Isolation of Bioactive Compounds and Free Radical Scavenging Activity of *Maclura Cochinchinensis* (Lour.) Corner Stem by Different Chromatographic Techniques

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Abstract

Antioxidants provide protection to human against free radicals through inhibiting and scavenging mechanism. This study was to determine the free radical scavenging activity of *Maclura cochinchinensis* Corner stem sample from different extraction methods to investigate the bioactive compound in stem extract. **Materials and methods:** The stem samples of *M. cochinchinensis* were extracted by sonication, maceration and soxhlet method with four different solvents; acetone, 50% ethanol, 80% ethanol and methanol. Each extracts was tested using 2,2-diphenyl 1-picryl hydrazyl (DPPH) radical scavenging activity assay with ascorbic acid and morin as positive control. **Findings:** For isolation of bioactive compound highest percentage yield of the crude extracts was from 50% and 80% ethanol(22.0 ± 1.35 % and 20.7 ± 2.29 %), followed by acetone (IC_{50} of 15.54 ± 1.34 µg/ml) and methanol (IC_{50} of 20.99 ± 1.07 µg/ml)extract by Soxhlet method not significantly different, also had higher radicle scavenging activity, DPPH test on 80% ethanol extract from soxhlet extraction was one of the highest antioxidant activity among the extracts.

Keywords: *Antioxidant, free radicals, bioactive compound, scavenging activity.*

Introduction

Antioxidant are needed for protection against free radical induced diseases [1, 2, 3]. Recent studies shown that anti-oxidant reduce the risk of getting diseases like cancer and heart diseases [4].

Free radicals are molecules with unstable unpaired electrons in its outer shells which react with other compound and pair their electrons to form a stable compound [5]. which keeps one electron via redox reactions. Free radicals are formed continuously in human body from by-product of adenosine triphosphate resulting in reactive oxygen species and reactive nitrogen species, At high concentration, free radicals generate oxidative stress [6].

In *M. cochichinensis* morin is the major active compound with effective antioxidant and antibacterial activity in mammals [7]. *It* has traditional medicinal value and was used to treat jaundice, anti-inflammatory, anti-diarrheal and antipyretic [8].

Flavonoids are bioactive compounds which scavenges the free radicals and protects the lipid bilayer from oxidants. The two major groups are flavonols and flavones are beneficial to plants and human health. Its effects are explained by their interaction with enzymes, transporters, receptors and signal transduction systems [9]. In order to investigate the free radical scavenging activity of flavonoids, the crude extract from the sample of *Maclura cochinchinensis* (Lour.) were tested using DPPH assay and column chromatography techniques.

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Materials & Method

Analytical reagent used are DPPH powder L-ascorbic acid silica gel Sephadex LH-20, silica gel TLC plate acetone, ethanol, hexane, dichloromethane, methanol distilled water, acetic acid and ethyl acetate.

Maclura cochinchinensis (Lour) Corner, the stem part was chosen and prepared from Pharmacognosy Laboratory.

Extraction Methods: Ten grams of *Maclura cochinchinensis* (MC) stem powder were used in each method. The solvents were acetone, 50% ethanol, 80% ethanol and methanol. After extraction, the samples were filtered using Buchner funnel with Whatman filter paper and vacuum pump. The filtrate was concentrated and dried under 48°C with reduced pressure.

Sonication: The samples were treated in ultrasonic bath at 40°C with frequency of 40% for 30 minutes. The supernatants were filtered. The remaining pallets were treated again with the same method.

Maceration: The solvents were placed on electronic shaker with 120 rpm for 72 hours at room temperature. The supernatants were filtered. This method was repeated twice with same solvent [10].

Soxhlet: The samples were placed in thimbles made from white cotton clothes,200 ml solvents were heated at boiling temperature for 3-9 hours, cooled at 4°C and the extraction method was carried out until exhausted.

DPPH Free Radical Scavenging Assay: The free radical scavenging activity of crude extract from each extraction method was evaluated using DPPH assay.

Separation Methods

Crude extract (10 g) of *Maclura cochinchinensis* stem sample obtained from soxhlet extraction with 80% ethanol was subjected to vacuum liquid chromatography (VLC).

Vacuum Liquid Chromatography

Ten grams of sample was dissolved in 25 ml of methanol and 10 ml of hexane. VLC was conducted with slurry of 140 g of silica gel with 300 ml of hexane. The sample was eluted with solvents of increasing polarity to form six fractions

Each of the fractions was concentrated using rotary evaporator, weight was recorded. The free radical scavenging activity were evaluated using DPPH assay and the compounds in the fractions were subjected to qualitative analyzed by TLC with morin as standard.

Flash Column Chromatography

Fraction-4 was selected as the sample, the flash column chromatography was done using Pump Module C-601/C-605 to reduce the time of separation.100mg of sample in 1 ml of the solvents was injected into the prepacked silica gel. The mobile phase -ethyl acetate and hexane with ratio of 50%:50%, 70%:30% were used .and 100%:0%. 100% methanol was used to wash the column.

Classic column chromatography was done using Sephadex LH-20 as the stationary phase and methanol as mobile phase.

Thin Layer Chromatography

Throughout the separation process, TLC procedures were carried out. The Rf value of each compound was calculated and compared with standard (morin).

Statistical Analysis

The results were reported as mean ± standard deviation (SD) (n = 3). The average percentage yield and IC_{50} of the extracts prepared by the different extraction methods using different solvents were statistically investigated using one-way analysis of variance (ANOVA) with least significant difference (LSD) by SPSS for Windows 21.0. A statistical probability (p value) less than 0.05 indicated a statistically significant difference between groups.

Results & Discussion

Percentage yield of crude extracts of *Maclura cochinchinensis* stem samples were subjected to three extraction methods and four types of solvents were used. Among the extraction methods, the highest percentage yield of the crude extracts was between 50% and 80% ethanol extracts, followed by acetone extract and methanol extract. However, the percentage yield among methanol, 50% and 80% ethanol were not significantly different.

Table 1: Percentage yield of crude extract of ten gram of *Maclura cochinchinensis* stem sample by different extraction methods with different solvents

Extraction method	Solvents	Total volume of solvents (ml)	Time for extraction	Percentage yield (%)			Average percentage yield ± SD (%)
				1st	2nd	3rd	
Sonication	Acetone	400	1 hour	6.5	11.2	17.0	11.6 ± 5.26a
	50% Ethanol	400	1 hour	20.2	27.1	21.5	22.9 ± 3.67b
	80% Ethanol	400	1 hour	21.9	21.2	21.6	21.6 ± 0.35b
	Methanol	400	1 hour	18.7	23.6	20.0	20.8 ± 2.54b
Maceration	Acetone	600	9 days	12.4	16.6	19.4	16.1 ± 3.52a
	50% Ethanol	600	9 days	22.1	23.3	23.0	22.8 ± 0.62b
	80% Ethanol	600	9 days	23.1	20.6	22.3	22.0 ± 1.28b
	Methanol	600	9 days	23.0	20.5	20.4	21.3 ± 1.47b
Soxhlet	Acetone	200	3 hours	14.0	17.3	12.5	14.6 ± 2.46a
	50% Ethanol	200	9 hours	21.9	20.7	23.4	22.0 ± 1.35b
	80% Ethanol	200	9 hours	21.7	22.2	23.7	22.5 ± 1.04b
	Methanol	200	6 hours	21.4	22.5	18.1	20.7 ± 2.29b

DPPH scavenging activity of crude extracts of *Maclura cochinchinensis* stem sample

Each of the extracts with different concentration (100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/ml) was tested with methanolic DPPH solution to evaluate the free radical's scavenger activity. Morin and vitamin C were used as positive control [1]. The interaction between the extract and methanolic DPPH solution causes the purple to yellow colour. High intensity of decolourisation of the sample means strong antioxidant activity [4].

Soxhlet extracts have higher radicle scavenging activity, Acetone extract from soxhlet extraction had the highest antioxidant activity but not significantly different from 50% and 80% ethanol. Acetone is an excellent solvent to extract high molecular weight flavonols [11]. Ethanol is a solvent that can extract much more compounds than acetone [11, 12].

Crude extract from appropriate extraction method and solvent for isolation and purification of pure compound

Crude extract of MC in 80% ethanol from soxhlet extraction method was chosen because of strong radical scavenging activity and high percentage yield; IC_{50} = 16.39 ± 1.94 µg/ml and 22.5 ± 1.04 % respectively. IC_{50} indicates the concentration of the sample required to inhibit 50% of the free radicles [5]. Lower IC_{50} indicates

lower concentration of the sample needed to scavenge the free radicles, hence the sample has high antioxidant activity.

DPPH test on 80% ethanol extract from soxhlet extraction was one of the highest antioxidant activities. The acetone extract from soxhlet method had low IC_{50} of 15.54 ± 1.34 µg/ml but it had low average percentage yield of 14.6 ± 2.46 %. 50% ethanol and methanol extract from soxhlet extraction both had high percentage yield of 22.0 ± 1.35 % and 20.7 ± 2.29 % but methanol extract had a slightly high IC_{50} of 20.99 ± 1.07 µg/ml. Both extracts from sonication and maceration methods had higher IC_{50} if compared to the extracts obtained from soxhlet method. Extraction yield and antioxidant activity depends on extraction methods and solvents used. Ethanol was chosen solvent for polyphenols extraction while methanol for low molecular weight of polyphenols [11].

The best conditions to extract flavonoids from mulberry leaves were at 80°C, using 80% alcohol, 3-5 number of extractions with 120-180 minutes [12]. Soxhlet extraction experiment used boiled solvent as the hot vapours condensed and return to liquid form, this method is not suitable in small scale [13].

The extracts from maceration method had the highest IC_{50} of 22.43 ± 1.54, 19.82 ± 0.87, 20.89 ± 1.46 and 25.59 ± 5.53 µg/ml for acetone, 50% and 80% ethanol and methanol extracts respectively, In Soxhlet, fresh solvent was used so that no loss of thermo labile volatile compound. The major drawback was its requirement of long hours for extraction and not suitable for thermo labile compounds [14].

DPPH scavenging activity of fractions of VLC and selection of VLC fraction for Separation and Purification methods.

Ten grams of crude extract of 80% ethanol from soxhlet extraction was used for VLC technique with four different solvents in increasing order of polarity: hexane, dichloromethane, ethyl acetate and methanol. Six fractions of VLC were collected, TLC and DPPH assay were done to each fraction except fraction-1 (eluted by hexane) to determine which fractions contain the interested compound (morin) and evaluate the free radical scavenging activity.

From six fractions, Fraction-4 (2.3g) eluted by 5100 ml ethyl acetate was chosen and separated by column chromatography as the fraction had low IC50 of 19.45 ± 1.72 µg/ml, indicating high free radical scavenging activity. From the TLC plate, the fraction contains a significant amount of morin, compound to be isolated.

Table 2: Quantitation of the amount of crude 80% ethanolic extract of *Maclura cochinchinensis* after VLC.

Fraction collected	Mobile Phase	Volume applied (mL)	Quantity (g)
Fraction-1	Hexane	250	0.29
Fraction-2	Dichloromethane	1150	0.73
Fraction-3	Ethyl acetate	500	5.11
Fraction-4	Ethyl acetate	5100	2.30
Fraction-5	Methanol	1000	3.40
Fraction-6	Methanol	950	0.90

The antioxidant activity of the extract depends on the compounds of eluted fractions. In **Fraction-2** eluted by dichloromethane had many blue bands which were phenolic compounds, had the highest I C_{50} of 60.52 ± 1.92 µg/ml and lowest percentage yield of 7.3%. In **Fraction-3** eluted by ethyl acetate which consists several phenolic compounds and morin has the lowest IC_{50} of 11.28 ± 1.24 µg/ml which indicates the highest antioxidant activity and highest percentage yield of 51.1%. **In Fraction-4**, the compounds found were less than Fraction-3 but has reasonable value of IC_{50}; 19.45 ± 1.72 µg/ml. This shows that morin had antioxidant activity along with other phenolic compounds. High antioxidant activity is because of the number of phenolic hydroxyl, methoxyl, or other functional groups [5,15].

The need to use four different solvent to elute the sample as it possessed. The different polarity enables the solvent to solubilize the polar and non-polar compounds separately [16] since the desired compound (morin) is polar the morin can be best eluted by ethyl acetate which is a polar solvent [17].

Based on TLC plate using mobile phase of ethyl acetate:hexane (1:1), F3, F4 and F5 had the same yellow band with Rf value of 0.07. F4 and F5 were subjected to another TLC with mobile phase: methanol:ethyl acetate (1:4) to examine the eluted compound. F4 was selected for further separated as the yellow band with the Rf value of 0.72 showed concentrated amount of interested compound (morin) in the fractions.

Isolation of Morin using Flash Column Chromatographic technique.

The separation method was started using 100 mg of Fraction-4 from VLC using silica gel as stationary phase and gradient elution technique of ethyl acetate:hexane (50%:50%), (70%:30%), (100%:0%) and methanol for cleaning the column. This method was repeated twice to increase the volume of compound.

The first and second flash chromatography (FC1 and FC2), 54 and 59 fractions were eluted with volume of 20 ml per fraction. All fractions were combined based on the similarities found in TLC plate, producing 9 fractions of each batch.

After the combination of the flash column chromatography fractions, they were chosen to further purify by classic column chromatography. From FC1, fractions 5, 6 and 7 with the weight of 5.2 mg, 2.2 mg and 15.8 mg respectively, and FC2, fractions 2, 3 and 4 with the weight of 1.6 mg, 4.6 mg and 4.3 mg respectively, were selected.

The stationary phase is silica gel while the mobile phase is non-polar. This set up was to separate the sample based on the polarity [18], to obtain the pure desired compound. Flash chromatography allows rapid elution to separation in short time and minimize dispersion of the compound in solution [19].

From the TLCs, it was found that the compounds in the fractions had not been separated yet, the selected fractions had been chosen carefully for another separation method of classic column chromatography in order to obtain pure compound of morin.

Conclusion

Maclura cochinchinensis (Lour.) Corner stem extracts had proven high free radicle scavenging activity. The best extraction method and solvent used were soxhlet extraction method with 80% ethanol. The bioactive compound was isolated with weight of 11.5 mg from ten gram of crude extract by using several column chromatography techniques and preliminary by TLC with standard of morin.

It would be interesting for researcher to test the activity of high purified fractions and isolate the responsible molecules that could be detected in different extracts by more efficient methods. It is important to highlight that majority of the test performed in vitro. To carry out in vivo studies, it is mandatory to investigate the important information for dietary interventions.

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