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Research Article

TOPICAL ANTI-INFLAMMATORY ACTIVITY TEST AND IDENTIFICATION OF ANTI-INFLAMMATORY ACTIVE COMPOUNDS OF ETHER EXTRACT OF STEM BARK AND LEAVES RESIN OF TENGGULUN PROTIUM JAVANICUM BURM

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A B S T R A C T

This research aimed to evaluate the topical anti-inflammatory activity of the ether extract of stem bark and resin of tenggulun leaves on the ears inflamed rats that were induced by croton oil. The ether extract of the stem bark and resin of tenggulun leaves was purified by gradient elution column chromatography. The extract resulted by the column chromatography was identified for the compound components by GCMS and then the test for the topical anti-inflammatory activity on the ears inflamed rats was carried out.
The GCMS result suggested that there were some secondary metabolites as follows: the dichloromethane fraction of the stem bark extract contained stigmasterol, lanosterol, cholesterol, ergosterol, picenon, lupeol and α-amyrin. The ethyl acetate fraction contained stigmasterol, lanosterol, cholesterol, ergosterol, lupeol, neogamaseron and stigmastenon. The dichloromethane fraction in the leaves resin contained limonene (61.74%), caryophyllene (23.07%), naphthalene (2.62%), isolongifolol (1.22%), spathulenol(11.35%). The ethyl acetate fraction contained limonene (13.09%), caryophyllene (14.85%), spathulenol (17.26%), lanosterol (31.50%) and epiglobulol (23.30%). The diethyl ether fraction contained caryophyllene (17.50%), spathulenol (28.67%), epiglobulol (18.51%), naphthalenol (0.69%), azulena (0.55%), bisabolen (6.19%), androstenon (6.19%) and dodecanediol (21.70%). The result of topical of anti-inflammatory activity test indicated that the stem bark and resin of tenggulun leaves could be used as an anti-inflammatory. The capability in inhibiting inflammation was more than 50%.

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INTRODUCTION

Inflammation is the body's attempt to inactivate organisms that attack, remove irritants and regulate tissue repair (Mycek *et al*., 2001). In response to inflammation, the body releases mediators such as histamine, bradykinin, serotonin, prostaglandins, and leukotrienes. The method of anti-inflammatory activity test can be conducted in vitro and in vivo. The in vivo test (in life) can be carried out by inflammatory induction method on ear inflamed rats (A Maral *et al*., 2009).

Burseraceae is a plant that has many benefits, such as traditional medicine for tonic and stimulant, as a cure skin diseases, anti-tumor, anti-allergic, anti-inflammatory, in the perfume industry, and as varnish (A.C Siani *et al*., 1999). In Brazil, the species of Proteum, hepthaphyllum is very popular as a drug for anti-inflammatory, antiurcerogen, antinoceptive, antineoplasic, and as carcaricidal (Bandeira *et al*., 2001).

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Otsuki M.F. *et al*. (2005) has carried out the anti-inflammatory activity test of ether extract of Protium klenii topically against the skin of rats induced by TPA. The active compounds identified as the anti-inflammatory were α-Amyrin pentacyclic triterpenoids. Martin S., *et al*. (1993) has examined the anti-inflammatory activity of volatile compounds of β-caryophyllene and α-pinene on the edema of foot rats induced by carrageenan and PGE_1. The test results showed that β- caryophyllene and α-pinene at a doses of 150 and 300 mg/kg provided a high percentage as anti-inflammatory.

Resin that were obtained from four plant species of Burseraceae family, such as Boswellia carteri, Boswellia dalzielli, Commiphora mukul and Commiphora incisa has been widely used in India and Africa as a treatment for various diseases one of which is used as anti-inflammatory. The active chemical compounds identified from that family were oktanordammarane triterpen, mansumbinon and mansumbinoic acid (Duwiejue M., *et al*., 1993)

Maria das G.B. Zoghbi and Jose G.S. Maia, 1995 has been successfully identified the essential oils contained in Burseraceae Protium heptaphyllum (Aubl.) March species by GCMS. Major components identified in the leaves were terpinolen (15.45%), β-element (22.09%), β-caryophyllene (11.11%) and in the rod is terpinolen (40.28%). Bandeira P.N. et al., 2001 have also been able to identify the components of the essential oils in the leaves, fruit and resin of the Protium heptaphyllum (Aubl.) March species with of GCMS. The major components identified in the leaves were mirsen (18.6%), β-caryophyllene (18.5%), while in the resin and the fruit were α-pinene (10.5%), limonene (16.9%), and terpinolen (28.5%).

Protium javanicum Burm, in Bali known as tenggulun, their leaves are used traditionally as an upset stomach, cough and diarrhea medicines. Their bark is used as edema and leprosy medicines (Nala, 1983 and Segatri, 1989). Sukmajaya, 2012 has examined the anti-inflammatory activity of essential oils of tenggulun leaves in which some active components were identified by GC-MS, namely β-ocimen, β-caryophyllene, germakren, humulena and caryophyllene oxide. Puspawati (2012), reported that the chemical contents of tenggulun leaves included the mixture of α and β-Amyrin, β-sitosterol and a series of long-chain alcohol compounds.

From the discussion above, therefore in this study the anti-inflammatory activity of the leaves resin and ether extract of tenggulun bark was investigated for finding out if the leaves resin and ether extract of tenggulun bark show anti-inflammatory activity topically to the skin inflammation models on rats ears, as well as the active components contained in the resin leaves and the ether extract of tenggulun bark were identified. This study aimed to obtain active isolates which act as a topical anti-inflammatory drugs.

Experimental

Materials and Equipment

The objects of the research were the leaves and stem bark of tenggulun plant (Protium javanicum Burm) obtained around Udayana University Campus, Bukit Jimbaran. The test subjects were some white male rats of Rattus novergicus Sprague Dawley of 150-200 g weigh.

The chemicals used were of analytical reagent grade: n-hexane, dichloromethane, diethyl ether, ethyl acetate, acetone, methanol, dexamethazon, and croton oil.

Equipment used were glass tools, a set of thin layer chromatography, column chromatography, a set of steam distillation, Olympus CX41 microscope (Japan) with 400 times magnification, and GCMS-QP2010 Ultra.

METHODS

Separation of volatile resin in leaves and extraction of active compounds in tenggulun stem bark

The essential resin in tenggulun leaves was separated by using steam distillation. The fresh tenggulun leaves was cut into small parts. The steam distillation equipment was assembled and filled with water, and then tenggulun leaves were put into the steam distillation apparatus. The volatile compound obtained was weighed and then stored in dark bottles at cold temperatures. The resin obtained was separated and purified by gradient elution column chromatography.

The active compounds in tenggulun bark were separated by maceration. Dry powder of tenggulun bark initially was macerated with ethanol. After the solvent was evaporated a concentrated ethanol extract was obtained. The ethanol extract was then dissolved in ether so that an ether soluble fraction was obtained. This ether fraction was then washed with water, therefore ether and water fractions formed. The solvent of ether fraction was then evaporated.

Separation and Purification of Ether Extract of Stem Bark and Leaves Resin of Tenggulun

The ether extract of stem bark and leaves resin of tenggulun were separated for their active components using gradient elution column chromatography. The stationary phase in the column was silica gel G 230 mess. Eluents used consecutively were n-hexane (500 mL), dichloromethane (200 mL), diethyl ether (200 mL) and ethyl acetate (200 mL). The fractions obtained were collected and then evaporated followed by the analysis of each fraction carried out by means of GCMS and examination of anti-inflammatory activity.

The Analysis of Compounds Components in Stem Bark and Leaves Resin of Tenggulun by GCMS

The fractions obtained from column chromatographic separation subsequently were identified for their active component by means of GCMS with the following conditions: helium carrier gas 1mL/min, Column Oven Temp 150^{o}C, Injection Temp 250^{o}C, Pressure: 102.6 kPa, Column Flow 1:11 mL/min, Oven temp Program 150 - 300^{o}C. The compounds were identified by comparing them to the Library data base: Wiley 7.LIB and Nisto8.LIB.

Anti-inflammatory Activity Examination by Rats Ears Tissues Histology Test

The treatment was given by dropping the sample on the outside of the right ear of the rats. The first treatment (KN) was on the right side of the rats ears which were dropped with croton oil of 0.4 mg in 80 mL acetone. The second treatment (KP) were of that the right side of the rats ears were dropped with 0.4 mg of croton oil, and then after 15 minutes the ears were smeared with 0.05 mg of dexamethazon. The third treatment (KE1) and the fourth (KE2) were of that the right side of the rats ears were dropped with 0.4 mg of croton oil, and then after 15 minutes each ear was dropped with 12 mg/80 mL acetone of the dichloromethane and diethyl ether extracts of tenggulun stem bark. The fifth treatment (KR1), sixth (KR2) and seventh (KR3) were of that the right side of the rats ears were dropped with 0.4 mg of croton oil, and then after 15 minutes each ear was dropped with 12 mg/80 mL acetone of the dichloromethane, diethyl ether and ethyl acetate resins. The eighth treatment (Ka), was that the rats ears were dropped with 80 mL of acetone. After 6 hours the tissue of the rats ears were cut for histology test.

The rats ears tissues resulted from biopsy which was of 5 mm in diameter and in the depth of up to subcutaneously were treated according to the fixation steps as follows: dehydration, clearing and embedding. Cutting was carried out with the use of a rotary microtome with a thickness of 5 micrometers followed by attachment to the object glass, and then incubation at 60 ° C for 2 hours. The observations were made by the method of digital microscope Olympus analysis CX41

(Japan), and recorded with camera Optilab Pro (Micronos, Indonesia).

RESULTS AND DISCUSSION

The results of GCMS analysis of the extract of stem bark and leaves resin of tenggulun resulted from the gradient elution column chromatographic separation

Essential resin and ether extract of tenggulun stem bark were purified by the application of gradient elution column chromatography. In gradient elution there were various solvents used such as n-hexane, dichloromethane, diethyl ether and ethyl acetate which have different polarity. Normal hexane was the most non polar solvent used in the first elution for eluting the compounds that are non-polar such as chlorophyll, waxes and fatty acids. Other solvent in gradient elution was semi-polar solvent in order to elute the secondary metabolites that are semi-polar. The results of GCMS analysis identified some secondary metabolites, as presented in Table 1 and Table 2.

Table 1 The results of GCMS analysis of dichloromethane and ethyl acetate fractions of tenggulun stem bark extract

No	Dichloromethane fraction		Ethyl acetate fraction	
	Retention time	Constituents	Retention time	Constituents
1	21,155	Stigmasterol	23,230	Stigmasterol
2	21,675	Cholesterol	21,175	Cholesterol
3	22,725	Lanosterol	24,935	Lanosterol
4	22,520	Ergosterol	22,505	Ergosterol
5	24,370	Picenon	-	-
6	24,575	Lupeol	25,545	Lupeol
7	24,885	Alpha amyrin	-	-
8	-	-	25,795	Neogamaseron
9	-	-	26,145	Stigmastenon

was the formation of double bonds on the carbon number 8 and 9 forming lanosterol. Lanosterol undergo hydrogenation reactions at carbon atom number 8 and 9 as well as on the C 24 and 25, also there was the formation of double bond at the C 5 and 6 with loss of the methyl group at C 4 and 14 forming cholesterol. Cholesterol turned into stigmasterol and ergosterol by the formation of a double bond in the carbon C 22 and 23 as well as the inclusion of an alkyl group at C 24. Lanosterol undergo cyclization process of cyclical 4 rings (ciclopentanoperhidrofenantreno) into cyclic ring 5 forming lupeol. Cyclopentane group on lupeol arranged themselves to form cyclohexane becoming compound of alpha amyrin.

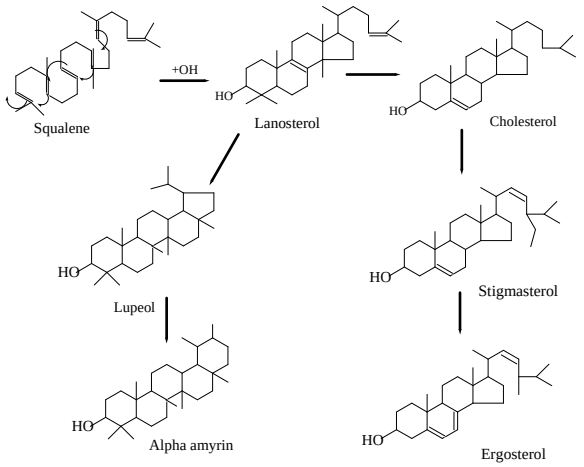

Figure 1 Biosynthesis of Secondary metabolites of some triterpene compounds

In tenggulun leaves resin, the fractions of dichloromethane, diethyl ether and ethyl acetate contained the same compounds, namely caryophyllene and spathulenol.

Table 2 The results of GCMS analysis of the results from column chromatographic separation of tenggulun leaves resin with dichloromethane, ethyl acetate and diethyl ether eluents

No	Constituent	Fractions					
		Dichloromethane		Ethyl acetate		Diethyl ether	
		Retention time	Percentage composition	Retention time	Percentage composition	Retention time	Percentage composition
1	Limonene	4.280	61.74	8.355	13.09	-	-
2	Caryophyllene	4.795	23.07	7.155	14.85	9.075	17.50
3	Naphthalene	5.940	2.62	-	-	-	-
4	Isolongifolol	6.030	1.22	-	-	-	-
5	Spathulenol	7.045	11.35	9.255	17.26	9.245	28.67
6	Lanostanol	-	-	9.250	31.50	-	-
7	Epiglobulol	-	-	10.137	23.30	10.145	18.51
8	Naphthalenol	-	-	-	-	10.385	0.69
9	Azulena	-	-	-	-	10.430	0.55
10	Bisabolene	-	-	-	-	10.540	6.19
11	Androstenon	-	-	-	-	10.695	6.19
12	Dodecanediol	-	-	-	-	11.035	21.70

The secondary metabolites contained in tenggulun stem bark extract of dichloromethane and ethyl acetate fractions were stigmasterol, lanosterol, cholesterol and ergosterol. The dichloromethane fraction contained picenon, lupeol and alpha amyrin that did not exist in the ethyl acetate fraction, whereas the ethyl acetate fraction contained neogarmaseron and stigmastenon which was not present in the dichloromethane fraction.

The secondary metabolites contained in the bark extract tenggulun was a triterpene class. The biosynthesis occurs from squalene cyclization process. At the time of cyclization, OH group entered the carbon atom number 3 and also there

The fractions of dichloromethane and ethyl acetate contained the compound of limonene whereas in diethyl ether fraction there was no limonene. Other components in dichloromethane fraction were naphthalene and isolongifolol, while ethyl acetate fraction contained lanostanol. Other components in ethyl acetate fraction were epiglobulol, naphthalenol, azulena, bisabolen epoxides, nerolidol, androtenon and dodecanediol.

The results of the anti-inflammatory activity test by histological test on the rats ears tissues induced by the extract of stem bark and leaves resin of tenggulun

From the histology test carried out it was found that the application of the stem bark extract and leaves resin of tenggulun on the ears of the rats induced by croton could inhibit the formation of inflammatory cells. The data obtained from the activity test is shown in Table 3.

CONCLUSIONS AND SUGGESTION

The conclusion that can be drawn from the results is that the secondary metabolites identified in the stem bark of tenggulun was a non-volatile compounds such as stigmasterol,

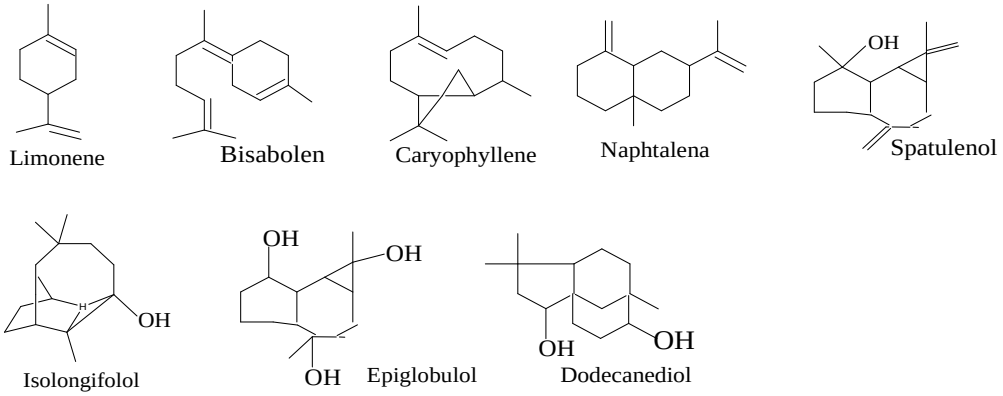

Figure 2 Essential compounds in tenggulun leaves resin

Table 3 The results of topical anti-inflammatory activity test by histology test on rats ears tissues

No	Treatment	Dose/ear	The number of inflammatory cell formation
1	Ka	80µL acetone	1
2	KN (croton oil)	0,4 mg/80µL acetone	43
3	KP(dexamethazone)	0,05 mg	12
4	KE1(bark, DCM)	12 mg/80µL acetone	13
5	KE2 (bark, diethyl ether)	12 mg/80µL acetone	16
6	KR1(leaves resin, DCM)	12 mg/80µL acetone	17
7	KR2 (leaves resin, diethyl ether)	12 mg/80µL acetone	21
8	KR3 (leaves resin, ethyl acetate)	12 mg/80µL acetone	22

It was observed that the ears of the rats induced by croton oil as an inflammatory agent resulted in edema on the ears of the rats. The edema is caused by the migration of inflammatory cells such as lymphocytes, monocytes, basophils, neutrophils and eosinophils to the sites of inflammation. It was evident that the inflammatory cells migrated to the sites of inflammation as a result of croton oil were detected as many as 43 inflammatory cells. The application of dexamethazone as an anti-inflammatorydrugs was able to inhibit the formation of inflammatory cells migrating to the sites of inflammation which was indicated by the formation of only 12 inflammatory cells. The fractions of dichloromethane (DCM) and diethyl ether of the stem bark extract of tenggulun were also able to inhibit the formation of inflammatory cells at the sites of inflammation. DCM fraction was able to inhibition the formation of inflammatory cells better due to the presence of alpha amyrin compound that has been proven as an anti-inflammatory. The fractions of DCM, ethyl acetate and diethyl ether of the leaves resin were also able to inhibit the formation of inflammatory cells. DCM fraction of the leaves resin of tenggulun inhibited inflammatory cells stronger due to the presence of caryophyllene compounds. From the research caryophyllene was proved to act as an anti-inflammatory. Caryophyllene could inhibit the formation of prostaglandins and enzymes phospholipase as arachidonic acid-forming.

lanosterol, cholesterol, ergosterol, picenon, lupeol, alpha amyrin, neogamaseron and stigmastenon which is a class of triterpen compounds. In leaves resin of tenggulun it was identified compounds that are essential from the class of diterpene and sesquiterpene such as limonene, caryophyllene, isolongifolol, spathulenol, epiglobulol, naphthalenol, azulena, bisabolen, nerolidol and dodecanediol. Histology test results showed that the extract of stem bark and leaves resin of tenggulun purified by gradient column chromatography could reduce the formation of inflammatory cells at the sites of inflammation well with the inhibition capacity of more than 50%.

It was evident that it is difficult to separate the fatty acids perfectly although a non-polar solvent hexane was used in excess. For this reason, it is necessary to find the right method to separate the fatty acids contained so that the purification becomes perfect. It is required to continue running the anti-inflammatory test by other methods.

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