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FORMULATION AND EVALUATION OF ANTIOXIDANT CREAM FROM METHANOL LEAVES EXTRACT OF *POLYGONUM MINUS*

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Abstract:

Oxidative stress is considered as one of the vital factor that causes serious ailments to humans. The free radicals speeds up the aging process and thus, the need of antioxidants gains importance as it will help to protect body from free radicals and their threats. Due to increase in demand for antioxidant products, the present study focuses on the formulation and evaluation of a herbal antioxidant cream from the methanol leaves extract of Polygonum minus. Leaves was extracted by continuous hot extraction method using hexane and methanol as solvent. The extracts were studied on its free radical scavenging activity using DPPH assay. Methanol leaves extract showed higher free radical scavenging activity with the Inhibition Concentration range between 31.25 to 62.5 µg/ml compared to hexane extract which is at 500 µg/ml. However, standard ascorbic acid have showed potential antioxidant activity when compared to sample extracts. Methanol leaves extract was selected for antioxidant cream formulation due to its phenomenal free radical scavenging activity. The invitro evaluation on the formulated cream was carried out on several parameters such as thermal stability, pH, viscosity, type of formulation and physical appearance. Evaluation parameters showed good results and the formulated cream was stable during the entire period of stability testing. The formulated antioxidant cream from the methanol leaves extract of P. minus proved potential free radical scavenging activity, which will enhance the importance of this plant to the future research community and increases its commercial visibility in the herbal and cosmetic industry.

Keywords: *Polygonum minus Huds. (P. minus), DPPH assay, IC50, Antioxidant Formulation.*

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INTRODUCTION:

Almost 80% of the world population depends on the traditional medicines in curing primary health problems. In recent years, the usage of medicinal plants was found to gaining its focus in pharmaceuticals, cosmetics and nutraceutical industries due to its reliable pharmacological actions and affordability to common people which makes them effective in control of various diseases.[4]

In recent days, the demand for antioxidant products keep increasing due to their ability in combating various factors that can leads to aging problem. Antioxidants react by destroying excessive free radicals and inhibit the initiation or propagation of oxidizing chain reaction. Thus, this will delay the oxidative damage process.[12] In addition, plant based antioxidants are more potent, safe and economic when compare to the synthetic antioxidant such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA).[1]

The plant used for this research study was *Polygonum minus* and the universal genus of *Polygonum* contains many medicinal plants which includes *Polygonum multiforum, Polygonum cuspidatum, Polygonum bistorta, Polygonum aviculare, Polygonum tinctorium.*[13] The plant has its origin from Malaysia, Thailand, Vietnam and Indonesia. Locally called as "kesum" in Malaysia, this plant was often consumed raw as 'ulam' for preventive health care.[9] It grows well on the lowlands, this shrubby plant may grow up to 1.0 m. Meanwhile, at hilly areas, it may grow to about 1.5m. With long and lanceolate leaves of about 5.7cm, it gives aromatic fragrant. In addition, it also has small white purpled-coloured flowers at the end of shoot. It also has the ability to survive on cool and hilly area where it commonly found in the damp areas such as the ditches, lakes and riverbanks.[8]

The ethanol leaves extract was found to contain high phenolic compound and furthermore, gallic acid, rutin and coumaric acid were also found in the leaves extract of *P. minus.*[2,10] The chemical composition of *P. minus* leaves extract contains high level of antioxidants, flavonoid and phenolic compound which contain several bioactive properties. The earlier research also supports strong antiviral activity against HPV- Type 1 but weak activity against vesicular stomatitis virus. However, extracts were also tested for its antioxidant, antimicrobial, plant pathogenesis, cytotoxicity, genotoxicity, anti-ulcer activities that has associated with its phytochemical composition Moreover, most edible plants especially spice and herbs contains high antioxidant properties due to rich content of ascorbic acid, carotenoids and phenolic compound such as flavonoids, phenolic acid, alcohol, tocotrienols, stilbenes and tocopherols these compounds have been reported to act as crucial antioxidant source.[5]

Aqueous extract of *P. minus* (200µg/ml) exhibited high antioxidant activity when analysed through lipid peroxidation inhibitory activity, superoxide scavenging activity and DPPH radical scavenging activity. Thus, it can be concluded that *P. minus* have great potential in preventing lipid peroxidation and scavenge access free radical, which may lead to aging process.[11] Comparing with synthetics antioxidant, plant based antioxidant have enormous therapeutics potential as they can serve the purpose with lesser side effects compare to the synthetic production. In view of the above, the present study will focus on the formulation of antioxidant cream by using leaves extract of *P. minus* whereby it enhances its commercial value in the cosmetic industry also draws the attention of the research community to explore more research opportunities with the selected plant.

MATERIAL AND METHODS:

Plant Collection

About 3 kg fresh leaves of the *P.minus* were collected at Changkat Jering, Perak, Malaysia. The collected plant was authenticated by Mr. Suhaimi bin Haji Din, Botanist from Plant Biosecurity Department of Kompleks Pertanian Bumbung Lima, Seberang Perai, Pulau Pinang, Malaysia. The plant leaves were shown in Fig.1. Upon identification, the selected fresh leaves were washed under running tap water to remove the residues if any. The washed leaves were subjected to drying at room temperature for 24 hours, followed by drying in hot air oven at controlled temperature for 48 h. Upon complete drying, the leaves were coarsely grounded using kitchen blender and was weighed and packed in airtight container for further study.

Fig. 1: Leaves of *Polygonum minus*

Plant Extraction

The weighed powdered leaf (500 g) was extracted using the method followed by Lay et.al, with slight

modification.[7] Plant material was extracted by using continuous hot extraction method with hexane as solvent for 48 h at 50o c. Upon complete extraction, solvent was cooled, filtered and the filtrate was concentrated under reduced pressure and controlled temperature using rotary evaporator. The collected hexane extract was weighed, packed in an airtight glass container and was stored in refrigerator until further process.

The extracted marc was dried and packed again in the soxhlet column for further extraction using methanol as solvent at 45o c for 72 h. Upon complete extraction the solvent was allowed to cool, filtered and concentrated using rotary evaporator under reduced pressure and controlled temperature. The collected methanol crude extract was weighed, packed in an airtight glass container and stored in the refrigerator for further studies.

Phytochemical Screening [6]

The stock solution was prepared with the corresponding crude extracts with 10 ml of their own mother solvents. The diluted crude extracts were subjected individually for the following phytochemical screening studies.

Test for Alkaloids

Dragendorff's test

Add 1ml of Dragendorff's reagent into the extract. An orange red precipitate indicates the presence of alkaloids.

Wagner's test

Add Wagner's reagent into the extract. A reddish-brown precipitate indicates the presence of alkaloids.

Mayer's test

Add 1ml of Mayer's reagent into the extract. A dull white precipitate indicates the presence of alkaloids.

Hager's test

Add 3ml of Hager's reagent into the extract. A yellow precipitate indicates the presence of alkaloids.

Test for Flavonoids

Magnesium turnings were added to the test extract, followed by addition of concentrated hydrochloric acid. A red colour indicate the presence of flavonoids.

Test for steroids and sterols

Libermann Burchard test

Extract was dissolved in 2ml of chloroform in test tube. Ten drop of acetic acid anhydride and 2 drops of concentrated sulphuric acid was added. The solution become red, blue and finally bluish green in colour indicate the presence of steroids.

Salkowaski test

Extract was dissolved in chloroform and equal volume of concentrated sulphuric acid was added. Bluish red to cherry red colour is observed in chloroform layer, whereas the acid layer assumes marked green fluoresces indicating the presence of steroids.

Test for Glycosides

Legal test

The extract was dissolved on pyridine. Sodium nitroprusside solution was added and made it into alkaline. Pink or red colour indicates the presence of glycosides

Baljet test

Sodium picrate was added to the extract. Yellow to orange colour indicate the presence of glycosides.

Test for Carbohydrates

Molisch test

1ml of alpha naphthol solution was added into the extract and concentrated sulphuric acid was added along the side the test tube. Purple or reddish violet colours at the junction between the two liquids indicate the presence of carbohydrate.

Test for Proteins

Biuret test

1ml of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution was added into the extract. A violet colour indicates the presence of proteins.

Test for Tannin

Ferric chloride is added to the extract. Dark blue or greenish black colour indicates the presence of tannins.

Potassium dichromate solution was added. The precipitate indicates the presence of tannins.

Free Radical Scavenging Activity using DPPH Assay

Preparation of DPPH Solution

Accurately weighed 22 mg of DPPH in 100 ml of methanol. From this stock solution, 18 ml was diluted to 100 ml with methanol to obtain 100 µml DPPH solutions.

Preparation of Test Solutions

21 mg of methanol and hexane leaves extracts were accurately weighed in a separate test tube and were dissolved with 1 ml of freshly distilled DMSO to obtain solutions of 21 mg/ml concentration respectively. Serial dilutions were made in order to get the final concentrations of 1000, 500, 250, 125, 62.5, 31.25, and 15.625 µg/ml on both the sample extracts respectively.

Preparation of Standard Solution
21 mg of ascorbic acid was weighed and it was dissolved in 1 ml of freshly distilled DMSO to obtain 21 mg/ml concentration and was serially diluted to obtain the lower concentrations as performed with sample extract solutions.

DPPH Assay
To 100 µl of each test and standard concentration solution, 2 ml of DPPH solution was added. The solutions were shaken vigorously and were incubated at 37 °C for 30 min and the absorbance of each solution was measured at 490 nm using UV spectrophotometer.

Formulation of antioxidant cream
Antioxidant cream was formulated using the listed composition stated in table 1.

Table 1: Composition of Antioxidant cream

Components	Amount % w/w
Active Ingredient	
Methanolic leaves extract of *P.minus*	3
Oily phase	
Stearic acid	11
Cetyl alcohol	4
Olive oil	4
Aqueous phase	
Glycerine	4
Triethanolamine	2
Methyl Paraben	0.05
Perfume	0.02
Distlled Water q.s to	100

The accurately weighed quantities of oily phase ingredients and aqueous phase ingredients were heated in the water bath to 700C in separate beakers. Upon complete melting of both phases, the aqueous phase ingredients was slowly added to oily phase and mixed thoroughly. Finally the extract, preservatives and few drops of perfume were mixed to the above warm mixture and mixed thoroughly until the cream reaches homogenous. The base cream was also formulated without adding the active ingredient.

Invitro Evaluation of Formulated Antioxidant Cream
The following parameter were used to evaluate the formulated antioxidant cream. The standard procedures was followed to evaluate all the parameters.[3]

Physical Evaluation
The cream was observed for colour, odour and appearance.

pH
The pH meter was calibrated using standard buffer solution. About 0.5 g of the cream was weighed and dissolved in 50 ml of distilled water and its pH was measured.

Type of Emulsion
Water solution test:-
1 g of cream was dispersed in distilled water inside the bottle. Then the bottle was shake and observed solubility of solution if the solution is homogenous state indicates the cream is o/w but if is not dissolved indicates the cream is w/o, to ensure the result the cream was dissolved in oil to identified the condition and the result will be determined vice versa by using distilled water.

Red dye test:-
The emulsion type was determined by using dye test. The scarlet red dye was mixed with the little quantity of the formulated cream. Small portion of the cream was placed on a microscopic slide covered with a cover slip and examined under a microscope. If the disperse globules appears colourless and the background is red, the cream is oil in water type. The reverse condition occurs in water in oil type cream i.e. the disperse globules appear red in the colourless background.

Appearance and Homogeneity
The formulations were tested for the homogeneity by visual appearance and by touch.

Spreadability (after feel effect)
Emolliency, slipperiness and amount of residue left after the application of the cream was checked.

Loss on Drying
1g of cream was taken in china dish and kept in an oven at 105 °C for 2 hours. Loss on drying is calculated by using this formula:

$$\% \text{ Loss on drying} = \frac{W2 - W3 \text{ (or) } Wn}{W2 - W1} \times 100$$

W1 = Weight of the empty bottle in grams.
W2 = Weight of the bottle with sample in grams (before drying).
W3 = Weight of the bottle with sample in grams (after drying) - as time specified.
Wn = Weight of the bottle with sample after additional 2 hours drying (constant weight).

Thermal Stability
Stability studies were carried out as per ICH guidelines for the formulated cream to access its stability parameters during its storage period. The cream filled bottle was kept in humidity chamber maintained 35+ 2 °C with 65 + 5 % RH for two months. At the end of the studies, samples were analysed for the physical properties.

Viscosity
10 g of cream was weighed and transferred into a 50ml beaker. Keep it impact for 1 hour. The beaker was inclined to one side see whether consistency has changed or not. The beaker was again tilted and checked for the pourability of formulated cream.

Test for Microbial Growth

The formulated creams were tested for its sterility. Small quantity of the cream was inoculated into Muller Hilton agar media through streak plate method and was at 37o C for 24 h. After the incubation period the plates were checked for any microbial growth by comparing with the control plate.

RESULTS AND DISCUSSION:

Percentage Recovery of the extracts

The *P.minus* leaves were extracted with hexane and methanol as solvent against 130 g of powdered leaves for each extraction. The percentage recovery and colour of the extracts were shown in table 2.

Table 2: Percentage recovery and colour of the extracts

P.minus extract	Colour	Percentage Recovery (%w/w)
Hexane extract	Brownish green	4
Methanol extract	Yellowish green	14

Phytochemical Screening
The quantitative phytochemical analysis of methanol leaves extract was tested and the results was shown in table 3. The presence of alkaloids, flavonoids, steroids, glycosides and tannins were confirmed from their corresponding test with methanol leaves extract. However, carbohydrates and proteins showed negative results.

Table 3: Phytochemical constituents of methanol leaves extract of P. minus

Phytochemical constituents	Methanol extract
Alkaloids	P
Flavonoids	P
Steroids	P
Glycosides	P
Carbohydrates	A
Proteins	A
Tannin	P

Free Radical Scavenging Activity using DPPH assay method
The antioxidant activity was compared on both methanol and hexane solvent extracts of *P. minus* leaves using DPPH free radical scavenging assay. The highest free radical scavenging activity between the extracts were analysed against standard ascorbic acid, and it was found that methanol leaves extract of *P. minus* recorded highest DPPH free radical scavenging activity with the concentration range between 31.25 µg/ml to 62.5 µg/ml, whereas the maximum free radical inhibition in hexane extract was recorded only at 500 µg/ml. However, the standard ascorbic acid showed phenomenal antioxidant activity at very lower concentrations. The comparison of IC50 results were shown in the table 4 and table 5 shows the report on statistical analysis of methanol leaves extract on its free radical scavenging activity.

Table 4: Comparison of Ic50 value of sample extract vs standard ascorbic acid

Concentration (µg/ml)	IC Value		
	Methanol extract	Hexane extract	Ascorbic acid
15.625	24.69	17.92	49.32
31.25	35.78	13.54	70.66
62.5	70.08	19.60	74.47
125	72.47	26.31	73.82
250	72.28	32.17	73.31
500	69.37	48.03	75.18
1000	59.90	22.24	72.99

Table 5: DPPH radical scavenging activity of methanol leaves extract of *P. minus*

Concentration (µg/ml)	S.D.	Mean of % inhibition	% Inhibition ± SEM
15.625	0.62	24.69	24.69 ± 0.62
31.25	0.19	35.78	35.78 ± 0.19
62.5	1.12	70.08	70.08 ± 1.12
125	0.56	72.47	72.47 ± 0.56
250	0.78	72.28	72.28 ± 0.78
500	0.34	69.37	69.37 ± 0.34
1000	0.11	59.90	59.9 ± 0.11

Thus, it can be concluded that methanol extract of *P. minus* possess high potential of antioxidant properties compared to the hexane extract. Moreover, observed antioxidant effects can be attributed majorly due to the presence of flavonoids and phenolic compounds in the *P. minus*. A comparison graph of DPPH radical scavenging activity of extract against standard was presented in the Fig. 2.

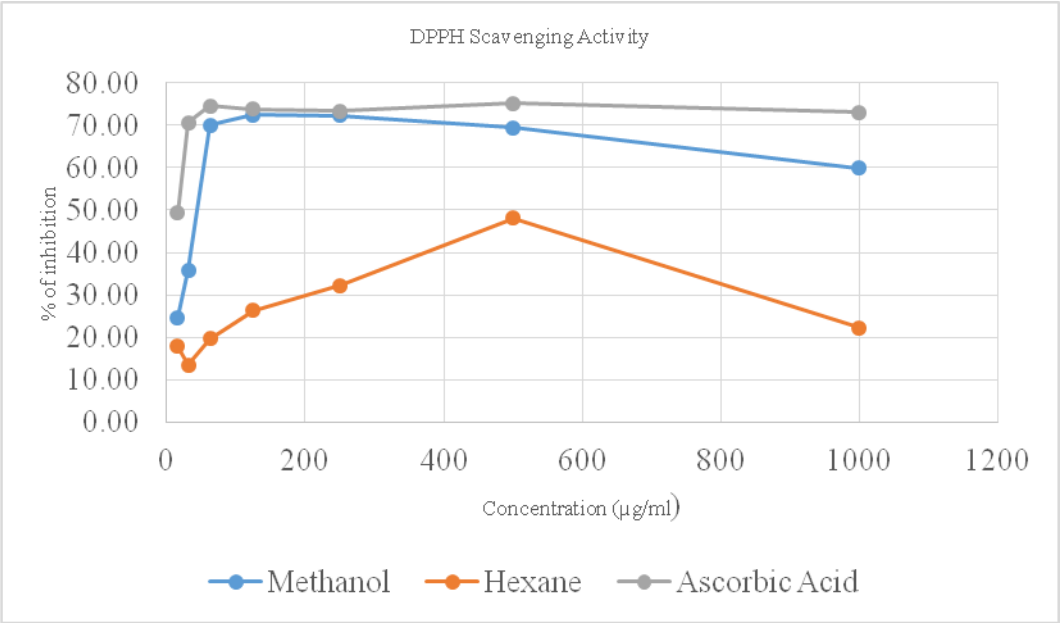

Fig. 2: DPPH scavenging activity of sample extracts vs standard ascorbic acid

Formulation of Antioxidant Cream

From the results of DPPH assay, the methanol leaves extract was chosen for further formulation as antioxidant cream due to its potential free radical scavenging effects. The formulated antioxidant cream was shown in Fig. 3.

Fig. 3: Formulation of antioxidant cream from the methanol extract of *P.minus*

Evaluation of antioxidant cream

The formulated antioxidant cream was subjected to various standard evaluation parameters and the results were shown in table 6. The dye test confirms that the formulated creams were o/w type of cream. The pH of the formulated creams was found to be 6.9 to 7.2.

The formulated cream showed acceptable odour and was in yellowish green colour. The formulated cream found non-greasy after application to the skin and was easily removable by washing with tap water. The cream showed homogenous distribution of extract in the cream which was confirmed by visual examination. There was no change in colour of formulated cream upon keeping it for long period during its entire stability testing. After feel test showed that the formulated cream was emollient and has slipperiness for easy application. Besides, the formulated cream was found to be non-newtonian which was confirmed through its viscosity testing. The loss on drying of the formulated cream was found to be within the limit as stated by standard procedure. All the physicochemical parameters were well maintained during the period of accelerated stability studies at temperatures 35+ 2 °C with 65 + 5 % RH in incubator and at 25°C ± 1°C, 40 °C ± 1 °C at room temperature for 7 weeks. The formulation showed good stability in colour and consistency until the end of accelerated study period.

Table 6: Physicochemical evaluation of the formulated cream

Parameter	Observation
Type of formulation	Oil in water type
Appearance	Yellowish green cream
Odour	Acceptable
pH	6.9-7.2
Homogeneity	Homogenous
Loss on drying	11.25 %
Spreadability	Good
After feel	Emollients and slipperiness
Removal	Easily removed
Stability	Stable until the entire period of study
Viscosity	Non-newtonian cream
Microbial test	No growth of colonies

CONCLUSION:

The demand of antioxidant product had increased in recent days among the cosmetic users due to its combating effects in various extrinsic factor that leads to aging process. The present prototype study proved that the methanol leaves extract was having higher free radical scavenging activity compared to that of hexane leaves extract by using DPPH assay method. Based on the parameters evaluated, the formulated cream from methanol extract revealed that it is safe to be used on the skin as an antioxidant cream. Moreover, the formulated cream showed good stability during the shelf storage. Apart from its efficacy, further study should has to be focused to enhance appearance of the cream colour to make it more appealing to the customers. In conclusion, the topical application of the formulated cream from *P. minus* extract will help in reducing the effects of aging and can give commendable antioxidant effect to our skin due to its high antioxidant values. Moreover, further studies is encouraged to investigate on the human volunteers for its efficacy and acceptance. The present prototype study will enhance the visibility of *P. minus* plant in the herbal and cosmetic industries.

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