

SHORT COMMUNICATION

CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF THE FRUITS EXTRACTS OF *PIPER CAPENSE*

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ABSTRACT. The fruits of *P. capense* from Ethiopia were hydro-distilled with Clevenger apparatus to furnish 2.6-2.8% yellowish oil which was analyzed with GC-MS. Results of the GC-MS analysis revealed the presence of twenty one components with the major ones are β -caryophyllene (5%), γ -muurolene (7.22%), germacrene D (9.30%), dihydroxy-isocalamendiol (11.6%), and cis-muurola-3,5-diene (15.6%). The fruits were also successively extracted with *n*-hexane and EtOAc to give 1.3% and 2.3% yield on dry weight basis, respectively. The EtOAc extract was screened for the presence of secondary metabolites which showed the presence of alkaloids, phenols, steroids, flavonoids, saponins and terpenoids while tannin was not detected. The EtOAc extract after silica gel column chromatography resulted in the isolation of 5-hydroxy-7,4'-dimethoxyflavone. The DPPH radical scavenging activity of the EtOAc extract, essential oil and 5-hydroxy-7,4'-dimethoxyflavone were evaluated and found to inhibit DPPH radical by 78%, 70% and 84% at 100 $\mu\text{g mL}^{-1}$, respectively. Likewise, the EtOAc extract, essential oil and 5-hydroxy-7,4'-dimethoxyflavone inhibited peroxidation of lipid by 74, 64 and 80%, respectively. The results are significant compared to ascorbic acid used as positive control suggesting the use of the fruit of *P. capense* as a natural antioxidant.

KEY WORDS: *Piper capense*, DPPH, Ferric thiocyanate, 5-hydroxy-7,4'-dimethoxyflavone

INTRODUCTION

Piper capense is an endemic medicinal plant of east Africa found in the genus *Piper* and family Piperaceae [1]. This plant is a popular hedge plant in Ethiopia. It is mostly cultivated and found in natural forest of some localized areas of Ethiopia including Keffa (Bonga) and Jimma zone. *P. capense* is known by different vernacular names in Ethiopia including *Turfo* (Afan Oromo and Kefinya) and *Timiz* (Amharic) indicating its wide distribution in these areas of Ethiopia. The fruits of *P. capense* are used as a spice in Ethiopia in many national dishes. It is identified by its sweet aroma. Its fruit is well known in Ethiopia's spice market and sold by its name "*abesha timiz*". The local peoples used the roasted fruits to flavor coffee, tea and butter [2]. Its cost is very cheap as compared to "*ferenj timiz*" which is imported from other part of the world [3].

The leaves and other parts of the plant are used in Ethiopia for the treatment of various diseases. For instance, the powdered leaves and stem barks of *P. capense* are used to treat urinary disorder in northern Ethiopia. Aerial parts are used as a remedy against fever, to improve appetite and stomach-ache. It is also used to cure both human and animal diseases including breathing and digestive problems. The essential oils, mainly comprising of terpenoids, were reported to have antimicrobial activity [4, 5]. The main constituents of essential oil of the fruits of *P. capense* from S. Tomé e Príncipe were β -pinene, sabinene and β -caryophyllene [6]. Furthermore an alkaloid piperine was previously reported from *P. capense* [7].

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Despite the extensive popular use of this plant as a spice and remedy against various diseases, there is no information describing the chemical constituents and antioxidant properties of the fruits of *P. capense* cultivated in Ethiopia. In view of these, this paper presents the results of the chemical constituents and antioxidant activities of the fruits of *P. capense*.

EXPERIMENTAL

Plant material. The fresh fruit of *P. capense* was purchased from local market in Adama town, East Shewa Zone in May 2016. The plant material was identified by Mr. Melaku Wendafrash of the Biology Department of Addis Ababa University and voucher specimen ED-01 was deposited in the National Herbarium of Addis Ababa University.

Chemicals and instruments. Analytical TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck) on aluminum plate. The spots were visualized after dipping in vanillin/H₂SO₄. Column chromatography was performed using silica gel (60-120 mesh) Merck. Samples were applied on column by either adsorbing on silica gel. Solvents were removed using rotary evaporator. The UV-Vis spectral measurements were done using UV-Vis on T 60 U spectrophotometer (PG instruments, UK) equipped with deuterium and tungsten lamps. NMR spectra were recorded using Bruker Avance 400 spectrometer operating at 400 MHz. The IR spectra of compounds were recorded using a Perkin-Elmer BX Spectrometer (400-4000 cm⁻¹) as KBr pellets.

Gas chromatography-mass spectrometry. The essential oil (1 mg) extracted by hydro-distillation was dissolved in *n*-hexane (10 mL) to give 0.1 mg/mL. 1 µL of this sample was injected using an auto sampler into a split mode injector with a 100:1 split ratio. The temperature program increased at the rate of 20 °C/min and was run from 60 °C to 300 °C using DB5-MS column (Agilent) (30 m in length, 250 µm internal diameter and 0.25 µm thicknesses). The MS parameters were set to scan for compounds 36–600 amu in size. EI was at 70eV. Identification of the constituents was based on search through mass\hunter\library\NIST11.L and mass\hunter\library\W9N11.L.

Extraction of essential oil. The cleaned and air dried fruits of *P. capense* (50 g) was powdered using grinder and subjected to hydro-distillation for 3 hours using Clevenger apparatus [8] to furnish 2.6-2.8% yellowish.

Extraction and isolation of compounds. The powdered fruits of *P. capense* (300 g) were extracted successively with each 1.5 L of *n*-hexane and ethyl acetate at room temperature for 72 hours. The resulting *n*-hexane and ethyl acetate extracts were filtered and concentrated under reduced pressure using rotary evaporator at 40°C to afford 4 g (1.3%) and 7 g (2.3%) on dry weight basis, respectively. The hexane extract was yellowish oil. The ethyl acetate extract (4 g) was fractionated over silica gel column chromatography with *n*-hexane:EtOAc of increasing polarities as eluent to afford thirty five fractions (each 50 mL). These fractions were combined based on their TLC profiles to furnish six combined fractions. The first, second, third, fourth, fifth and sixth fractions were eluted with hexane, hexane:EtOAc (9:1), hexane:EtOAc (4:1), hexane:EtOAc (7:3), hexane:EtOAc (1:1) and hexane:EtOAc (2:3), respectively. Each combined fractions were concentrated *in vacuo*. Fraction 6, eluted with hexane: EtOAc (2:3), was identified as compound **1**.

Phytochemical screening of the EtOAc extract of fruit of P. capense. The ethyl acetate extract of the fruits of *P. capense* were screened for the presence of secondary metabolites including flavonoids, phenols, terpenoids, alkaloids, saponins, steroids and tannins following previously developed standard procedure [9].

DPPH radical scavenging activity. The DPPH radical scavenging activity of the extracts and constituent of the fruits of *P. capense* was done following previously developed procedure with slight modification [10]. The EtOAc extract was dissolved in methanol to furnish 1 mg/mL. This was used as stock solution which was serially diluted with methanol to obtain concentrations of 500, 250, 125, and 65 $\mu\text{g mL}^{-1}$. Diluted solutions (1 mL each) were mixed with 4 mL of 2,2-diphenyl-1-picryl hydrazyl (0.004% in methanol) in a brown vials to afford 100, 50, 25 and 12.5 $\mu\text{g mL}^{-1}$. After an incubation period of 30 min at 37 °C in an oven, the absorbance was determined against a blank at 517 nm [10]. The above procedure was repeated for the essential oil and compound **1**. The percent of DPPH discoloration of the samples was calculated according to the formula [12, 13]:

$$(\%) \text{ inhibition} = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \times 100$$

where A control was the absorbance of the DPPH solution and A sample was the absorbance in the presence of plant extract [12]. Samples were analyzed in triplicate. Ascorbic acid was used as positive control.

Ferric thiocyanate method. The anti-lipid per-oxidation potential of the essential oil, ethyl acetate extract and compound **1** were evaluated following standard procedure in the literature [14]. 0.1 mg of the ethyl acetate extract, 100 μL of linoleic acid, EtOH (5 mL) and phosphate buffer (5 mL, 0.05 M, pH = 7) in water were separately added in to a vial and incubated at 40 °C in an oven. After 24 h, 0.1 mL were taken and added in to a vial containing 75% aqueous EtOH (7 mL), 30% of NH_4SCN (0.15 mL) and 0.15 mL of 0.02M FeCl_2 in 3.5% HCl. It was then subjected to UV-Vis spectrophotometry to record the absorbance at 500 nm. Similar procedure as above was repeated for the essential oil and compound **1**. Absorbance of the blank and ascorbic acid were done in the same fashion.

The percentage inhibition using ferric thiocyanate method was calculated according to the following formula: % inhibition = $100 - (A_s/A_b) \times 100$ where A_s is absorbance of the sample and A_b is absorbance of the blank [15].

RESULTS AND DISCUSSION

Phytochemical screening of the fruit extract of *P. capense*. Phytochemical screening results of the EtOAc extract of the fruit of *P. capense* revealed the presence of alkaloids, phenols, steroids, flavonoids, saponins and terpenoids. However, the presence of tannins was not detected. The presence of these secondary metabolites in the fruits of *P. capense* is significant as they may contribute for the traditional use of this plant for the treatment of various ailments.

Radical scavenging assay. DPPH radical scavenging assay is a simple method for finding antioxidants by measuring absorbance at 517 nm due to the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [15]. When the radical is scavenged by antioxidants to produce neutral hydrazine, the absorbance at 517 nm is reduced. The antioxidant activity of the ethyl acetate extract, essential oil and compound **1** (Table 1) of the fruits of *P. capense* were measured by bleaching of the purple-colored solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) at four different concentrations (100, 50, 25 and 12.5 $\mu\text{g mL}^{-1}$). The DPPH assay indicated that the ethyl acetate extract and 5-hydroxy-7,4'-dimethoxyflavone displayed pronounceable free radical scavenging activity. The essential oil also showed significant radical scavenging activity with percent inhibition of 70, 58, 50 and 45% at 100, 50, 25 and 12.5 $\mu\text{g mL}^{-1}$, respectively. As revealed from the results, 5-hydroxy-7,4'-dimethoxyflavone (Table 1) had the highest (84%) radical scavenging activity which turned out to be comparable with ascorbic acid (90%) used a

positive control. This is most likely due to its strong ability of donating an electron and phenolic hydrogen to DPPH radical, which was visualized by immediate discoloration of the purple DPPH solution to yellow compared to the essential oil and the ethyl acetate extract of the fruits of *P. capense*. Therefore the antioxidant activity of the ethyl acetate extract partly accounts to the presence of 5-hydroxy-7,4'-dimethoxyflavone.

Table 1. DPPH radical scavenging activity of EtOAc extract, essential oil and 5-hydroxy-7,4'-dimethoxyflavone obtained from the fruits of *P. capense*.

Sample concentration ($\mu\text{g/mL}$)	% of DPPH scavenging activities of samples		
	EtOAc extract	Essential oil	5-Hydroxy-7,4'-dimethoxyflavone
100	78	70	84
50	64	58	71
25	53	50	56
12.5	40	45	44

Results are reported as the average of triplicates experiments.

Ferric thiocyanate method. On oxidation, lipids having many unsaturation sites undergo deterioration producing a number of toxic metabolites [16] which are known to interact with biological materials thereby causing cellular damage [17]. The degree of lipid peroxidation can be used to measure the antioxidant potential of compounds or extracts. In the course of this work, the ethyl acetate extract, essential oil and 5-hydroxy-7,4'-dimethoxyflavone obtained from the fruits of *P. capense* were evaluated for their anti-lipid peroxidation potential (Table 2).

Table 2. Anti-lipid peroxidation activities of the ethyl acetate extract, essential oil and 5-hydroxy-7,4'-dimethoxyflavone.

Sample name	% inhibition	Remark
Blank	-	
Ascorbic acid	84	
<i>P. capense</i> EtOAc extract	74	
Essential oil from <i>P. capense</i>	64	
5-Hydroxy-7,4'-dimethoxyflavone	80	

Ascorbic acid was used as positive control.

As shown in Table 2, 5-hydroxy-7,4'-dimethoxyflavone was found to exhibit the best antioxidant activity in the ferric thiocyanate method with percent inhibition of 80%, demonstrating the potential of this compound in preventing the formation of lipid peroxides. The anti-lipid per-oxidation displayed by the extract and 5-hydroxy-7,4'-dimethoxyflavone were comparable with ascorbic acid. Phenolics and flavonoids were known to act as scavengers of peroxide radicals and prevent oxidative damages [18]. This agrees well with the phytochemical results of this report since the extract contains both phenolics and flavonoids. Furthermore, the EtOAc extract, essential oil and hydroxy-7,4'-dimethoxyflavone from *P. capense* might be employed to retard autoxidation chain reactions in oils and fats which further indicate the potential of the fruits of *P. capense* as natural antioxidants.

Characterization of compound 1. Compound **1** was isolated as a light yellowish solid from the ethyl acetate extract of the fruits of *P. capense*. The melting point was 169-170 °C. TLC (hexane:EtOAc, 7:3) gave rise to a spot at $R_f = 0.65$, visualized as a yellowish spot after dipping in vanillin/ H_2SO_4 . The UV-Vis spectrum (MeOH) of compound **1** showed absorption maxima at 283 nm and 340 nm suggesting the presence of flavonoid skeleton in the structure of the compound [19]. The IR spectrum displayed absorption band at 1682 cm^{-1} attributable to α,β -

unsaturated carbonyl. The presence of C-C double bond and C-O stretching were evident from the observed absorption bands at 1602 cm^{-1} and 1164 cm^{-1} , respectively. The absorption band at 2924 cm^{-1} and 2850 cm^{-1} indicates the presence of C-H stretching.

The $^1\text{H-NMR}$ spectrum of compound **1** revealed signals at δ 3.90 (3H, s) and 3.91 (3H, s) suggesting the presence of two methoxy groups. The aromatic protons at δ 6.39 (1H, d, $J = 2.4$ Hz) and 6.51 (1H, d, $J = 2.4$ Hz) were evident for the presence of *meta* coupled protons on the A-ring of flavonoid. A characteristic olefinic signal of flavonoids is evident at δ 6.61 (1H, s). The proton signals at δ 7.03 (2H, d, $J = 8.8$ Hz) and 7.88 (2H, d, $J = 8.8$ Hz) are due to symmetrically placed hydrogens with AAB B' spin pattern on unsymmetrically *para* substituted B ring of flavonoid.

The proton decoupled $^{13}\text{C-NMR}$ spectrum of compound **1**, analyzed with the aid of DEPT-135 spectrum showed eight quaternary carbons which are attributed to five oxygenated aromatic carbons at δ 165.4 (C-7), δ 164.0 (C-2), δ 162.6 (C-5), and δ 157.7 (C-9); two aromatic carbons at δ 123.5 (C-1') and δ 105.5 (C-10), δ 162.2 (C-4') and an α,β -unsaturated carbonyl carbon at δ 182.4 (C-4). Four methine carbon signals are also observed in the aromatic region at δ 92.6 (C-8), δ 98.0 (C-6), δ 114.5 (C-3', 5') and δ 128.0 (C-2', 6'). The latter two carbon signals are due to symmetrically placed carbon on unsymmetrically *para*substituted ring B of the flavonoid. The presence of methine carbon is evident at δ 104.3 due to C-3. Furthermore the spectrum demonstrated the presence of two methoxy groups at δ 55.5 and 55.8. The upfield chemical shift values of C-6 (δ_c 98) and C-8 (δ_c 92) compared to other aromatic methines is additional evidence that these two methines are located in between 1,3-diortho oxygenated quaternary carbons in agreement with the proposed skeleton (Ring A). The spectral data generated for compound **1** agreed well with 5-hydroxy-7,4'-dimethoxyflavone [20, 21] available in literature. This compound was not yet reported from this species with its structure as shown in Figure 1.

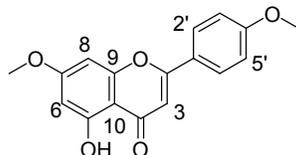


Figure 1. Chemical structure of 5-hydroxy-7,4'-dimethoxyflavone.

Essential oil constituents of the fruits of P. capense. On hydrodistillation, the fruits of *P. capense* afford 2.6-2.8% yellowish oil which was analyzed with GC-MS. It is higher than the values reported for other plants as source of essential oils including lavender (0.8-1.8%), laurel (0.1-0.35%), *Mentha rotundifolia* (4.33%) and *Mentha pulegium* (2.33%) [22]. Twenty one components were identified in the essential oil of *P. capense* (Figure 2). The major components were found to be terpenes which agree well with the previous reports on the constituents of essential oil of the same species from S. Tomé e Príncipe [6].

Safrole (**2**), σ -cubebene (**3**), σ -copaene (**4**), β -caryophyllene (**5**), germacrene D (**6**), Tetramethylbicyclo[8.1.0]undeca-2,6-diene (**7**), α -muurolene (**8**), 1,5,9-cyclododecatriene (**9**), β -bisabolene (**10**), isoshyobunone (**11**), β -myrcene (**12**), aromandendrene (**13**), 1,5-hexadiene (**14**), bicyclo[4.4.0]dec-1-ene (**15**), σ -cadinol (**16**), and 3-tetradecen-5-yne (**17**), γ -muurolene (**18**), dihydroxy-isocalamendiol (**19**), *cis*-muurola-3,5-diene (**20**) and (+)-epi-bicyclosesquiphellandrene (**21**) (Figure 3) are the constituents found in appreciable quantities in the essential oil of the fruits of *P. capense*. Results from the percent area showed that the major components of the oil of *P. capense* were β -caryophyllene (5%), γ -muurolene (7.22%), dihydroxy-isocalamendiol (11.6%), *cis*-muurola-3,5-diene (15.6%), germacrene D (9.30%). The result is not in agreement with the reported value of the major constituents of the essential oil of

P. capense from S. Tomé e Príncipe which comprises of β -pinene, sabinene and β -caryophyllene [6]. This is likely due to geographical locations.

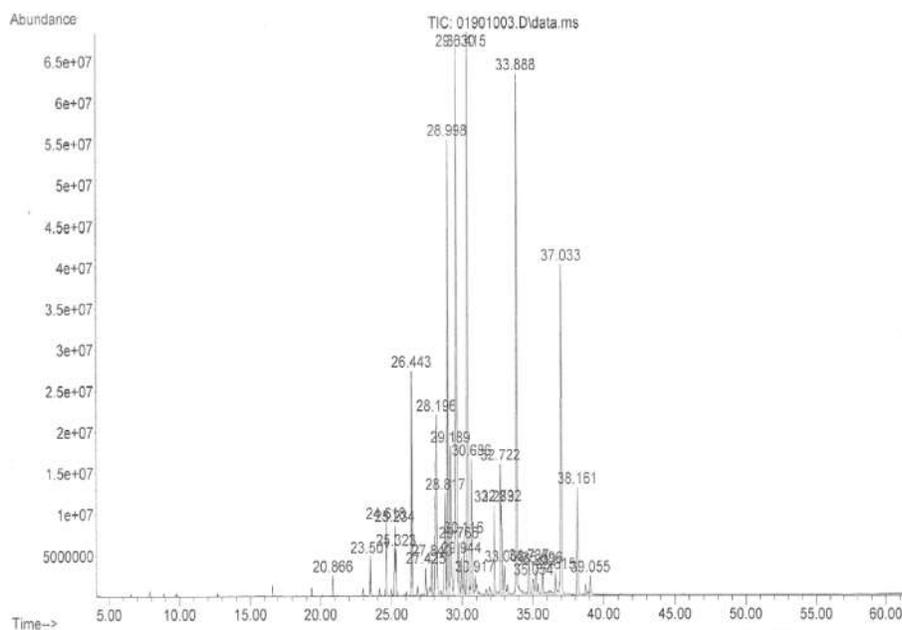


Figure 2. Chromatogram of the fruits of *P. capense* essential oil.

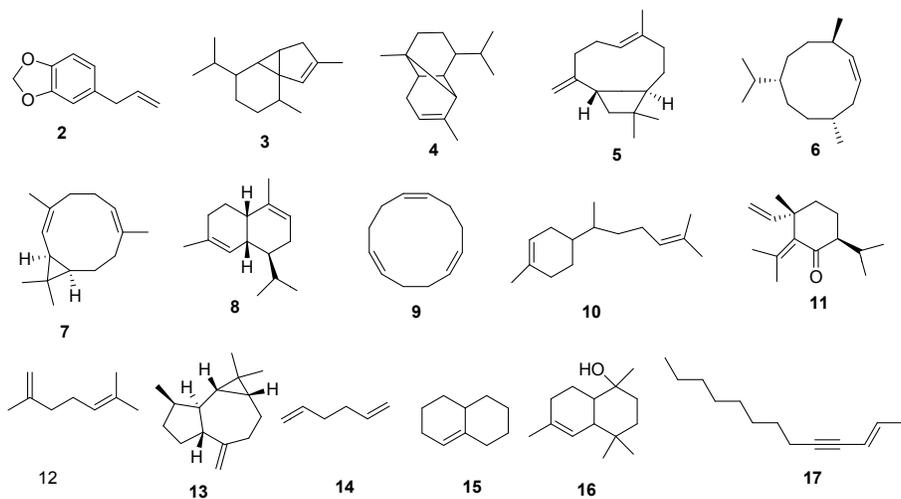


Figure 3. Chemical structures of some constituents of the essential oil of *P. capense*.

CONCLUSION

The fruits of *P. capense* on hydrodistillation furnished about 2.6-2.8% yellowish oil which after GC-MS analysis gave 21 constituents. This report shows that the essential oil is rich in β -caryophyllene, γ -muurolene, dihydroxycisocalamendiol, cis-muurola-3,5-diene and germacrene D. The latter four compounds were not been reported from the fruits of *P. capense*. The essential oil and the ethyl acetate extract were found to exhibit appreciable antioxidant activities against DPPH and ferric thiocyanate methods. The EtOAc extract after silica gel column chromatography affords 5-hydroxy-7,4'-dimethoxyflavone. The compound had strong antioxidant activities against DPPH and ferric thiocyanate methods. Therefore, the EtOAc extract and essential oil from the fruits of *P. capense* can be employed to retard autoxidation chain reactions in oils and fats which further indicate the potential of the fruits of *P. capense* as natural food and antioxidants.

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