Global Journal of Pharmacology 13 (1): 01-09, 2019
ISSN 1992-0075

DOI: 10.5829/idosi.gjp.2019.01.09

Evaluation of Liquid Volatile Matter Antifungal Activities from Pyrolysis of Cashew Nut Shell on Cocoa Seeds

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Abstract: The superior seeds of cocoa plants are prepared from quality seeds that are free of fungi. The purpose of this study was to determine the activity of antifungal compounds of Liquid Volatile Matter (LVM) derived from pyrolysis of cashew nut shell (CNS) on cocoa seeds. The research method consisted of preparation and pyrolysis of CNS at temperatures of 365-600°C with 4°C/minute flow rate, purification of LVM by filtration method using zeolite and activated carbon followed by distillation at 60-120°C, analysis of LVM chemical compounds with Gas Chromatography-Mass Spectroscopy (GC-MS) and finally activity tests of the LVM as antifungals on cocoa seeds using the Total Plate Count (TPC) method. Pyrolysis of CNS produced LVM, tar and charcoal with rendemen 25.06 ± 1.90%; 18.50 ± 1.90% and 30%, respectively. GC-MS analyses of non-distilled LVM revealed that the substance contained pyrimidine derivatives, amines, azoles, carboxylic acids, carbamate acids and furans, while distilled LVM, phosphoric acid esters derivatives, pyridine, siloxane, ketones, furans and aldehydes were identified. Non-distilled LVM predominantly contained 5-methylpyrimidin-2(1*H*)- one (45.14%), whereas the distilled LVM predominantly contained dimethyl vinyl phospate (21.84%). Antifungal observations after 10 days of storage, cocoa seeds given the distilled LVM inhibited fungal growth effectively with as many colonies as 1.6×10^3 CFU/g. This number was below the threshold required by the national standard, SNI 7388: 2009, which is equal to 1×10^4 CFU/g. In other words, the cacao seeds have fulfilled the national-standard requirement.

Kay words: Antifungal · Cashew nut shell · Cocoa seed · Liquid Volatile Matter · Pyrolysis

INTRODUCTION

Theobroma cacao L. is claimed to be the only commercially cultivated and most prominent in the market among the 22 species of the *Theobroma* genus [1]. Cocoa is one of the main stay commodities that plays an important role in the Indonesian economy, especially in terms of farmers' income and the country's foreign exchange resources. Most cocoa production is exported in the form of seeds (raw materials) while exports in the form of processed products have only reached 17-20%. The development of cocoa plantations experienced an increasing trend in line with the demand for cocoa seeds. The increase of cocoa price since 2001 has also caused the increase of the farmers' desire in dealing with cocoa plants [2].

Three countries, Cote D'Ivoire, Ghana and Indonesia together cultivate and produce 61 and 67%, respectively of globally traded cocoa. Global cocoa production is estimated at 4.59 million tonnes for 2017/2018 [3]. In 2016, the annual production of cocoa, in decreasing order, by the eight largest cocoa producing countries were Côte D'Ivoire, Ghana, Indonesia, Nigeria, Ecuador, Cameroon, Brazil and Malaysia. These countries together produced about 4.23 million tonnes, representing ~ 95% of the world production [4].

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The growth of cocoa plants depends not only on variety and environment but also on the quality of cocoa seeds. Until now, damage to cocoa beans caused by the presence of fungi is still a major obstacle in efforts to improve the quality of cocoa seeds; damages on cocoa seeds due to fungal attack, especially mold can reach 80%. Three of the major cocoa diseases ((black pod, pod rot and cocoa pod borer), also known as the disease trilogy, affect the pod specifically resulting in significant crop loss [5]. Several initiatives have therefore been undertaken to counter the severe crop loss, for example, development of varieties with thicker cuticle that are more resistant to the common black pod rot and/or other pathogens [6].

Efforts made by farmers and the government to control fungal attacks in preparing cocoa seeds are using synthetic pesticides. Apart from less economical, the use of synthetic can endanger health if they leave residues in food ingredients and are not environmentally friendly because they are not easily degraded. Therefore, the solution proposed to reduce the negative impact of synthetic pesticide uses is to develop bio-pesticides from natural materials or organic waste. These pesticides will be easily decomposed and not pollute the environment and are relatively safe for humans. One of the raw materials for making bio-pesticides is CNS. Biomass as a renewable resource is considered to be the only raw material for sustainable production of chemicals [7]. Recently, biomass used for chemical production has been derived from waste biomass. Waste biomass includes municipal solid waste, livestock and poultry manure, agricultural crop residues, forestry residues and industrial waste [8]. In this research, we utilize an agricultural crop residue generated from a cashew plantation in Indonesia. The shell of cashew nuts nowadays is still a waste produced by cashew plantations (*Anacardium occidentale Linn*.) containing about 50% of oil consisting of phenolic compounds in the form of 90% of anacardic acid ($C_{18}H_{23}O_3$) and 10% of cardol and cardanol. Anacardic acid, cardol and cardanol are phenolic compounds with long side chains [9].

The potential application of the LVM from CNS pyrolysis as a pesticide is a new field of study. The Pyrolysis is the decomposition of the chemical components of the biomass by heating or incomplete combustion so that the compound can be broken down into compounds with shorter chains. In this process, in the presence of limited combustion heat and oxygen, the raw material will produce the three material states; solid (called char), condensable liquid (some time called bio-oil or tar) and non-condensable (gas). The pyrolysis is a technology for thermal treatment of biomass to recover a new material and energy [10-12]. The amount of material produced is influenced by the type of material, method and condition of pyrolysis. Due to in complete combustion in pyrolysis, complex carbon compounds containing cellulose, hemicellulose and lignin in raw materials would not be oxidized to carbon dioxide [13].

The analysis of chemical compounds' content of the LVM from CNS pyrolysis by GC showed chemical compounds including hexane, hydrazine, acetic acid, propanone, aldehyde, ketone, organic acid, alcohol, ester and phenol [14-16]. Purification of the LVM is needed to obtain the desired functional properties and also to eliminate the Polycyclic Aromatic Hydrocarbons (PAH) component and its derivatives which are carcinogenic [17]. LVM purification, in this study, was carried out by filtration using zeolite and activated carbon and followed by fractional distillation. Chemical compound content of LVM was identified using GC-MS. Purified LVM produced an extract containing chemical compounds that have antifungal activities, therefore the substances may be utilized as raw materials for bio-fungicides on cocoa seeds.

MATERIALS AND METHOD

Cashew nuts shell preparation: Wet CNS was collected from cashew plantation in Southeast Sulawesi by Tim researcher at The University of Halu Oleo (UHO), Kendari, Indonesia. The shell of cashew nuts was dried in the sun for 2-7 days. The dried CNS was then chopped with a size of ± 1 cm length.

Pyrolysis: As many as 1000 grams of cashew nut shell was weighed then put into a pyrolysis reactor equipped with a series of condenser devices.The pyrolysis reactor was equipped with a thermocouple connected to the readout meter. The smoke channel pipe was made of 2 inch diameter of stainless stell with a length of 120 cm, connecting the reactor to the condenser. Condenser and reactor were made of stainless steel having the size of 86 cm in height and 60 cm in diameter.

Electric heaters in the form of a reactor sheath with a current of 10 A. The heating process occurred at temperatures of 365-600°C with a flow rate of 4°C/minute producing smoke that passed through the condenser where it was converted into liquid. The CNS was pyrolyzed to the temperature of 600°C. Pyrolysis was stopped when no more liquid smoke dripped into the shelter. Pyrolysis was repeated three times.

The LVM Preparation: The LVM of the pyrolysis was filtered using whatmann filter paper and gauze, then using zeolite and activated carbon adsorbent. Furthermore, the filtrate was placed in the fractional distillation flask then heated at 60 to 120°C. The LVM distillate was collected until no more samples evaporated and the color of LVM changed from dark brown to yellowish clear [18].

The Analysis of LVM by *Gas Cromatograhpy-Mass Spectroscopy* (GC-MS): The analysis of LVM chemical compound content was carried out using GC-MS instrument. There were two types of samples; first, LVM underwent filtration only and second, LVM underwent filtration and distillation. Each of them, as much as 1 µL was injected into Thermo Scientific GC-MS Trace 1300 GC/ISQ with ionizing type EI (Electron Impact) 70 ev, injector temperature 290°C and Triple Quadrupole Detector, column temperature 70°C to 280°C, 30 m column length, 25 mm diameter in column, 5°C temperature rise per minute, 100 kPa Helium carrier gas, flow rate of 60 mL/minute.

The LVM Activity Test as an Antifungal in Cocoa Seeds Provision of Cocoa Seedlings: The seeds and pod husk of a cocoa fruit sample were separated to each other and the beans from the middle part only were used for the seeds.The pulp was removed from the beans by using sawdust then the beans were washed cleanly with water. The clean beans were then separately immersed into a 20% of LVM sample, 20% of synthetic fungicides as a positive control and distilled water as a negative control for 30 minutes. After that the cocoa beans were collected and dried with the help of a fan then stored it 10 days [19].

The LVM Test as an Antifungal on Cocoa Plant Seeds: The LVM testing as an anti fungal on the seeds of cocoa plants during the storage period included several stages, namely the preparation and manufacture of media, sterilization of tools and media and the calculation of the number of molds that grow on the media.

Preparation and Creation of the Media: The media used were Potato Dextrose Agar (PDA), consisting of PDB (Potato Dextrose Broth) and agar. Solid PDA media was created by mixing 6 grams of PDB with 5 grams of agar into an Erlenmeyer flask containing 250 mL of distilled water. The flask was then heated and stirred using a hot plate equipped with a magnetic stirrer until the media became homogeneous. During this process, the Erlenmeyer flask was covered in cotton and aluminum foil. After the heating process completed, the media was then sterilized before use [20].

Calculation of Number of Molds: As many as 1 g of sample of cocoa plants' seed was aseptically mashed using mortal and put it in a shaker bottle containing 9 mL of sterile distilled water, shaked and this called a 10^{-1} diluted solution**.** From this solution, 1 mL was pipetted and dissolved with 9 mL of sterile distilled water in a shaker bottle, shacked to make a 10^{-2} diluted solution. To obtain a 10^{-3} diluted solution, a same procedure was employed. Each suspension was taken as many as 1 mL and put it into a petri dish followed by pouring the PDA media that had been thawed at a temperature of 45°C to 50°C. The sample was homogenized by rotating the petri dish on the table with a movement forming the number eight. After the media was solidified, the dishes were incubated at room temperature (26-28°C) in an upside down position [21, 22]. After incubating, the calculation of the number of mold colonies of each isolate that grew on the media was carried out using the formula of Total Plate Count (TPC) [23].

$$\text{TPC} = \text{Number of colonies} \times \frac{1}{\text{dilution factor}} \quad (1)$$

where: dilution factor = diluent factor × inoculated volume

RESULTS AND DISCUSSION

The section deals with the optimization of pyrolysis conditions and examines the liquid, gas and char yields as a function of pyrolysis temperature. Pyrolysis of CNS at temperatures of 365-600°C with the heating flow rates of 4°C/minute produced LVM, tar and charcoal components with rendement of 25.06 ± 1.90%; 18.50 ± 1.90% and 30%, respectively. The LVM yield was a strong function of temperature, peaking at 365–600°C [24]. The effect of pyrolysis temperature on the liquid, gas and char yields is shown in Fig. 1.

LVM filtration process from pyrolysis using zeolite and activated carbon was intended to eliminate PAH levels in LVM [25]. Furthermore, the distillation process was carried out to obtain purer LVM or grade 2 LVM, which in turn, this could be applied for an antifungal material on food.

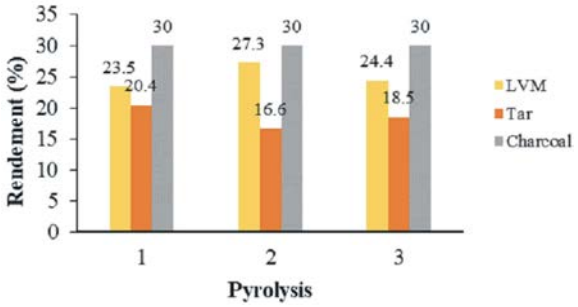
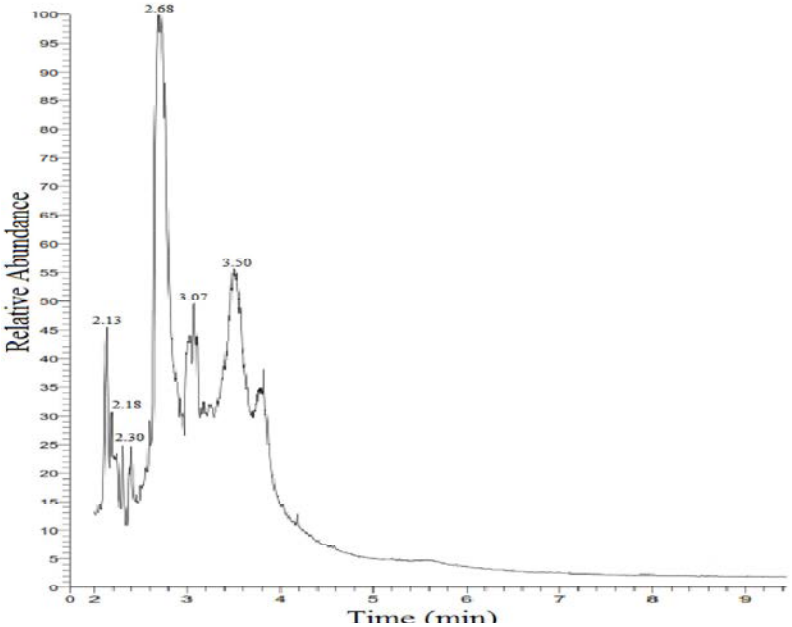


Fig. 1: Pyrolysis data of CNS.



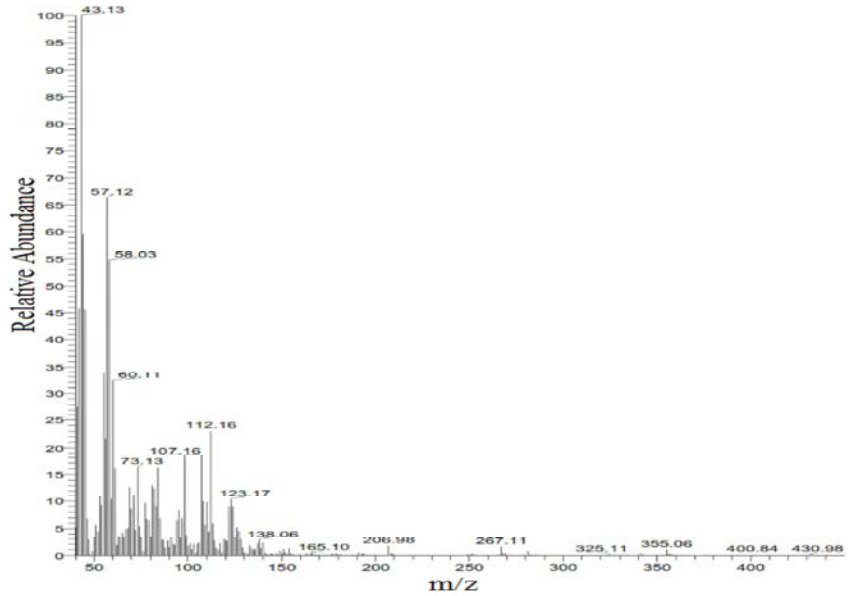


Fig. 2: GC-MS chromatogram (a) and MS spectrum (b) of CNS LVM after filtration with zeolite and activated carbon.

Table 1: Chemical compounds found in CNS LVMafter filtration with zeolite and activated carbon

No.	Retention time (minute)	% Area	Compound name*	Chemical formula	m/z
1	2.13	6.82	4-methyl-4*H*-1,2,4-triazol-3-amine	$C_3H_6N_4$	98
2	2.18	3.65	Isopropyl N,N-difluorocarbamate	$C_4H_7F_2NO_2$	139
3	2.30	4.58	1,6:2,3-dianhydro-4-deoxy-á-d-ribo-hexopyranose	$C_6H_8O_3$	128
4	2.68	45.14	5-methyl pyrimidin-2(1*H*)-one	$C_5H_6N_2O$	110
5	3.07	11.42	Furan-2,5-dicarbaldehyde	$C_6H_4O_3$	124
6	3.50	21.57	2-(aminooxy)propanoic acid	$C_3H_7NO_3$	105

*The compounds' content was determined by using standard NIST MS software.

Table 2: Chemical compounds found in CNS LVM after filtration and distillation

No.	Retention time (minute)	% Area	Compound name	Molecule formula	m/z
1	2.11	8.35	*N*-methyl pyridin-4-amine	$C_6H_8N_2$	108
2	2.20	21.84	dimethyl vinyl phospate	$C_4H_9O_4P$	152
3	2.27	5.39	4-Trimethylsilyl-9,9-dimethyl-9-silafluorene	$C_{17}H_{22}Si_2$	282
4	2.46	7.54	Formohydrazide	CH_4N_2O	60
5	2.79	4.37	2-(aminooxy)propanoic acid	$C_3H_7NO_3$	105
6	3.17	2.00	Cyclopentane-1,2,3,4,5-pentaol	$C_5H_{10}O_5$	150
7	3.66	9.71	1,3-Dioxolane, 2-(3-bromo-5,5,5-trichloro-2,2-dimethylpentyl)	$C_{10}H_{16}BrCl_3O_2$	352
8	3.82	3.92	2-amino-8-methy- 6,7-diphenyl-7,8-dihydropteridin-4(3*H*)one	$C_{19}H_{17}N_5O$	331
9	4.13	1.36	2-Butanone, 3-methoxy-3-methyl	$C_6H_{12}O_2$	116
10	4.29	3.61	exo-1,2-O-Ethylidene-á-d-erythrofuranose	$C_6H_{10}O_4$	146
11	5.60	7.65	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)	$C_{20}H_{40}$	280

*The compounds' content was determined by using standard NIST MS software.

The Chemical Compounds Structure Analysis of LVM from CNS Pyrolysis: The content characteristics of LVM chemical compounds were identified using the GC-MS instrument and the chemical compound structures were determined using the NIST MS software standard. The results of the GC-MS analysis are presented in Figures 2 and 3 as well as in Tables 1 and 2.

Table 1 shows the content of chemical compounds found in LVM before distillation consisting of pyrimidine derivatives, amines, azoles, carboxylic acids, carbamate acids and furan. The most dominant compound is 5-methyl pyrimidine-2(1*H*)-one with an area of 45.14%. Pyrimidine compounds are an integral part of DNA and RNA that have pharmacological properties and are effective as bactericides and fungicides [26].

Table 2 shows the compounds contained in LVM after filtration with zeolite, activated carbon and distillation consisting of derivatives of pyridine; dimethyl vinyl phosphate; 4-trimethylsilyl-9, 9-dimethyl-9-silafluorene; formohydrazide; phosphoric acid esters; 1, 3-dioxolane, 2-(3-bromo-5, 5,5-trichloro-2, 2-dimethylpentyl); 2-amino-8-methy-6,7-diphenyl-7,8-dihydropteridin-4(3*H*)one and cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl). The group of compounds shown in Tables 1 and 2 can function as anti-fungi Based on the table, dimethyl vinyl phosphate is the most dominant compound,with an area of 21.84%. This compound is a vinyl derivative of organophosphates that is effective as an insecticide. Insecticides of organophosphate (OP) class produce neurotoxic effects in inhibiting the acetylcholinesterase enzyme (AChE) [27]. The group of pyrimidine and pyridine compounds act as sterol biosynthesis inhibitors (SBI class I: demethylation inhibitors, DMI) and are systemic acropetal. Systemic acropetal is a type of pesticide that is absorbed by plant tissue, then translocated through wood vessels upward following the movement of water from the ground [28].

The difference in the content of the compounds produced between LVM before and after distillation is due to the effect of heating involved in the distillation process causing the chemical compounds in the form of gases to be easily separated while compounds with low boiling points are easily changing to become more stable compounds. When steam is produced from a mixture, it contains more volatile components so that the process of separating components from the mixture can occur. Therefore, the temperature and time duration of distillation can also affect the chemical composition of the LVM produced. Since the compounds contained in LVM have different boiling point, the LVM can be fractionated to get the desired functional properties. The LVM distillation process can also eliminate unwanted compounds, such as tar compounds and PAH.

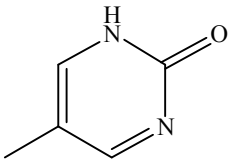


Fig. 3: 5-methylpyrimidine-2(1*H*)-one.

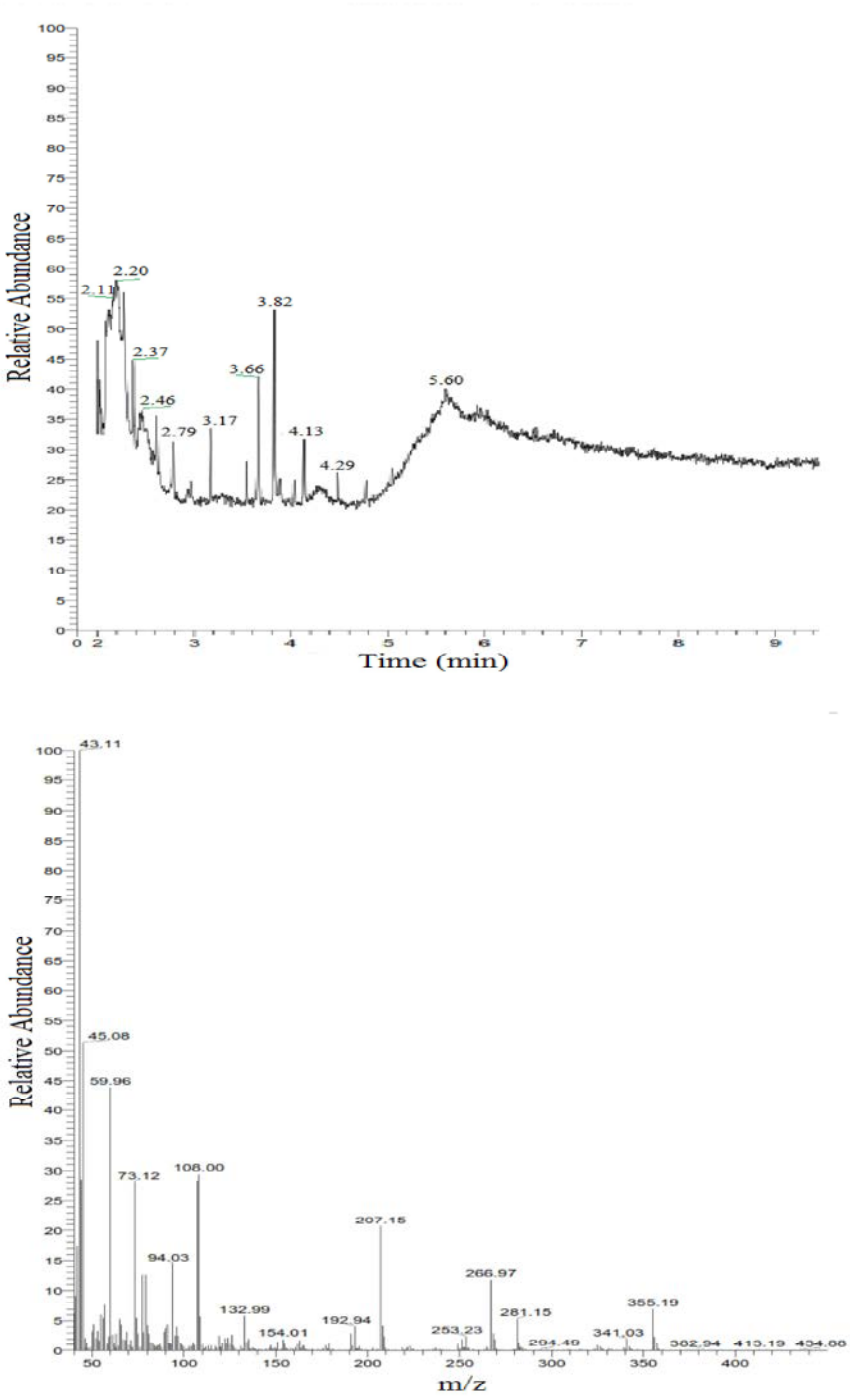


Fig. 4: GC-MS chromatogram (a) and MS spectrum (b) of CNS LVM after filtration and distillation.

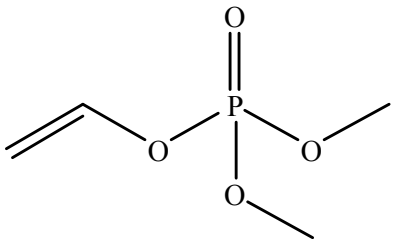


Fig. 5: Dimethyl vinyl phospate.

Table 3: Total amount of mold colonies (CFU/g) grown on cocoa seeds after 10 days of storage.

Type	Types of Seed Treatment	Total number of colonies (CFU/g)
A	Non-distilled LVM, packed after being coated with activated carbon	3.5×10^3
B	Non-distilled LVM, packed without being coated with activated carbon	4.7×10^3
C	Non-distilled LVM, unpacked after being coated with activated carbon	4.5×10^3
D	Non-distilled LVM, unpacked and without being coated with activated carbon	4.7×10^3
E	Distilled LVM, packed after being coated with activated carbon	1.6×10^3
F	Distilled LVM, packed without being coated with activated carbon	3.8×10^3
G	Distilled LVM, unpacked after being coated with activated carbon	3.2×10^3
H	Distilled LVM, unpacked and without being coated with activated carbon	4.0×10^3
I	Control (+)	1.1×10^4
J	Control (-)	5.0×10^4

The Antifungal Activity of Liquid Volatile Matter from Cashew Nut Shell: The Minister of Agriculture of the Republic of Indonesia in 2013 has issued the requirements of cocoa seed quality, namely the condition of seeds are perfect, not defective; maximum storage time is 10 days after harvest; and seeds are soaked in a solution of fungicide. To get good and healthy cocoa beans, provision of quality seeds commences from the selection of cocoa fruits. The fruits have to be normal, healthy and not wrinkled in shapes and sizes. Cocoa beans that are deformed, bruised, young and soft are not processed further. Fleshy seeds, containing pulp, are excreted from the fruit and cleaned since the fleshy seeds contains high water that can stimulate the fungus to grow quickly. After the seed selection, cocoa beans were soaked with LVM and synthetic fungicide as a positive control with each a concentration of 20%. Cocoa beans were then stored after being coated with activated carbon to maintain moisture levels [29]. Observations were carried out for 10 days in tightly closed plastic storage at room temperature [30].

The LVM of CNS was used as a bio-fungicide to protect seeds from fungal attack because the fungus needs to be inhibited due to its mitotoxin growth. Obtained data on total number of mold colonies in seeds (CFU/g) was shown in Table 3.

Table 3 shows that seeds given LVM with a variety of treatments (seeds in treatment A-H) showed a lower total number of fungi (mold), namely 1.6-4.7 x 10^3 than seed given synthetic fungicide (control +), i.e. 1.1×10^3. This indicates that LVM is better at inhibiting fungal growth in cocoa seeds than synthetic fungicide. Table 3 also show that cocoa seeds that were given LVM distillation (E-H treatment), the number of molds that grew lower, i.e. 1.6-4.0 $\times 10^3$ compared to seeds given LVM before distillation, indicated LVM distilled better in inhibiting fungi (mold) compared to LVM without distillation. For example, H results of treatment of seeds with LVM after distillation are better in inhibiting fungi (mold) compared to D results of treatment of seeds with LVM before distillation. Seed treatments for D and H are the same, unpacked and without being coated with activated carbon. In this case, the amount of colony found in H was moderately lower than in seed D. This also occurred to the activated carbon coated and packed seeds, such as seed A and seed E. By applying distilled LVM on seed E, the amount of mold colony decreased more than 50% compared to the amount of colony found in seed A.

Based on the data in Table 3, it is also known that seed coating with activated carbon and packaging can reduce the growth of mold colonies in cocoa seeds compared to those without activated carbon or packaging. Moulds are simple microorganisms that thrive almost everywhere, provided the climatic conditions are met [31]. Coating the seeds with activated carbon and packaging it are two different kinds of treatment, however the effects caused should be supportive to each other. This can be seen from the cacao seeds in the treatment of seed

coating with activated carbon and packaging (A and E), the number of molds that grow is lower than seed treatment which is only covered by activated carbon (C and G) or only packaged seeds (B and F). Therefore, cocoa seeds which are carried out by active carbon and packaging better. This treatment is based on the assumption that if the seeds are only covered with activated carbon without packaging or vice versa it will cause an imbalance between the moisture content of the seeds and the humidity of the surrounding air so that the fungus will grow more easily.

Based on the results of this study, it is known that the provision of LVM distillation, coating of activated carbon and packaging on cocoa seeds is most effective in inhibiting fungal growth in cocoa seeds, which is indicated by the least amount of molds in the treatment (E) compared to other seed treatments. This shows that LVM is very efficient as an antifungal and activated carbon and packaging helps provide environmental conditions that are not suitable for fungal growth, so that the fungus cannot grow well on cocoa seeds.

Anti-fungal material is able to inhibit fungal growth by working to affect cell walls, cytoplasmic membranes and cell nucleic. The mechanism of the reaction between the fungal cell wall chemical compounds and the active side of the carboxylic acid derivatives forms a complex compound [32]. The results of this study are in accordance with SNI 7388:2009. It was found that cocoa seeds immersed in distilled LVM, coated with activated carbon and packaged contained less than the maximum threshold of colony number of fungal allowed in cacao seeds, which is according to that SNI, the amount should not exceed 1×10^4 CFU/g [33,34].

CONCLUSIONS

After conducting the research, several facts were prevailed. First, LVM resulted from the pyrolysis of CNS containe chemical compounds that are capable of being antifungal. Identification of the chemical compound content of the LVM using GC-MS revealed that before distillation, 5-methylpyrimidin-2(1*H*)- one was the dominant compound (45.14%) and dimethyl vinyl phospate (21.84%) was the dominant content of distilled LVM. Furthermore, both non distilled and distilled LVM from the pyrolysis of CNS indicated good antifungal activity on cocoa seeds. The total number of colonies in cocoa seeds during 10-day storage period for distilled and non-distilled LVM were different. Apart from the distillation of the LVM, the treatments on seeds were identical which are coated with activated carbon followed by being packed in plastic bag. In fact, it was found that the number of TPC when non-distilled LVM used was 3.5×10^3 CFU/g and this number decreased significantly to 1.6×10^3 CFU/g when distilled LVM was utilized.

ACKNOWLEDGEMENT

The authors are grateful to the Ministry of Research, Technology and Higher Education of the Republic of Indonesia for funding this research through the PSN-Institution and Applied research program of the 2018-2019 budget year and also we thank to Halu Oleo University for any support and facility provided so that this research was successfully carried out.

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