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FORMULATION AND *IN VITRO* EVALUATION OF SUNSCREEN GEL FROM THE METHANOLIC RIPEN FRUITS PULP EXTRACT OF *PHALERIA MACROCARPA*

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Abstract:

Exposure of ultraviolet light at cellular level in our skin will lead to sunburn, that results in the formation of free radicals which in turn causes serious illness like skin cancer. However, sunscreen protectors helps to prevent sunburn and reduces the risk of skin cancer and also helps in prevent early sign of skin aging. The current study was designed to formulate and evaluate the sunscreen gel from the methanolic fruit extract of Phaleria macrocarpa. The free radical scavenging activity was initially compared between methanolic extracts of unripen and ripen fruits pulp of P.macrocarpa using DPPH assay. However, the potential free radical scavenging extract was chosen for further formulation into a sunscreen gel. Free radical scavenging activity of the methanolic fruit pulp extract of P. macrocarpa exhibited IC_{50} at a concentration range between 62.50 µg/ml and 125 µg/mL when compared with the unripen fruits pulp extract which resulted IC_{50} at the range 125 µg/mL and 250 µg/mL. Nevertheless, the standard ascorbic acid showed highly potential IC_{50} range between 15.625 µg/mL and 31.25 µg/mL. The formulated sunscreen gel from the ripen fruit extract was evaluated for its Sun Protection Factor (SPF) and was found to be 8.58. The physiochemical evaluation on the formulated gel was analysed, that showed good results on the following parameters like viscosity, opacity, extrudability, spreadability, stability and pH. Thus, the formulated sunscreen gel from the ripen fruit pulp extract of P. macrocarpa can be used as a potential UV rays barrier due to its commendable free radical scavenging activity and its effective sun protection factor.

Keywords: *P. macrocarpa fruits pulp, DPPH, IC_{50}, SPF, UV rays.*

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INTRODUCTION:

Located in the equatorial region, the climate of Malaysia is essentially hot and humid for the whole year. Excessive heat exposure can increase melanin count and cause skin to dehydrate which leads to the formation of freckles, wrinkles, sunburns and pigmentation. Ultraviolet ray exposure at least results in 50% of induced damage to the skin causing the free radical formation [1]. The ultraviolet ray will facilitate the aging process and thus causes decreases skin elasticity. Furthermore, the aging also causes the inner body to accelerate cell death which can lead to carcinogenesis[2]. The free radicals are incomplete and unstable molecules with a loss of an electron that causes it to become extremely reactive towards other molecule. Thus, attacking a normal cell causes destruction and accelerates cell death [3]. Although our body was protected with endogenous defence mechanism which helps in producing antioxidative enzyme and nonenzymatic antioxidative molecules that can reduce and neutralize the free radicals, some of the antioxidant can be inhibited by ultraviolet light [4]. The focus on medicinal plant is gaining importance in cosmetic industries due to its potential proven free radical scavenging effects. *P.macrocarpa* extract has benzophenone group and was responsible for its strong antioxidant activity [5]. Biological active compounds in natural products have been widely used in phytocosmetics and the use of topical formulation containing antioxidants extracted from medicinal plant proved to neutralize some of the resulting free radicals, thus consequently minimize or hinder the signs of aging skin [2].

P. macrocarpa is a species of Thymelaceae family and was believed to have its origin from Papua Island, located in Indonesia. In Malaysia this fruit is called as Mahkota dewa or God's Crown [6]. Moreover, Thymelaceae family has history of ethno pharmacological usage and been use for a long time ago by Malaysian and Indonesian as an indispensable medicinal plant [7]. The fruits are small, round shape with 3-5 cm in diameter and have a smooth surface. The ripen fruit is red in colour while the young unripen fruit is green in colour as shown in Fig 1 & 2. It has a white fleshy pulp with watery and fibrous texture. The plant can grow from 1 to 6 meter tall and capable of growing throughout the year in various conditions from lowland to highland and its productivity is reported to be able to reach for ten years [8].

Natural antioxidants shows commanding uses in cosmetic industry at recent years, due to its benefits when compare to synthetic based formulation. The objective of this study was to formulate and evaluate the sun screen gel from the methanolic fruits pulp extract of *P. macrocarpa*. The present study has focused to identify and select the potential free radical scavenger between the unripen and ripen methanolic fruits pulp extract of *P. macrocarpa*. Furthermore, the potential extract will be formulated as a sunscreen gel. The traditional uses of *P. macrocarpa* was reported to treat diabetes mellitus, hypertension, allergies, heart failure, kidney problem, blood disease, stroke and skin diseases [9]. According to previous biological screening of *P. macrocarpa* fruit, it was found that this fruit has antibacterial, antioxidant, anti-inflammatory, anticarcinogenic, and antimutagenic activity. [10]. The fruits were reported on its antibacterial properties due to its constituents like flavonoid, triterpenoid and alkaloid compound in the ethanol extraction [11]. The phenolic and flavonoid compound were found in the majority with kaempferol, myricetin, naringin, quercetin, and rutin which contribute in the antioxidant properties. [6]. *In vivo* study upon diabetic rat shown a decrease in blood glucose level contributed by the terpenoid, tannins and flavonoid group presence in the fruits [12]. The extraction of *P. macrocarpa* leaves using ethyl acetate contain 2,6,4'-trihydroxy-4-mmethoxy benzophenone which showed a potent antioxidant properties on 2,2-diphenyl-1-picrylhydrazyl with IC_{50} of 10.57 ± 0.72 µg/mol [5].

The phytochemical screening using methanol extract of *P. macrocarpa* it was found that this solvent yield the highest flavonoid content which is 9.33 ± 0.8 mg/ml compare to other type of fractionation [13]. The antioxidant extract in pericarp, mesocarp and seed using ferric thiocyanate method and the results show that pericarp contains the highest phenolic and flavonoid compound which is 59.2 ± 0.04 and 161.3 ± 1.58. The antioxidant activity evaluated using free radicle scavenging reported that pericarp gives the highest scavenging activity; 71.97%[6]. Tray drying method is used in preserving the alkaloid compound as it can be degraded by UV irradiation during direct sun drying. This method also gave the largest antioxidant yield of 0.200 µmol 2,2-diphenyl-1-picrylhydrazyl/mg [14]. Thus, the selected plant has wide range of uses both in medicinal and cosmetics, an attempt was made in the current study to increase the visibility and commercial value of this selected plant parts by formulating as a Sunscreen gel.

MATERIALS AND METHODS:

Collection and Identification

Each 5 kg of unripen and ripen *P. macrocarpa* fruits was collected from a local market in Kedah, Malaysia. The fruits were authenticated by the botanist Mr. Suhaimi at plant biosecurity division Pulau Pinang, Malaysia and the herbarium was recorded and stored for future refrence. Care was taken to select healthy fruits and the selected fruits were then washed thoroughly using tap water to remove dust and foreign materials. The washed fruits were wiped off to remove excess moisture, peeled and sliced to separate pulp from the seed. The

collected fruits pulp was subjected to tray drying at 40° C for 72 h. Dried pulp of ripen and unripen fruits were grounded respectively to a coarse powder using the blender. The powdered samples were collected, weighed and stored accordingly in air tight containers until further study.

Fig 1: Ripen Fruits of *P. macrocarpa*

Fig 2: Unripen Fruits of *P. macrocarpa*

Extraction

The grounded coarse powders of ripen and unripen fruits pulp were packed in an individual soxhlet extraction column and was subjected to continuous hot extraction using methanol as solvent at 40° C for 48-72 h. Upon complete extraction, both the extracted solvents was cooled and made to concentrate in a rotary evaporator under controlled temperature and pressure. The percentage yield of the extract was calculated and recorded. The concentrated extracts were collected, labelled, stored in a well closed container and kept in refrigerator until further use.

Qualitative Phytochemical Analysis

The stock solution was prepared from the crude extracts by dissolving it in 8 ml of its mother solvent. The obtained stock solutions were subjected to preliminary phytochemical screening using standard method [15].

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.

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 Carbohydrates:

Molisch test

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

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 fixed oils:

Spot test

A small quantity of extract was pressed between two filter papers. Oil stains on filter paper indicated presence of fixed oil.

Test for Tannins:

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 tannin the presence of tannin.

Test for Flavonoids:

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

Free radical scavenging Assay Using DPPH

Antioxidant was proven to have an inhibitory or delaying effect during oxidation process. This in

vitro method is based on inhibition whereby samples were added to the generating system and the antioxidant activity is measured indirectly by measuring the inhibition of the free radical action. Both the extracts and standard ascorbic acid were tested for its in vitro antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) using free radical scavenging assay. The final concentration of the extract and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml. The absorbance was measured using spectrophotometer against the corresponding blank solution.

The percentage of free radical scavenging was calculated using the following formula.

Scavenging activity % = $(A_{control} - A_{sample}) / A_{control} \times 100$

$A_{control}$: absorbance of control reaction

A_{sample} : absorbance in present extract

IC_{50}, which is the concentration of the sample required to inhibit 50% of free radicals was calculated.

DPPH Assay

The present study on estimation of free radical scavenging activity of ripen and unripen pulp extracts of *P. macrocarpa* on 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical was analyzed using standard assay method.

Preparation of Reagents

2, 2-Diphenyl-1-picryl hydrazyl solution (DPPH, 100 µg):

22 mg of DPPH was weighted accurately and added into100 ml of methanol. 18 ml from the stock solution prepared was pipetted out using 10ml pipette and diluted to 100 ml with methanol to obtain 1000 µg DPPH solution.

Preparation of Extract Solutions

21 mg of each extracts was weighed and dissolved in 1 ml of freshly distilled $DMSO_4$ to obtain solutions of 21 mg/ml concentration. These solutions were serially diluted separately to obtain the lower concentrations.

Preparation of Standard Solutions

21 mg of ascorbic acid was weighed and dissolved in 1 ml of freshly distilled $DMSO_4$ to get 21 mg/ml concentration. These solutions were serially diluted separately to obtain the lower concentrations.

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

Formulation of Sun Screen Gel

Methanolic extract of ripen *P. macrocarpa* was used to prepare the sun screen gel. The compositions of the gel were shown in Table 1.

Table 1 : Composition of sun screen gel.

Ingredients	Amount (% w/w)
Ripen fruits pulp extract of *P. macrocarpa*	1.0%
Methyl paraben	0.02%
Propyl paraben	0.2 %
Polyethylene glycol	4 %
Methanol	3 %
Carbopol 940	1 %
Triethanolamine	1%
Distilled water q.s	100 %

The sunscreen gel was formulated by using methanolic pulp extract of ripen *P. macrocarpa* fruits, carbopol 940, methyl paraben, propyl paraben, polyethylene glycol, triethanolamine and distilled water. Water was divided into two parts, to one part added carbopol 940, mixed and allowed to stand for approximately 2 hours to swell completely and the remaining part of water was used to dilute the methanolic extract followed by addition of polyethylene glycol, methyl paraben and propyl paraben.Upon complete mixing, the mixture was transferred into the beaker containing carbopol. Furthermore, required quantity of tri-ethenolamine was added to above mixture and mixed thoroughly to obtain good gel consistency. The formed gel was sonicated in order to remove the air bubbles from the formulation. The formulated sunscreen gel was shown in Figure 3. The base gel formulation was also formulated omitting the extract.

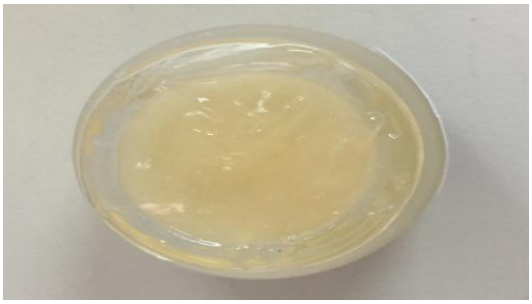

Fig 3: Formulated Sunscreen Gel

***In Vitro* Evaluation of formulated Sunscreen gel**

Following parameters were evaluated invitro on the formulated sun screen gel.

Physical properties:

The prepared gel formulation was inspected for its colour, odour and appearance.

Determination of pH:
The pH meter was calibrated using standard buffer solution prior to testing the sample. To 0.5 g of formulated sunscreen gel added 50 ml of distilled water, mixed well and pH was measured.

Homogeneity and Appearance:
The formulated gel was examined for its homogeneity by visual appearance and by touching.

After Feel Effect:
Slipperiness, emolliency and amount of residue left after the application of gel on hand was checked.

Loss on Drying:
Weighed accurately 1 g of formulated gel and transferred into a petri dish and kept in an oven at 105 °C for 2 hours. Upon completion, allowed the petri dish to cool before it is weighed. The percentage loss of drying was then calculated using the following formula
Percentage loss of drying: Initial weight – weight after drying / initial weight × 100

Spreadability:
Spreadability of the formulated sunscreen gel was evaluated by taking the final diameter of 0.5 g gel which was placed on the pre-marked 1 cm diameter, being pressed between two horizontal glass plate with a loading weight of 500 g for 5 five minutes and observed.

Centrifugation test:
To 10 g of the formulated gel, transferred into a tapered test tube and centrifuged at 3000 rpm for 30 minutes at room temperature. Any changes to the consistency of the gel were observed and reported.

Extrudability test:
Aluminum foils was used to make a cone like container mimicking a cone tube and 10 g of gel was placed in the foil container. The end of foil container was sealed to prevent any rollback and a hole of standard diameter was made at the tapered end. The gel was pressed firmly for 10 second at the sealed end and the weight of gel removed from the container was calculated and reported.

Opacity test:
Small amount of gel was spread smoothly on a slide. A hollow light was directed to the front part of the slide and the penetration of light rays was observed.

Sterility test:
Small quantity of the formulated gel was tested for its stability using spread plate method in the nutrient agar medium and incubated for 24 h at 37^0 c and observed for any colony growth.

Viscosity test:
The formulated gel was subjected for its viscosity determination using Modular Compact Rheometer 202 without dilution. Shear rate versus shear stress was applied for 1-6 shear rate per second with one second measuring point. The viscosity range was analysed from 2350 to 580 pascal per second by applying Carreu-Yasuda constant.

Stability Studies:
The formulated sunscreen gel was subjected to stability studies in order to evaluate the formulation stability as per standard guidelines. To the weighed quantity of the formulated gel in a glass container, incubated at a temperature of 37°C for two months. At the end of the studies, samples were analyzed for the physical properties, pH, homogeneity and appearance, after feel effect, spreadability, extrudability, sterility, opacity and viscosity.

Evaluation of Sun Protecting Factor (SPF) on the formulated gel:
The SPF of formulated sunscreen gel was evaluated for its potential UV barrier effect [16]. To 0.5 g of formulated gel, mixed with 100 ml of distilled water and ultra-sonicated for 5 minutes to ensure homogeneity of the prepared solution. The dispersed solution was then filtered using whatman filter paper and few ml of the filtrate was discarded. To 2 ml of the fresh filtrate, diluted to 50 ml with distilled water and were subjected for SPF testing. The SPF of the ripen fruits pulp extract was determined by taking absorbance of sample using spectrophotometer in the range of 290- 320 nm with 5nm intervals using distilled water as blank. The data obtain was calculated to determine the SPF by using the equation:

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

SPF = Sun Protecting Factor

CF = Correction Factor = 10

EE (I) = Erythema Effect Spectrum

I (I) = Solar Intensity Spectrum

ABS =Absorbance or sample value of EE(I) is constraint = 1

RESULTS AND DISCUSSION:

Extraction and Qualitative Phytochemical Studies
The nature and percentage yield of both the extracts were shown in Table 2. From the qualitative phytochemical analysis of both pulp extracts, it was found that both the fruits pulp extracts contains alkaloid, glycosides, flavonoids, tannins and phenolic compounds, carbohydrates and proteins. However, fixed oils and amino acids were absent. The results shown in Table 3.

Table 2: Nature and Percentage Yield of the Extract

Extract	Nature	Percentage Yield
Crude methanol extract of ripen *P.macrocarpa* fruit	Darkish brown semisolid	28.48 %
Crude methanolic extract of unripen *P.macrocarpa* fruit	Darkish yellow semisolid	51.14 %

Table 3: Phytochemical analysis of the extract

Phytoconstituents	Ripen fruits extract	Unripen fruits extract
Alkaloids	P	P
Proteins	P	P
Carbohydrates	P	P
Glycosides	P	P
Fixed oils	A	A
Tannins and Phenolic compounds	P	P
Flavonoids	P	P
Amino Acids	A	A

A = Absent, P = Present

DPPH Free Radical Scavenging Assay

DPPH assay was used to determine the free radical scavenging activity of both fruits pulp extracts. It is noted that lower IC_{50} value equals a higher scavenging activity. The scavenging activity was presented as the percentage of inhibition of DPPH free radicals in Table 4 & 5. Thus, it can be concluded that methanolic ripen fruits pulp extract of *P. macrocarpa* possess remarkable antioxidant properties when compared to unripen pulp extract. The IC_{50} was found to be 62.5 µg/ml to 125 µg/ml and 125 µg/ml to 250 µg/ml respectively. However the standard Ascorbic Acid showed a very potential free radical scavenging effect at IC_{50} between 15.625 µg/ml and 31.25 µg/ml.

Table 4: DPPH radical scavenging activity of ripen and unripen *P.macrocarpa* fruits extract.

Concentration (µg/mL)	Percentage of inhibition of ripen fruit (%) ± SEM	Percentage of inhibition of unripen fruit (%) ± SEM
15.625	33.85±0.12	27.65±0.51
31.25	35.99±0.12	32.69±0.51
62.50	44.10±0.13	41.59±1.39
125	68.66±0.07	51.13±0.06
250	71.76±0.06	66.15±0.58
500	76.98±0.05	72.53±0.17
1000	83.75±0.22	79.88±0.06

Table 5: IC_{50} value of standard vs sample extract

Extracts / Standard	IC_{50} (µg/ml)
Ascorbic acid	Range between 15.625 µg/mL and 31.25 µg/mL
Ripen *P. macrocarpa* fruits pulp extract	Range between 62.50 µg/mL and 125 µg/mL
Unripen *P. macrocarpa* fruits pulp extract	Range between 62.50 µg/mL and 125 µg/mL

Table 6: Sun Protection Factor Evaluation of the formulated gel

No	ƛ	EE.I	(EE.I.A)
1	290	0.0150	0.082
2	295	0.0817	0.619
3	300	0.2874	2.667
4	305	0.3278	2.901
5	310	0.1864	1.525
6	315	0.0837	0.711
7	320	0.0180	0.078
		SPF	8.583

Sun Protection Factor Evaluation

In vitro analysis using UV spectro-photometer was performed to evaluate the sunscreen protection factor on the formulated sunscreen gel from the ripen fruits pulp methanolic extract of *P. macrocarpa*. Based on determination of SPF, it was prooved that *P. macrocarpa* sunscreen gel displayed commendable UV barrier activity as a topical formulation. The SPF value of formulated gel was 8.583. Thus, the UV barrier effect may be due to presence of Benzophenone glycoside (4,5-dyhydroxy,4'-metoxybensophenone-3-O-β-D- glycoside) in the fruits of *P. macrocarpa* that allows to absorb the UV light. The evaluation of SPF was shown in Table 6.

Evaluation of Formulated Gel

The formulated sunscreen gel from the methanolic extract of ripens fruits pulp of *P.macrocarpa* was subjected to the standard evaluation parameters and the results were discussed below.

The pH of the formulated gel was found to be 7.3 which are recommended as suitable pH in cosmetics skin formulations. The formulated sunscreen gels were evaluated for several physicochemical tests and the results were shown in Table 7. The detailed report on viscosity test parameter was displayed in Table 8 and Table 9. Figure 4 details the graphical representation on the viscosity testing. The formulated gel showed a good acceptable odour and was in pale yellowish in colour. After application of the gel to the skin, the formulation did not show any greasiness and it was easily removed by washing under the running tap water. The gel formulation showed homogenous distribution of extract which was confirmed by visual examination. After feel test showed that the formulated gel were emollient and has good slipperiness. The loss on drying of the formulated gel was found to be 2.37 %. The formulated gel is opaque which was confirmed by light penetration test and the formulated gel had a good extrudability as the quantity of gel extrude from the collapsible aluminium foil cone was found to be 0.791 g per second. The gel is easily spreadable by small amount of shear without runoff. The shear thinning system of the gel was found to be pseudoplastic with an increasing shear rate. The sterility test shows no growth of colonies on the agar plates. The centrifuge test shows no separation on the formulated gel and was homogenous. The formulated sunscreen gel showed good stability throughout the entire study period and the post stability study results of physiochemical evaluation of the formulated gel was given in Table 10.

Table 7: Physicochemical Evaluation of the formulated Sunscreen gel before stability testing

Evaluation Parameters	Results
pH	7.3
Homogeniety	Homogenous
Appearance	Yellowish Brown
Spreadability	9.4 cm
Extrudability	0.791g per second
Odour	Good
Loss on Drying	2.37 % w/w
Opacity	Clear
After Feel Effect	Emollient / Slipperiness
Ease of Removal	Easy for removal
Viscosity	Pseudoplastic
Shear rate	Increase Shear rate
Centrifugation Test	No Separation
Sterility test	No growth of colonies

Table 8: Viscosity test results on the formulated sunscreen gel

Measuring Points.	Shear Rate (1/s)	Shear Stress (Pa)	Viscosity (Pa . s)	Speed (1/min)	Torque (µNm)
1	0.00274	6.43	2350	0.000451	26.3
2	0.00486	17.3	3560	0.000799	70.8
3	0.012	30.7	2550	0.00198	125
4	0.0326	35.8	1100	0.00536	146
5	0.0463	37.2	803	0.00762	152
6	0.0681	39.5	580	0.0112	162

Table 9: Comparison on Shear rate, Viscosity and Zero shear viscosity

Measuring Points	Shear Rate (1/s)	Viscosity (Pa.s)	Zero Shear Viscosity (Pa.s)
1	0.00274	2720	2720
2	0.00486	2720	2720
3	0.012	2520	2720
4	0.0326	1110	2720
5	0.0463	809	2720
6	0.0681	573	2720

Table 10: Physicochemical Evaluation of the formulated gel after the stability testing period

Parameters	Results
pH	6.9
Homogeniety	Homogenous
Appearance	Yellowish brown
Spreadability	6.4 cm
Extrudability	0.821 g per second
Odour	Good
Opacity	Clear
After Feel Effect	Emmolients / Sliperiness
Removal	Easy removal
Loss on Drying	2.5% w/w
Sterility test	No growth of colonies

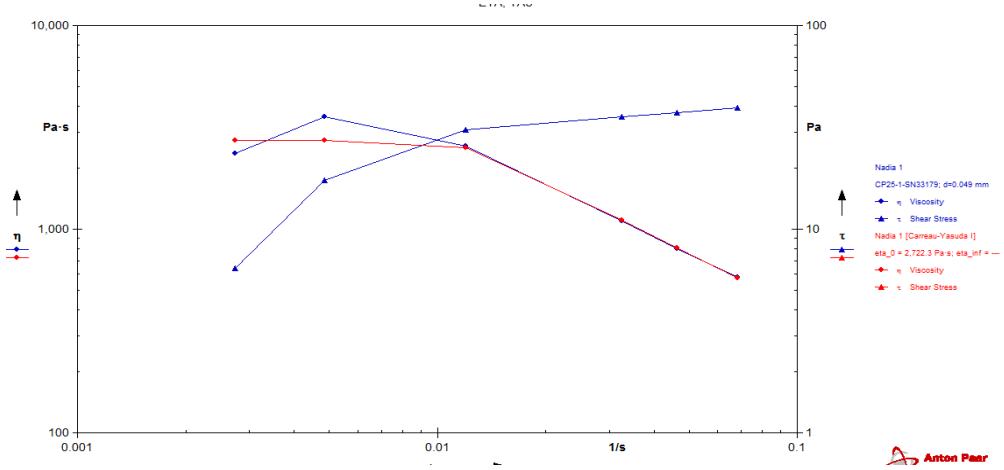

Fig 4: Viscosity graph of the formulated sunscreen gel

CONCLUSION:

The traditional use of *Phaleria macrocarpa* was reported to treat various ailments like hypertension, diabetes, skin and lung diseases. However, the novelty of the present study was proved by formulating as a sunscreen gel with a commendable antioxidant activity and Sun Protection Factor (SPF). Furthermore, the findings of present study exhibited that formulated sunscreen gel were safe to use in skin as a UV barrier. Moreover, this research increases the visibility of the fruits of *Phaleria macrocarpa* in the field of cosmeceuticals and enhances the commercial value of the plant in herbal industries.

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