Isolation and Identification of Flavone Compound from the Leaves of *Ocimum sanctum* Linn.

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ABSTRACT

The leaves of *Ocimum sanctum* Linn (Local name-Pinsein-net) were selected for this research. From the phytochemical tests of the crude extracts, the sample contains many chemical constituents such as alkaloids, flavonoids, terpenes, steroids, glycosides, reducing sugars, polyphenols, saponins and tannins respectively. The antimicrobial activities of the crude extracts of the sample were tested by Agar well diffusion method on six selected microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomas aeruginosa, Bacillus pumilus, Candida albicans* and *E.coli*. The compound (I) was isolated as pale yellow feather shape crystal by thin layer and column chromatographic separation techniques. In addition, the complete structure of flavonoid compound (I) was elucidated by applying 1D and 2D NMR spectroscopy as well as EI-Mass spectrometry. The name of the isolated compound could be assigned as 5-hydroxy-2-(4-(3-hydroxyphenoxy)-3-methoxyphenyl)-6, 7-dimethoxy-4H chromen-4-one.

Keywords : *Ocimum sanctum* Linn., 1D and 2D NMR spectroscopic methods, EI- MS spectrometry, flavone compound

1 INTRODUCTION

Plants and plant extracts have been used as a major source of medicines since ancient time and that is why a large number of drugs are derived from different kinds of plants in the world. [1] The medicinal plants are rich in secondary metabolites which are potential sources of drugs. [2] The uses of medicinal plants are effective, safe due to low side effects, low costs, and ease of availability. [1, 2, 3] From the survey (1993) of World Health Organization (WHO), the medicinal practitioners treat about 85% of patients in Myanmar, 90% in Bangladesh and 80% in India. [2]

Among the plants known for their medicinal value, the plants of genus Ocimum belonging to family Labiatae are very important for their therapeutic potentials. [2] It is widely distributed in Asia, Africa, South America, and the Mediterranean but widely cultivated in many countries in natural and green house conditions in order to maximize the yield and obtain a regular supply of the material. It is a very important medicinal plant and culinary herb. [4] Several medicinal properties have been attributed to *Ocimum sanctum* Linn. Different parts of this plant are known to possess therapeutic potentials and have been used, by traditional medical practitioners, as expectorant, analgesic, anticancer, antiasthmatic, antiemetic, diaphoretic, antidiabetic, antifertility, anti antifungal, antimicrobial, hepatoprotective, cardio-protective, antiemetic, antispasmodic, hypotensive, hypolipidmic and antistress agents. [2, 5, 6]

It has also been used in treatment of fever, bronchitis, arthritis, convulsions etc. It is also a popular home remedy for many ailments such as wound, bronchitis, liver diseases, catarrhal fever, lumbago, hiccough, ophthalmia, gastric disorders, genitourinary disorders, skin diseases, various forms of poisoning and psychosomatic stress disorders. [7] Aqueous decoction of the leaves is given to patients suffering from gastric and hepatic disorders. Herbal preparations containing *Ocimum sanctum* L. have been suggested to shorten the course of illness, clinical symptoms and biochemical parameters in patients suffering from viral hepatitis. The leaf juice of *Ocimum sanctum* L. along with Triphala is used in Ayurvedic eye drop preparations recommended for glucoma, cataract, chronic conjunctivitis and other painful eye diseases. The juice of fresh leaves is also given to patients to treat chronic fever, dysentery, hemorrhage and dyspepsia. The leaves also check vomiting and have been as anthelmintic. [2, 7]

Ocimum sanctum has a specific aromatic odour because of the presence of an essential oil, concentrated mainly in leaves. This aromatic volatile oil contains a variety of terpenes with phenols and aldehydes groups, differring their chemical composition according to studies in different parts of the world. [8, 9, 10] In this research work, one of the Myanmar traditional indigenous medicinal plants, Pinsein-net, (*Oci-*

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mum sanctum) grown in Pyin Oo Lwin Township, Mandalay Region was selected for chemical investigation due to its different medicinal properties.

2 MATERIALS AND METHODS

2.1 Materials

Analytical grade reagents and solvents were used without further purification. The leaves of *Ocimum sanctum* Linn were collected from Pyin Oo Lwin Township, Mandalay Region, Myanmar. Precoated siligica gel (Merk. Co. Inc, Kiesel gel 60 F_{256}) plate was applied for thin layer chromatography. Iodine vapor and UV- lamp (Lambda – 40, Perkin – Elmer Co, England) were used as visualizing agents in column chromatography. Melting Point Apparatus, SMP30 was employed to measure the melting point of the compound. UV spectrometer (Perkin Elmer (Lambda 25) UV/VIS spectrometer) was applied to confirm the isolated compound as flavonoid compound. FT-IR spectrometer (Shimadsu, Japan) was used for the identification of the functional groups of the isolated compounds. ^{1}H-and ^{13}C NMR spectroscopy was carried out on JEOL at 500MHz and 125MHz respectively. Chemical shifts values are given in δ-value (ppm) with tetramethylsilane (TMS) as internal standard. EI-mass spectrometer (JEOL, JMS-600 MHz) was employed to assign the molecular formula of the compound.

2.2 Preliminary Phytochemical Tests of the Leaves of *Ocimum sanctum* Linn.

The phytochemical tests of the leaves of *Ocimum sanctum* were carried out at Department of Chemistry, University of Mandalay, Myanmar to detect the different kinds of chemical constituents in the sample. [11] [12] [13] [14]

2.3 Determination of Antimicrobial Activity of Crude Extracts from the Leaves of *Ocimum sanctum* Linn.

The antimicrobial activities of the crude extracts of the sample were tested using Agar-well diffusion method on six selected microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomas aeruginosa, Bacillus pumilus, Candida albicans* and *E.coli* in PRD (Pharmaceutical Research Department), Yangon, Myanmar. [15]

2.4 Extraction and Isolation of Pure Organic Compound

The air-dried powdered leaves (335 g) were extracted with 95% ethanol at room temperature (3.8 L, 2 months). The combined extract was concentrated to give 2.2 g. The ethanol crude extract was successively extracted with ethyl acetate.1.51 g of the ethyl acetate soluble part was packed with 50g of silica gel in a column (75cm x 2.0cm) and eluted with n-hexane; n-hexane: ethyl acetate mixtures (19: 1- 1: 19) and ethyl acetate. The progress of separation was monitored by thin layer chromatography using n- hexane: ethyl acetate mixtures. Twelve combined fractions with same R_f values were obtained.

The combined fraction V (36.6 mg) was subjected to repeated column chromatography over silica gel eluted with n- hexane/ EtOAc (3:2, 1:1, 2: 3, 1: 3, 1: 4 and 1: 9) to afford a pale yellow feather shape crystal 10.00 mg, mp 184- 186 °C. TLC using solvent system (n-hexane: EtOAc- 1:4) gave a single spot with R_f (0.58). [16] Compound I was identified by ^{1}H-NMR (ID&2D), and ^{13}C-NMR (ID&2D), HSQC, DEPT, HMBC.

3 RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Tests of the Leaves of *Ocimum sanctum* Linn.

According to the phytochemical tests of the crude extracts from the leaves of *Ocimum sanctum*, the sample contains many chemical constituents such as alkaloids, flavonoids, terpenes, steroids, glycosides, reducing sugars, polyphenols, saponins and tannins respectively.

Table (1) Results of Phytochemical Tests of the Leaves of *Ocimum sanctum* Linn.

No.	Tests	Reagents	Observation	Results
1.	Alkaloids	Dragendorff's reagent	Pale Orange ppt	+
2.	Flavonoids	conc: HCl, Mg tunning	Brown color solution	+
3.	Terpenes	Pet ether, Acetic anhydride, conc: H_2SO_4, $CHCl_3$	Reddish brown color solution	+
4.	Steroids	EtOH, conc: H_2SO_4	Greenish color solution	+
5.	Glycosides	10 % lead acetate	Yellow ppt	+
6.	Reducing Sugars	Benedict's solution	Brick Red ppt	+
7.	Polyphenols	10 % $FeCl_3$, $K_3[Fe(CN)_6]$	Greenish blue color solution	+
8.	Saponins	Shaken vigorously	Froth formation	+
9.	Tannin	1% $FeCl_3$	Brown ppt	+

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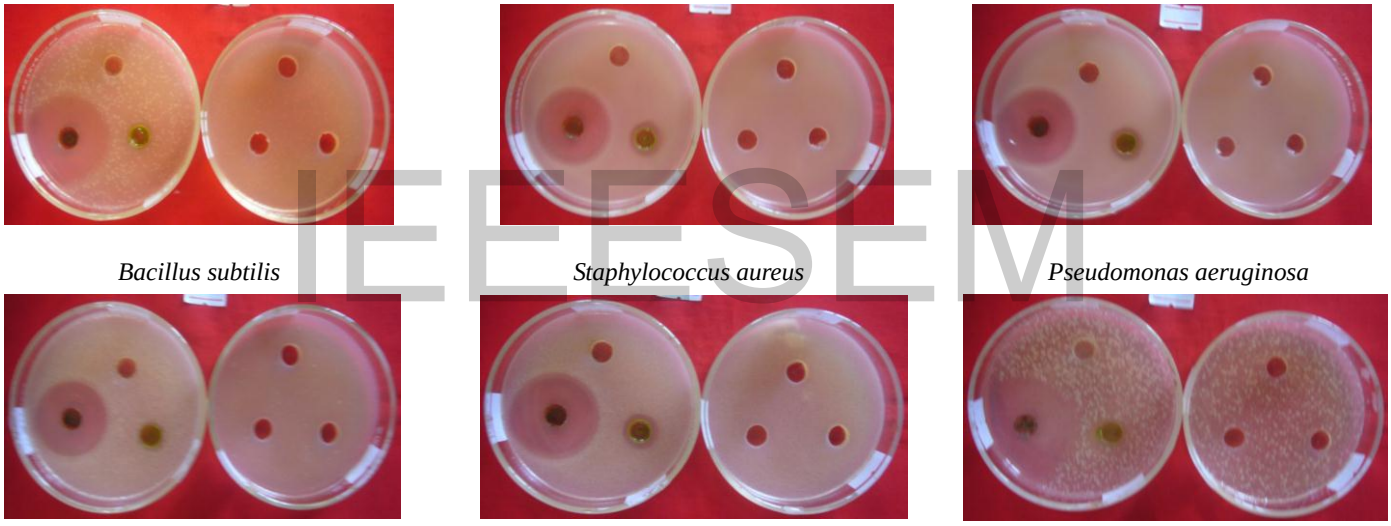
3.2 Antimicrobial Activities of the Crude Extracts of the Sample

The results of the antimicrobial test relevant to different types of organisms are tabulated in table (2). From the test results, the ethyl acetate extract of the sample responds high activity on all microorganisms. The ethanol extract shows median activity on *Staphylococcus aureus, Pseudomonas aeruginosa, and Candida albicans* and low activity on *Bacillus subtilis, Bacillus pumilus and E.coli.* N- hexane extract has no activity on all tested organisms. Therefore, the ethyl acetate extract is valuable for further studies.

Table (2) Results of Antimicrobial Activities of Crude Extracts of the Sample

Crude Extract	Diameter of Inhibition Zone (mm)					
Solvent	I	II	III	IV	V	VI
n- hexane	-	-	-	-	-	-
EtOAc	35(+++)	28 (+++)	30 (+++)	27 (+++)	28 (+++)	35 (+++)
EtOH	12(+)	15 (++)	15 (++)	12(+)	15(++)	14(+)

Microorganisms
I = *Bacillus subtilis* II= *Staphylococcus aureus* III = *Pseudomonas aeruginosa*
IV = *Bacillus pumilus* V = *Candida albicans* VI = *E. coli*
Agar well- 10 mm, 10 mm ~ 14 mm (+); 15 mm ~ 19 mm (++); 20 mm above (+++)



Bacillus subtilis *Staphylococcus aureus* *Pseudomonas aeruginosa*

Bacillus pumilus *Candida albicans* *E. coli*

Figure 1. Antimicrobial activities of the crude extract of the sample

3.3 Structure Elucidation of Isolated Compound (I)

The concentrated EtOAc extract of the leaves of *Ocimum sanctum* Linn was chromatographed on silica gel by using n-hexane and ethyl acetate as eluents from non-polar to polar. Compound I was obtained as a pale yellow feather shape crystal.

The UV spectrum showed absorption bands at 276 and 344 nm characteristic of a flavonoid nucleus. The molecular mass of the isolated compound is 436 Da according to EI-MS spectrum, indicating the molecular formula ($C_{24}H_{20}O_8$). [17] The FT-IR spectrum indicated the characteristic signal bands of flavonoid compound [(3448.5 cm^{-1}, O-H stretching), (3085.9 cm^{-1}, sp^2 hydrocarbons), (1654.8 cm^{-1}, C=O stretch of aromatic carbonyl), (1600.8 cm^{-1}, C=C stretch of aromatic hydrocarbons), (1033.8 cm^{-1}, C-O-C stretching of ether)]. [18]

The ^{13}C-NMR spectrum revealed totally 23 carbon signals indicating 24 carbons in the compound. The low field signals at 182.16 ppm was due to the carbonyl group at C-4. Eight signals between δ 164 and 132 represented aromatic carbon atoms directly connected with oxygen atoms where that at δ158.55 represented two carbon atoms. The spectrum showed eleven additional signals between δ 129 and 91 represented aromatic carbon atoms. The ^{1}H-NMR spectrum reflected three methoxyl groups (δ_H 3.74, 3.90 and 3.94 ppm) corresponding to δ_C 59.98, 55.99 and 56.42 ppm. The existence of flavonoid type compound could be confirmed by the ^{13}C- NMR spectral data. The singlet signal at δ_H 6.83 ppm in C-3 position of the compound indicated that the compound is the flavone type compound. The other singlet signal at 6.94 ppm in C-8 position showed the presence of three substituted carbons at C-5, 6 and 7 of the flavonoid nucleus.

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Careful interpretation of the cross signals in the COSY, HMQC and HMBC spectra combined with the coupling constants of the signals in the ^{1}H NMR spectrum giving the complete structure assignment of the compound (I). [19, 20]

Table (3) ^{1}HNMR, ^{13}CNMR, DEPT, HMQC, COSY and HMBC Spectral Data of the Compound (I) (at 500 and 125 MHz, resp. in DMSO; 296 K; δ in ppm, J in Hz)

Position C/H	DEPT	δ_H	δ_C	J correlation (HMBC)	^{1}H-^{1}H COSY correlation
2	C		161.54	H-3, H-2', H-6'	
3	CH	6.83 (s)	102.55	H-8	
4	C		182.16	H-3, H-8	
5	C		152.02	H-8	
6	C		131.85	OMe, H-8	
7	C		158.55	OMe, H-8	
8	CH	6.94(s)	91.59		
9	C		152.59	H-8	
10	C		105.04	H-8	
1′	C		121.09	H-2', H-6'	
2′	CH	6.93(d, J= 1.7Hz)	115.99		
3′	C		148.11	H-2', OMe	
4′	C		164.01	H-5', H-4"	
5′	CH	7.96 (d, J=8.5Hz)	128.46		H-6'
6′	CH	6.92 (dd, J=8.5Hz, 1.7 Hz)	115.81		H-5'
1″	C		151.25	H-5", H-6"	
2″	CH	6.91(d, J=1.7Hz)	91.53		H-6"
3″	C		158.55	H-2", H-4"	
4″	CH	6.92 (dd, J= 8.7Hz, 1.7Hz)	102.87		H-5"
5″	CH	7.61 (d, J=8.7Hz)	120.50	H-4", H-6"	H-4", H-6"
6″	CH	7.59 (dd, J=8.7Hz, 1.7Hz)	110.24	H-5"	H-2", H-5"
OMe at 6	CH_3	3.74 (s)	59.98		
OMe at 7	CH_3	3.94 (s)	56.42		
OMe at 3′	CH_3	3.90 (s)	55.99		

(s = singlet, d = doublet)

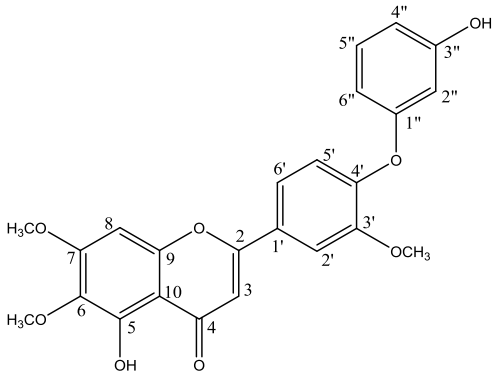

Figure 2. Structure of flavone type compound (I)

4 CONCLUSION

In this research, the leaves of *Ocimum sanctum* Linn were selected to determine the phytochemical constituents, to test the bioactivity of the crude extracts, and to isolate the organic compound for its structure assignment. The leaves of this plant contain alkaloids, flavonoids, terpenes, steroids, glycosides, reducing sugars, polyphenols, saponins and tannins respectively. The compound (I) could be isolated by thin

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layer and column chromatographic separation techniques. Phytochemical test for compound (I) was done and it showed a positive test for flavonoid. The melting point of this compound was found to be 184-186 ºC. The yield percent of the compound was 0.66% based upon the ethyl acetate crude extract.

The FT- IR spectrum showed the characteristic signal bands of flavone [(3448.5 cm^{-1}, OH stretch of hydroxyl group), (1654.8 cm^{-1}, C=O stretch of aromatic carbonyl), (1033.8 cm^{-1}, C-O-C stretch of ether)]. The MS displayed (M+) at *m/z* 436 (corresponding to $C_{24}H_{20}O_8$) and significant peak at *m/z* 344 showed cleavage of one aromatic ring from the flavonoid derivative skeleton. According to the FT-IR, ^{13}C-NMR, and HMBC spectrum, there was a carbonyl group at C-4 of ring C (δ 182.16 ppm in ^{13}C NMR spectrum and at 1654.8 cm^{-1} in FT-IR) in the compound (I). In addition, DEPT and HMBC spectra indicated the presence of two hydroxyl groups of this compound (one at C-5 of ring A and other at C-3 of ring D). Three sp3 methoxy groups (at δ 158.55ppm, 131.85ppm, and 148.11 ppm) were identified by using β ^{1}H- ^{13}C long range connections of HMBC spectrum. The name of the compound could be assigned as 5-hydroxy-2-(4-(3-hydroxyphenoxy)-3-methoxy phenyl)-6, 7-dimethoxy-4H-chromen-4-one.

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