BIDANG ILMU: MIPA (BIOLOGI)

EXECUTIVE SUMMARY

HIBAH PENELITIAN HIBAH BERSAING

Efektivitas dan Produksi Massal Biopestisida Nabati Minyak Essensial Rimpang Dringu sebagai Pengendali Hama Penggerek Buah Kopi (*Hypotenemus hampei* Ferrarri) (Coleoptera: Scolytidae)

Tahun ke 1 dari rencana 3 Tahun

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Abstract

Effects of *Acorus calamus* rhizome extract were studied for their efficacy on the *Hypotenemus hampei.* The powder of *A. calamus* rhizome was extracted using the ethanol as an organic solvents and then was separated by using hexane and methanol solvents. Based on the Thin Layer Chromatography methods, it indicated that α and β asarone were predominatly present in the extracts. These results were confirmed by using High Performance Liquid Chromatography (HPLC) method. The insecticidal activities were examined using residual contact method on the coffee berry. In a test with adults of *H.hampei, a*fter a week application, the hexane fraction caused more mortality of insect than the methanol fraction.

Key words: *Hypotenemus hampei, Acorus* rhizome, Asarone, polar and non polar solvent

1.Introduction

The roles of secondary metabolites of plants are very important for their contribution to plant defence against insect pests since they can significantly affect pest behaviour. Scientists believe that these secondary metabolites are based on phytochemicals which originate from the continuous interaction between plant and insect. A variety of chemical components can act as an insect antifeedant and also disrupt insect growth and reproduction (Gao et al. 2004; Koul et al. 2007).

The extraction process has been a challenging study to maximise the yield of active compounds. This is because the potential insecticidal compounds from plant material should be extracted to determine their biological active compounds. One of the techniques of extraction method is using wide range of solvents polarity to ensure that the wide polarity range of plant compounds can be extracted. Some of organic compounds in plants are polar and hence soluble in polar solvents and others are non polar and soluble only in non polar solvents.

To date, some publications have been reported that *A. calamus* L. (Sweet flag) rhizome which mostly has been extracted by using non polar solvents such as petroleum ether had phenylpropanes, monoterpenes, and thermolabile sesquiterpenoids with asarone as major chemical constituents of the essential oils of Sweet flag (Motley 1994). All these groups of chemical compounds are known for their antifeedant activity and repellent activity (Saxena & Koul 1978; Paneru et al. 1997; El-Nahal et al. 1989; Sharma et al. 2008), regulation of insect development (Mathur & Saxena 1975; Nair & Thomas 2001; Poplawski et al. 2000; Saxena et al. 1977). It also has been reported that the *A. calamus* essential oil is effective as an insecticide and as an insect repellent against houseflies (*Musca domestica* L.); inhibits the growth of the tobacco caterpillar, *Spodoptera litura* F., and variegated cutworm, *Peridroma saucia* Hubner (Chopra et al. 1986).

A literature search revealed little information dealing with selection of organic solvents for the plant extraction process and in particular *A. calamus* material from Indonesia. This information is important as the successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedures (Eloff 1998). In this study, the efficacies of extracts of *A. calamus* rhizome on *H. hampei* are reported.

2.Material and Methods

2.1.Plant material and Chemicals

Rhizomes of *A. calamus* were collected from Slawu-Jember district, East Java, Indonesia in January 2014 and were authenticated by Umiyah, Biology Department, Botany Laboratory, University of Jember, Indonesia. This material is representative of the Indonesian area genotype of *A. calamus* and as such almost certainly differs from genotype arrays from other geographical areas. The rhizomes were cut thinly and dried at room temperature for one week. Dried rhizome was then powdered mechanically using a commercial stainless steel blender. Α and β asarone were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Triton X-100. Also were purchased from Sigma- Aldrich.

2.2.Insects

H. hampei were initially obtained from a coffee plantation in Distric Durjo, Jember. Rearing was done in a secure Controlled Environment (CE) room, 14:10 h (light:dark) at 25±2°C and 54±10% relative humidity in the laboratory of zoology, The University of Jember. Coffee berry that infested by *H. hampei* were put in the small container (ca.10 cm x 5 cm x 10 cm) with coffee berry without exposure of any insecticides. They were kept caged until adults emerged and mated. After the females oviposited, coffee berry with eggs were removed from the container and kept caged until the eggs hatched. The coffee berry with

newly-hatched larvae, were transferred to the new container filled with new berry where they were kept until the larvae pupated and adult emerged.

2.3.Plant extraction and chemical analysis

Extraction and fractionation of extracts was done in the Botanical laboratory and Organic Chemical Laboratory at department of Biology and Chemistry of University of Jember. Five hundred gram lots of powdered *A. calamus* were subjected to extraction using two litres of ethanol for 24 hours at room temperature. The mixtures were filtered using no. 1 Whatman filter papers and vacuum of 27 kPa on a Buchner funnel. Each of the filtrates was concentrated under reduced pressure at 40-50˚C in a flask evaporator at around speed 5 to yield about 50 ml of extract and the residue thus obtained stored at 4˚C.

The separation of *Acorus* extract then was conducted by using separation flask. The solvents used were methanol which has polar characteristic and hexane which represented non polar solvent. Two extracts solvent were obtained. Each of them were analysed their chemical substances and their efficacy.

For identification of the main compounds in *A. calamus* extracts in each solvent, TLC and HPLC analysis were done. The reference solutions for TLC and HPLC were obtained by dissolving 60µg/ml of α asarone and β asarone in toluene. The methanol and hexane fractions were separated by TLC using a silica gel plate (20x20 cm; Merck's 60G F_{254}) as a stationary phase and a mixture of toluene and ethyl acetate (93:7) as a mobile phase. The fractions were detected by UVC-lamp (λ = 280-100 nm). Two spots of the standard solution and two spots of each of the *A. calamus* solutions were applied about 1.5 cm from the edge of the TLC plate using a fine capillary tube. The plates were then developed up to 10 cm in the mobile phase.

For further separation of the main compounds of the *A. calamus* samples, HPLC analysis (Shimadzu Corporation, Tokyo, Japan) was used. The reverse phase C18 column (kinetex 1.7µ XB-c18 100A, size

50x2.1mm) was employed; the injection volume being 1 µl. The mobile phase consisted of Solvent A (MilliQ water) and Solvent B (acetonitrile) in the following program: a gradient run of 40% B to 60% B for 4.5 mins, a 0.1min to 95% B and held for 1.4 min and then run at 5% B in an isocratic mode for 1 min. The flow rate was maintained at 0.4 mL min^{-1} at 40°C. The drying gas was at 250°C, the gas flow at 15 L/min, the nebulising gas flow at 3 L/min, and the heating block was at 400°C. Nitrogen was used as a drying and nebulising gas, and the capillary voltage was 4.5 KV. The asarone was optimised for solvent extraction using direct flow injection analysis in a positive mode with a single ion scan of 208.2. The dwell time was 50 ms for all

2.4.Insecticidal activity of A. calamus rhizome extracts on H. hampei

The potential of the toxic effect of the Acorus extracts was determined by the mortality effect. Residual contact method was used for *H.hampei.* Ten aduls were transferred into container (dia:5cm, height:5cm, wide:5cm) containing filter paper. Each fraction of *A. calamus* rhizome-derived fraction i.e. hexane and methanol fraction was suspended in distilled water with Triton X-100 (0.1 ml/L). Controls receieved water only. Test material solutions were applied at a concentration 0.1%-3%. After drying for 10 min, 10 adults of *H.hampei* were placed into the container.Treated and control insects were held at the same conditions used for colony maintenance. Mortality were determined 7 weeks after application. All treatments were replicated five times.

2.5. Statistical analysis

The percentage mortality was determined for analisys variance. Treatment means were compared and separated by Least Significant Difference test at p 0.05 (Zar, 1993)

3. Results

3.1.Plant extraction and chemical analysis

The main compounds from *A. calamus* extracted by ethanol solvents used in this current study were likely to be similar. Using TLC methods it was observed that there were big blue spots which were asummed to be asarone as it is a typical compound found in *A. calamus* (Figure 1a). These biologically active compounds were characterised as two asarone isomers – α and β asarone – which were reported to have antifeedant and insecticidal activity. In this study, the isomers could not be separated by TLC method however; the retention factors (Rf) of 0.35 of the *Acorus* compounds may indicate the presence of β-asarone (Table 1).

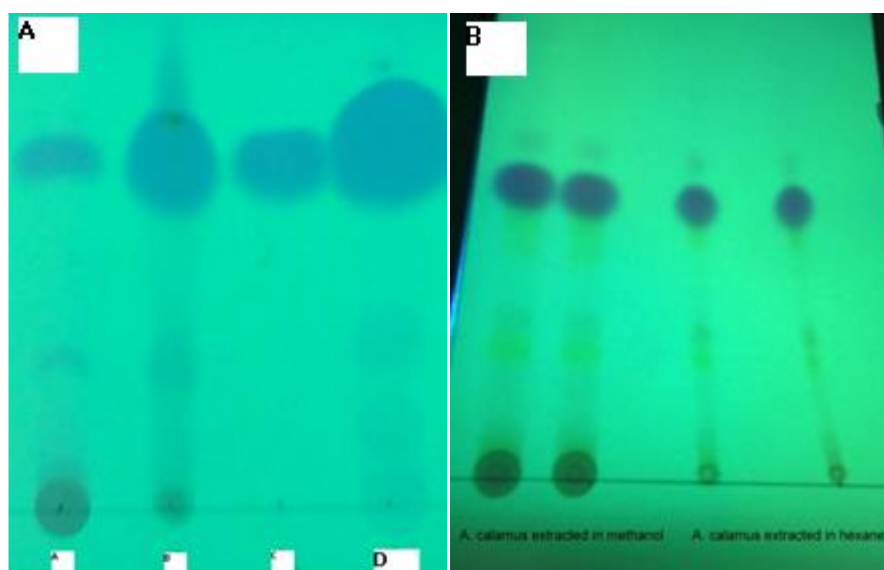

Figure 1A. Thin Layer Chromatograph of ethanol extract of Acaros (A, C were ethanol extract of Acorus; B α asarone; D.β asarore). 1B. Methanol fraction and Hexane fraction of Acorus extract

Table 1. Retention factor (Rf) of *Acorus calamus* extract compared with α and β asarone

Sampel	Developing pelarut (Y) (mm)	Spot dari fraksi Acorus (X) (mm)	Rf= X/Y	Rf

Methanol	100.50	35.50	0.35	0.35
	100.50	35.50	0.35	
Hexane	101.00	35.50	0.35	0.35
	101.00	35.50	0.35	
α-asarone	104.00	33.00	0.32	0.32
	104.00	33.00	0.32	
β-asarone	99.50	35.00	0.35	0.35
	99.50	35.00	0.35	

Further separation of the compounds of the *A. calamus* extracts was undertaken by HPLC analysis to confirm this claim. The results obtained from the HPLC are shown in Figure 2. The asarone was separated into its two isomers. While the α-asarone was purchased as a dry powder it produced a single peak, however, the β-asarone arrived in a liquid form and perhaps due to a poor storage area or a short shelf life it appeared as a double peak, it is assumed that the sample carries both species as the secondary peak relates to the retention time of the α-asarone. In addition, they all had similar area, which suggests similar extraction capacity. This results similar with Purwatiningsih (2013) which reported that α and β-asarone had similar area by using UHPLC-MS.

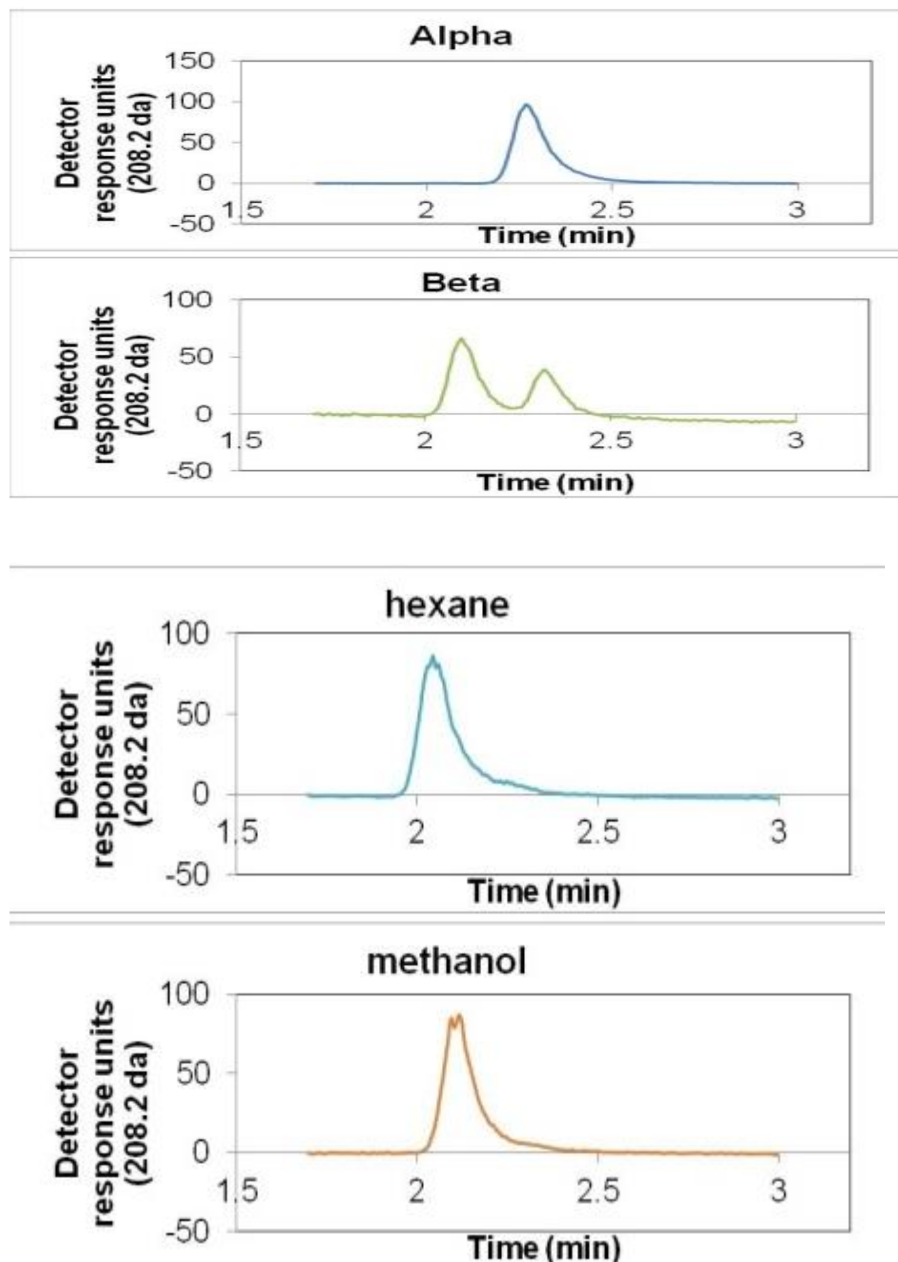

Figure 1. HPLC analysis of *A.calamus* extracts

This finding may confirm that *A. calamus* of Indonesian origin could be categorised as tetraploid genomic character because the predominant compound found in the extract is β-asarone (Motley 1994). The portion of β-asarone is still high and similar even though the rhizome extract has been extracted in

polar and non polar organic solvents. In addition, this finding could give valued information about the benefit of using polar organic solvents other than non polar solvents to extract *A. calamus* as they are cheaper and safer. This result was consistent with a number of studies of *A. calamus* origin from other Asian countries such as India and Korea. It was reported that β-asarone is the predominant active constituent in *A. calamus* rhizomes (Saxena et al. 1977; Koul et al. 1990; Motley, 1994; Lee et al. 2002).

3.2. Insecticidal activity of A. calamus rhizome extracts on H. hampei

The toxicity of each fraction of *A.calamus* extract against *H.hampei* was evaluated by using residual contact method. Significant differences were observed in hexane fraction (F=30.65, p=0.0001) while methanol fraction shown not significant differences (F=1.20, p=0.33). Hexane fraction showed mortality ranged from 90% to 98% at ranged of concentration used, whereas methanol fraction showed mortality around 20% to 48% at the same concentration (Table 2).

Table 2. Toxicity of fractions of *A. calamus* to *H.hampei*

Hexane Fraction		Methanol Fraction	
Concentration (%)	Mortality % (mean ± sd)*	Concentration (%)	Mortality % (mean ± sd)
0	16 ± 0.55^{a}	0	16 ± 0.55^{b}
0.1	90 ± 1.73^{ab}	0.1	26 ± 2.60^{a}
0.2	86 ± 1.07^{a}	0.2	48 ± 3.60^{a}
0.4	96 ± 0.55^{ab}	0.4	42 ± 2.20^{a}
0.8	92 ± 0.84^{ab}	0.8	28 ± 1.09^{a}
1	96 ± 0.89^{ab}	1	38 ± 1.60^{a}
1.5	94 ± 1.34^{ab}	1.5	32 ± 1.09^{a}
3	98 ± 0.45^{ab}	3	42 ± 2.48^{a}

*Means within column followed by the same letter are not significantly different at p=0.05 (lsd test)

4. Discussion

The extraction and separation of the compounds of *A. calamus* rhizome extract yielded α and β-asarone. TLC analysis was identified that both of asarone isomer cannot be separated as they all had similar area, which suggests similar extraction capacity. However, HPLC and the Rf of TLC showed that β-asarone more predominantly than α-asarone. This finding may confirm that *A. calamus* of Indonesian origin

could be categorised as tetraploid genomic character because the predominant compound found in the extract is β-asarone (Motley 1994). The portion of β-asarone is still high and similar even though the extract has been separated in polar and non polar organic solvents. In addition, this finding could give valued information about the benefit of using polar organic solvents other than non polar solvents to extract *A. calamus* as they are cheaper and safer.

This result was consistent with a number of studies of *A. calamus* origin from other Asian countries such as India and Korea. It was reported that β-asarone is the predominant active constituent in *A. calamus* rhizomes (Saxena et al. 1977; Koul et al. 1990; Motley, 1994; Lee et al. 2002).

The plant chemical substance is somewhat contain both polar and non polar organic compounds. Since different organic solvents show different polarity gradient in dissolving the toxic component in the plant, it indicates that the bioactive component for responsible in toxic effect has similar gradient polarity. In this present study, Acorus extract showed more toxic in hexane fraction than methanol fraction. Purwatiningsih (2013) have also made similar observation in her studies using Acorus extracted in for different solvent ranged from polar to non polar i.e. methanol, chloroform, hexane and benzene against *Plutella xylostella.* It showed that non polar solvent had more influenced on growth and development of *P. xylostella* than non polar solvent

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