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The Antihyperpigmentation Activity Test In Vitro And The Gel Formulation's Development From Ethanol Extract Of Pine Bark As A New Natural Product.

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ABSTRACT

The pine bark has a high antioxidant activity by protecting skin from sunlight's radiation than can induced the hyperpigmentation abnormality. The antihyperpigmentation activity from an ethanol extract of pine bark gel showed by IC_{50} value of this ethanol extract of pine bark to tirosinase enzyme activity. In this research we have been made the gel formulation from ethanol extract of pine bark by various concentration : 1.0%, 2.0% and 3.0%. Based on the evaluation of the gel formulation we concluded that an ethanol extract from pine bark can be formulated as gel formulation and has been fulfill the essential of pharmaceutical formulation. From the three gel formulation we found that those three formulation has characterictic such as the stability for eight week storage time either in cold temperature (5^0 C) or room temperature (15^0 C), organoleptically, homogenity and pH. The result of this research, the IC_{50} value from an ethanol extract of pine bark gel is 229.14 µg/ml and the standard M® IC_{50} value is 183.43µg/ml. This value shows that the antihyperpigmentation activity from an ethanol extract of pine bark gel is lower than the standard M®. But a single antihyperpigmentation activity from ethanol extract of pine bark it self has IC_{50} value 131.08 µg/ml.

Keywords: Antihyperpigmentation, enzyme tirosinase, extract of pine bark.

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INTRODUCTION

Introduction is the disturbance in face skin because of the melanine over production or unsmooth distribution of melanine. In this condition, the skin could be seen darken and the emerge of light brown to dark brown spot on cheek, forehead, upper lip, nose and chin. Beside the sunlight there are some trigger factor of melanine overproduction, eg : hormonal changes because of pregnancy, contraception usage, the cosmetic usage and the use of certain drugs(1).

One of the herbal which has the high antioxidant activity is pine bark. The pine bark can protect the capiler blood vessel, blood clotting prevention, and also can reduce the cholesterol concentration. *Pinus merkusii* Jungh & De Vriese is only pine naturally growth in Indonesia, *P. merkusii* Jungh & De Vrise is the multi function tree which the planting is develop widely in the future to produce the wood, sticky plant-sap production and plantation conversion. Almost every part of the tree has advantage , eg : the stem will produce sticky plant-sap that can be process as gondoruken and terpentine (2).

Before this research was done, a similar research of an ethanol extract of maritime pine bark that grow in French has also been done, the standardized French Maritime Pine Bark (FMPB) is pycnogenol. Based on this research, this FMPB extract has antioxidant activity that can protect the skin from UV radiation which formed the hyperpigmentation abnormality (3).

The popular semisolid formulation today and widely use topically is gel. The gel formulation was choose because this formulation can disperse easily and smoothly on the skin, not stick, not oily, comfortable to use , give moisture and shinning effect because the high water concentration compare with cream formulation.

Based on the above explanation, we do the developmental research of formulation from an ethanol extract of pine bark (*P. merkusii* Jungh & De Vriese) as natural antihyperpigmentation.

MATERIALS AND METHODS

An ethanol extract of pine bark, eathanol, HPMC, propilenglikol, methyl paraben, DMSO, DPPH, kuersetin, tirokinase enzym, L-dopa, phosphate buffer pH 6.8, hexana, aethyil acetat.

The Antioxidant Qualitative Test From An ethanol Extract Of Pine Bark (*P Merkusii* Jungh & De Vriese) by DPPH Method using Thin Layer Chromatoghraphy (4).

The an ethanol extract of pine bark and kuersetin as standard was spot to thin layer chromatoghraph plate (the silica gel of constant phase F254) by using the hexan : aetil acetate (3:2) as mobile phase. The silica plate was put into chromatography chamber that already been saturated by mobile phase, the form spot was disperse by 0.2% DPPH in an ethanol solution, the spot was checked every 30 minute after the dispersion time.

Tirosinase Inhibition Test (5)

A. The 2.5 mM L-Dopa Solution Making
L-Dopa was weigh about 12,4 mg; then dissolved by phosfat solution (pH = 6.8) in 25 ml erlenmeyer. In preparation's time untill tirosinase inhibitory test being done, this solution was protected for light.

B. The 50 mM, pH 6.8 Phosfat Solution Making
The potassium dihidrogen phosfat was weigh about 1.74 gram; then dissolved by 100 ml of aquadest in erlenmeyer. Add drop by drop NaOH 0.2 N to this solution until it reach 6.8 pH then add aquadest to 200 ml.

C. The Tirosinase Solution Making

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The tirosinase was weigh about 1,16 mg; then dissolved by 10 ml phosfat pH 6,8. After the preparation 's time untill tirosinase inhibitory test, this solution was kept in low temperature condition (2-8^{0}C).

D. The Maximum Length Wave Absorbance Measurement

To measure the maximum wave length we use 2.4 ml phosfat solution 50 mM (pH 6.8) and 666 µl L-Dopa solution (2.5 mM) pipette into the reaction tube. This solution then being incubated in room temperature in 10 minute. Then add 184 µl tirosinase solution (496 unit/ml) to the reaction tube. This solution then being incubated again in room temperature in 25 minute so that the reaction can occur. The inhibitory activity of tirosinase was determined by measuring the dopachrome absorbance using th UV-Vis spectrofotometer in 400-800 nm wavelength.

E. The Measurement of Inhibitory Activity By IC_{50} Test

The principal solution was made 500 ppm by weigh about 50 mg the an ethanol extract of pine bark and dissolve in 100 ml DMSO. Based on principal solution was made in series of 80, 100, 120, 140 and 160 ppm. The IC_{50} measurement was done to various inhibitory concentration in % inhibitory activity test.

$$\% \text{ inhibitory of tirosinase} = [(A-B)/A]$$

A is absorbance value without the inhibitor
B is absorbance by the adding of inhibitor

Tabel 1: The Gel Formula Of An ethanol Extract Of Pine Bark

Composition	FO (%)	F1 (%)	F2 (%)	F3 (%)
An ethanol Extract Of Pine Bark	0	1	2	3
Propylene glycol	5	5	5	5
Methyl paraben	0,2	0,2	0,2	0,2
HPMC	5	5	5	5
Aquadest ad	100	100	100	100

F0 : Gel base Formulation
F1 : Gel Formula From 1% An ethanol Extract Of Pine Bark
F2 : Gel Formula From 2% An ethanol Extract Of Pine Bark
F3 : Gel Formula From 3% An ethanol Extract Of Pine Bark

The Gel Formulation Evaluation (For 8 week period of time)

The Tirosinase Inhibitory Test On Gel Formulation

The F3 gel formula was weigh about 1.67 g (or equal to 50 mg an ethanol extract of pine bark), this solution then dilute by DMSO to 100 ml (an ethanol extract of 500 ppm pine bark), and being sentrifuge for 2 minute, and being filter to separate between sediment and filtrate solution. This sample solution then being dilute into several concentration eg : 80, 100, 120, 140 and 160 ppm and then measure the absorbance value. The IC_{50} is the amount of sample concentration which needed to inhibit 50% of tirosinase enzyme activity. The measurement of IC_{50} value was done by variety of inhibitory concentration in % inhibitory activity. The IC_{50} value of gel in this research was compare to IC_{50} antihyperpigmentation formulation being sale in the market, the M® formula; and to make the 500 ppm solution, the standard formulation being weigh is 2.5 g because it contains 2 % of hydroquinone.

RESULT AND DISCUSSION

The Evalution Of An ethanol Extract Of Pine Bark

The evaluation that has been done to the thick an ethanol extract of pine bark are : organoleptic, phytochemistry, pH, solubility, dry spillage and dust concentration test. The organoleptic test of an ethanol extract of pine bark shows some characteristic such as : the thick consistency, the brown colour, the aromatic smell and the acidic taste. The phytochemistry test shows that the an ethanol extract of pine bark consist of flavonoid, phenolic, saponin and terpenoid substance. The acidity degree of this extract is 4.10 pH; this means that this an ethanol extract of pine bark is acidic substance. The solubility test of this extract showed that this an ethanol extract of pine bark has a little solubility in water, easy soluble on an ethanol and propylene glycol solution.

Lost on drying (LOD) test of an ethanol extract of pine bark, we get the value of 9.25%. This result is quite good because according to Indonesian Materia Medika handbook the LOD less than 10% so it can reach the optimum stability. and the storage time relatively longer because of the reduction of microbiotic stain The dust concentration test was 2.51%; this value was determined to obtain the mineral content in the sample which represent the value of anorganic content that will left in the form of dust.

The Antioxidant Qualitative Analysis From An ethanol Extract Of Pine Bark

To determine the an ethanol extract of pine bark consist of antioxidant compound or not, we do the antioxidant qualitative analysis test using the thin layer chromatograph (TLC) with DPPH. The identification result shows that the an ethanol extract of pine bark gives 5 spot in TLC plate with different RF. The suspected antioxidant substance is the second spot with 0.34 RF value, because this spot gives the yellow colour after being disperse by DPPH solution compound. This spot is the same with quersetin which RF is 0.30.

The Antihyperpigmentation Activity Test From An ethanol Extract Of Pine Bark(8)

Hyperpigmentation is the disturbance of face pigment because of the melanine hyperproduction. Melanine is biopolymer from tirosinase amino acid, produced in melanosit cell and distributed between keratonoid in epidermic layer of skin. The melanine is produce by complex metabolic pathway controled by tirosinase enzyme. The tirosinase works in dehydroxilation reaction of tirosine into 3,4 dihydroxy phenilalanine (L-dopa) and then formed the dopaquinone. Dopaquinone is concert into dopachrome and then by autooxidation reaction dopachrome is convert into dihydroxy indole (DHI) or dihydroxy indole carboxy acid (DHICA) which then formed into eumelanine (brown pigment). Higher melanine production will cause the skin more dark, and to inhibit the eumelanine production we need the tirosinase as inhibitor. Tirosinase will reduce the eumelanine effect and then reduce the production of dopachrome (6).

The substrate selection is the important factor to consider because it will affect the measurement result. In enzymatic reaction there are two important substrate, L-tyrosine and L-dopa. In this reaction, we choose L-dopa because this substrate will directly form dopachrome and can be measure by spectrofotometer UV-Vis in 481 wave length. If we choose L-tyrosine, then the formed product is L-dopa , dopaquinone then dopachrome, so the longer time and the higher tirosinase enzyme are needed.

Based on this research, the formation of dopachrome marked by the colour changes, the non colour L-Dopa will change into orange after the tirosinase enzyme adding, this solution then inkubated for 25 minute so that the reaction will formed completely, then the formed dopachrome being measure. The maximum absorbance wave lenght measurement of dopachrome is the highest peak absorbance in 481 nm.

The inhibitory melanine activity measurement from an ethanol extract of pine bark by the determination of IC_{50} value. The measurement of IC_{50} value was done by variety of concentration of an ethanol extract of pine bark from standard solution in 500 ppm concentrate. The standard solution was made by weigh 50 mg of extract then diluted in 100 ml DMSO. Based on this standard solution we makes a series of concentrations : 80; 100; 120; 140 and 160 ppm, then we determined the absorbance and we calculate the inhibition percentage. The calculation result then being plot into a curve between extract concentration and inhibitory percentage. The linier regression equation of the curve is $y = -22.092 + 0.550 x$ by relation coeficient is 0.9980.

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Based on the above linier regression equation, the IC_{50} is 131.076 µg/ml. The IC_{50} is the amount of extract concentration with inhibitory activity concentration of tirosinase is 50 %. According to Kim (2004) the IC_{50} is important to acknowledge so that we can determined the potency of inhibitor to inhibit the tirosinase enzyme activity (7). Based on earlier research using the fruit-tree bark extract the obtainable IC_{50} is 142.37 µg/ml, and in the research using combination of mangove, *alamanda* and *binohang* tree extract the obtainable IC_{50} is 146.87 µg/ml. From the above research showed that an ethanol extract of pine bark has higher inhibitory potention to inhibit the tirosinase activity because the IC_{50} was obtained in lower concentration.

Table 2: The Recapitulation of Evaluation Data From An ethanol Extract of Pine Bark Gel

No	Evaluation	Formula			
		F0	F1	F2	F3
1	**Organoleptic**				
	Form	Ss	Ss	Ss	Ss
	Colour	T	B	B	B
	Smell	-	Sp	Sp	Sp
2	Homogenity	H	H	H	H
3	The stability measurement in 5^0C temperature	stable	stable	stable	stable
4	The measurement in room temperature	stable	stable	stable	stable
5	The pH measurement	6.14	4.34	4.35	4.65
6	Iritation Test	-	-	-	-
7	Particle size analyzer	-	30.40 µm	25.70 µm	21.66 µm

From the recapitulation table of evaluation's result from the gel above, we concluded that an ethanol extract of pine bark can be formulated as gel formulation. To obtained the effect of formulation type to antihyperpigmentation activity to an ethanol extract of pine bark, we also do the inhibitory test to tirosinase enzyme or antihyperpigmentation activity test. This research was only developed for one gel formulation because it was done to approximately 50 mg an ethanol extract of pine bark so it will has the same concentration with antihyperpigmentation activity test that has been done earlier, the M® formula using the approximate concentration.

In this research, we used the F3 formula because the F3 formula consist of the highest concentration of an ethanol extract of pine bark, the 3% formula, so the amount sample formula is not much. This condition makes the dilution of gel and the attraction of an ethanol extract of pine bark from gel base using DMSO is easier. To omit the effect of gel base, the gel's solution was centrifuge for 2 minute to separate the polimer from an ethanol extract of pine bark.

From the above research we got the IC_{50} value from an ethanol extract of pine bark is 229.14 µg/ml, and the standard IC_{50} is the M® formula is 183.43µg/ml The antihyperpigmentation activity of an ethanol extract of pine bark is smaller than the standard M®. Yet, from the IC_{50} from an ethanol extract of pine bark, 131,08 µg/ml, we can concluded that the an ethanol extract of pine bark activity is higher than the M® formula. The decreasing antihyperpigmentation activity from an ethanol extract of pine bark in gel formulation is presume because the active component release from gel base happen unperfectly.

Table 3: Determination of IC50 extract, standart M®, gel of extract of pine bark

Sample	Sampel consentra-tion(µ/ml)	Sample absorbance	% inhibition	IC 50 (µ/ml)	Regresion equation
Extract of pine bark	80.0	0.617	20.99	131.076	Y=-22.09+0.55X
	100.0	0.515	34.05		
	120.0	0.439	43.79		
	140.0	0.346	55.44		
	160.0	0.271	65.30		

Gel of extract 3%				229.14	
Standart (M®)				183.43	
blank		0.781			

CONCLUSION AND SUGGESTION

Conclusion

The an ethanol extract of pine bark can be formulated in gel formulation and has fulfill the gel formulation requirement and we got the IC_{50} value from gel an ethanol extract of pine bark 3% is 229.14 µg/ml, Standar M® is 183.43 µg/ml and extract of pine bark is 131.08 µg/ml.

Suggestion

For the next researcher to examine the best formulation and method to attract the an ethanol extract of pine bark so that the obtainable anti-hyperpigmentation activity is the same with the antihyperpigmentation activity of an ethanol extract of pine bark.

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