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A STUDY ON IDENTIFICATION OF ORGANIC CONSTITUENTS FROM ETHANOL EXTRACT OF EUPATORIUM ODORATUM LINN. AND EVALUATION OF SOME BIOLOGICAL ACTIVITIES

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Abstract

One of the Myanmar indigenous medicinal plants, *Eupatorium odoratum* Linn.(Taw-bizat) leaves was studied in bioactive organic constituents and its antioxidant activity. Firstly, pet-ether extract and defatted 70% ethanol extract were prepared by percolation method. One of the bioactive flavonoid compounds, 4'-methoxy -5, 7- dihydroxy flavanone (0.05 g, 0.02%, R_f =0.33, PE: EtOAc, 8:1) was isolated from 75% ethanol extract of *Eupatoriun odoratum* L. by silica gel column chromatographic separation method. It was identified by modern spectroscopic methods such as UV-Visible, FT-IR, ^{1}HNMR, NOE, and EI-MS spectroscopy. Moreover, in the evaluation of the antioxidant activity, the IC_{50} value of isolated compound A from ethanol extract of *Eupatorium odoratum.* L. was observed (13.07 µg/mL) that more potent activity than ethanol and watery extract by DPPH assay method.

Keywords: *Eupatorium odoratum.* , bioactive flavonoid , 4'-methoxy -5,7-dihydroxy flavanone,

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Introduction

Eupatorium odoratum Linn.

Eupatorium odoratum L. is a Christmas bush, also known as bitter bush, baby tea, is a scram bling shrub. The plants are maintained by a system of abundant, yellowish, fine lateral roots. The leaves are aromatic when crushed. These flowers are white, flowering time from August to October. It is distributed throughout Indian, Indochina and common in open country. In Myanmar, it can be widely distributed in anywhere (Ahmad, 1969). *Eupatorium odoratum* L. has acquired a reputation as a medicinal herb for a variety of ailments including malaria, fever, and the aqueous extract of the plant is used as antiseptic for wound dressing in traditional system, (Bose, 1973). *Eupatorium odoratum* L. has been made to establish the scientific validity to investigate the possible wound healing activity of different formulations such as ointments and gets made from the dried method extract of *Eupatorium odoratum* L. leaves in animal models. The part of leaves are used externally in traditional medicine as a wound healing, skin abscess, diuretic, cathartic, intermittent fever, ulcers, bilious, catarrh and influenza (Biswal,1997). According to the reported data, steroids, terpenoids and flavonoids were observed in this plant. This plant was so found that rich in flavonoids. There is no report concerning with investigation of flavonoid in this plant so far in Myanmar. Therefore, in this study the phytochemical constituents, isolation, identification and antioxidant activity of *Eupatorium odoratum* Linn. was conducted (Baruah ,1978).

Materials and Methods

Sample Collection

The leaves of *Eupatorium odoratum* L. were collected from Nwe Kway Ywa, Htauk Kyant Township, Yangon, Myanmar. After cleaning, the leaves were air-dried at room temperature for three weeks. Then they were ground into powder by grinder. The dried powdered samples were stored separately in air-tight containers to prevent moisture changes and other contamination. These plants were identified at the Department of Botany, University of Yangon.

Figure 1 The plant of *Eupatorium odoratum* L.

Phytochemical Examinations

Phytochemical tests for the selected indigenous medicinal plant *Eupatorium odoratum* L. was carried out to examine the presence or absence of organic compounds such as alkaloids, glycosides, carbohydrates, α-amino acids, steroids, flavonoids, phenolic compounds, saponins, terpenoids, tannins and cyanogenic glycosides (M-Tin Wa,1972). The preliminary phytochemicals tests were carried out according to the appropriate reported as the test tube method (Harbone,1984).

Preparation of Crude Extracts by Successive Extraction

The dried powdered leaves of *Eupatorium odoratum* L. sample (ca 300 g) were percolated in pet-ether (60-80 $^{\circ}$C) 1000 cm^3 with occassional shaking for one week and filtered. This procedure was repeated for three times. The combined filtrate was concentrated under vacuum rotatory evaporator to obtain pet-ether crude extract. Some of the pet-ether crude extract was decolourized by charcoal to give decolourized pet-ether crude extract.

The defatted residue was then extracted by percolation with 70 % EtOH (1000 cm^3) for one week and filtered. This procedure was repeated for three times. The total filtrate was concentrated under vacuum rotatory evaporator to obtain 70% EtOH crude extract. The ethanol crude extract was decolourized by charcoal to obtain the decolourized EtOH crude extract. The prepared extracts were kept for separation, isolation and screening of biological activities.

Separation and Isolation of Phytoconstituents from Active Decolourized Ethanol Extract of *Eupatorium odoratum* Linn.

Decolourized active 70% EtOH extracts (5 g) was partitioned between EtOAc and water. The EtOAc layer was concentrated under reduced pressure using vacuum rotatory evaporator. The ethyl acetate extract (3 g) was then separated by column chromatographic method eluting with PE:EtOAc (8:1 v/v) solvent system. On chromatographic separation, 340 fractions (3cm^3/ fraction) were collected. From the inspection of TLC chromatograms viewed over UV lamp, the fractions having the same appearance on TLC chromatograms were combined to give five fractions: F **I** (F_{51-121}), F**II** ($F_{122-265}$), F**III** ($F_{266-318}$), F**IV** ($F_{319-331}$) and F**V** ($F_{332-340}$). After removal of the solvents, fractions F **I** gave a colorless solid,

compound (**A**), and it was purified by recrystallization using PE/EtOAc to give a colorless crystal (0.05 g, 0.02%).

Characterization of Isolated Compound A from *Eupatorium odoratum* Linn.

One of the isolated compounds (**A**) from 70% EtOH extracts of Taw-bizat leaves was characterized by visualizing under UV-light, by determination of melting points, R_f values and some colour tests. The melting point was examined on a Gallenkamp melting point apparatus. The colour tests was carried out by spraying with 5% $FeCl_3$ solution, $K_4Fe(CN)_6$ solution, aqueous NaOH solution, conc: H_2SO_4 and by exposure to NH_3 vapour on precoated Aluminium TLC plate after developing in PE:EtOAc = 8:1. And R_f values were determined. In addition, their R_f values were also examined by using cellulose plates developed with HOAc solvent system. In addition, the physico-chemical properties of isolated compounds **A** was tabulated in Tables **1** and **2**.

Identification of Isolated Compound A from *E. odoratum* Linn.

Compound **A** was identified by modern spectroscopic techniques such as UV-Visible, FT-IR, ^{1}HNMR, NOE and EI-MS spectroscopy and also by comparing with reported spectral data.

UV-Visible Spectroscopy

The UV spectra of the isolated compound **1** was recorded on a Shimadzu UV-240 UV-Visible spectrophotometer at URC, Yangon. Spectra were recorded directly on methanolic solutions immediately. A small amount of the isolated compound was dissolved in methanol and this solution was introduced into a silica cuvette of 3 cm^3capacity and the UV spectrum was taken against methanol blank. For the flavonoids compound **1,** other shift reagents than NaOH namely $AlCl_3$ and $AlCl_3$/HCl, NaOAc and NaOAc/H_3BO_3 were also added before the respective UV-Visible spectra were recorded.

FT-IR Spectroscopy

The infrared spectra of isolated compounds were as recorded on a Perkin Elmer Spectrum GX FT-IR spectrophotometer at URC, Yangon. The isolated compound was sampled as a 1% KBr pellet.

^{1}H NMR Spectroscopy

The ^{1}HNMR spectra of isolated compound were recorded in $CDCl_3$ with TMS as internal standard at the Department of Chemistry, Kanazawa University, Japan.

Mass Spectrometry

Electron impact mass spectra (EI-MS) and Electrospray ionization mass spectra (ESI-MS) of the isolated flavonoids were recorded on a JEOL SX-102, a mass spectrometer at the Department of Chemistry, Kanazawa University, Japan.

Antioxidant Activity of *Eupatorium odoratum* L.

Antioxidant activity of 95 % ethanol, ethanol extracts and isolated compound **A** were carried out by DPPH (1,1-Diphenyl, 2-Picryl, Hydrazyl) radical scavenging as using UV visible spectrophotometer. Then IC_{50} (50 % oxidative inhibitory concentration) values were also calculated by linear regressive excel program (Kahlonene, 1999).

Results and Discussion

Phytochemical Tests

In order to know the types of phytoorganic constituents present in the dried leaves powdered samples, the phytochemical investigation was preliminarily carried out according to the conventional methods. The phytochemical tests revealed that the secondary metabolites such as terpenoids, steroids, phenolic compounds, tannins, α- amino acids, flavonoids carbohydrates, glycosides, saponins and alkaloids were present but cyanogenic glycosides was not found in this sample. In addition, were found to be present only in Taw-bizat leaves. From this observation it can be inferred that Taw-bizat may generally possess a wide range of biological activities such as antioxidant, antimicrobial, enzyme inhibition, genotoxic, allergenic, antitumor, anticancer, anti-inflammatory, hemolytic and estrogenic activities due to the presence of the above secondary metabolites, (Ahmad, 1969).

Preparation of Different Crude Extracts

After carrying out preliminary phytochemical tests, in order to screening the antioxidant activity and to isolate the phytoorganic compounds from the non-polar and polar crude extracts, pet-ether extracts and defatted 70% EtOH extracts for all samples were firstly prepared according to the procedure depicted in Figure **2**, including the decolourized crude extracts obtained after treating with charcoal. Here in, the decolourization of crude extracts using charcoal was carried out due to the fact that since all samples tested were being leaves which contain a lot of chlorophylls. That can disturb the separation of crude extract. In the case of 70% EtOH decolourized crude extract, ethyl acetate soluble portion of decolourized 70% EtOH crude extract was first prepared by solvent extraction between ethyl acetate and water. Solvent extraction should be done before column chromatographic separation so as to manipulate easily.

Isolation of Compound A from *Eupatorium odoratum* L.

To isolate the active compound from decolourized ethanol extract, was fractionated by silica gel column chromatographic method using PE:AcOEt (8:1) as eluent. 7 cm^3 of each fraction was collected and checked on TLC using appropriate solvent system. Fractions 51-121 gave similar TLC pattern and therefore they were combined. The combined fraction was concentrated to 3 cm^3 and left overnight. The solid was precipitated out and recrystallized from PE/EtOAc to provide colourless crystal (0.05g, 0.02%) and was denoted as compound **A**.

Characterization of Isolated Compound A from *Eupatorium odoratum* L.

The yield percents of isolated compounds **A** are summarized in Table **1**. The physico-chemical properties of compounds **A** are summarized in Table **2**. According to the characters of the compounds **1** appeared on TLC chromatograms when viewed over longwave UV light (365 nm) and, treated with NH_3 vapour and with 5% $FeCl_3$, all of these compounds gave the same characters of flavonoids. Therefore the compounds **1** may be flavonoids. However, compounds **A,** was found to give pink colour with Mg/ HCl.

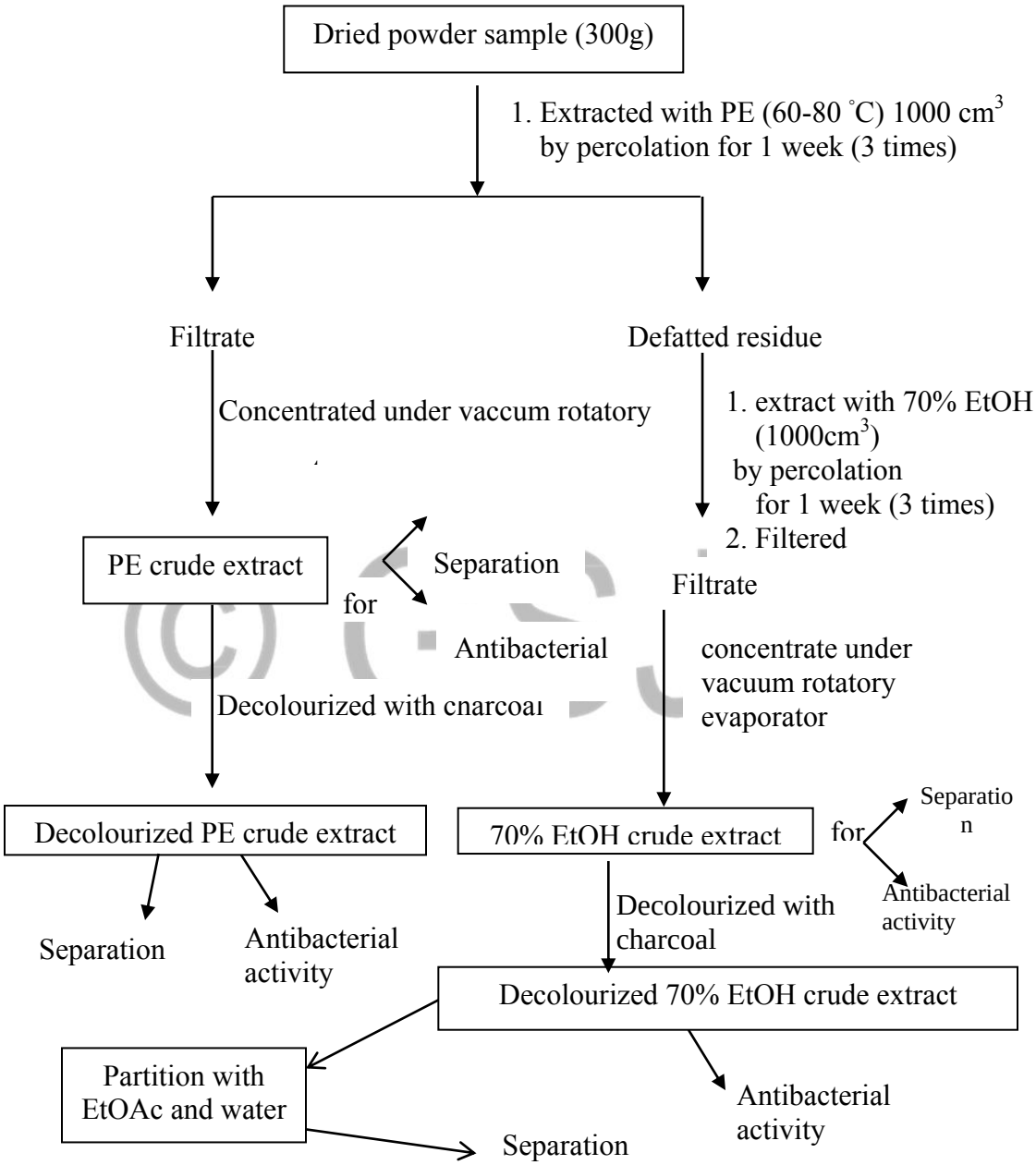


Figure 2 Flow diagram for the preparation of crude extracts

Table 1 Yield Percents, R_f Values and Melting Points of Isolated Compounds A from 70% Ethanol Extract

Isolated Compounds	Yield (%)	R_f	mp / ºC	Appearance
A	0.02	0.33 (PE:AcOEt, 8:1)	195-196 (PE/AcOEt)	Colourless crystal

Table 2 Some Physico-Chemical Properties of Isolated Compound A

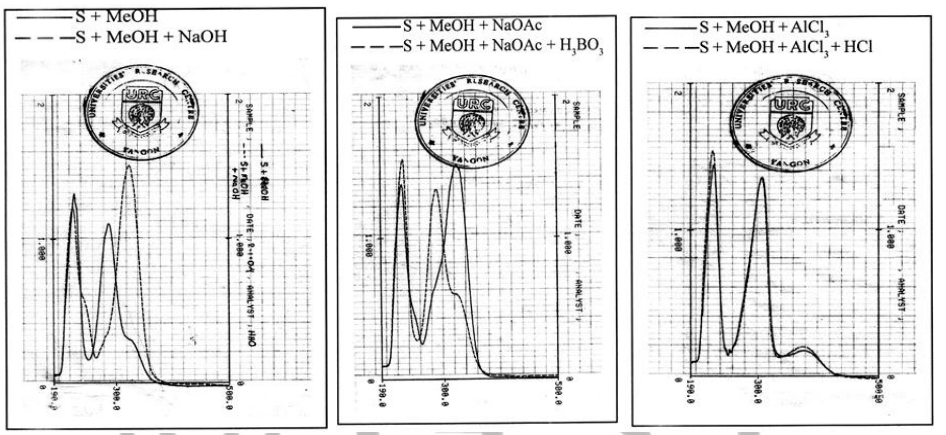
Isolated compound	UV(365 nm)	5%$FeCl_3$	NH_3(TLC)UV	$K_3Fe(CN)_6$TLC	Mg/HCl	Aqueous NaOH	Conc H_2SO_4	R_f (HOAC)
A	deep purple	brownish	No change	brownish	pink	orange	orange	0.33

Identification of Compound A Isolated from Ethanol Extract of *Eupatorium odoratum* L.

Isolated compound A from ethanol extract was observed as a deep purple spot under UV (365 nm) at R_f of 0.33 on cellulose plate with HOAc solvent system indicating that compound **1** may be isoflavone or flavanone (Markham, 1982). In addition no color change occurred after exposure to NH_3 under UV (365 nm) (Table **3**). This observation also confirmed that compound **A** may be an isoflavone or flavanone with 5-OH (Mabry, 1970).

The structure of isolated flavonoid **A** was then analyzed by six UV-Visible spectra taken by adding with diagnostic reagents such as NaOMe, NaOAc, NaOAc / H_3BO_3, $AlCl_3$, $AlCl_3$ / HCl in methanol. The UV-Visible spectrum taken in MeOH [Figure **3** (**a**)] and Table **3** exhibited a major absorption peak at λ_{max} of 290 nm and a low intensity peak at λ_{max} of 320 nm,

indicating that compound **A** may be a flavanone or dihydroflavonol which gave and intense peak (Band II) with a shoulder (Band I) in the range of 270-295 nm and 300-400 nm respectively (Markham, 1982). According to the observation from R_f and TLC behaviour under longwave UV light, it can be informed that compound **1** may be a flavanone compound. The presence of NaOMe changed the main absorption band (Band II) with increasing intensity. This compound **A** may therefore possess OH groups at 5 and 7 positions of A ring (Markham, 1970).



(a) **(b)** **(c)**

Figure 3. UV-Visible of Spectrum of Compound A from 70 % ethanol extract

(a) MeOH, NaOH/MeOH
(b) NaOAc / MeOH
NaOAc / H_3BO_3/ MeOH
(c) $AlCl_3$ / MeOH
$AlCl_3$ / HCl / MeOH

Table 1. UV-Visible Spectral Data of Compound A

No	Solvents & Additives	λ_{max}/nm
1	MeOH	230 (II) (Band II),327(sh) (Band I)
2	MeOH + NaOH	225, 250 (sh), 280 (sh), 325 (II)
3	MeOH + NaOAc	230, 285 (sh), 328 (II)
4	MeOH + NaOAc + H_3BO_3	230, 290 (II), 330 (sh) (I)
5	MeOH + $AlCl_3$	230, 310 (II), 380 (I)
6	MeOH + $AlCl_3$ + HCl	228, 310 (II), 375 (I)

In addition, 38 nm bathochromic shift of Band II in NaOAc and MeOH [Figure **3** (**b**)] also confirmed that compound **A** possesses 5-OH and 7-OH groups. As Band II in MeOH was not shifted by adding NaOAc/ H_3BO_3 that there is no ortho-hydroxy groups in A ring. Both $AlCl_3$ and $AlCl_3$ / HCl reagents cause a 20 nm bathochromic shift of Band I [Figure **3**(**c**)] indicating the presence of 5-OH. According to the UV spectral analysis, R_f value and TLC spot appearance, the isolated compound **A** may be 5,7-dihydroxy flavanone.

Assignment of FT-IR spectrum of compound **A** (Figure **4** and Table **4**) exhibited the presence of functional groups: OH (3422 cm^{-1}), aromatic ring (3010, 1459, 1162, 1088, 874, 832 and 713 cm^{-1}), OMe group (2929, 2850 cm^{-1}) and =C-O-C (1253, 1025 cm^{-1}). Therefore, compound **2** may be methoxylated 5, 7- dihydroxy flavanone.

From the ^{1}HNMR spectroscopic determination, it was observed that (Figure **5** and Table **5**) there were 14 protons including one methoxy group appeared as a singlet at chemical shift 3.38 ppm and two hydroxyl groups appeared at 10.25 ppm for 7-OH and at 12.0 ppm for 5-OH respectively. 5-

OH proton as a singlet appeared at downfield due to H-bonding with carbonyl oxygen. The signal at 5.98 ppm and 5.99 ppm appeared as doublets with coupling constant 2.2 Hz were assigned due to H-6 and H-8 protons from A ring respectively. Two double doublets splitting with coupling constants (3.2 Hz, 17.2 Hz) and 12.8 Hz, 17.2 Hz) were assigned due to two H-3 protons, i.e H-3 (cis) and H-3 (trans) at 2.74 ppm and 3.08 ppm respectively.

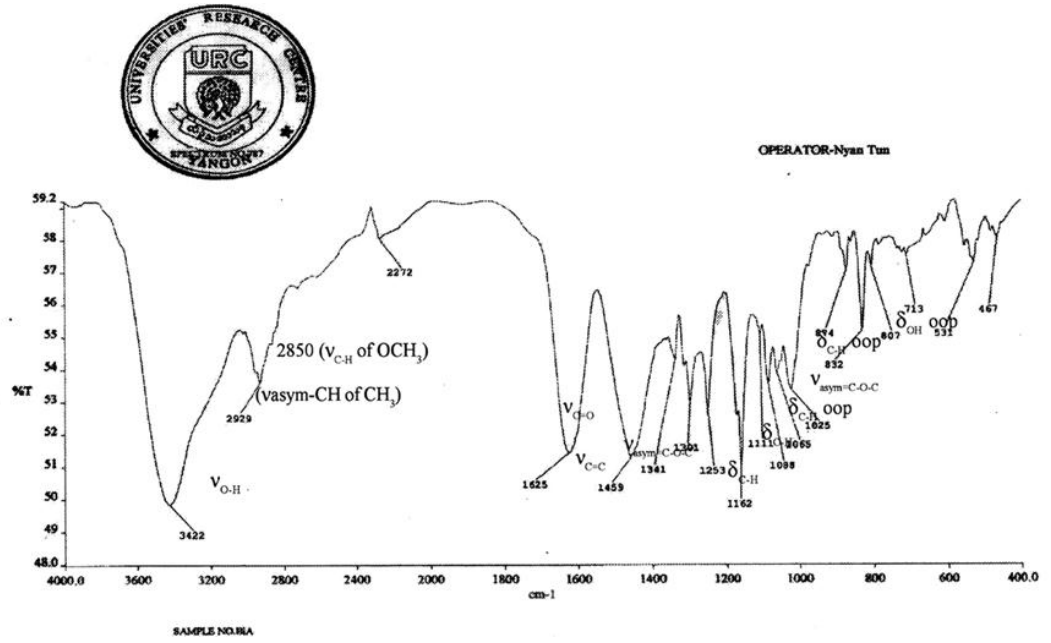


Figure 4. FT-IR spectrum of isolated compound A (KBr)

Table 4. FT-IR Spectral Data of Isolated Compound A

Wave Number (cm^{-1})	Assignment
3422	OH stretching vibration
3010	aromatic ν_{C-H}
2929	ν_{C-H} of CH_3 (asymmetric)
2850	ν_{C-H} of the OCH_3 gp
1625	ν_{C-H} (α,β-unsaturated carbonyl group)
1459	$\nu_{C=C}$ (aromatic)
1162	δ_{C-H}(in plane) of aromatic
1088	δ_{C-H}(in plane) of aromatic
1253	$\nu_{=C-O-C}$ (antisymetric)
1025	$\nu_{=C-O-C}$ (symmetric)
874	$\delta_{C-H(oop)}$ isolated H of aromatic
832	$\delta_{C-H(oop)}$ adjacent H
713	δ_{O-H}(oop) phenol deformation

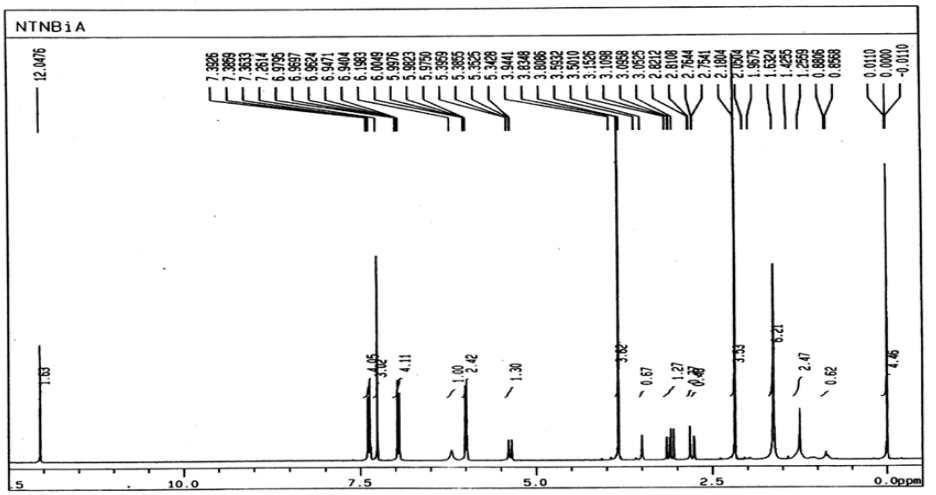


Figure 5. 1HNMR spectrum ($CDCl_3$, 400MHz) of isolated compound A

Table 5. ^{1}HNMR ($CDCl_3$, 400 MHz) Spectral Data of Compound A

δ/ppm (Chemical shifts)	**Multiplicity**	**Remark**
2.74	dd, (J = 3.2 Hz,17.2 Hz)	H–3 (cis) (1H)
3.08	dd, (J=12.8Hz, 17.2 Hz)	H–3 (trans) (1H)
3.83	s	O–CH_3 (3H)
5.37	dd, (J =3.2 Hz,12.8 Hz)	H–2 (1H)
5.98	d (J =2.2 Hz)	H–6
5.99	d (J =2.2 Hz)	H–8
6.95	d, (J = 8.8 Hz)	H – 3′& H – 5′ (2H)
7.39	d, (J = 8.8 Hz)	H – 2′& H – 6′ (2H)
10.25	s (br)	7 – OH (1H)
12.0	s	5 – OH (1H)

Total Protons = 14

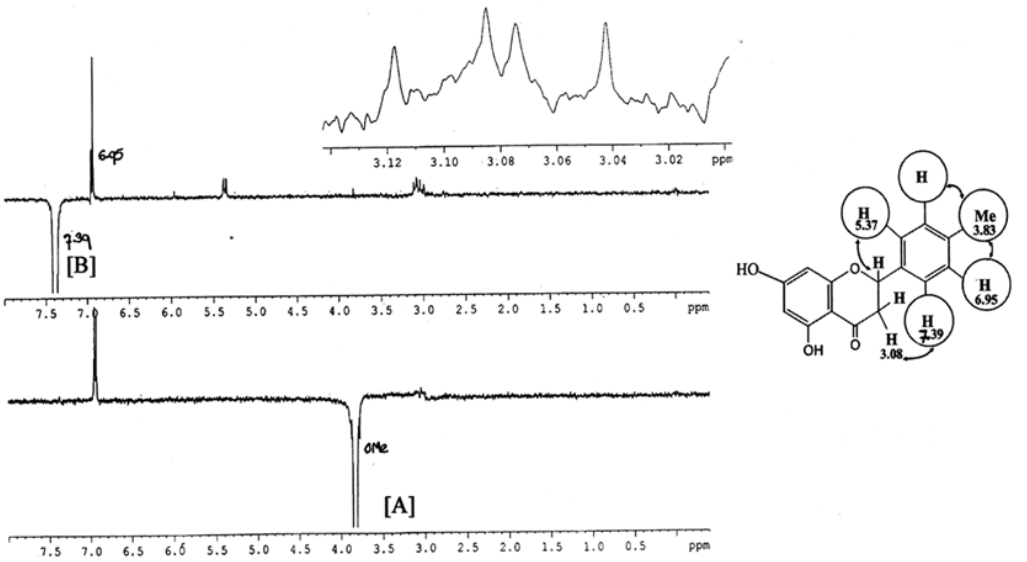


Figure 6. NOE difference spectra of compound **A**
A shows irradiation of (H-2' & H -6') protons 7.39 ppm
B shows irradiation of (H-3' & H -5') protons 6.95 ppm

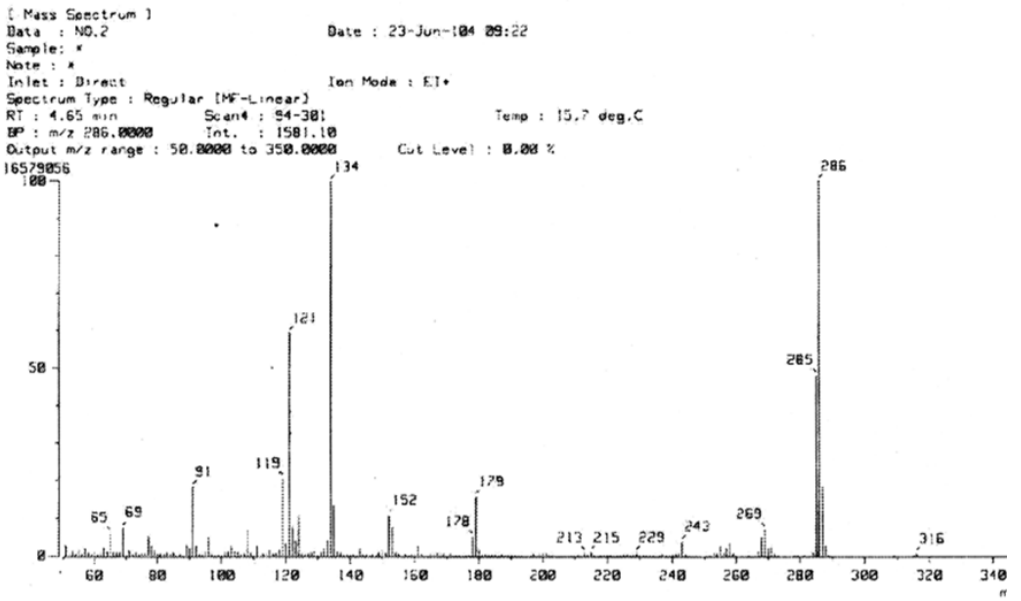


Figure 7. EI-MS spectrum of isolated compound **A**

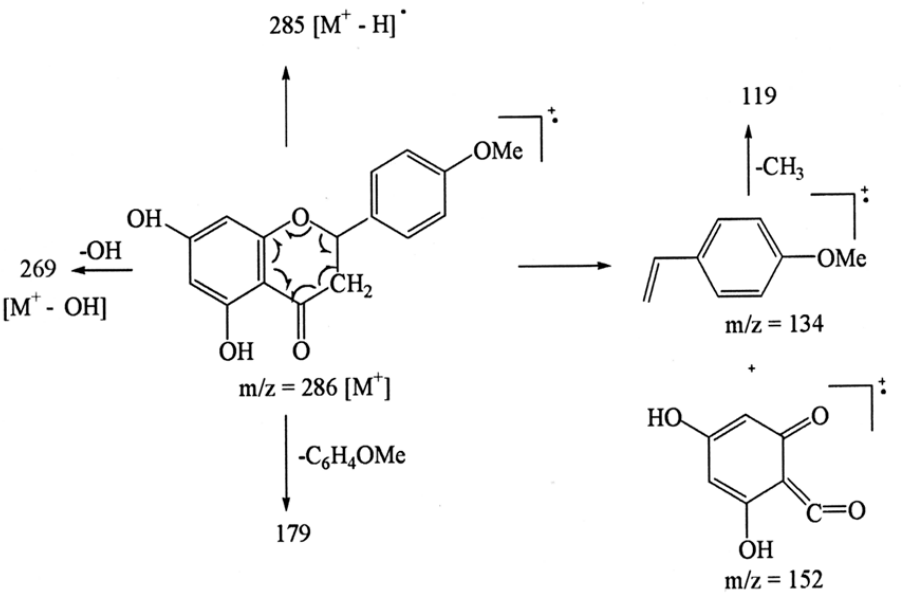


Figure 8. The fragmentation pattern of compound A

Again, the doublet peak (5.73 ppm) with coupling constants (12.8, 3.2 Hz) was assigned as splitting due to one H-2 protons. The correlation with H-2,H-3 (trans) and H-3 (cis) was also studied by NOE experiment (Figure **6**). Irradiation of proton at 5.37 ppm was found to enhance the intensity of the signals of both protons at 2.74 ppm and 3.08 ppm. Two doublet peaks at 6.95 ppm and 7.39 ppm splitting with coupling constants 8.8 Hz represented respectively (H–3′& H-5′) and (H-2′ & H-6′) protons of para substituted B-ring. The positions of protons were also confirmed by NOE spectra as shown in Figure **6**.

Irradiation of methoxy protons at 3.83 ppm enhanced only the intensity of the signal at 6.95 ppm (A). In addition, NOE enhancement by irradiation of protons of 7.39 ppm occurred at 3.08 ppm, 5.37 ppm and 6.95 ppm (B). These observations indicated that the signal at 6.95 ppm must correspond to (H-3′ & H-5′) protons, that at 7.39 ppm to (H-2′ & H-6′) protons, that at 3.83 ppm to 4′-methoxy protons, that at 3.08 ppm to H-3(trans) and, that at 5.37 ppm to H-2 proton. Therefore, the complete structure of isolated compound **A** was assigned as 4′-methoxy -5, 7-dihydroxy flavanone.

Identification of isolated compound **2** was also studied by EI-MS spectrum (Figure **7**). The molecular ion [M^+] peak occurred at m/z = 286 indicated that the molecular formula of compound1 is $C_{16}H_{14}O_5$. The structure of compound **A** could also be confirmed by the following fragmentation pattern depicted in Figure **8**.

Antioxidant Activity of *Eupatorium odoratum* L.

The antioxidant activity was expressed as 50% oxidative inhibitory concentration (IC_{50}). The lower the IC_{50} values, the higher the antioxidant activity of the sample. By using DPPH free radical scavenging assay, the compound A was more potent antioxidant activity than 95% ethanol and watery extracts. The results of antioxidant activity are shown in Table 6 and Figures 9 and 10.

Table 6. Oxidative Percent Inhibitions and IC_{50} Values of Crude Extract and Isolated Compound A of *Eupatorium odoratum* L. and Standard BHT

Sample	% Inhibitions (Mean ± SD) in various Concentrations (µg/ml)								IC_{50} (µg/ml)
	3.125	6.25	12.5	25	50	100	200	400	
95 % EtOH	38.095 ± 3.863	36.395 ± 2.567	30.272 ± 7.167	27.347 ± 18.395	37.551 ± 4.081	54.762 ± 4.248	52.381 ± 5.802	59.372 ± 15.587	56.14
Watery	31.748 ± 0.601	35.034 ± 0.601	41.497 ± 1.202	64.966 ± 0.601	80.952 ± 0.601	94.217 ± 1.202	93.197 ± 0.601	93.061 ± 1.323	24.09
Compound 1	15.646 ± 0.601	29.232 ± 0.601	53.334 ± 1.202	55.799 ± 0.601	62.148 ± 0.601	69.247 ± 0.601	72.135 ± 0.601	79.428 ± 0.531	13.07
Standard BHT	43.301 ± 1.40	53.582 ± 2.49	65.53 ± 1.132	74.82 ± 0.621	83.321 ± 0.782	87.412 ± 2.372	91.516 ± 1.113	94.702 ± 0.692	3.16

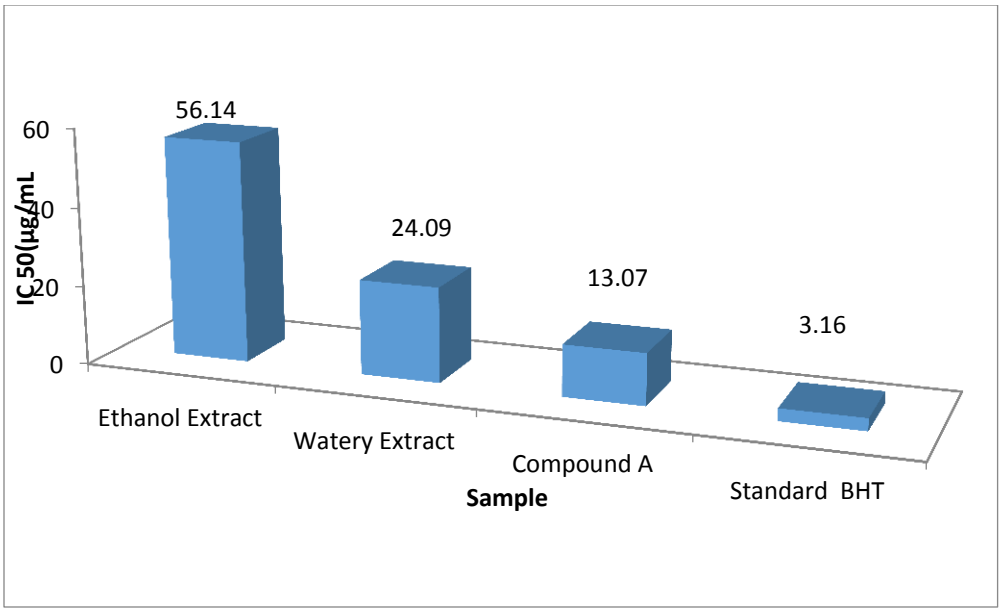


Figure 9. A bar graph of IC_{50} (µg/ml) values of different concentration of watery, EtOH extracts and isolated compound A from *Eupatorium odoratum* L.

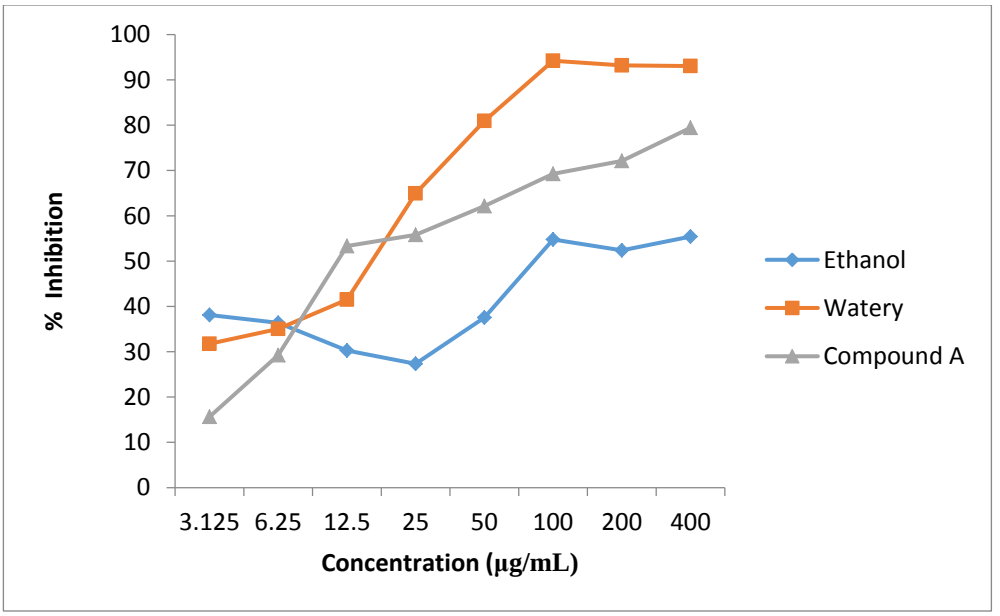


Figure 10. Percent inhibition of different concentration of watery, EtOH extracts and isolated compound A from *Eupatorium odoratum* L.

Conclusion

The overall assessments of the research work, the preliminary phytochemical investigation indicated that alkaloids, α-amino acid, carbohydrates, glycosides, flavonoids, phenolic compounds, saponins, steroids, tannins and terpenoids were present in *Eupatorium odoratum* L. Among them, cyanogenic glycosides was not detected in *Eupatorium odoratum* L. In order to find out the bioactive organic constituents from active decolourized ethanol extract, silica gel column chromatographic separation using PE/ EtOAc solvent system with various ratio were carried out. One of the bioflavonoid compounds **A**: 4´- methoxy - 5, 7, dihydroxy flavanone (0.02%, R_f = 0.33, mp = 195-196 °C) was totally isolated from *Eupatorium odoratum* L. by column chromatographic separation. This compound was identified structurally by modern spectroscopy:UV, FTIR, ^{1}HNMR, NOE and EI-MS. From the determination of antioxidant activity, the IC_{50} value of compound **1 (** 13.07 of μg/mL**)** was more potent activity than the remaining watery and 95% ethanol extract. Consequently, it can be deduced that *Eupatorium odoratum* L. can be contributed in the areas of diseases related to oxidizing actions and effective drug in Myanmar Traditional medicine.

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