ISOLATION AND IDENTIFICATION OF SOME PHYTOCONSTITUENTS FROM LEAVES OF *MORUS ALBA* L. AND SCREENING OF ANTIOXIDANT ACTIVITY

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Abstract

This research work deals with investigation of some bioactive phytoconstituents of *Morus alba* L. (Po-sa) plant collected from Pyin-Oo Lwin Township, Mandalay Region. By silica gel column chromatographic separation technique, three compounds were isolated from selected plant. Umbelliferone (**1**,0.0045%) Scopoletin (**2**, 0.0009 %)m.pt 201ºC) and protocatechuic acid (**3,**0.0036 %) from ethyl acetate extract of Po-sa leaves. The isolated compounds were identified by physic-chemical properties and modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR and EI MS spectrometry as well as by comparing with their reported data. Antioxidant activity of Po-sa (leaves) was also investigated by using DPPH (1, 1'-diphenyl-2-picrylhydrazyl) radical scavenging assay. The IC_{50} values of EtOAc, EtOH and watery extracts of leaves were 2.39 µg/mL, 2.11 µg/mL and 2.15 µg/ml respectively. The EtOH and watery extracts of leaves showed more radical scavenging activity than EtOAc extract. In addition, Umbelliferone, scopoletin and protocatechuic acid isolated from Po-sa (leaves) also showed radical scavenging activity determined by using semi quantitative DPPH staining method.

Keywords: Umbelliferone, Scopoletin, Protocatechuic acid, Antioxidant activity

Introduction

Morus alba is a plant species that belongs to *Moraceae* family and high economic worldwide (Donno *et al.,* 2015). Its fruits are source of alkaloids, anthocyanins, flavonoids and phenolic acids, being used as human food while its foliage contains unique nutrients for silkworm development (Butt *et al.,* 2008). This plant species is native to Asia, but due to its wide adaptation in the tropics(Europe, north and south America, Africa and India it has been considered cosmopolitan nowadays (Khan *et al*., 2013).Sericulture was the main process that drove the improvement of *Morus alba* species leading to the development of varieties that represents valuable plant genetics resources. Leaves are a good source of ascorbic acid and rich in calcium (Wealth of India, 1962). Phenolic compounds are secondary metabolites that protect plants against damage from pathogens and environmental stress. The consumption of these substances has been correlated with minimizing damaging effects of uncontrolled free radicals production, duetothei, antioxidanta ctivity (Yangthong *et al*., 2009). Mechanisms of antioxidant action are very complex, including electrons donation, which act accelerating lipids oxidation (Huang *et al*., 2005). Other mechanisms include hydrogen donation to reduce reactive oxygen species (Villaño *et al*., 2007) related to alterations in DNA and cancer. Phenolic compounds concentration and, consequently, antioxidant activity of any plant is greatly affected by environmental conditions, storage, geographicregion, varietyand the part of plant used in the biological evaluation Stem possesses antirheumatic, antispasmodic, diuretic, hypotensive and pectoral activities. They are used in the treatment of rheumatic pains and spasms, especially of the upper half of the body, high blood pressure. A tincture of the bark is used to relieve

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toothache. The branches are harvested in late spring or early summer and are dried for later use (Kim *et al*., 1999).A fiber is obtained from one-year old stem, it is used in weaving clothes etc. The stem bark is fibrous and is used in China and Europe for paper making.

Materials and Methods

Plant Material

Po-sa (Leaves) was collected from Pyin Oo Lwin Township, Mandalay Region. After collection, the scientific names of *Morus alba* L. were identified by the authorized botanists at Botany Department, University of Yangon. The sample was washed with water and dried at room temperature. The dried samples were ground to get a fine powder. The dried powder were then stored in an air-tight container.

Preparation of Crude extracts

The powdered sample (700 g) of Po-sa (Leaves) was extracted with 80 % EtOH. The filtrate was evaporated to get 25 g of crude extract. And then partitioned with PE, 5 g of PE extract was obtained. After that partitioned with EtOAc , 8 g of EtOAc crude extracts was obtained.

Isolation of Compounds from Po-sa (Leaves)

The 95 % ethanol extract was extracted with pet-ether (60 °C - 80 °C), the soluble matter of pet-ether was obtained. The defatted alcohol soluble portion was then partitioned between ethyl acetate and water by using separatory funnel. After removal of the solvent from organic layer, ethyl acetate soluble extract was obtained. The ethyl acetate extract (8 g) was separated by column chromatographic separation techniques Gradient elution was performed successively using PE:EtOAc in the ratios of 5 : 1,3:1 and 1:1 v/v. From this separation, three main fractions F_1 to F_3 were collected. From the condensed fraction F_2, compound **1** (0.005 g, 0.0045 %) and **2** (0.0015 g, 0.0009 %) were collected by paper chromatography with the solvent of formic/A: H_2O (2:98).The last fraction F_3 was purified by paper chromatography with formic/A: H_2O(1:99) solvent system, compound **3** (0.003 g, 0.0036 %) were obtained. The isolated compounds were characterized by melting point determination and TLC, UV, FT IR, ^{1}H NMR and mass spectroscopic methods.

Antioxidant Activity Test

For the examination of *in vitro* antioxidant activity of Po-sa (leaves), DPPH staining method and spectrophotometric method were used. In DPPH staining method, for each diluted sample was applied as a dot on a TLC plate followed by staining solution. The appearance of white coloured spots has a potential value of antioxidant activity (Soler-Rivas *et al.*, 2000). In spectrophotometric method, the sample solutions were measured by using spectrophotometer (Marinova and Batchvarov, 2011).

Results and Discussion

Identification of the Isolated Compounds

Compounds 1 (5 mg, 0.0045 %), compound 2 (1.5 mg, 0.0009 %) and compound 3 (3 mg, 0.0036 %) were characterized by physical and chemical methods and then identified by spectroscopic techniques.

Compound 1 (Umbelliferone): UVλ_{max}^{MeOH}nm; 220, 329; UV$\lambda_{max}^{MeOH+NaOH}$nm: 221,363 (Figure 1). FT IR υ_{max}^{KBr} cm^{-1}: 3398 (υ_{O-H}), 1705 ($\upsilon_{C=O}$), 1616-1562 ($\upsilon_{C=C}$), 1234-1053 ($\upsilon_{asy-C-O}$), 840 ($\delta_{oop-C-H}$) (Figure 2). ^{1}H NMR δ (ppm) 6.2 (3H, d, J=9 Hz), 8.0 (4H, d, J=9Hz), 7.3 (5H, d, J=8Hz), 6.9 (6H, dd, J=8 Hz), 6.8 (8H, d, J=1 Hz) (Figure 3). MS m/z; 161 (M-H), 133.1, 92.9 (Figure 4). All of these chemical shifts are very similar to those in the spectrum of umbelliferone (Harborne, 1989; Mya Thandar Aung, 2007).

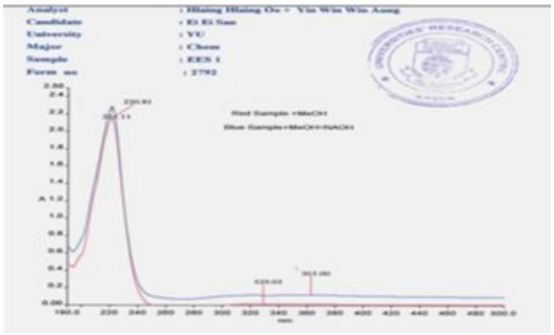


Figure 1 UV spectrum of the isolated compound1 (Solvent: MeOH and MeOH+ NaOH)

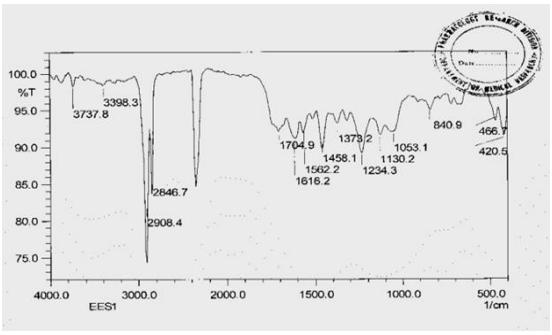


Figure 2 FTIR spectrum of isolated the compound 1(KBr)

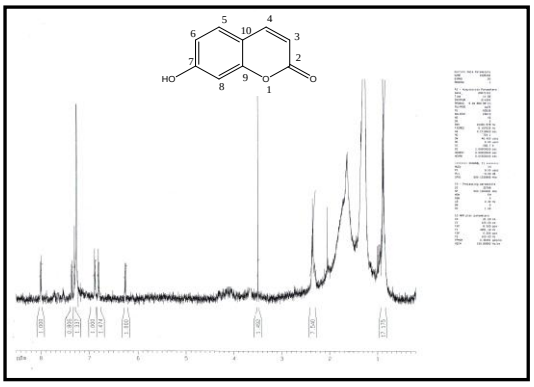


Figure 3 ^{1}H NMR spectrum of the isolated compound 1

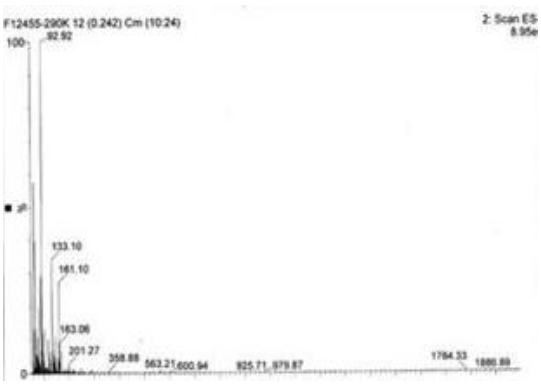


Figure 4 ESI-mass spectrum of the isolated compound 1

Compound 2 (Scopoletin): Colourless crystal, m.p. 201 °C. UV λ_{max}^{MeOH}nm: 215, 344;UV $\lambda_{max}^{MeOH+NaOH}$ nm: 218, 390 (Figure 5). FT IR $\upsilon_{max}^{KBr}$$cm^{-1}$: 3325 ($\upsilon_{O-H}$), 3055 ($\upsilon_{C-H}$ of C=CH), 2920 (υ_{C-H} of $-CH_3$, CH_2), 2850 (υ_{C-H} of $-OCH_3$), 1705 ($\upsilon_{C=O}$) of δ lactone, 1604($\upsilon_{C=C}$ of aromatic ring) (Figure 6). ^{1}H NMR δ(ppm): 6.2 (3H, d, J = 9 Hz), 7.6 (4H, d, J= 9Hz), 6.8 (5H, S), 6.9 (8H, S), 3.7 (6H-OMe, S) (Figure 7). All these spectral data of compound 2 were agree with the reported data of scopoletin(Merck Index, 2001; Harborne, 1993; Khin Tar Yar Myint, 2010)

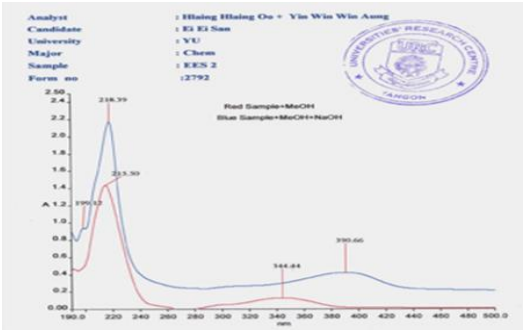


Figure 5 UV spectrum of isolated the compound 2 (Solvent MeOH and MeOH+NaOH)

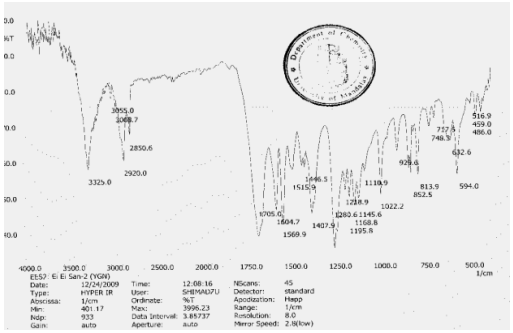


Figure 6 FTIR spectrum of the isolated compound 2 (KBr)

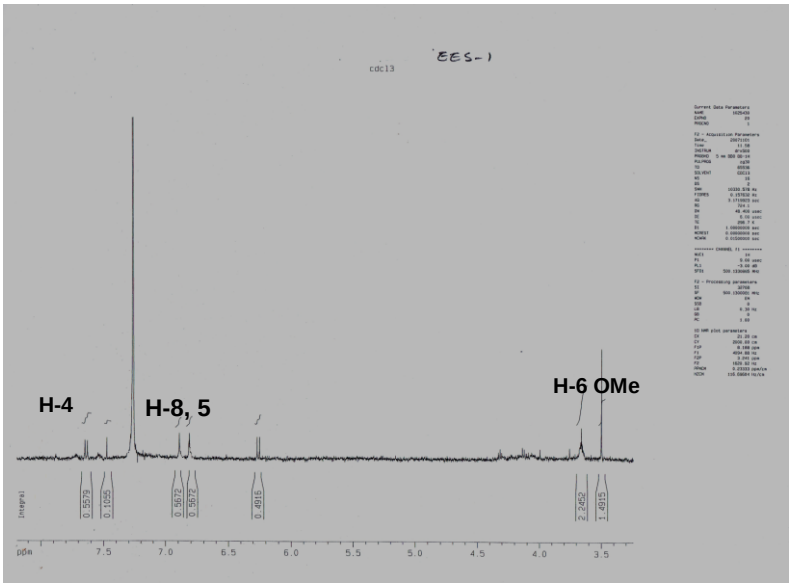
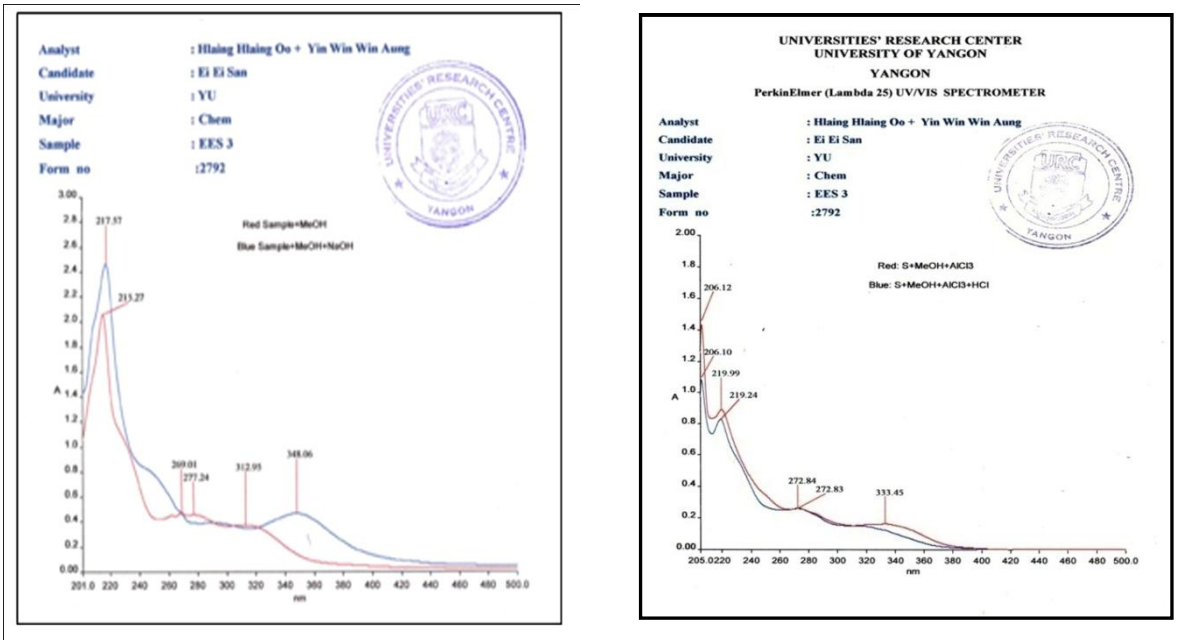


Figure 7 ^{1}H NMR spectrum of isolated compound 2

Compound 3 (Protocatechuic Acid):(UV λ_{max}^{MeOH}nm; 313, UV$\lambda_{max}^{MeOH+NaOH}$nm: 348,UV λ_{max}^{AlCl3}nm; 333, UV $\lambda_{max}^{AlCl3+HCl}$nm: 313) (Figure 8). FT IR υ_{max}^{KBr}cm^{-1}: 3028 (υ_{O-H}), 2923 (υ_{C-H} of C=CH), 1685 ($\upsilon_{C=O}$) 1596 ($\upsilon_{C=C}$ of aromatic ring) (Figure 9). ^{1}H NMRδ(ppm): 7.34 (2H, d, J = 3 Hz), 6.97 (5H, d, J= 9Hz), 7.04 (6H, dd, J= 3,9 Hz) (Figure 10).The proton signals of compound 3 was reported protocatechuic acid (Myint Myint Khine,2006).



(a) MeOH (red) and MeOH/ NaOH (blue) (b) $AlCl_3$ (red) and $AlCl_3$/HCl (blue)

Figure 8 UV spectra of isolated compound 3

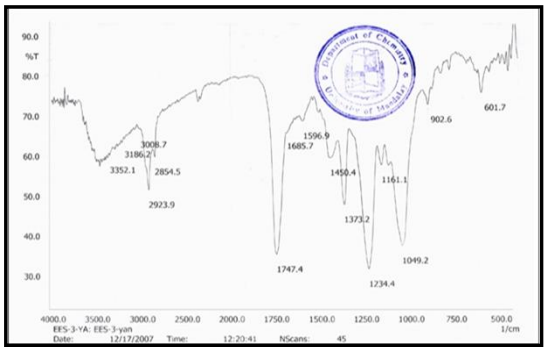


Figure 9 FT IR spectrum of isolated compound 3

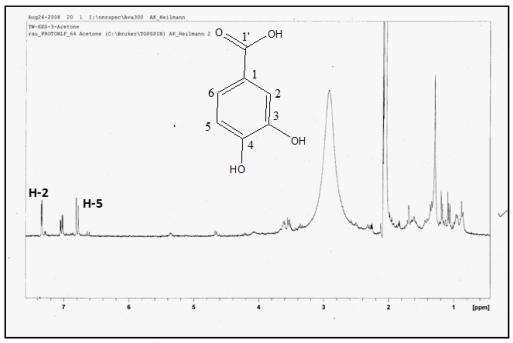


Figure 10 ^{1}H NMR spectrum of isolated compound 3

Rapid Screening of Antioxidant Activity of Two Isolated Compounds by DPPH staining method

It was observed that compounds 1, 2 and 3 from *Morus alba* L. (leaves) showed antioxidant activity on the TLC plates. After staining, white spots with strong intensity was found by staining with the amount of (6.25-200 µg) of dry matter for compounds (Figure 11). The intensity of white colour depends upon the amount and nature of radical scavenger present in the sample.

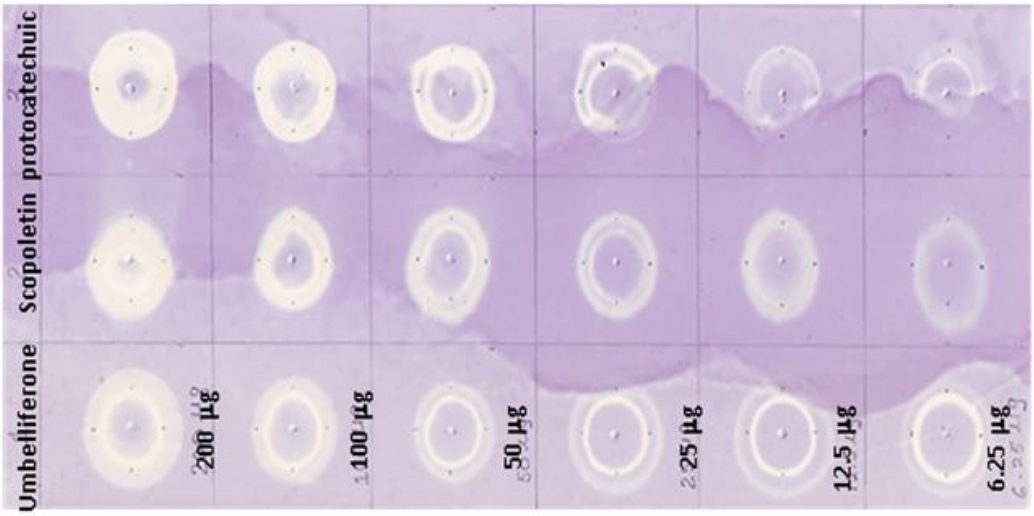


Figure 11 Screening of antioxidant activity of the isolated compounds from Po-sa (leaves) by DPPH Dot-Blot assay

DPPH Radical Scavenging Activity by Spectrophotometric Method

The antioxidant activity of EtOAc, 80%EtOH and H_2O extracts was tested according to DPPH radical scavenging activity assay by using spectrophotometric method. The H_2O extract was found to be more potent than the other extracts compared with standard BHT (Table1). From these results, increase in concentration showed increase in percent inhibition, i.e. increase free radical scavenging activity. The lower IC_{50} value indicates the greater antioxidant activity.

Table 1 Oxidative Inhibition % in Various Concentrations and IC_{50} Values of Extracts of Po-sa (Leaves)

Extracts	% Inhibition in various concentrations (µg/mL)					IC_{50} (µg/mL)
	0.625	1.25	2.5	5	10	
Po-sa -EtOH (Leaves)	21.70	40.42	50.97	57.15	69.03	2.39
Po-sa-H_2O (Leaves)	18.97	32.24	58.16	63.61	68.91	2.11
Po-sa-EtOAc (Leaves)	37.94	44.79	52.06	64.85	76.12	2.15
BHT	14.04	54.82	74.22	77.13	87.40	1.17

BHT = Butylated Hydroxy Toluene

Conclusion

On silica gel column chromatographic separation, three compounds were isolated from ethyl acetate extract of the Po-sa leaves. Umbelliferone (0.0045 %), scopoletin (0.0009 %) and protocatechuic acid (0.0036 %) were obtained from ethyl acetate extract of Po-sa (leaves) by column chromatography on silica gel using PE:EA (1:1). In antioxidant activity, ethanol extract (IC_{50}=2.11 µg/mL) and isolated compound ,protocatechuic acid (6.25-200 µg) were found to be more potent than the other extracts and other isolated compounds. The findings of the present study suggest that Po-sa leaves is a potential source of natural antioxidant that could have great importance as therapeutic agents or in slowing down of aging and its related degenerative diseases.

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