing

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Antioxidant Power (FRAP). The structure of the pure compound was elucidated using 1D-(^{1}H and ^{13}C), GC-MS and UV–vis analysis.

MATERIALS AND METHODS

Plant materials and sample preparation

The ginger powder (*Zingiber officinale* - India) was purchased from a local market in Sfax-Tunisia. Two extractions were monitored.

Extraction 1. About 20 g of fine powder in 200 mL of *n*-hexane were left under agitation for 24 h at room temperature to remove the lipophilic compounds. Then, the solid residue was suspended in a 200 mL of ethyl acetate followed by methanol.

Extraction 2. About 20 g of fine powder was suspended in a methanol–water mixture, 20/80 (v/v), at room temperature for 24 h under constant stirring.

Each resulting extract was filtered, the solvent was completely removed using a rotary evaporator, and the extract was stored at + 4 °C until further use. Before testing, each extract was freshly reconstituted in the appropriate solvent at a final concentration of 400 mg/mL which was used further to prepare serial dilutions from 400 mg/mL to 50 mg/mL.

Total phenolic content

The amount of total polyphenols was determined using the Folin–Ciocalteu's method according to the methods described previously by Rigane *et al*.[8,9] Total phenol content was expressed as mg gallic acid per gram of methanolic extract (mg GA/g extract). All samples were analyzed in triplicate.

Total flavonoid content

Total flavonoids were measured colorimetrically according to Rigane *et al.*[8,9] Total flavonoid content was expressed as mg quercetin per gram of extract (mg QE/g extract). All samples were analyzed in triplicate.

GC-MS analysis

GC-MS analysis was performed with a HP model 5975B inert MSD, equipped with a capillary HP5MS column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness). The carrier gas was He used at 1 mL min^{-1} flow rate. The oven temperature program was as follows, 1 min at 100 °C, from 100 to 260 °C at 4 °C min^{-1} and 10 min at 260 °C. For the silylation procedure, a mixture of pyridine (20 μL) and BSTFA (100 μL) were added and vortexed in screw cap glass tubes and consecutively placed in a water bath at 80 °C for 45 min. From the silylated mixture 1 μL was directly analyzed by GC-MS.

NMR and UV analysis

^{1}H NMR and ^{13}C NMR were recorded by a Bruker AV 400 (400 MHz) spectrometer with $CDCl_3$ as solvent. Chemical shift (δ) are reported in parts per million (ppm) using the solvent peak. Tetramethylsilane (TMS) was used as an internal standard. UV data were measured on a Shimadzu UV-1800 spectrometer.

Solid phase extraction

The optimized procedure was as follows: The silica cartridge (Silica Gel 60, 0.06–0.2 mm, 70–230 mesh ASTM, and 300 mm x 20 mm) was conditioned with 150 mL of *n*-hexane-diethyl ether 70/ 30 before the application of two grams of aqueous methanol extract. The collected fractions were evaporated under reduced pressure at room temperature to afford the marker compound as pale yellow oil (30 mg). The dry residues were dissolved in the appropriate solvent for GC-MS analysis.

Chemical characterization

Pale yellow oil: UV (MeOH) λ_{max}: 226 and 283 nm (Figure not shown); ^{1}H and ^{13}C NMR data (See Table 2); MS data (sylilated compound) *m/z*: 348.

Antioxidant activities

DPPH radical scavenging activity: The free radical-scavenging capacity was measured using the DPPH method described in a previous study.[8,10] The synthetic antioxidant reagent butylated hydroxytoluene (BHT) was used as positive control and all tests were carried out in triplicate.

Table 1

Total phenolic and flavonoids content

Bioactive compounds	Yields (%)	Total phenolic content (mg GAE/kg of extract)	Flavonoids content (mg QE/kg of extract)
Hexane extract		-----	-----
Ethyl Acetate extract	1.83±0.23[a]	682.94±30[!]	45.51±0.00[$]
Methanol extract	2.31±0.15[b]	1079.41±14.70[!!]	71.12±0.00[¥]
H_2O/MeOH(80/20,v/v)extract	9.02±0.21[c]	1100±18.82[#]	80±0.00[*]

Results are expressed as mean ± standard deviation of 3 determinations. Means with different letters and symbols were significantly different at $p < 0.05$.

Ferric reducing antioxidant power (FRAP) assay: FRAP assay was estimated following the procedure originally described by Pulido *et al*.[10] Results are expressed as millimolar Trolox equivalents (TE).

Antibacterial activity

In order to determine the antibacterial activity of 6-shogaol various Gram-positive and Gram-negative bacteria were used: *Escherichia coli* (ATCC 10536), *Salmonella enterica* (CIP 80.39), *Staphylococcus aureus* (ATCC 9144), *Listeria monocytogens, Pseudomonas savastanoi* and *Agrobacterium tumefaciens.*[11]

Statistical analysis

Results of the analytical determinations were expressed as mean ± standard deviation (SD) of 3 measurements. Statistical differences were calculated using a one-way analysis of variance (ANOVA) employing the Student's t-test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Extract yields, total polyphenols and flavonoids contents

The phytochemical analysis of rhizome extracts of *Zingiber officinale Rosco* showed the presence of different groups of secondary metabolites. Quantitative estimation of the percentage of crude chemical constituents in ginger was summarized in Table 1. The aqueous methanolic extract yields (9.02±0.21%) was the highest compared to the other extracts.

The amount of total phenolic compounds was higher in aqueous-methanolic extract (1100±18.82 mg GAE/kg of extract) than in ethyl acetate and methanolic extracts (682.94±30 and 1079.41±14.70 mg GAE/kg of extract, respectively). These results were in agreement with the reports of Hertog *et al*.,[12] Yen *et al*.[13] and Boukhris *et al*.,[14] which proved that methanol is the most suitable solvent for the extraction of phenolic and flavonoids compounds. These results were different from those reported by Pawar *et al*.[2] who studied the total phenolic content from different ginger cultivars growing in India. They found that total phenolic content for the ginger cultivars were highest in Rajasthan 1.58 g while 0.886 g tannic acid eq/100 g of dry weight in Udaipur was the lowest. Moreover, Shamina *et al*.[15] determined the variability in total phenols in ginger using 25 cultivars. This could be explained also by the protocol of extraction used in order to extract the major of bioactive compounds.

On the other hand, the total flavonoids content from rhizome of ginger was rather important in aqueous methanol extract, 80 mg QE/kg of extract (Table 1). Different studies were undertaken on the determination of the content of flavonoids compounds. Study of Pawar *et al*.[2] revealed that flavonoids content from 12 different ginger varieties were expressed as quercetin equivalent per gram: The highest content was observed in cv. Rajasthan (0.38 g quercetin eq/100 g) while cv. Udaipur showed the lowest one (0.13 g quercetin eq/100 g of dry weight).

One step purification of 6-shogaol

In our search for bioactive compounds from medicinal plants, Silica gel column was employed to purify the aqueous methanolic *Zingiber officinale Rosco* extract. Direct application of aqueous phenolic extract on the Silica gel column, eluted by a mixture of *n*-hexane-diethyl ether (70/30, v/v), permitted separation of one fraction with a yield of 1.5% (30 mg) which was analyzed by GC-MS apparatus, this technique, *i.e*, gas

chromatography coupled with mass spectrometry was well known as a routine analytical tool to check the purity of some fractions. To analyze by GC-MS, the fraction was sustained to a silylation procedure as it mentioned in the GC-MS section. Based on the data, which was summarized in Table 2, the pure compound was identified as 6-shogaol (N) (Fig. 1). The structure of the compound was further confirmed by comparing its spectral data with the reported values.[16.17]

It should be noted that the optimum isolation of 6-shogaol was achieved using a mixture of *n*-hexane-diethyl ether (70/30, v/v). The repeatability of the optimized isolation method, checked for an 80% aqueous methanol extract (n = 5) at two random working days, was found satisfactory (Data not shown). This made possible the modification of the method suggested by Mallavadhani *et al*.[18] in order to evaluate the effect of extraction methodology on the yield of 6-shogaol, a biomarker of *Z. officinale* using UPLC-Q–TOF-MS. In this report, Mallavadhani and co-workers[18] mentioned that using a column chromatographic purification of the methanol extract (10 g) on Silica gel column using the gradient elution of *n*-hexane and ethyl acetate. Based on TLC, the column fractions of *n*-hexane–ethyl acetate (90:10) were combined and purified further by column chromatography to afford the 6-shogaol with a yield of 0.25 %.

Table 2

NMR data of 6-shogaol

Position	^{13}C NMR (δ ppm) Our data	Kim *et al*. [16] Lu *et al*. [17]	^{1}H NMR (δ ppm) Our data	Kim *et al*. [16] Lu *et al*. [17]
1	29.87	29.80.	2.86-2.84 (4H, m)	2.86 – 2.84 (4H, m)
2	42.00	41.90		
3	199.94	199.80		
4	130.31	130.30	6.84 -6.82 (2H, m)	6.86 -6.78 (2H, m)
5	146.41	146.40		
6	32.49	32.40	2.20 (2H, m)	2.20 (2H, dq, 7.2, 1.6)
7	31.36	21.30	1.44 (2H, m)	1.44 (2H, m)
8	27.78	27.70	1.30 -1.25 (4H, m)	1.32 -1.25 (4H, m)
9	22.45	22.40		
10	13.99	13.90	0.90 (3H, t, 6.8)	0.89 (3H, t, 6.8)
1'	133.27	133.20		
2'	111.07	111.10	6.71 (1H, d, 2)	6.71 (1H, d, 2)
3'	148.02	147.90		
4'	143.83	143.80		
5'	114.36	114.30	6.10 (1H, dd, 1.6, 16.0)	6.09 (1H, dd, 1.6, 16)
6'	120.79	120.70	6.67 (1H, dd, 2, 8)	6.68 (1H, dd, 2, 8)
7'	55.88	55.80	3.87 (3H, s)	3.87 (3H, s)

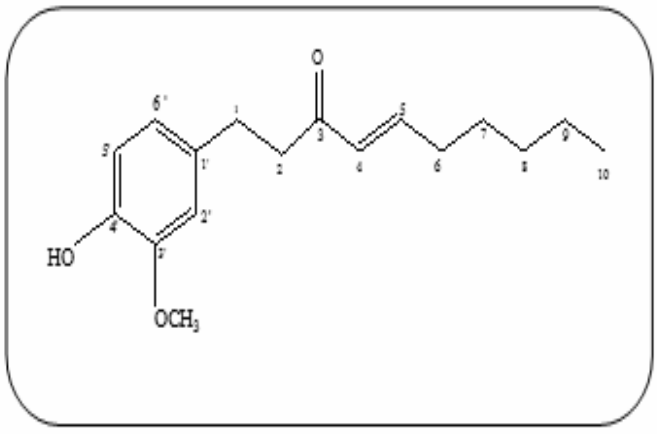

Fig. 1 – Structure of compound N: 6-shogaol.

Table 3

Antioxidant activity of the extracts of ginger and 6-shogaol by DPPH radical scavenging and FRAP assays

	DPPH (IC_{50} µg.mL^{-1})	FRAP (mM of Trolox)
Methanol extract (100 %)	0.15±0.01^{a}	0.47± 0.00*
Methanol extract (20 %)	0.19±0.00^{b}	0.44± 0.01$^{\#}$
Ethyl Acetate extract	0.66 ±0.00^{c}	0.24± 0.00$^{!}$
6-shogaol	0.13±0.04^{d}	0.68±0.02$^{¥}$
BHT	0.08±0.01^{e}	1.22 ± 0.03$^{\$}$

Results are expressed as mean ± standard deviation of 3 determinations. Means with different letters and symbols were significantly different at $p < 0.05$.

Table 4

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) obtained with shagoal against reference bacteria

Bacteria	MIC (mg/mL)	MBC (mg/mL)
Staphylococcus aureus	[1.25-0.625]	1.25
Pseudomonas savastanoa	[1.25-0.625]	1.25
Sallmonella enterica	[2.5-1.25]	2.5
Listeria monocytogenes	[1.25-0.625]	2.5
Escerichia coli	[1.25-0.625]	2.5
Agrobacterium tumefaciens	[0.3125-0.156]	0.3125

Antioxidant activity

Several methods have been proposed to evaluate antioxidant characteristics and to explain antioxidants mechanisms and actions. Of these, free synthetic radical scavenging like $DPPH^{\cdot}$ or $ABTS^{\cdot+}$ superoxide anion scavenging, reducing power and antioxidant assay using β-carotene linoleate system are most commonly used for the evaluation of the total antioxidant behavior of extracts and essential oils.[19] The antioxidant properties of ethyl acetate, methanol (100 %), methanol (20 %) extracts as well as 6-shogaol were determined as DPPH radical scavenging ability and ferric reducing/antioxidant power (FRAP method). Appreciable antioxidant potential of all extract and especially to the pure compound (N) was observed and related to the presence of a mixture of polyphenol compounds with good antioxidant properties (Table 3). An extremely correlation was confirmed between the free radical scavenging ability (IC_{50}) and the content of polyphenols (R^2 = 0.999), and the reducing power of extracts (FRAP) and the content of polyphenols (R^2=0.994) (Figure not shown).

A significant difference was found between the different studied extracts of ginger rhizomes (Table 3). In fact, the results pointed out that the methanol extract (100 %) showed the highest antiradical capacity with an IC_{50} about 0.15 µg/mL while the ethyl acetate extract showed the lowest one (IC_{50} = 0.66 µg/mL). It should be noted also that the radical scavenging activity of BHT was 1.625 fold higher than 6-shogaol (Table 3).

These results are in accordance with previous reports on antioxidant capacities of medicinal plants which exhibit polyphenols to play important role in the activity.[2] Accordingly, the superiority of the methanol extract (100 %) is probably due to its polarity which allows the accumulation of a variety of antioxidant compounds (polyphenols, flavonoids and other bioactive molecules). On the other hand, Lu and co-workers[17] studied the influence of side chain structure changes on antioxidant potency of the 6-gingerol related compounds. They concluded that a comparison of shogaols with gingerols, or dehydroshogaols with dehydrogingerols clearly indicates that the C4–C5 double bond is superior to the 5-OH in increasing the activity, in agreement with the previous results that 6-shogaol can more effectively scavenge the DPPH than 6-gingerol.[19,20]

As for the other antioxidant assays, the TEAC values, as shown in Table 3, are significantly different between the four fractions with a TEAC varying from 0.24 to 0.68 mM of Trolox. As for the radical-scavenging activities, the methanol (100%) extract showed the best activity with the highest TEAC value (0.47 mM of Trolox), while the ethyl acetate one displayed the lowest activity

with a TEAC value equal to 0.24 mM of Trolox. Furthermore, the 6-shogaol presented a moderate iron reducing power when compared to the TEAC related to BHT (Table 3). These observations may suggest that both solvent polarity and plant phenolic natures greatly influence the antioxidant activity estimation. Similar results were also observed by Lu *et al.*[17] who expressed their results as the number (n_e) of donated electrons per molecule. On the basis of the n_e values, the electron-donating ability decreases in the order of shogaols > gingerols > dehydroshogaols > dehydrogingerols for two series compounds, in agreement with the result obtained from the DPPH -scavenging assay.

Antimicrobial activity

The evaluation of the antimicrobial activity is based on the determination of MICs and MBCs. Obtained results are grouped in Table 4. According to the values of MIC and MBC, the action of the product is efficient towards the whole range of tested bacteria. All bacterial strains were found sensitive to the antibacterial activity induced by this product, but they have not shown the same degree of sensitivity. As the results show quite different inhibitory and minimum bactericidal concentrations. In general, the ratio MIC/MBC approaches the value 1 which shows the bactericidal effect of the product and effectiveness as an antibacterial agent. It has been reported that ginger had interesting antibacterial activities.[22] This activity is linked to the richness of ginger on active biomolecules like zingerone and shogaol. For example, it has been shown that gingerol and shogaol have interesting anti-bacterial activities that could help solve the stomach problems.[23] The evaluation of antibacterial activity of purified shows relatively low MICs which are almost equal or close to the MBCS reflecting the bactericidal effect of this molecule against most of the tested pathogenic bacteria.

CONCLUSIONS

All the extracts from ginger as well as 6-shogaol showed potent biological activities. Phenolic concentration in the rhizome of *Zingiber officinale Rosco* was very high especially for the methanolic extract (20%) and was probably responsible for its important biological activities. It should be noted that using only one silica cartridge, it is possible to prepare fractions quantitatively rich in 6-shogaol. The procedure was found to be reliable, fast and allowed recovery of the compound in order to use it as a food additive.

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