

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF DIFFERENT PARTS FROM FIVE MALAYSIAN HERBS

ABSTRACT

Natural drugs play important and vital role in the modern medicine. It is usually used to cure some ailments which may not be treated by conventional medicine. Natural drugs may exhibit many biological activities, such as antimicrobial, anticancer, anti-diabetic and antioxidant. As the prevalence of the diseases due to free radicals increases over the years, the study of antioxidants, which is substances used to fight against the free radicals has stimulates the concerns of the public and researchers. Thus, the objective of the study is to extract and analyse different parts of 5 chosen Malaysian herbs. The ethanolic extracts of seeds of *Carica Papaya*, leaves of *C. nutans*, and *S. crispa*, fruit of *M. charantia*, and roots of *T. cordifolia* were used in the study to identify and compare their potential antioxidants properties by studying their effect on the standard free radical, 2,2-Diphenyl-1-picryl-hydrazyl(DPPH). Vitamin E was used as the positive control of the study. Based on the results obtained, it was showed that the percentage of radical scavenging activity increases when the concentration of plant extracts changes from 0.01mg/mL to 0.1mg/mL and to 1mg/mL. Besides that, among the five plants, the radical scavenging activity of *S. crispa* (93.75%±1.10) were highest and the seeds of *C. papaya* was the lowest (67.45%±9.04) compared to the other four plants when concentration of to 1 mg/mL. At concentration of 2.69 mg/mL, vitamin had 29.67%±4.59 of radical scavenging activity. The concentrations of plants extracted required to cause 50% loss of DPPH activity were also recorded and it showed that the plant with lowest IC₅₀ was *S.crispa* (30.84µg/mL±1.07) and the highest was *C.papaya*(521.3µg/mL±1.10). The results show that *S.crispa* was the most potent antioxidants among the fivesamples and *C.papaya* was the least potents. Preliminary phytochemicals screening were also conducted to identify the presence of various phytochemicals, such as carbohydrate, protein, fats and oils, alkaloids, tannins, saponins, steroids, glycosides and flavonoids. This study is useful to aid in the development of drugs to treat various diseases, especially those due to free radicals.

Key words: Natural drugs, seeds of *Carica Papaya*, leaves of *C. nutans*, and *S. crispa*, fruit of *M. charantia*, and roots of *T. cordifolia*, and DPPH essay

1. INTRODUCTION

Natural products play an important roles of drug discovery process include provide basic compounds affording less toxic and more effective drug molecules, serve as extremely useful natural drugs, exploration of biologically active prototypes towards newer and better synthetic drugs and modification of inactive natural products by suitable biological or chemical means into potent drugs. (Ashutosh K., et al 2007).

Malaysia is one of the countries where vast areas of tropical rainforest are located. According to WWF (World Wide Fund for Nature), forests in Malaysia still cover about 59.5% of the total land area even though deforestation has been increasing tremendously over the years. This vast area of the forests has made Malaysia to become a country which has abundant natural resources and there are over six thousand species of tropical plants all over the country (Zakaria M., et al 2010).

Antioxidants are substances that are used to fight against the free radicals which has involved in food and chemical material degradation and oxidize nucleic acids, proteins and lipids which will cause oxidative stress and initiating degenerative diseases such as cancer, Alzheimer's disease, Parkinson's diseases and some cardiovascular diseases Over the years, prevalence of the diseases result from oxidative stress has been increasing over the year (Pisoschi, et. al., 2009).

Hence, the aim of this part of the study was to extract and analyse the antioxidant property of different parts of five chosen plants (seeds

of *C. papaya*, leaves of *C. nutans*, and *S. crispa*, fruit of *M. charantia*, and roots of *T. cordifolia*)

2. MATERIAL AND METHODS

2.1 Materials

The herbal samples used in this study were; different parts of five chosen plants (seeds of *C. papaya*, leaves of *C. nutans*, and *S. crispa*, fruit of *M. charantia*, and roots of *T. cordifolia*) collected from nursery at Klang, Malaysia. The 1,1-diphenyl-2-picrylhydrazyl (α, α -diphenyl- β -picrylhydrazyl; DPPH:1) and Vitamin E obtained from BDH (Dorset, England) and Merck (Darmstadt, Germany) respectively. All other solvent and chemical used were of analytical grade from J. T. Baker (Phillipsburg, NJ, USA).

2.2 Instrumentation

Soxhlet apparatus, first described in 1879, is a versatile tool that can be used to separate a single gram to hundreds of gram with 100 % recovery. The basic procedure calls for a solid sample to be placed in a porous container and allowing the condensed solvent to extract continuously. There are 3 basic components of a Soxhlet apparatus: a) condenser: to cool the solvent vapor and cause it to condense and turn back into liquid, b) porous container: to hold the liquid sample and allow for the condensed solvent to saturate and pass through thereby extracting active material, c) distilling pot: to hold the solvent and serve as reservoir for the concentrated material. Another instruments used were Rotary Evaporator, digital balance, and UV-visible Spectrophotometer.

2.3 Collection and identification of plants materials

Fresh leaves of *Strobilanthes crispa* and *Clinacanthus nutans* were obtained from Jinjang Utara, Kuala Lumpur. Fresh seeds of *Carica papaya* was obtained from Kepong, Kuala Lumpur. Dried roots of *Tinospora cordifolia* and dried fruits of *Mormodica charantia* were obtained from Ethno Herbs Resources, Sungai Buloh, Selangor. All the plants were sent to Institut Biosains, Universiti Putra Malaysia for species identification.

2.4 Extract preparation

The selected parts of the fresh plants were washed with running tap water and dried under sunlight for few weeks. All the dried plants were grinded by using blender to powdered-like substances. The powdered-like substances were extracted with absolute ethanol for 24 hours using Soxhlet apparatus. The extracts were then dried by using rotary evaporator until semi-solid is obtained. (See figure 1)

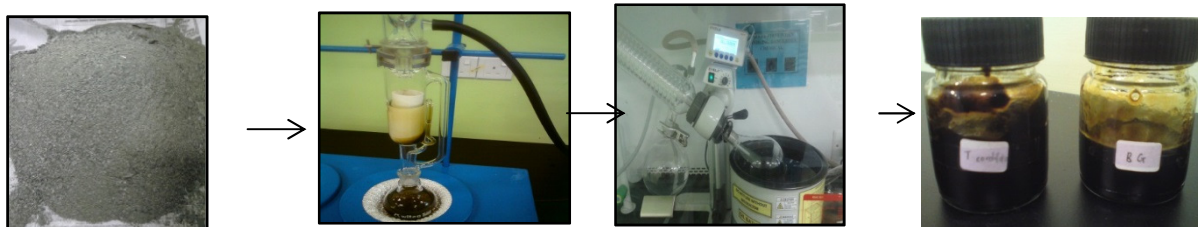


Figure 1: Steps in Extract Preparation which includes grinding to form powder-like material, soxhlet extraction and until rotary evaporation

2.5 Preliminary phytochemical screening

It involves the testing of the extracts of different Malaysian herbs to identify the various phytoconstituents. The methods for the screening will be carried out by following the standard procedures described by Kokate et.al. (2009) and Khandelwal KR (1995) with some modifications. The tests for phytochemical screening include:

a) Test for Carbohydrates (Molisch's Test)

Extracts were dissolved individually in 5 ml distilled water and filtered. Filtrates were treated with 2 drops of alcoholic α -naphthol solution in a test tube. Add 0.2mL of con. Sulfuric acid slowly through the sides of the test tube, a purple to violet color ring appears at the junction.

b) Test for Proteins & Amino Acids (Millons test)

Test solution with 2mL of Millons reagent (Mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate appears, which turns red upon gentle heating.

c) Test for Fats & Fixed Oils

a. Stain test

Press the small quantity of extract between two filter papers, the stain on I filter paper indicates the presence of fixed oils.

b. Saponification test

Add a few drops of 0.5N of alcoholic potassium hydroxide to small quantities of various extracts along with a drop of Phenolphthalein separately and heat on a water bath for 1-2 hrs. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

d) Test for Alkaloids (Hager's test)

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. Filtrates were then treated with Hager's reagent (saturated picric acid solution). The presences of alkaloids were confirmed by the formation of yellow coloured precipitate.

e) Test for Phytosterols(Salkowski test)

Treat extract in Chloroform with few drops of cone. Sulfuric acid, shake well and allow standing for some time, red color appears at the lower layer indicates the presence of Steroids and formation of yellow colored lower layer indicates the presence of Triterpenoids.

i. Diterpenes (Copper acetate Test)

Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

f) Test for Tannins and Phenolic compounds (Ferric Chloride Test)

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

g) Test for Flavonoids (Lead acetate Test)

Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

h) Test for Glycosides (Bromine water test)

Extracts were treated with bromine water gives yellow precipitate.

i) Test for Specific Glycosides

a) Saponin Glycosides - Frothing Test

Extract was dissolved in water and shaken vigorously. Froth which last for a long time shows the presence of saponins.

b) Anthraquinone Glycosides – Borntragers Test

Extracts were treated with 5mL chloroform and shaken for 5 minutes. The extract were filtered and filtrate was added with equal volume of 10% ammonia solution. A pink violet or red colour was observed for the presence of anthraquinone.

2.6 Antioxidant assay

In order to compare the antioxidant properties of the samples, DPPH method was used. In this method, the free radical scavenging activity of extract was measured by the method described by Chan, et. al (2008) with some modifications. One gram of semi-solid extracts of *S. crispaw* was dissolved in 10 mL absolute ethanol to make up stock solution S_0 . Stock solution S_0 is then diluted ten times to make up stock solution S_1 . Stock solution S_2 was made by dilution S_1 by ten times dilution. The selected stock solution of extracts were dissolved in absolute ethanol at specific volume as listed in Table 1 to make up concentrations of 1 mg/mL, 0.1 mg/mL and 0.01 mg/mL to be used as working solution. The solutions were then mixed well with 40 μ L of 2mM DPPH dissolved in absolute ethanol to make up a total volume of 1.6 mL in each plain tube. A background for each concentration was also prepared by mixing the stock solution with ethanol without DPPH. The same procedures were repeated for other plant extracts.

Concentration	Volume of DPPH (μ L)	Volume of Ethanol (μ L)	Volume of Stock Solution (μ L)
S_3 (1 mg/mL)	40	1400	160 of S_1
S_4 (0.1 mg/mL)	40	1400	160 of S_2
S_5 (0.01 mg/mL)	40	1544	16 of S_2
DPPH (blank)	40	1560	0

Table 1: Various Concentration of working solution for antioxidant assay

The final solution was allowed to react at dim light for 30 minutes at 30 °C. It was centrifuged at the speed of 4000rpm for 5 minutes. The absorbance of the supernatant was measured at 517 nm with a UV spectrophotometer. Ethanol were served as the blank, DPPH in ethanol without the extracts were served as negative control and vitamin E (2.69mg/mL, 5.2 mg/mL, 10.4 mg/mL) were served as positive control. The radical scavenging activity of the extract, expressed as percentage inhibition against DPPH, was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[A_0 - (A_1 - A_s)]}{A_0} \times 100$$

Where A_0 is the absorbance of the control solution containing only DPPH after incubation; A_1 is the absorbance in the presence of plant extract in DPPH solution after incubation; and A_s is the absorbance of sample extract solution without DPPH for baseline correction arising from unequal color of the sample solutions (optical blank for A_1). The reading for each sample was repeated 3 times and the test was carried out in duplicates. The mean was calculated.

Half maximal inhibitory concentration (IC_{50}), the amount of sample extracted into 1 mL solution necessary to decrease by 50% the initial DPPH concentration, is then derived from the percentage of radical scavenging activity (% inhibition) versus sample concentration plot. (Norshazile S, et al., 2010) See figure 2.

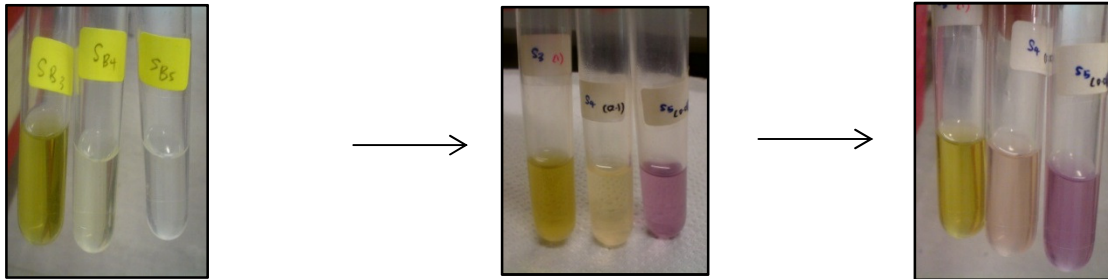
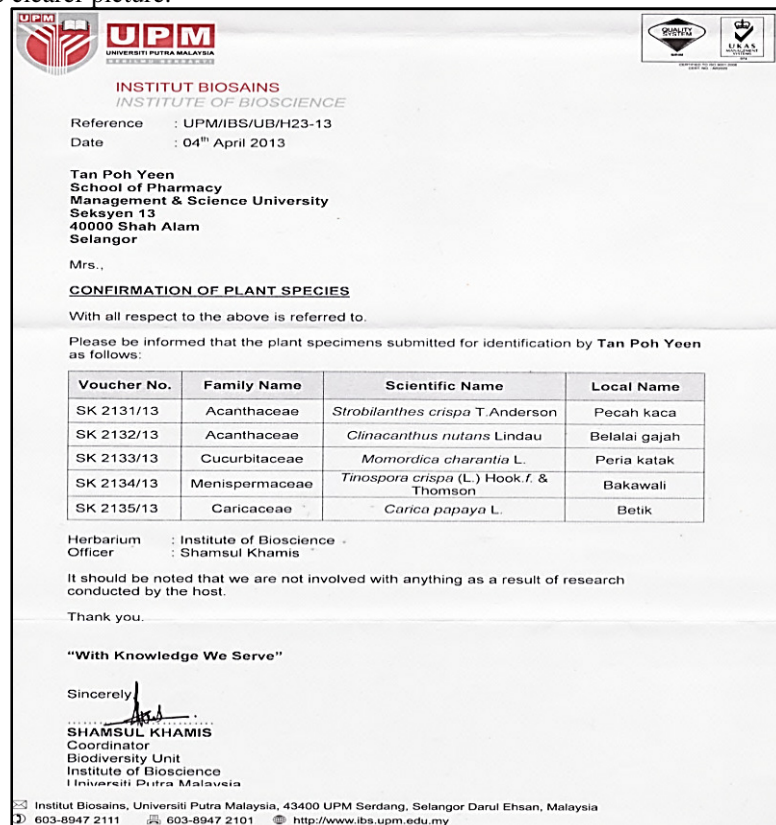


Figure 2: Steps in DPPH assay where the extracts was diluted with alcohol and treated with 2mMDPPH reagent and incubated for 30 minutes. Absorbance will be measured after that at $\lambda=517\text{nm}$ by using UV spectrophotometer

3. RESULT & DISCUSSION

3.1 Plant authentication

The authentication result of the five samples which were send to Institut Biosains, Universiti Putra Malaysia, confirm that the samples are *Strobilanthes Crispa*, *Clinacanthus nutans*, *Tinosporacordifolia*, *Mormodicacharantia* and *Carica papaya*. Figure 4 shows the certificate of authentication. See Apendix for the clearer picture.



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Reference : UPM/IBS/UB/H23-13
Date : 04th April 2013

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Mrs..

CONFIRMATION OF PLANT SPECIES

With all respect to the above is referred to.

Please be informed that the plant specimens submitted for identification by Tan Poh Yeen as follows:


Voucher No.	Family Name	Scientific Name	Local Name
SK 2131/13	Acanthaceae	<i>Strobilanthes crispa</i> T. Anderson	Pecah kaca
SK 2132/13	Acanthaceae	<i>Clinacanthus nutans</i> Lindau	Belalai gajah
SK 2133/13	Cucurbitaceae	<i>Momordica charantia</i> L.	Peria katak
SK 2134/13	Menispermaceae	<i>Tinospora crispa</i> (L.) Hook. f. & Thomson	Bakawali
SK 2135/13	Caricaceae	<i>Carica papaya</i> L.	Betik

Herbarium : Institute of Bioscience
Officer : Shamsul Khamis

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you.

"With Knowledge We Serve"

Sincerely,

SHAMSUL KHAMIS
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Figure 4: Certificate of Authentication

3.2 Extraction yield

The selected plants are extracted with ethanol by using soxhlet extractor. The percentage of yield is calculated by using the formula:

$$\text{Percentage of Yield (\%)} = \frac{\text{Amount of extract yield (g)}}{\text{Amount of dried plants used (g)}} \times 100$$

The results were summarized in the following Table 2

Sample	Amount of dried plants used (g)	Amount of extract yield (g)	Percentage of Yield (%)
StrobilantesCrispa	50.44	8.48	16.80
ClinacanthusNutans	48.23	12.51	25.94
TinosporaCordifolia	52.14	11.77	22.57
MomordicaCharantia	69.86	10.59	15.16
Carica Papaya	50.81	10.94	21.53

Table 2: Extraction yield of the selected plants

3.3 DPPH radical scavenging activity of sample

DPPH radical scavenging activity (%) can be calculated by using the formula as mentioned previously. The values obtained are tabulated in Table 3 and it is expressed as mean \pm standard deviation.

SAMPLE	DPPH radical scavenging activity (%)		
	0.01mg/mL	0.1mg/mL	1mg/mL
S.crispa	7.60 \pm 4.75	93.16 \pm 2.72	97.35 \pm 1.10
C.nutans	8.19 \pm 2.22	36.11 \pm 3.25	97.07 \pm 2.35
T.crispa	2.65 \pm 2.11	38.69 \pm 3.95	94.44 \pm 3.82
M.charantia	7.74 \pm 1.04	10.43 \pm 1.37	89.53 \pm 3.94
Papaya seeds	4.70 \pm 2.20	13.19 \pm 4.79	67.45 \pm 9.04

- Values are expressed in mean \pm standard deviation

Table 3: DPPH radical scavenging activity of various plant extracts

Based on the results obtained, a graph also can be made. Figure 5 showed the percentage of radical scavenging activity of various plant extracts at different concentration. It shows the comparison of the ability of various concentration of plant extract to scavenge the free radical, DPPH.

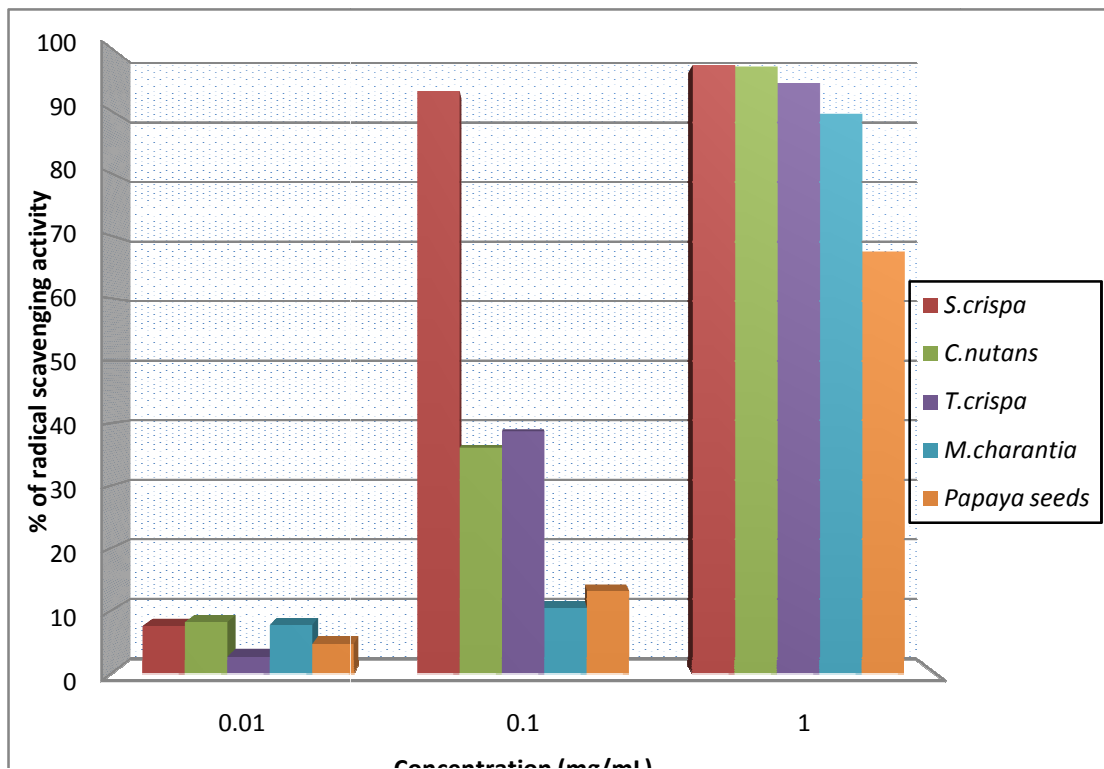


Figure 5: Percentage of Radical Scavenging Activity of Various Plant Extracts at Different Concentrations

3.4 Half maximal inhibitory concentration (IC₅₀)

Based on the data obtained from the DPPH scavenging activity, IC₅₀ (half maximal inhibitory concentration), which is the amount of sample extracted into 1 mL solution necessary to decrease by 50% the initial DPPH concentration, is then derived from the percentage of radical scavenging activity (% inhibition) versus sample concentration plot. The results obtained are tabulated in Table 4.4 and graph of IC₅₀ vs. plant sample are plotted as Figure 4.

Sample	Half Maximal Inhibitory Concentration, IC ₅₀ (μG/mL)
<i>S. crispa</i>	30.84 ± 1.07
<i>C. nutans</i>	142.7 ± 1.07
<i>T. cordifolia</i>	138.1 ± 1.04
<i>M. charantia</i>	315.3 ± 1.09
<i>C. papaya</i>	521.3 ± 1.10

- Values are expressed in mean ± standard deviation

Table 4: Half Maximal Inhibitory Concentration, IC₅₀ of various plants extracts

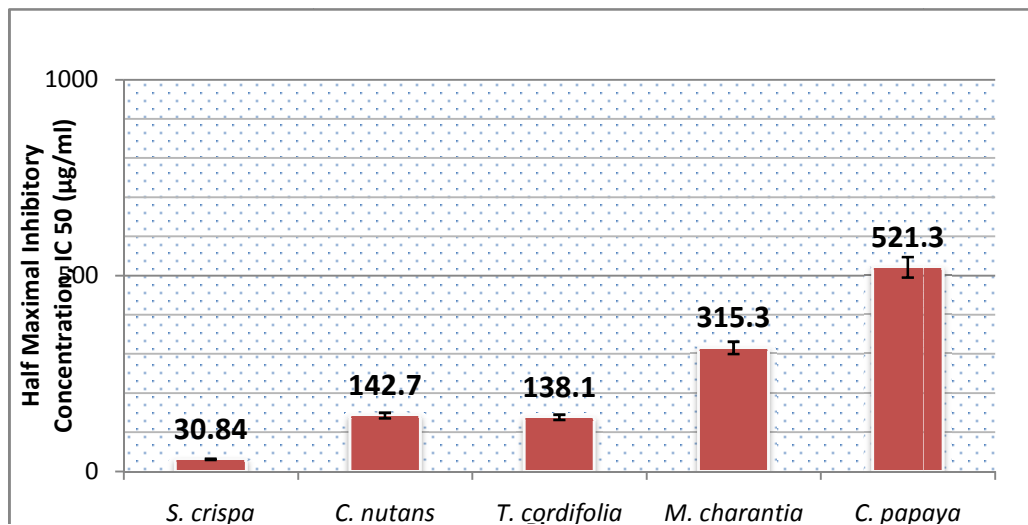


Figure 6 Half Maximal Inhibitory Concentration, IC₅₀(µg/mL) of various plants extracts

3.5 Preliminary phytochemicals screening

Different phytochemicals test were used to identify the phytochemicals that are present in the sample. The results of the tests are shown in Table 5.

Phytochemicals	<i>S. crispa</i>	<i>C. nutans</i>	<i>M. charantia</i>	<i>T. cordifolia</i>	<i>C. Papaya</i>
1) Carbohydrate					
a) Molisch Test	-	+	+	+	+
2) Protein					
a) Millon test	-	-	+	-	+
3) Fats and oils					
a) Stain Test	-	-	+	+	+
b) Saponification Test	-	-	+	+	+
4) Alkaloid					
a) Hager test	+	+	+	+	+
b) Mayer test	+	+	+	+	+
5) Steroids					
a) Salkowski reaction	+	+	-	-	+
6) Triterpenoids					
a) Salkowski reaction	-	-	-	+	-
7) Diterpenes					
a) Copper acetate test	+	+	-	+	-
8) Tannin					
a) Ferric Chloride reduction test	-	+	-	-	-
9) Flavonoids					
a) Lead Acetate test	-	+	+	-	-

10) Glycosides					
a) Bromine water test	-	+	+	+	+
11) Anthraquinone glycosides					
a) Borntrager's test	-	-	-	-	-
12) Saponin					
a) Frothing Test	+	+	+	-	-

Table 5: Summary of the phytochemical screening of the five samples

4.0 CONCLUSION

As antioxidants play an important role in fighting against many diseases, especially those that are due to oxidative stress, it is important to identify which natural plants are effective in fighting against the free radicals. Based on the results obtained, the radical scavenging activity is concentration-dependent, as it increases when the concentration changes from 0.01 mg/mL to 0.1 mg/mL and to 1 mg/mL. Among the 5 plants being tested, ethanolic extract of *Strobilanthes crispus* possesses the highest antioxidant activity compared to the other 4 plants. Besides that, its antioxidant activity is also higher compared to standard antioxidant, vitamin E, as a higher concentration of vitamin E is required to achieve the same percentage of antioxidant activity when tested with DPPH assay. Thus this result could be useful in the future for the drug development for treating or preventing diseases.

Besides that, the antioxidant activity of the plants can be resulted from the activity of various phytochemicals present in the plant. However, not all the phytochemicals are responsible for the antioxidant activity, which means that even if a plant tested positive for most of the phytochemicals, it is not necessarily that it could show the highest antioxidant activity. Thus it is important to identify which phytochemicals are responsible for the antioxidant activity.

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