A STUDY OF SOME BIOACTIVITIES AND ISOLATION OF PURE ORGANIC COMPOUNDS FROM THE BARK OF *DOCYNIA INDICA* (WALL.) DECNE (PIN-SEIN)

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Abstract

In the research work, the bark of *Docynia indica* (Wall.) Decne (Pin-Sein) was collected from Pang Wa Village, Loi Mwe Township, Shan State. Phytochemical constituents present in the bark of Pin-Sein were investigated according to the general methods. Two kinds of nutritional values (moisture and ash) of Pin-Sein bark were determined by oven dry method and AOAC method. In addition, the elemental contents in this sample were determined by EDXRF. The antimicrobial activities of the various crude extracts were tested by agar well diffusion method on six selected microorganisms (*Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Bacillus pumilus, Candida albicans* and *Escherichia coli*). Moreover, antioxidant activity of crude extract of Pin-Sein bark was measured by DPPH free radical scavenging assay method. The pure organic compounds, TMK-1 and TMK-2 were isolated from the bark of Pin-Sein by thin layer and column chromatographic techniques. Finally, these isolated compounds were identified by phytochemical tests and their respective melting point. The functional groups of pure organic compounds were identified by FT IR spectral data.

Keywords: *Docynia indica* (Wall.) Decne, phytochemical constituents, antimicrobial activity, antioxidant activity, DPPH assay

Introduction

Pin-Sein is one of the well-known Myanmar indigenous medicinal plants. Its botanical name is *Docynia indica* (Wall.) Decne. It belongs to the genus *Docynia* and species *indica* in the family rosaceae. *D. indica* is a plant that is abundantly found in the Shan State in Myanmar. *D. indica* is an evergreen tree, which distributes widely in southwest China and Southeast Asia. The *D. indica* leaves were widely used as tea or a drug for the healing of fever, cancer, empyrosis and rheumatic disease by the local ethnic minorities in southwest China, with lipid-lowering and weight-loss effects (Deng *et al*., 2014). The fruits were consumed by the locals. Previous studies have demonstrated that the content of polyphenols and flavonoids in *D. indica* was much higher, which exhibited significant bioactivities, including antioxidant, antitumor, anti-obesity, and anti-bacterial activities (Loan *et al*., 2011). Dietary supplementation of natural substances with antioxidant activity was now regarded as a safe and effective strategy for the prevention and treatment of obesity, which could be natural active substances instead of medicine (Hogan *et al*., 2010).

Polyphenols are the biggest group of phytochemicals and many of them have been found in plant-based foods. Polyphenol-rich diets have been linked to many health benefits. Polyphenols are strong antioxidants that complement and add to the functions of antioxidant vitamins and enzymes as a defense against oxidative stress caused by excess reactive oxygen species (ROS). Polyphenols have also beneficial for health as anti-carcinogenic, anti-ulcer, anti-atherogenic, anti-thrombotic, anti-inflammatory, immune modulating, antimicrobial and for its analgesic effect (Loganayaki *et al*., 2010). Therefore, the bark of Pin-Sein was selected to investigate some

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bioactivities and isolation of some organic compounds in this research paper. Plant, flower, fruit and bark of *D. indica* are shown in Figure 1.

Figure 1 Plant, flower, fruit and bark of *Docynia indica* (Wall.) Decne

Materials and Methods

Sample Collection and Preparation

The bark of Pin-Sein was collected from Pang Wa Village, Loi Mwe Township, Shan State, in January, 2020. The species was identified by Department of Botany, Kyaing Tong University.

The collected sample was cut into small pieces and allowed to be dried in air for 10-14 days. The air-dried samples were stored in well-stoppered bottles and used throughout the experiment.

Preliminary Phytochemical Screening of Bark of Pin-Sein

Preliminary phytochemicals tests were carried out according to the general methods (Harborne, 1984).

Determination of Moisture Content

The dry powder sample (2 g) was placed in a pre-weighed crucible. Then it was heated in an oven at 103 °C- 105 °C for 2 h. Just after being removal from the oven, the sample was allowed to cool at room temperature in desiccator for 1 h. The crucible and the dry sample were weighed again. This process of heating, cooling and weighing was repeated until a constant weight was obtained.

Determination of Ash Content

Clean porcelain crucible with a lid was heated in a Muffle furnace at 550 °C for 1 h. After cooling in desiccator for 30 min, the crucible was weighed. The dry powder sample (2 g) was weighed into pre-weighed crucible and heated at a low temperature to prevent sputtering. Then the crucible with lid were transferred to a Muffle furnace and ignited at 550 °C for about 6 h, until the residue was uniformly grayish to white. During heating, the lid was removed. The lid was put on after complete heating to prevent loss of ash. Next, the temperature of furnace was maintained at 105 °C for 20 min. It was cooled in desiccator and then weighed again. The process of heating, cooling and weighing was repeated until a constant weight was achieved.

Determination of Elemental Contents of the Sample

The air dried sample was ground to a fine powder. It was used to detect the elemental contents by EDXRF (Energy Dispersive X-ray Fluorescence) Spectrometry.

Preparation of Crude Extracts from Pin-Sein Bark

The air-dried sample (25 g) was added into separate conical flasks containing 100 mL of solvents (Pet-ether, EtOAc, EtOH and H_2O) and stand for 48 h with occasional stirring. The content of flask was filtered through sterile Whatman No. 1 filter paper and evaporated to dryness. These various solvent crude extracts of selected sample were used for the investigation of antimicrobial activity.

Screening of Antimicrobial Activity of Crude Extracts from Pin-Sein Bark

The nutrient agar medium was prepared according to the method described by Cruickshank, 1975. Nutrient agar was boiled and then 25 mL of this agar medium was poured into the test tube and plugged with cotton wool and autoclaved at 121 ºC for 15 min. Then, the tubes were cooled down to 30-35 ºC and poured into sterilized petri-dish and 0.02 mL of spore suspension was also added into the dishes. The agar was allowed to set for 2 h after which 10 mm plate agar disc was made with the help of sterilized cork borer. After that, about 0.1 mL of sample was introduced into the agar-disc and incubated at 37 ºC for 24 h. The inhibition zone (clear zone) that appeared around the agar-disc indicated the presence of antimicrobial activity.

Antioxidant Activity of Watery and Ethanol Extracts from Bark of Pin-Sein

The control solution was prepared by mixing of 3 mL of 0.002 % DPPH solution and 3 mL of 95 % ethanol using vortex mixer. Blank solution was prepared by mixing 3 mL each of crude extracts solution (25, 50, 100, 200, 400 μg/mL) and 3 mL of ethanol. Test sample solution was prepared by mixing 3 mL each of crude extracts solution (25, 50, 100, 200, 400 μg/mL) and 3 mL of 0.002 % DPPH solution. All these solutions were allowed to stand at room temperature for 30 min. The absorbance of these solutions was measured at 517 nm by UV-visible spectrophotometer and the percentage of the radical scavenging activity (% RSA) was calculated.

Isolation of Pure Organic Compounds from Pin-Sein Bark

The sample (300 g) was percolated with ethanol (1200 mL) for about one month at room temperature. During percolation, the whole mixture was frequently shaken to achieve maximum extraction of sample. After percolation time, the solution was filtered. The filtrate was evaporated to dryness at room temperature and the ethanol extract was obtained. The ethanol extract was re-extracted with ethyl acetate. The solution was filtered and then the filtrate was evaporated to dryness at room temperature. Ethyl acetate crude extract (6.02 g) was obtained. Ethyl acetate crude extract (2 g) was separated by column chromatography applying silica gel (70-230 mesh) as an adsorbent with n-hexane and ethyl acetate solvent system with various ratios from non-polar to polar. Total fractions were collected in clean containers. Each fraction was checked by TLC with suitable solvent system. The fractions with the same R_f value were combined to give total combined fractions and then evaporated at room temperature. The combined fractions were checked on TLC plate under UV detector. The R_f values of pure organic compounds (TMK-1 and TMK-2) were measured. The amounts of pure organic compounds were weighed and then the yield percent was calculated based on the ethyl acetate crude extract.

Identification of Isolated Pure Organic Compounds by TLC

Thin layer chromatography was conducted on 0.25 mm pre-coated silica gel (60 F_{254} Merck). It was cut into small plates (1×5 cm in size). The isolated pure organic compounds were checked by TLC with specified solvent systems (Stahl, 1965).

Polyphenol Test for Pure Organic Compound

A small amount of pure organic compound was tested with 1 % ferric chloride solution and then 1 % potassium ferric cyanide solution was added into the mixture. Formation of the colour of the filtrate was examined to decide the presence or absence of phenolic compounds (Marini-Bettolo *et al*., 1981).

Flavonoid Test for Pure Organic Compound

A small amount of pure organic compound was tested with three drops of concentrated hydrochloric acid and small pieces of magnesium ribbon. Observations were carried out to see whether the colour of the solution turned or not within 10 min.

Determination of Melting Point for Pure Organic Compounds

A few crystals form of pure organic compounds TMK-1 and TMK-2 were inserted into the capillary tubes and the melting points were determined by melting point apparatus.

Identification of Pure Organic Compounds by FT IR Spectroscopy

The FT IR spectral data of isolated pure organic compounds (TMK-1 and TMK-2) were recorded by Shimadzu Fourier Transform Infrared Spectrometer, at the Department of Chemistry, Taunggyi University. The resultant IR spectra were applied for the identification of functional groups for` the pure organic compounds.

Results and Discussion

Phytochemicals Present in the Bark of Pin-Sein

The respective colour indicates the presence or absence of phytochemical constituents in the Pin-Sein bark. It contains phytochemical constituents such as glycosides, reducing sugars, saponins, tannins, α-amino acids, polyphenols, flavonoids, carbohydrates and steroids. Alkaloids were not detected.

Moisture Content of Pin-Sein Bark

The moisture content of Pin-Sein bark was found to be 6.2 %. If the moisture content is greater than 10 %, the chance for growth of microorganism and the degradation of chemical compositions of nutrients will be higher.

Ash Content of Pin-Sein Bark

The ash content of Pin-Sein bark was found to be 0.54 %. So, Pin-Sein bark is a good source of mineral elements.

Elemental Contents in the Bark of Pin-Sein by EDXRF

EDXRF spectrum of the sample is shown in Figure 2. The relative elemental compositions are presented in Table 1. It was found that calcium was the highest amount (1.432 %) and potassium (0.327 %), the second highest amount the bark of Pin-Sein. According to the data, the inorganic minerals such as calcium, potassium, sulphur, barium, iron, strontium, copper and rubidium were present in this sample.

Table 1 Elemental Contents in the Bark of Pin-Sein

Symbol	Elements	Content (%)
Ca	Calcium	1.432
K	Potassium	0.327
S	Sulphur	0.042
Ba	Barium	0.009
Fe	Iron	0.005
Sr	Strontium	0.004
Cu	Copper	0.002
Rb	Rubidium	0.001

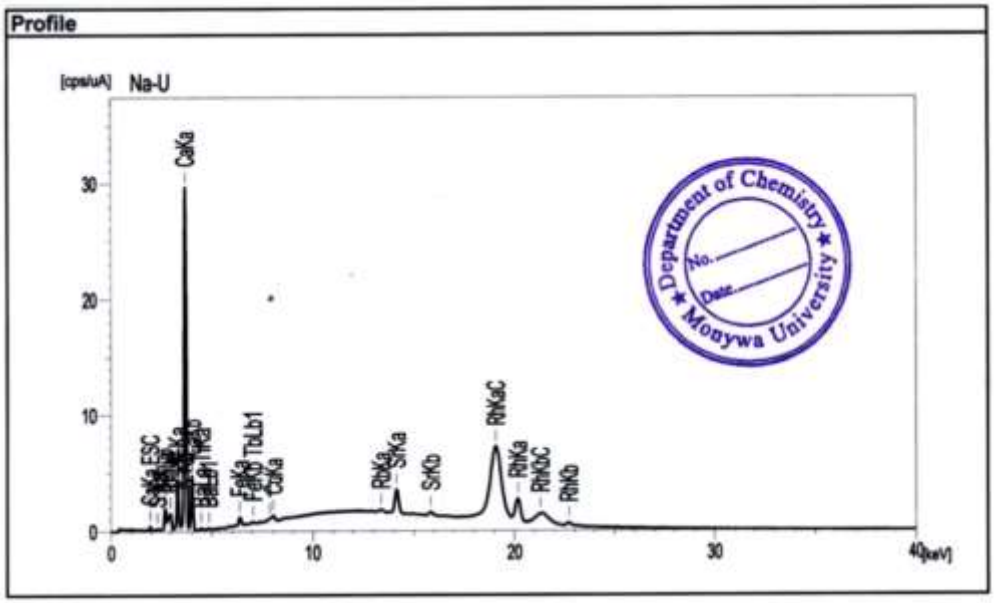


Figure 2 EDXRF spectrum of the bark of Pin-Sein

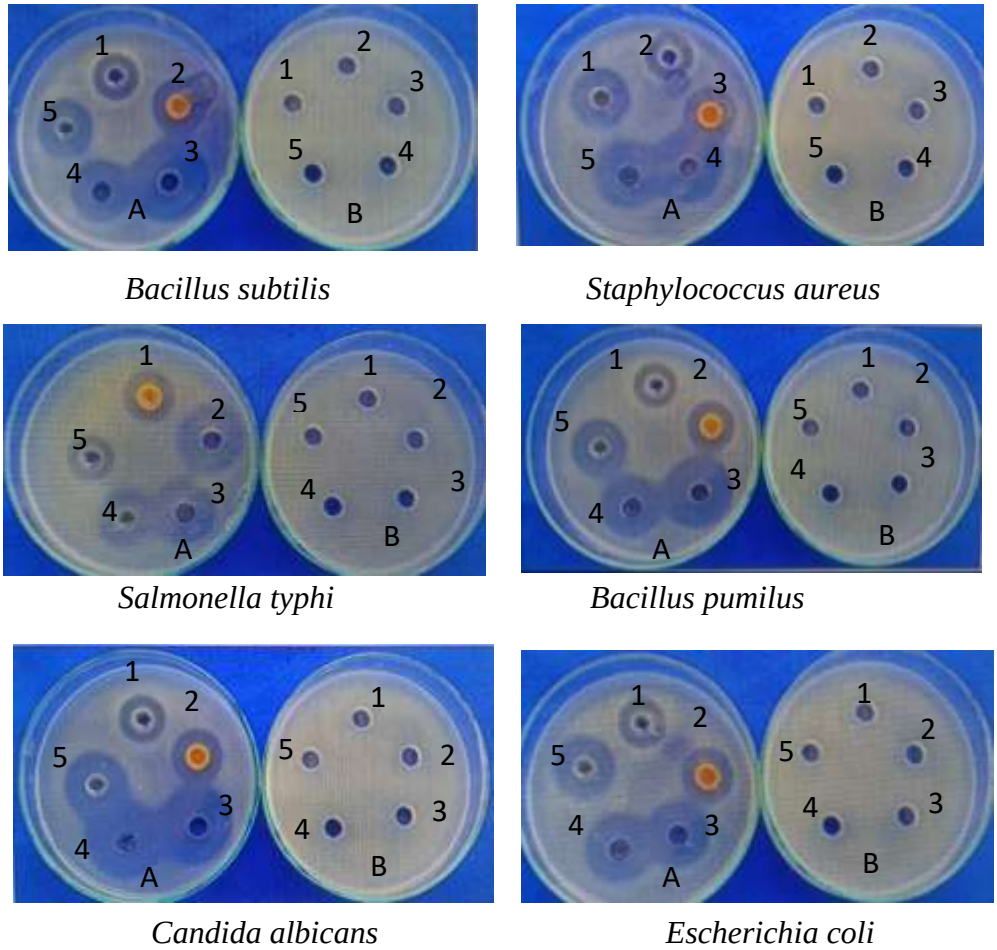
Antimicrobial Activities of Crude Extracts from Bark of Pin-Sein

The antimicrobial activities of various crude extracts from the bark of Pin-Sein are tabulated in Table 2. According to these data, the Pin-Sein bark was high in antimicrobial activity with inhibition zone diameter range, 18-31 mm in Figure 3. Three extracts of ethanol, ethyl acetate and chloroform showed high activities on six selected microorganisms. Among the various crude extracts of Pin-Sein bark, ethyl acetate extract exhibited the potent antimicrobial activity with inhibition zone diameter range, 29-31 mm. So, ethyl acetate extract of Pin-Sein bark sample was selected for the isolation of pure organic compounds by column separation method. It was observed that the Pin-Sein bark sample has a potential source of new classes of antibiotics that could be useful for infectious diseases chemotherapy and control.

Table 2 Antimicrobial Activities of the Various Crude Extracts of the Pin-Sein Bark

Microorganisms	Inhibition zone diameter (mm)				
	Pet-ether	**EtOAc**	**EtOH**	**$CHCl_3$**	**H_2O**
Bacillus subtilis	22.53	30.50	21.76	24.66	19.68
Staphylococcus aureus	23.66	29.47	24.06	26.60	21.56
Salmonella typhi	19.56	29.78	21.68	24.91	18.47
Bacillus pumilus	22.20	31.98	23.41	24.50	20.10
Candida albicans	23.35	29.43	21.22	29.64	20.50
Escherichia coli	22.98	25.40	23.11	25.97	20.68

Agar well (8 mm); 9 mm ~ 14 mm (+) -mild activity;
15 mm ~ 20 mm (++) -medium activity; 21 mm above (+++) -highest activity



Bacillus subtilis *Staphylococcus aureus*

Salmonella typhi *Bacillus pumilus*

Candida albicans *Escherichia coli*

1- Pet-ether, 2- EtOAc, 3- EtOH, 4- $CHCl_3$, 5- H_2O, A- Sample, B- Control

Figure 3 Antimicrobial activity of the bark of Pin-Sein

Antioxidant Activity of Crude Extracts from Pin-Sein Bark

It was found that the higher the concentration of crude extract, the lower the absorbance of DPPH solution and the greater the radical scavenging activity of the extract. The results are shown in Table 3. The plot of % inhibition versus concentrations of different extracts is shown in

Figure 4. So, the lower value of IC_{50} indicates the higher antioxidant activity. Therefore, ethanol extract (IC_{50}= 95.89 µg/mL) showed more potent antioxidant activity than watery extract (IC_{50}= 226.30 µg/mL) of the sample but lower activity in compare with standard ascorbic acid (IC_{50}= 3.70 µg/mL) in Figure 5. According to these data, the Pin-sein bark sample showed good antioxidant activity.

Table 3 Percent Inhibitions and IC_{50} Values of Watery and Ethanol Extracts of Pin-Sein Bark

Extracts	Concentration (µg/mL)	Percent inhibition (%)	IC_{50} (µg/mL)
Watery	25	33.27	226.30
	50	32.05	
	100	42.68	
	200	49.65	
	400	63.06	
Ethanol	25	8.29	95.89
	50	28.94	
	100	88.13	
	200	87.18	
	400	96.74	
Ascorbic acid	3.125	41.51	3.70
	6.25	87.01	
	12.5	88.91	
	25	89.92	
	50	90.10	
	100	93.55	

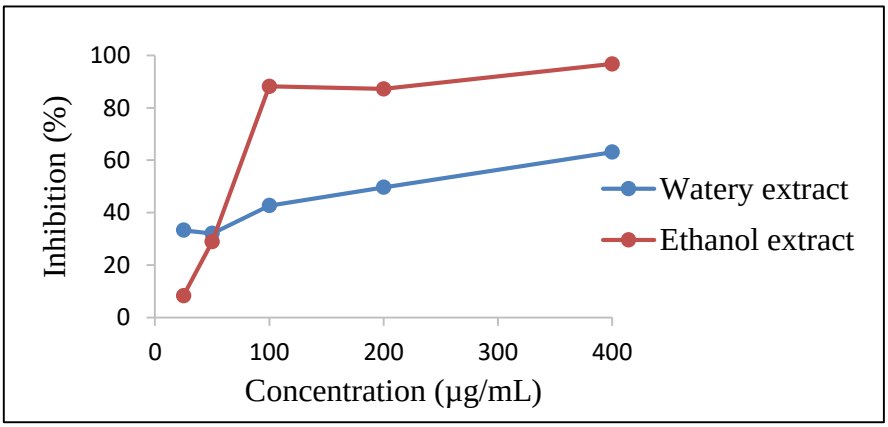


Figure 4 Plot of percent inhibition Vs different concentrations of crude extracts from the bark of Pin-Sein

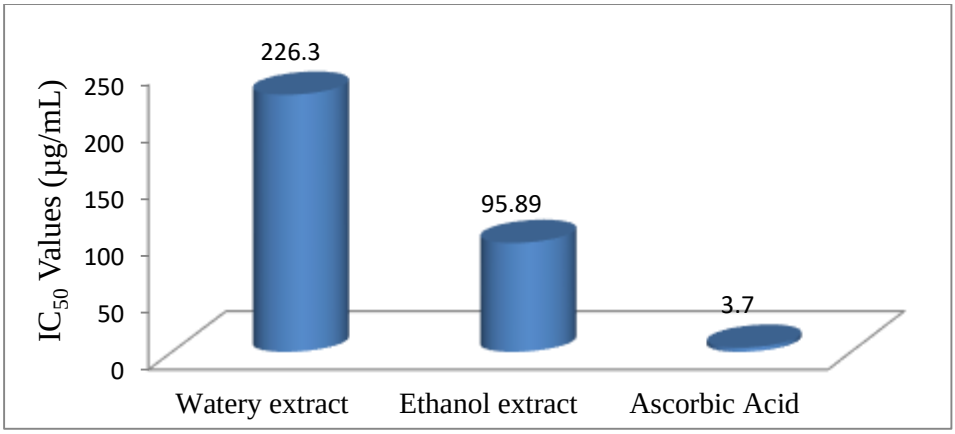


Figure 5 IC_{50} values of crude extracts of the Pin-Sein bark and standard ascorbic acid

Isolation of Pure Organic Compounds

From the column chromatographic separation of sample, totally 107 fractions were obtained. The fractions with the same R_f value were combined to give eight combined fractions. The fraction 6 gave 27 mg of pure organic compound, TMK-1, (R_f = 0.55) as a pale yellow crystal form and the yield percent was 1.35 % based on the ethyl acetate crude extract. The fraction 8 gave 31 mg of pure organic compound, TMK-2, (R_f = 0.64) as a yellow crystals form and the yield percent was 1.55 % based on the ethyl acetate crude extract.

FT IR Assignments of Pure Organic Compound, TMK-1

The FT IR spectrum of TMK-1 is shown in Figure 6, and the spectral data are tabulated in Table 4. TMK-1 consists of O-H stretching vibration of hydroxyl group, unsymmetrical and symmetrical CH stretching vibration, C=O stretching vibration, C=C stretching vibration of aromatic ring, C-O-C stretching vibration of ether group, =CH out of plane bending vibration of cis or Z and trans or E alkenic group, =CH out of plane bending vibration of aromatic ring and OH out of plane bending vibration, respectively. These functional groups are consistent with the structure of polyphenol (Silverstein e*t al.*, 2003). The occurrences of these functional groups imply that the isolated compound TMK-1 may be polyphenol.

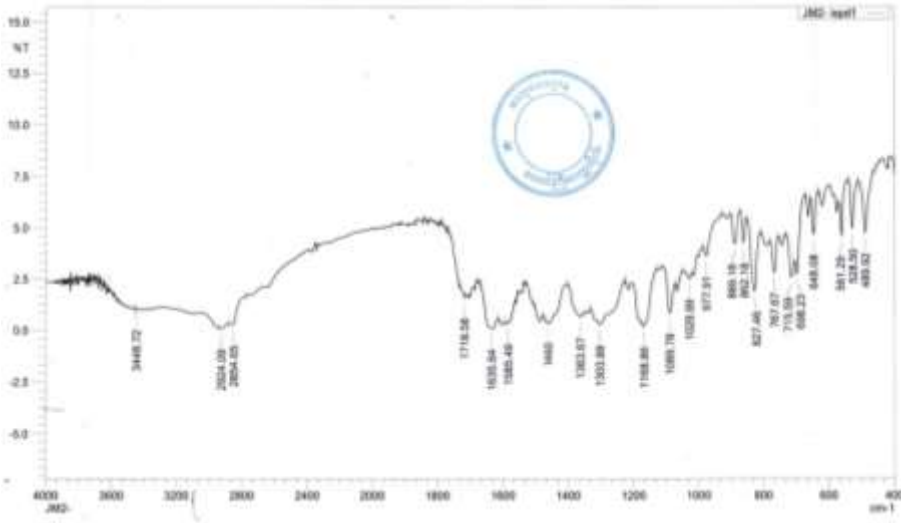


Figure 6 FT IR Spectrum of pure organic compound, TMK-1

Table 4 FT IR Assignments of Pure Organic Compound, TMK-1

Absorption band(cm^{-1})	Assignments (functional group)
3448	O-H stretching vibration of hydroxyl group
2924, 2854	unsymmetrical and symmetrical stretching vibration of sp^3 hydrocarbons
1718	C=O stretching vibration
1635, 1585	C=C stretching vibration of aromatic ring
1460, 1363	C-H in plane and out of plane bending vibration of sp^3 hydrocarbons
1303	OH in plane bending vibration
1168	C-O stretching vibration of hydroxyl group
1089	C-O-C stretching vibration of ether group
977	=CH out of plane bending vibration of trans or E alkenic group
889, 862	=CH out of plane bending vibration of aromatic ring
827	=CH out of plane bending vibration of cis or Z alkenic group
767, 715	C=C and OH out of plane bending vibration

FT IR Assignments of Pure Organic Compound, TMK-2

Figure 7 shows the FT IR spectrum of pure organic compound, TMK-2. These data are described in Table 5. The pure organic compound, TMK-2 consists of O-H stretching vibration of hydroxyl group, unsymmetrical and symmetrical stretching vibration of sp^3 hydrocarbons, C=O stretching vibrations, C=C stretching vibration of aromatic ring, C-O-C stretching vibration of ether group, =CH out of plane bending vibration of aromatic ring, =CH out of plane bending vibration of cis or Z alkenic group, C=C out of plane bending vibration and OH out of plane bending vibration, respectively. These functional groups are consistent with the structure of flavonoid (Silverstein e*t al.*, 2003). According to these functional groups, isolated pure organic compound, TMK-2 may be flavonoid.

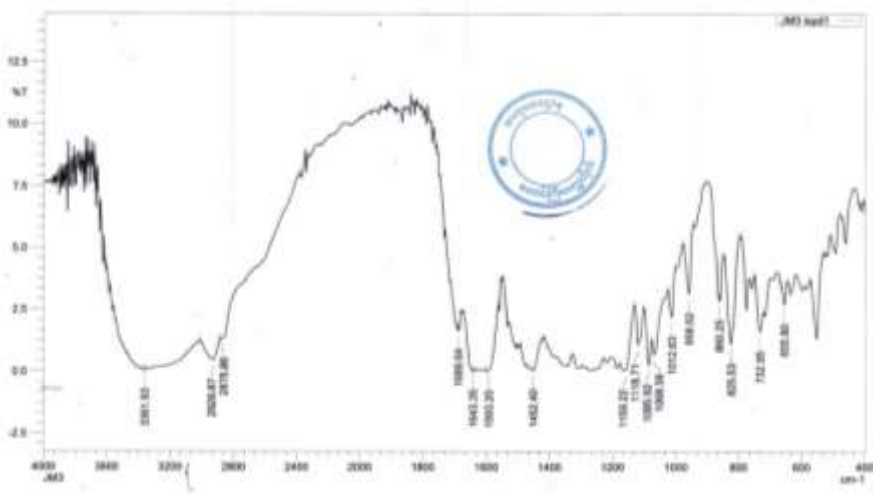


Figure 7 FT IR spectrum of pure organic compound TMK-2

Table 5 FT IR Assignments of TMK-2

Absorption band (cm^{-1})	Assignments (functional group)
3361	O-H stretching vibration of hydroxyl group
2929, 2875	unsymmetrical and symmetrical stretching vibration of sp^3 hydrocarbons
1689	C=O stretching vibration of carbonyl group
1643, 1593	C=C stretching vibration of aromatic ring
1452	C-H in plane bending vibration of sp^3 hydrocarbons
1159	C-O stretching vibration of hydroxyl group
1118, 1085	C-O-C stretching vibration of ether group
1068	O-H in plane bending vibration
1012; 958, 860	=CH in plane and out of plane bending vibration of aromatic ring
825	=CH out of plane bending vibration of cis or Z alkenic group
732	C=C out of plane bending vibration of aromatic ring
655	O-H out of plane bending vibration

Pure organic compound, TMK-1 gave the blue black colour solution for polyphenol test and TMK-2, reddish pink colour solution for flavonoid test. So, two isolated compounds may be polyphenol and flavonoid. The melting points of polyphenol (TMK-1), 253-256 ºC and flavonoid (TMK-2), 298-301 ºC, were observed, respectively.

Conclusion

In this research work, the bark of *Docynia indica* (Wall.) Decne was a good source of phytochemicals and mineral elements. It was found to contain 6.2 % moisture and 0.54 % ash. Antimicrobial activity of Pin-Sein bark was high activity on six selected microorganisms with inhibition zone diameter range of 18-31 mm. Among the crude extracts, antimicrobial activity of ethyl acetate extract was found to be the highest and water extract was the lowest in the bark of Pin-Sein. Therefore, Pin-Sein bark was less soluble in water. In addition, IC_{50} values of 95 % ethanol and watery extracts were 95.89 µg/mL and 226.30 µg/mL. So, ethanol extract had more potent antioxidant activity than watery extract of this sample. According to these data, the Pin-sein bark sample showed good radical scavenging activity and inhibition ability. It might be considered as a potential source of antioxidants. Polyphenol and flavonoid, pure organic compounds were isolated from Pin-Sein bark by column chromatography. The yield percent of polyphenol (TMK-1) was 1.35 % and of flavonoid (TMK-2), 1.55 % based upon the ethyl acetate crude extract. R_f values of polyphenol and flavonoid were 0.55 and 0.64. Melting point was found to be 253 -256 ºC of polyphenol, and 298-301ºC of flavonoid. The functional groups present in these two compounds were identified by FT IR spectral data. Due to its bioactivity and bioactive constituents: polyphenol and flavonoid, the bark of Pin-Sein may be useful as antioxidant for the treatment of oxidative stress related diseases such as diabetes, cancers, tumors, hypertension, liver diseases, inflammatory etc.

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References

Cruickshank, R. (1975). *Handbook of Bacteriology*. London: 11th Ed., E. and S. Livingstone Ltd., pp. 888

Deng, X., X. Zhao, Z. Lan, J. Jiang, W. Yin, and L. Chen. (2014). "Anti-tumor Effects of Flavonoids from the Ethnic Medicine *Docynia delavayi* (Franch.) Schneid. and its Possible Mechanism". *J. Med. Food*, vol.17 (7), pp.787-794

Harborne, J. B. (1984). *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis.* New York: 3rd Ed., Chapman and Hall ltd, pp. 120-160

Hogan, S., **C. S.** Canning, X. Sun, and K. Zhou. (2010). "Effects of Grape Pomade Antioxidant Extract on Oxidative Stress and Inflammation in Diet Induced Obese Mice". *J. Agric. Food Chem.*, vol. 58, pp. 11250-11256

Loan, N. T. T., H. T. M. Tan, V. T. H. Tam, C. L. Luan, L. M. Huong, and D. N. Lien. (2011). "Antiobesity and Body Weight Reducing Effect of *Docynia indica* (Wall.) Decne Fruit Extract Fractions in Experimentally Obese Mice". *VNU J. Sci. Nat. Sci. Technol.*, vol. 27, pp. 125-133

Loganayaki, N., S. Perumal, and M. Sellamuthu. (2010). "Antioxidant Activity of Two Traditional Indian Vegetables: *Solanum nigrum* L. and *Solanum torvum* L.". *Food Sci. Biotechnol.,* vol.19 (1) pp. 121-127

Marini-Bettolo, G., M. Nicoletti, M. Patamia, C. Galeffi, and I. Messana. (1981). "Plant Screening by Chemical and Chromatographic Procedures under Field Conditions". *J. Ethanopharmacol*. pp. 121-123

Silverstein, R. M., F. X. Webster, and D. J. Kiemle. (2003). *Spectroscopic Identification of Organic Compounds*. New York: 7th Ed., John Wiley & Sons, Inc.

Stahl, E. (1965). "Effect of Low Temperature and Use of Gradients in Thin Layer Chromatography''. *J. Pharm. Belg.* vol. 20, pp. 159-168