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Phytochemical Studies of the Ethyl Acetate Extract of the Fruit of *Piper capense*

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Abstract

Natural products have been a major source of drugs for centuries. The fruit of *Piper capense* is used traditionally as spice and for the treatment of infectious diseases in different parts of Ethiopia. The main objective of this work is to carry out phytochemical study of the EtOAc crude extract of the fruit of *P. capense*. Phytochemical screening on this crude extract revealed the presence of phenols, alkaloids, steroids, terpenes, saponins and flavonoids. After silica gel column chromatography the crude extract led to the isolation of compound PC1 identified as 5-Hydroxy-7, 4'-dimethoxyflavone. Characterization of this compound was achieved via spectroscopic methods (NMR, UV and IR). The structure of compound PC2 was elucidated, the spectral data showed the presence of more than one compound one of which is partially characterized and proposed to be rotenone.

Keywords: *Piper capense*; Phytochemicals; Phytochemical screening

Introduction

The natural products as medicinal agents presumably predate the earliest recorded history as the earliest humans used various, but specific plants to treat illness [1]. The genus *Piper* is among the medicinal plants comprised of an estimated 2000 species [2]. It is distributed in the tropical regions of both hemisphere. Piper species are often shrubs, herbs or lianas commonly found in forest undergrowth [3]. The Piper species have long been known for their various ethno medical uses. The widespread ethno medical use of Piper species has led to an increased interest in the search for active compounds from these species and it has been found that many of these plants contain a number of biological activities [4]. The chemistry of the genus *Piper* has been widely investigated and the phytochemical investigations from all parts of the World have led to the isolation of a number of physiologically active compounds. A number of physiologically active compounds have been isolated from the Piper species: alkaloids/amides, propenyl phenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones, dihydrochalcones, flavones and flavanones [5]. Piper capense is among the species of piper found in Ethiopia which is locally known as "abesha timiz" in a reference to its special shape The flavor of timiz (Piper Capense) is described as less strong in 'pepper taste' but with different aroma. Timiz is essentially found in the Bonga's coffee forest of the South West of Ethiopia. It is linked to Ethiopian culture in several different aspects: it is gathered in a unique ecosystem, transformed in traditional ways and the fact that it is used in many national dishes [6]. Phytochemical analyses have shown that phenolics are the most frequently isolated compounds found in P. capense and it can be hypothesized that these compounds could be responsible for the antimicrobial activity. Alkaloids are the second most abundant group of compounds found in P. capense [7,8].

Materials and Methods

Plant material

The fresh fruit of *P. capense* was collected from Bonga's coffee forest of the South West of Ethiopia, on September, 2016. The plant material was identified by plant taxonomist Mr. Melaku Wendafrash of the Biology Department of Addis Ababa University, Addis Ababa, Ethiopia. Specimens have been deposited at National Herbarium of Addis Ababa University with Voucher number Eyob D.1.

Chemicals, apparatus and instruments

The apparatus used in the course of this work include Clevenger apparatus, digital melting point apparatus, heat gun, Whatman no-1 filter paper, etc. Chemicals and solvents used are vanillin, n-hexane, chloroform, ethyl acetate, methanol, distilled water, ferric chloride, hydrochloric acid, sulfuric acid, acetic acid anhydride, iodine in potassium iodide. Analytical TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck) on aluminum plate. Spraying agent used was 1% vanillin-sulfuric acid solution. Column chromatography was performed using silica gel (60-120 mesh) Merck. Samples were applied on column by either adsorbing on silica gel or dissolving in appropriate solvent. Solvent was 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.

Extraction and Isolation

Extraction

The fruit of *P. capense* was washed with water to remove dusts, air dried and grinded using grinder. The powdered fruits (300 g) were

extracted successively with 1.5 L of n-hexane and ethyl acetate at room temperature for 72 hours. The resulting extracts were filtered by whatman no.1 filter paper and concentrated under rotary evaporator. The extraction and isolation process of the fruit of *P. capense* is summarized in the Figure 1.

TLC analysis

Samples of the crude extract in µg amount was dissolved in its extraction solvent and analyzed using TLC plate (5 × 20 cm, 0.25 mm thickness) and developed in n-Hexane: EtOAc: MeOH with different ratio as a solvent system. After development, the spots were sprayed with 1% vanillin-sulfuric acid solution to visualize the presence of spots. TLC profile of the crude extract indicated the presence of a number of spots likely indicating the presence of many phytochemicals.

Phytochemical screening of the EtOAc crude extract of the fruit extract of *P. capense*

Phytochemical screening test is the most important step in the detection of phytoconstituents of plants; this has a major use in the discovery and development of drugs. EtOAc crude extract of the fruits of the plant was screened for the presence of secondary metabolites like flavonoids, phenols, terpenoids, alkaloids, tannins etc. using standard methods which are presented below.

Test for Alkaloids (Wagner's Test): 0.5 g of the extract was dissolved in dilute HCl and filtered. The filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). The formation of brown/reddish precipitate was checked as a positive test for the presence of alkaloids [9].

Test for Terpenoids (Salkowski test): 2 mL of chloroform was added to 0.5 g sample. Then, 3 mL concentrated sulfuric acid was carefully added to form a layer and examined if a reddish brown coloration of the interface is formed as a positive test for the presence terpenoids [9].

Test for Saponins: To 0.5 g of crude extract 5 mL of distilled water was added and shaken and then heated to boil. The formation of Frothing (appearance of creamy miss of small bubbles) was checked as a positive test for the presence saponins [9].

Test for Flavonoids: To 0.5 g crude extract 10 mL of ethyl acetate was added and heated with a steam bath for 3 min. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution and, the formation of a yellow coloration was checked as a positive test for the presence flavonoids [9].

Test for Phenols: 0.5 g of the crude extract was treated with a few drops of 2% of $FeCl_3$; the formation of bluish green or black coloration was checked as a positive test for the presence Phenols [9].

Test for Tannins: 0.5 g of the crude extract was boiled in 10 mL of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added then examined if brownish green or a blue-black coloration is formed to confirm the presence of tannins [9].

Test for Steroids: 0.5 g of the crude extracts was dissolved in 5 mL of methanol. 1 ml of the extract was treated with 0.5 mL of acetic acid anhydride and cooled in ice. This mixture was mixed with 0.5 mL of chloroform and 1 mL of concentrated sulfuric acid was then added carefully by means of a pipette. At the separations level of the two liquids, a reddish-brown ring formation was checked to confirm the presence of steroids [9].

Isolation of compounds from the EtOAc extract of the fruits of *P. capense*

A 4 g portion of the EtOAc extract was fractionated by using column chromatography with silica gel (60-120 mesh) Merck (150 g) eluted with increasing polarity of n-Hexane: EtOAc: MeOH.

Sixty fractions (each with 50 mL volume) were collected. The eluents that showed the same profile on TLC were combined to give nine fractions. Purification of a combined fractions (F_{32}-F_{37}) (72 mg) was carried out using CC with silica gel, eluted with 95% n-hexane-EtOAc in 5% polarity increment and twelve subtractions (each with 10 mL volume) were collected, out of them subfraction 1 and subtraction 2 were further analyzed by TLC and single spots were observed. It was finally concentrated and yield compounds PC2 (23 mg) and PC1 (12 mg). CC fractionation of the EtOAc extract of the fruits of *P. capense* is summarized in Table 1.

Fractions		Eluent (ratio)	Amount in mg
F1-5		Hexane	55
F6-9		Hexane: EtOAc (9:1)	43
F10-14		Hexane: EtOAc (4:1)	27
F15-21		Hexane: EtOAc (7:3)	35
F22-31		Hexane: EtOAc (1:1)	28
F32-37		Hexane: EtOAc (2:3)	72
F38-45		Hexane: EtOAc (1:4)	94
F46-53		EtOAc	44
F54-60		Me OH	63

Table 1: Column chromatographic fractionation of the EtOAc extract of the fruits of *P. capense.*

F32-37 (72 mg) was analyzed with TLC using n-hexane: EtOAc (4:1) as eluent. It gave three distinct spots visualized after dipping in vanillin-H_2SO_4 followed by heating over heat gun. An attempt was made to purify the mixture using silica gel column chromatography which resulted in twelve fractions. Out of this subtraction, subfraction-2 (12 mg) was recrystallized in petroleum ether and filtered.

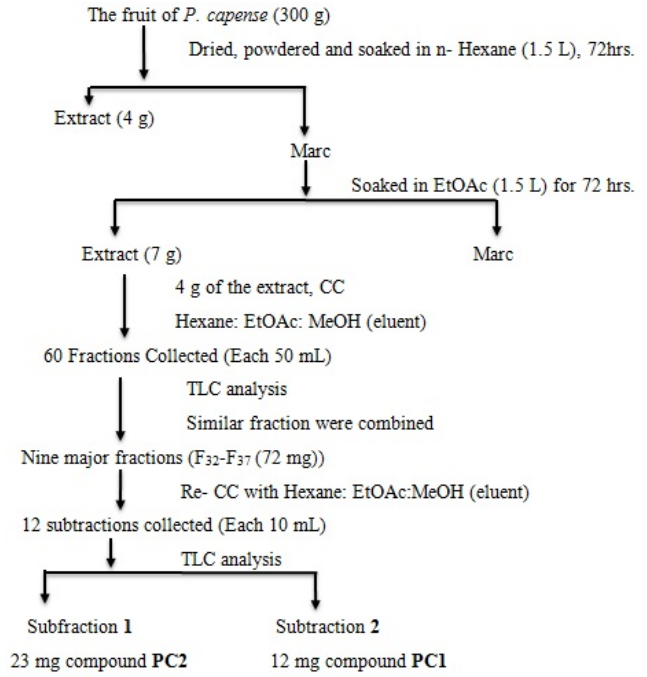

Figure 1: Extraction and isolation of compounds from the EtOAc extract of the fruits of *P. capense*.

Results and Discussion

Phytochemical screening of the fruit extract of *P. capense*

Phytochemical screening on the EtOAc extract of the fruit of *P. capense using standard methods* revealed *the presence* of alkaloids, phenols, steroids, flavonoids, saponins, terpenoids and absence of tannins. 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. The whole results are depicted in Table 2.

Phytochemical	Alkaloids	Terpenoids	Saponins	Flavonoids	Phenols	Steroids	Tannins
Results	+	+	+	+	+	+	-
Key: (+) Present; (-) Absent							

Table 2: Phytochemical screening test of EtOAc extract of the fruit of *P. capense*.

Characterization of compound PC1

Compound PC1 was isolated as a light yellowish solid from the ethyl acetate extract of the fruits of *P. capense*. The melting point was measured to be 169.1-170.2 with Rf value of 0.65 using hexane: EtOAc (7:3) as eluent. It was visualized as a yellowish spot after dipping in vanillin/H_2SO_4 followed by heating over heat gun. The UV-Vis spectrum (MeOH) of compound *PC1* showed absorption maxima at 283 nm and 340 nm attributed to n→ ∏ * and δ →∏* respectively suggesting the presence of flavonoid skeleton in the structure of the compound. 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 1H-NMR spectrum of compound *PC1* (Table 3) 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. The proton signals at δ 6.61 (1H, s) is ascribed to the proton signal on the C-ring of the flavonoid. 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 AABB' spin pattern on unsymmetrically para substituted B ring of flavonoid. The first peak is due to the protons at 3'and 5' position while the second one is due to the protons at position 2'and 6' of aromatic nucleus. The proton decoupled ^{13}C-NMR spectrum of compound *PC1*, analyzed with the aid of DEPT-135 spectrum (Table 3) 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 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 δ104.3 due to C-3. Furthermore the spectrum demonstrated the presence of two methoxy groups at δ 55.5 and δ 55.8. The upfield chemical shift values of C-6 (δc 98) and C-8 (δc 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 value of the two methoxy below 60 ppm suggests that none of them are located at C-5 position and don't experience peri-effect of carbonyl. The down field chemical shift of one of the quaternary carbon at δ 164.0 suggests that this carbon chemical shift is for β-

position (C-2) of the, β-conjugation of flavonoid skeletal in C ring. The spectral data generated for compound *PC1* agreed well with 5-hydroxy-7, 4'-dimethoxyflavone [10] available in literature (Table 3).

Carbon No	Observed		Markham (1989) [10]	
	δ H	δC	δC	DEPT-135
2	-	164.0	163.9	-
3	δ 6.61 (1H, s)	104.3	104.2	104.3
4	-	182.4	182.4	-
5	-	162.5	162.0	-
6	δ 6.39 (1H, d, J=2.4 Hz)	98.0	98.0	98.0
7	-	165.4	165.3	-
8	δ 6.51 (1H, d, J=2.4 Hz)	92.6	92.5	92.6
9	-	157.7	157.6	-
10	-	105.5	105.4	-
1'	-	123.5	123.4	-
2' and 6'	δ 7.88 (2H, d, J=9.2 Hz)	128.0	127.9	128.0
3' and 5'	δ 7.03 (2H, d, J=9.2 Hz)	114.5	114.3	114.5
4'	-	162.2	162.5	-
OMe	δ3.90 (3H, s)	55.5	55.6	55.8
OMe	δ3.91 (3H, s)	55.8	55.5	55.4

Table 3: ^{1}H-NMR, ^{13}C-NMR and DEPT-135 (in $CDCl_3$) spectral data of compound PC1 (5-hydroxy-7, 4'-dimethoxyflavone) and literature data.

All the above spectral data agrees well with 5-hydroxy-7, 4'-dimethoxyflavone. The structure of this compound is shown below in Figure 2.

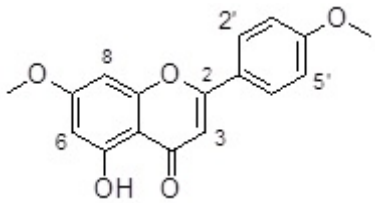

Figure 2: The proposed structure of compound PC1 (5-hydroxy-7, 4'-dimethoxyflavone).

Partial characterization of compound PC2

The UV-Vis spectrum (MeOH) of compound PC2 showed absorption maxima at 290 nm due to δ →∏* absorption, suggesting the presence of conjugation in the structure of the compound. The IR spectrum displayed absorption band at 3434 cm^{-1} attributable to the presence of O-H stretching. The presence of methine C-H stretching was evident from the observed absorption bands at 2924 cm^{-1} and 2850 cm^{-1}. Absorption at 1666 cm^{-1} is due to C=O stretching and absorption at 1606 cm^{-1} is due to C=C stretching. Absorption band at 1272 cm^{-1} is due to C-O stretching. ^{13}C-NMR spectrum *showed* signals at δ 202.8 indicate the presence of aldehyde or ketone which, not α, β-conjugated. Signals between δ 144 to δ 163 likely indicate the signal of an oxygenated aromatic carbon. Signal at δ 147.5 and δ 145.1 indicate vicinal sp^2 oxygenation pattern. Signal at δ 163.8 indicated sp^2 quaternary oxygenated carbons. Signals at δ 118.1, δ 100.5 indicated the presence of olefinic CH_2. The signal in the region δ 100, δ 134 is due to the presence of olefinic or aromatic group. The compound also displayed methoxy signals δ 57.1 and δ 57.4.

The remaining 8 signals are due to aliphatic carbons. Though the spectra shows mixture of samples compound PC2 looks like some rotenone pattern. The proposed structure is shown below in Figure 3.

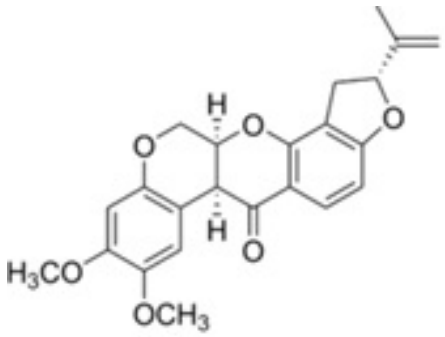

Figure 3: The proposed structure of compound PC 2 (rotenone).

Conclusion

In this study, the fruits of *P. capense* were sequentially extracted with n-hexane and EtOAc. The EtOAc crude extract was screened for the presence of secondary metabolites using standard procedures. The phytochemical screening revealed the presence of alkaloids, saponins, flavanoids, terpenoids, phenols and steroids. The presence of these secondary metabolites in the fruits of P. capense may accounts for the traditional use of this plant for the treatment of various diseases. The EtOAc extract after silica gel column chromatography led to the isolation of two compounds of which Compound PC1 was identified as 5-hydroxy-7, 4'-dimethoxyflavone based on spectroscopic evidence and by comparison with literature values . The NMR spectrum of compound PC2 looks as it contains impurities. Furthermore, the TLC profile of the EtOAc extract showed the presence of many spots which are not identified in this study the. These findings suggested that the EtOAc extract and the essential oils of the fruit of *P. capense* contain numerous compounds having medicinal property. Further study is required to explore other chemical constituents of the EtOAc and methanol extracts of the fruits of *P. capense* using more advanced chromatographic techniques such as HPLC, 2D-NMR spectroscopic techniques such as HSQC, HMBC, H-H COSY and MS to fully characterize the second compound PC2. Furthermore it is also necessary to study antimicrobial activity of the extract and pure constituents of the fruits of *P. capense*.

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