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Functional Food, Myin-khwa and its Antioxidant Activity and Acute Toxicity

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Abstract

In this research, *Hydrocotyle asiatica* Linn. (Myin-khwa) has been chosen for chemical analysis. From phytochemical analysis, Myin-khwa was known to contain polyphenols, flavonoids and phenolic compounds, so it was chosen to determine antioxidant activity and acute toxicity. Nutritional values including moisture (7.75), ash (8.66 %), protein (8.89 %), fat (1.68 %), fibre (1.46 %) and carbohydrate (4.11%) of Myin-khwa residues were also investigated. Mineral contents of Myin-khwa were analyzed by using AAS method. From this study, the adequate amount of Ca, Fe, K, Mg, Mn, Na and Zn were present in dry Myin-khwa. Acute toxicities of ethanolic extract of Myin-khwa was determined by using Wistar Strain Rats and antioxidant activities of Myin-khwa extracts with four kind of solvents such as distilled water, ethanol, n-hexane and ethylacetate were determined by using DPPH (1,1-diphenyl-2-picryl hydrazine) assay. The highest radical scavenging effect was observed in Myin-khwa with IC_{50} - 0.66μgml^{-1}. The potency of radical scavenging effect of EtOH extract of Myin-khwa was greater than standard antioxidant ascorbic acid. From the acute test, it was found that the median lethal dose (LD_{50}) was more than 18g/kg when administered orally.

Key Words: *Hydrocotyle asiatica* Linn., antioxidant activity, acute toxicity, Wistar Strain Rats, Nutritional values, Mineral contents

Introduction

Millions of people across the world find it is impossible to achieve a sufficient amount and variety of safe food each day. Others may have access to enough food but still lack the knowledge to make good dietary and lifestyle choices to get the best from their food. Both situations can lead to poor nutrition, diseases related to diet and poor health.

Nowadays, consumers become more interested in healthy life. They believe that food is one of the important factors for improving the quality of their lives. Functional ingredients from plant origin provided phytochemicals and dietary fiber, non-

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nutrients; promote health benefits such as reduction of cholesterol in blood, prevention of degenerative diseases such as cancer, cardiovascular diseases and diabetes (Kritchevsky, 1995, Premier. 2002).

Myin-khwa is utilized as a medicine for thousands of years for wound healing, memory improvement, treating hypertension, toxic fever, leprosy, ulcer and cancer (Kartnig, 1988, Sahu, 1989, Farnsworth, 1992, Goh, 1995).

The major phytochemicals that provide the benefits in Myin-khwa are tri-terpenes as well as polyphenols, particularly flavonoids. (Grimaldi, 1990, Inamdar, 1996, Zainol, 2003). Fresh Myin-khwa, abundantly available all year round is consumed as a side dish of spicy minced meat salad, papaya salad, fried noodles and chilli paste, and can be used as an ingredient of salad and curry.

The Myin-khwa residue is considered interesting to be investigated the remaining phytochemicals as well as free-radical-scavenging activity. Therefore, the aim of the research work is to study the pharmacological and chemical properties of Myin-khwa residue as a functional ingredient.

Botanical Description

Botanical Name	-	*Hydrocotyle asiatica* Linn.
Family	-	Umbelliferae (Apiaceae)
English name	-	Asiatic pennywort
Local name	-	Myin-khwa
Distribution	-	Myanmar, Malaysia, China, India, Thailand, Indonesia, Madagascar, Australia, Southern and Northern of Africa

Toxicology and Acute Lethality

Toxicology is the study of the adverse effect of chemicals on living organisms. The variety of potential adverse effects and the diversity of chemicals present in our environment combine to make toxicology, a very broad science (Bulletin, 2001**)**.

The first toxicity test performed on a new chemical is acute toxicity. The acute toxicity tests give (1) a quantitative measure of acute toxicity (LD_{50}) for comparison to other substances, (2) identify the clinical manifestations of acute toxicity, and (3) give dose-ranging guidance for other studies (Klassen, 1995, Loomis, 1968).

Material and Methods

Sampling

The whole plant of H*ydrocotyle asiatica* Linn. (Myin-khwa) was collected from Aung Thar Village, Sagaing Division. Firstly, the sample was dried in the shade and cut into small pieces.

Preliminary Phytochemical Analysis

Preliminary detection of phytochemical compounds present in *Hydrocotyle asiatica* Linn. was carried out according to the general methods mentioned in phytochemical methods (Priestman, 1953, Finar, 1964, and Vogel, 1956, 1966).

Determination of pH and Nutritive Values in Myin-khwa

The pH of dried Myin-khwa was measured by using pH meter. The nutritional values of Myin-khwa were determined by using the following methods.

Nutritional Value	Method
Moisture	Moisture Analyser
Ash	Muffle furnace
Protein	Kjeldahl Analyser
Fat	Soxhlet extraction
Fibre	Kjeldahl Analyser
Carbohydrate	UV-vis spectrophotometer
Phosphorus	UV-vis spectrophotometer

Determination of Mineral Contents

Mineral contents present in Myin-khwa were determined by atomic absorption spectro-photometric method at Ministry of Industry (1), Myanmar Pharmaceutical Industries, Myanmar Pharmaceutical Factory, Sagaing, Myanmar.

Determination of Acute Toxicity

Preparation of Plant Extracts

Site of Study - Pharmacology Research Division, Department of Medical Research, Upper Myanmar.

About 55 g of air dried Myin-khwa leaves were stored in a stoppered bottle and percolated with 0.8 L of 95% ethanol. After 10 days, it was filtered with filter paper and the filtrate was evaporated at room temperature. Then 95% ethanol (0.7 L) was added to the residue for about 5 days. And then, it was also filtered and the filtrate was concentrated at room temperature. The entire residue was discarded. Totally 6 g of ethanolic extract was obtained. The dried extract obtained was stored in the desiccator.

For testing the effect of Myin-khwa, on experimental animals, required amount of extract was taken and mixed with distilled water to get the required dosage of extract.

Animal Selection

Strain	-	Wistar Strain rat
Weight	-	180 $\pm$ 20 g
Sex	-	both sexes
Quantity	-	40 numbers

A total of 40 Wistar Strain Rats of both sexes (body weight 160-180 g) used in this study were randomly divided into four groups with 10 animals in each. Three were tested groups and one was control group. 10 albino rats in each group were fasted overnight before administration of the ethanolic extract of Myin-khwa. The acute toxicity study was carried out with an oral administration of ethanolic extract of Myin-khwa at increasing doses of 12g/kg, 16g/kg and 18g/kg on the three test groups and 10ml/kg of distilled water on control group. After administrating the extract orally, each group containing rat was kept in individual cage with

free access to food and water and was observed toxic effects daily for two weeks.

Observation and Termination

Daily observation was carried out for clinical sighs of toxic effects such as changes in appearance, behavior, the rate of body weight gain and mortality for fourteen days.

At the end of fourteen days, animals dying during the study period, as well as surviving to the end of the observation will be autopsied.

Determination of Antioxidant Activity

Preparation of Plant Extracts

Dried powders of Myin-khwa (*Hydrocotyle asiatica* Linn.) were weight about (50 g) in each. These powders were percolated with different solvents such as ethanol, n-hexane, and ethyl acetate refluxed with (each 250 ml) at room temperature for 2 months and then filtered. Complete removal of solvent under reduced pressure provided crude extracts. The crude extracts were partitioned with ethanol 100 ml.

Preparation of Aqueous Extract

About 50 g of air dried Myin-khwa was extracted with 500ml of distilled water by using continuous hot extraction at 60ºC. After 6 hours of extraction, it was cooled at room temperature and then the mixture was filtered using filter paper. The filtrate was concentrated at 50ºC to get the constant weight in an evaporating basin. The crude extract was partitioned with ethanol 100 ml.

Preparation of 60 μM DPPH and Test Sample Solution

DPPH powder (2.364 mg) was dissolved in 95% ethanol and made the volume to 100 cm^3. The solution was freshly prepared in the brown coloured flask and kept in refrigerator for no longer than 24 hours.

2 mg of the extract to be tested was dissolved in 50% ethanol and made the volume to 10 cm^3. The solution was then filtered to get a stock solution. The desired concentration of test sample

solutions (1.25, 2.5, 5, 10 and 20 μg/ml) were prepared by dilution the stock solution with 50% ethanol.

Procedure

(i) Control solution was prepared by mixing 60μM DPPH solutions
($1.5\ cm^3$) and 95% ethanol ($1.5\ cm^3$)

(ii) Similarly, the blank solution was prepared by mixing test solution
($1.5\ cm^3$) and 50% ethanol ($1.5\ cm^3$)

(iii) The sample solution was also prepared by mixing the test solution
($1.5\ cm^3$) with 60μM DPPH solution ($1.5\ cm^3$)

All these solutions were allowed to stand at room temperature for 30 minutes. Then, the absorbance was measured at λ 517 nm using UV-160 spectrophotometer. Absorbance of individual solutions was measured its triplicate and calculated % inhibition by using the following formula.

$$\%\ \text{inhibiiton} = \frac{\text{DPPH alone - (sample - blank)}}{\text{DPPH alone}} \times 100$$

$$\text{average } \overline{X} = \frac{x_1 + x_2 + \text{-------} + x_n}{n}$$

Then, IC_{50} value was calculated by linear regressive excel program and standard deviation was calculated by the following formula.

$$\text{Standard Deviation} = \sqrt{\frac{(\overline{X} - X_1)^2 + (\overline{X} - X_2)^2 + \text{------} + (\overline{X} - X_n)^2}{(n - 1)}}$$

DPPH = absorbance of control solution
Sample = absorbance of sample solution
Blank = absorbance of blank solution
x = average % inhibition
$x_1, x_2.., x_n$ = % inhibition of test sample solution
n = number of times

Results and Discussion

According to the preliminary phytochemical tests, most of phytochemical constituent contains in Myin-khwa except saponinn (cf. Table 1). The suspension of dried Myin-khwa residue in distilled water was measured by pH value. The result shown that pH of dry sample residue was slightly acidic (5.19 $\pm$ 0.01). The specified pH of cellulose and insoluble fibre should be between 5.0 and 7.5. The dried Myin-khwa residue could be applied in dietary with minimal effects on the functional properties of food. And the microorganism does not grow in the dried sample, Myin-khwa because of low moisture content. The nutritive values of Myin-khwa are given in Table 2. Moreover, the nutritional values of Myin-khwa are suitable amount to use it as a functional food.

Mineral Contents in Myin-khwa

Mineral contents of Myin-khwa were investigated by Atomic Absorption Spectroscopic Method. Calcium, Iron, Potassium, Magnesium, Magnese, Sodium and Zinc in Myin-khwa were found to be 6334 ppm, 1263 ppm, 23727 ppm, 5825 ppm, 120 ppm, 873 ppm and 49 ppm (cf. Table 3). These minerals are important and essential for building and repairing body process.

Acute Toxicity of Ethanolic Plant Extract *in Vivo*

The mice administrated with 12 g/kg, 16 g/kg and 18 g/kg doses of ethanolic extract of *Hydrocotyle asiatica* Linn. were kept under observation for two weeks. After two weeks, all the mice were alive and did not show any toxic symptoms such as body weight loss and restlessness. So, it was found that 18g/kg dose of ethanolic extract of *Hydrocotyle asiatica* Linn. showed confidence dose and considered as safe. Therefore it was concluded that the median lethal dose (LD_{50}) was more than 18g/kg when administrated orally (cf. Table 4).

In Vitro Antioxidant Activities of Myin-khwa

To thoroughly examine the antioxidant activities of Myin-khwa, *in vitro* radical scavenging activity screening was carried out by using DPPH assay. This assay was chosen to apply because of its simplicity and applicability either an antioxidant in its pure state or in a mixture (e.g., natural extract). This method based on the

capability of test sample to inhibit stable free radical (DPPH) that was followed by decrease in absorbance at λ_{max} 517 nm. Low absorbance value of test sample indicates high % inhibition against DPPH radical which in turn reveals high radical scavenging activity of that sample at constant concentration.

In this experiment, five concentrations (1.25, 2.5, 5, 10 and 20 μg/ml) for each extract were prepared and determined their radical scavenging activity. Ascorbic acid was used as a standard antioxidant for comparison purpose.

The absorbance of DPPH solution after exposing individual concentration of extracts (distilled water, ethanol, n-hexane and ethyl acetate) of *Hydrocotyle asiatica* Linn. are shown in Table (5). Similarly, the results obtained from ascorbic acid are also reported along side. It was found the higher the concentration of the Myin-khwa extract, the lower the absorbance of DPPH solutions and the greater the radical scavenging activity. On the basis of absorbance value, % inhibition of individual extract at different concentration was calculated and from which IC_{50} values for individual extract were derived and reported as Table (6).

The plot of concentration versus % inhibition of individual extracts is shown in Figure (2). Comparison of radical scavenging activity (IC_{50} values) of different crude extracts of Myin-khwa wa done. Among them, radical scavenging activity of ethanolic extract (IC_{50} = 0.66 μg/ml) was found to be highest, it was found to be higher than that of ascorbic acid (IC_{50} = 0.89 μg/ml) standard. According to these results, especially $^{*}LD_{50}$ and $^{**}IC_{50}$ values, Myin-khwa is non-toxic plant and is suitable for food.

* LD_{50} is the statistically derived single dosage of a substance that can be expected to cause death in 50% of the animals.

** IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radical.

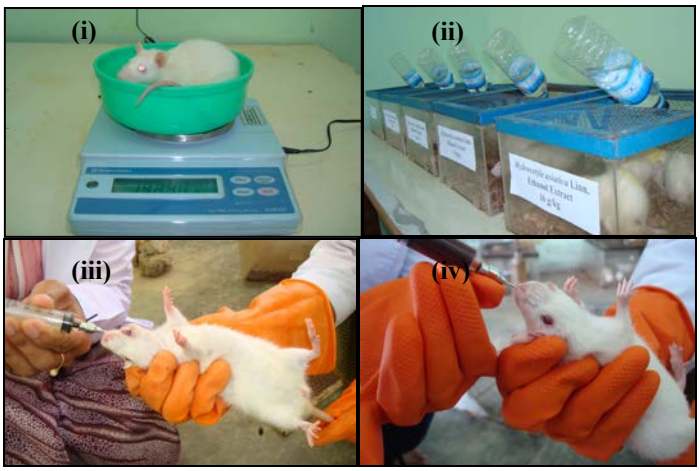

Figure (1) i) Weighing
ii) Wistar Strain Rats of Both Sexes
iii) Administration of Distilled Water
iv) Administration of Drug Solution

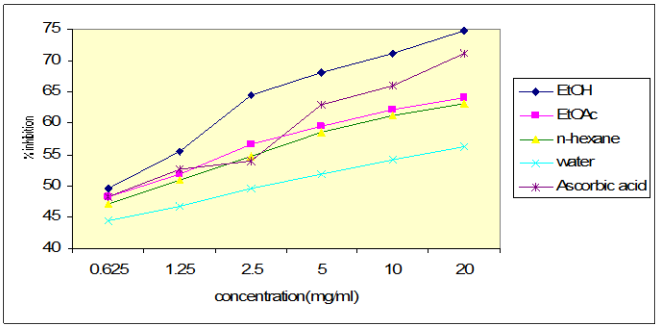

Figure (2) Plot of Concentration Vs % Inhibition of Crude Extract

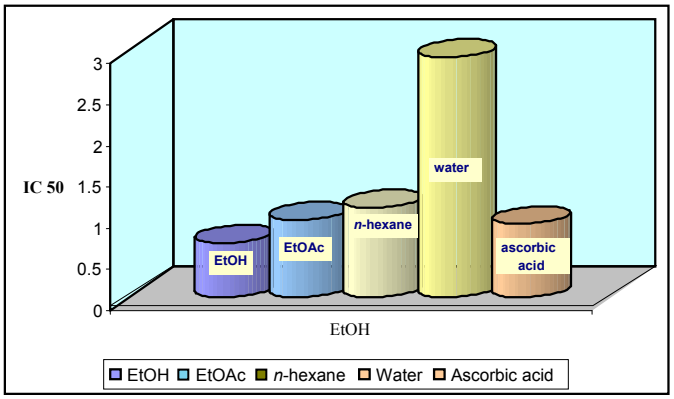

Figure (3) Comparison of IC_{50} Values of Different Crude Extracts

Table (1) Result of Preliminary Phytochemical Test on Myin-khwa

No.	Constituent	Reagents	Observation	Myin-khwa
1	Alkaloid	Mayer's solution	cream ppt	+
2	Flavonoid	Zn (or)Mg, dil HCl	color changes	+
3	Glycosides	NaOH	yellow ppt	+
4	Saponins	10 drops of $NaHCO_3$	Nil	-
5	Polyphenol	10% $FeCl_3$	Blue color	+
6	Steroid	Acetic anhydride,H_2SO_4 (conc;)	greenish blue color	+
7	Phenolic compound	10% $FeCl_3$	purplish color	+
8	Carbohydrate	Benedict's	red ppt	+

(+) = presence of constituent (-) = absence of constituent

Table (2) Nutritive Value of Dry Myin-khwa Residue

No.	Nutritional Value	Values (%)	Method
1	Moisture	7.75 ± 0.13	Moisture Analyser
2	Ash	8.66 ± 0.11	Muffle furnace
3	Protein	20.97 ± 0.27	Kjeldahl Analyser
4	Fat	1.68 ± 0.17	Soxhlet extraction
5	Fibre	1.46	Kjeldahl Analyser
6	Carbohydrate	4.11	UV-vis spectrophotometer
7	Phosphorus	1.392	UV-vis spectrophotometer

Table (3) Mineral Contents of Myin-khwa

No.	Parameter	Symbol	Measuring Value (ppm)
1	Calcium	Ca	6334
2	Iron	Fe	1263
4	Potassium	K	23727
3	Magnesium	Mg	5825
4	Manganese	Mn	120
5	Sodium	Na	873
6	Zinc	Zn	49

Table (4) Lethal Activity of Ethanolic Extract of Myin-khwa Showing Number of Death, Survivals and Percent of Death

group No.	Dose of alcoholic extract of Myin-khwa	No. of rats used	No. of rats dead	No. of rats surviced	% dead
1	12g/kg	10	0	10	0
2	16g/kg	10	0	10	0
3	18g/kg	10	0	10	0
control	10ml/kg	10	0	10	0

Table (5) Comparison of % Inhibition for 4-crude Extracts with Ascorbic Acid

Concentration (μg/ml)	EtOH	EtOAc	n-hexane	water	Ascorbic Acid
0.625	49.63	48.15	47.17	44.44	48.14
1.25	55.55	51.88	50.94	46.66	52.59
2.5	64.44	56.60	54.71	49.63	57.03
5	68.15	59.43	58.50	51.85	62.96
10	71.11	62.26	61.32	54.07	65.92
20	74.81	64.15	63.20	56.30	71.11

Table (6) Absorbance, % Inhibition of Various Concentrations of Crude Extracts and IC_{50} Values

Extracts	Concentration (μg/ml)	Mean Absorbance	Mean % Inhibition	IC_{50} (μg/ml)
Ethanol	0.625	0.109	49.63	0.66
	1.25	0.102	55.55	
	2.5	0.092	64.44	
	5	0.087	68.15	
	10	0.084	71.11	
	20	0.080	74.81	
Ethyl acetate	0.625	0.110	48.15	0.94
	1.25	0.106	51.88	
	2.5	0.101	56.60	
	5	0.097	59.43	
	10	0.094	62.26	
	20	0.092	64.15	
n-hexane	0.625	0.111	47.17	1.09
	1.25	0.107	50.94	
	2.5	0.103	54.71	
	5	0.098	58.50	
	10	0.095	61.32	
	20	0.092	63.20	
Water	0.625	0.114	44.44	2.19
	1.25	0.112	46.66	
	2.5	0.109	49.63	
	5	0.106	51.85	
	10	0.103	54.07	
	20	0.101	56.30	
Ascorbic acid	0.625	0.111	48.14	0.89
	1.25	0.105	52.59	
	2.5	0.100	54.03	
	5	0.093	62.96	
	10	0.090	65.92	
	20	0.084	71.11	

Conclusion

The whole plant (Myin-khwa) *Hydrocotyle asiatica* Linn. (Umbelliferae) was selected for pharmacological investigation. Myin-khwa contains flavonoid, polyphenol, and phenolic compounds which are antioxidant activity. The pH of Myin-khwa was determined; it is slightly acidic 5.19 $\pm$ 0.01. According to the nutritional values, Myin-khwa has low moisture content, low inorganic contents and suitable amount of fiber, fat, protein, carbohydrate and phosphorus. From AAS study, Myin-khwa contains adequate amount Ca, Fe, K, Mg, and Na which are essential mineral for human health. In acute toxicity study, 18 g/kg of ethanolic extract of *Hydrocotyle asiatica* Linn. on Wistar Strain Rats did not cause any death within 24 hours and for two weeks. So, it was found that 18g/kg dose of ethanolic extract of *Hydrocotyle asiatica* Linn. showed confidence dose and considered as safe. Therefore it was concluded that the median lethal dose (LD_{50}) was more than 18g/kg when administrated orally. The antioxidant activities of crude extracts were done by using 1, 1-diphenyl-2-picryl hydrazine (DPPH) assay. The ascorbic acid was used as a standard compound in this assay. When the % inhibitions of 4-crude extracts were compared with Ascorbic acid, ethanolic extract was found to be the highest than standard ascorbic acid and other three solvent extracts. Therefore, the ethanolic extract has the highest antioxidant activity, the IC_{50} value of ethanolic extract is 0.66 mg/ml. According to the observation of this research work, it can be seen that Myin-khwa has not any toxic upon human and it has enough antioxidant content to support the human requirements.

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