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Jurnal Siliwangi Vol. 1. No.1. Nov. 2015
Seri Sains dan Teknologi
ISSN 2477-3891

IDENTIFICATION OF ANTIBACTERIAL ACTIVITIES FROM CALLUS CULTURE OF SOYBEAN INDUCED BY 2.4-D AND NAA

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Abstract; The objective of this research was to find the best induction media using 2.4-D (2.4-dichlorophenoxy acetic acid) and NAA (naphthalene acetic acid) for in vitro growth of soybean callus, and to identify the founded antibacterial compounds through callus culture induced by 2,4-D and NAA. This study consisted of three experiments, which were (1) callus culture of soybean using MS basal medium suplemented by 2.4-D and NAA, (2) Extraction of active fraction from callus and test of antibacterial activities using bacterial test of Bacillus substilis dan Escherichia coli, and (3) GC-MS analyses to identify the antibacterial compunds obtained. The research was set up in Paired and Unpaired Test Design with different replications and two treatments of media (MS + 2,4-D 40 mg/l and MS + 2,4-D 5 mg/l + NAA 5 mg/l). The significant differences among treatment means were calculated by the T test ($\alpha = 0.05$). The results showed that MS + 2,4-D 5 mg/l + NAA 5 mg/l was the better media for inducing callus soybean with immature cotyledone explants. Hexane fraction from callus of MS + 2,4-D 5 mg/l + NAA 5 mg/l exhibited higher antibacterial activity compared to that of MS + 2,4-D 40 mg/l and other fractions. GC-MS analyzes to hexane fraction showed the identified antibacterial compounds, that were : beta-sitosterol, cholesta-3,5-dien-7-one dan phenol (from callus source of MS + 2,4-D 40 mg/l), and oleic acid and cholesta-3,5-dien-7-one (from callus source of MS + 2,4-D 40 mg/l). Antibacterial activities of hexane fraction against Bacillus substilis was more higher than that of Escherichia coli.

Keywords: Antibacterial activities, Soybean, 2,4-D, NAA, in vitro culture

Abstrak; *Penelitian ini bertujuan untuk mendapatkan media induksi terbaik menggunakan 2.4-D (2.4-dichlorophenoxy acetic acid) dan NAA (naphthalene acetic acid) untuk pertumbuhan kalus kedelai secara in vitro, dan mengakaji aktivitas antibakteri yang dihasilkan, serta mengidentifikasi senyawa-senyawa bersifat antibakteri dari ekstrak kalus yang dihasilkan. Penelitian ini terdiri dari : (1) kultur kalus dengan media dasar MS yang diberi perlakuan kombinasi 2,4-D dan NAA, (2) Ekstraksi fraksi aktif dan pengujian aktivitas antibakteri menggunakan bakteri uji Bacillus substilis dan Escherichia coli, dan (3) Analisis GC-MS untuk mengidentifikasi senyawa bersifat antibakteri yang dihasilkan. Percobaan kultur kalus disusun berdasarkan Paired dan Unpaired Test dengan jumlah ulangan berbeda, dan menggunakan uji T untuk melihat perbedaan diantara perlakuan pada* $\alpha = 0{,}05$*. Hasil penelitian menunjukkan media yang lebih baik untuk menginduksi pertumbuhan kalus kedelai in vitro dengan eksplan kotiledon muda adalah MS + 2,4-D 5 mg/l + NAA 5 mg/l. Fraksi heksan hasil ekstraksi metode I (langsung+heksan) dari sumber kalus MS + 2,4-D 5 mg/l + NAA 5 mg/l menghasilkan aktivitas antibakteri tertinggi dibanding fraksi yang sama pada sumber kalus MS + 2,4-D 40 mg/l dan fraksi lainnya. Hasil analisis GC-MS terhadap fraksi heksan didapatkan senyawa bersifat antibakteri yaitu beta-sitosterol, cholesta-3,5-dien-7-one dan phenol (dari sumber kalus media MS + 2,4-D 40 mg/l) serta asam oleat dan cholesta-3,5-dien-7-one (dari sumber kalus media MS + 2,4-D 40 mg/l). Dari kedua bakteri uji, aktivitas*

antibakteri (dari fraksi heksan) terhadap Bacillus substilis lebih tinggi dari pada Escherichia coli.

Kata kunci: *Aktivitas Antibakteri, Kedelai, 2,4-D, NAA, Kultur in vitro*

INTRODUCTION

Soybean (*Glycine max*) is an important legume crop, known for its high quality protein and oil content, and beneficiary secondary metabolites (Sakthivelu *et al*., 2008). According to Duke (2002) soybean also produce antibiotic or antibacterial secondary metabolites such as : lactate acid, kaemferol, beta-cytosterol, xanthatin, gossipol, squalene, ethanol and malate acids.

Antibiotics may be used to treat, or in some cases prevent, bacterial infections**.** It can be produced by animal, plant and microorganims. The use of plant as antibiotic producer is become more important and urgently because of the expensive price of synthetic or imported antibiotic coming to Indonesia.

The secondary compounds product can be produced directly from plant *ex vitro* or from plant *in vitro* or by tissue culture techniques. Cell and callus cultures is mostly culture choices to be used in producing secondary metabolites from plants. In cell culture, the environment and metabolic process can possibly be regulated, and hence, the continuity and high production of secondary metabolites can be guaranted and controlled (Wetter and Constabel, 1991). The use of callus culture, besides for any other experiments, also for the experiment to study secondary metabolites productions (Smith, 1992).

The success of plant tissue culture to produce secondary metabolites compounds commercially started at 1983 in Japan in producing shikonin compound (anti inflammantory, antibacteria, colouring matter) from koshikon plant (*Lithospermum erythrorhizon*) (Tjondronegoro, 1991). Other countries, such as USA and European countries have also been develop plant tissue culture methods to produce secondary compounds. Studying about secondary metabolites from tissue culture also was reported by Wiedenfeld, *et al*. (1997) who investigate the presence of Camptothecin dan 10-hydroxycamptothecin in callus and shoot (plantlets) of *Camptotheca acuminata*. Furthermore, organogenesis and shoot regeneration of *Duboisia myoporoides* treated with cytokinin/auxin produced tropane alkaloid (Khanam, at al., 2000), and somatic embryogenesis and shoot culture of *Huperzia* selago in determining the contained huperzine A (neural and degeneratif protections) (Szypula, *et al*., 2005). In case of finding antibacterial compounds, Taraszkiewicz *et al*. (2012) demonstrated that extracts of *D. gigantea* from *in vitro* cultures contain antibacterial compounds that can be used against the bacterial fruit tree pathogens *P. syringae*.

In soybean, immmature cotyledone (originated from immature pods) is frequently used as the explants for callus culture, and it require plant growth regulator (PGR) such as 2,4-D (*2,4-dichlorophenoxy acetic acid*) and NAA (*naphthalene acetic acid*) to induce callus formation of soybean. The types and the concentration of PGR is the main factor that control callus formation in culture media (Smith, 1992). The combination of 2,4-D and NAA may potentially be used to enhance callus production, and in turn to high production of secondary metabolites resulted. Plant growth regulator, besides as stimulant and inhibitor, chemically and physically agents, also it is the factor that control the accumulation of sesqueterpene

lacton metabolite by its mode of action in bioshyntetic pathway or in accumulation stages (Goleniowski and Silva, 1993).

Antibacteria compound that resulted from in vitro culture will hopely have the ability to eliminate the bacteria or to inhibit the growth of bacteria, either Gram positive bacteria or Gram negative bacteria, such as *Bacillus substilis* dan *Escherichia coli*. *Bacillus substilis* is involved and responsible in hemolysis (blood cell lysis) and septichemically problem (the bacteria developed during blood cell transport and be pathogenic). *Escherichia coli* is known to be the cause of infection diseases in digestion channel, urinaria system and neural system (Gupte, 1990).

More information regarding the isolation of antibacteria compound from the soybean is still required, especially about the possibility of the presented antibacterial compounds from extract soybean callus in vitro, besides directly from seed extraction. Hence, the aim of this study was to find the best induction media using 2.4-D (*2.4-dichlorophenoxy acetic acid*) and NAA (*naphthalene acetic acid*) for *in vitro* growth of soybean callus, and to identify the founded antibacterial compounds through callus culture induced by 2,4-D and NAA.

MATERIALS AND METHODS

The research was conducted from October 2014 to December 2014 in Laboratory of Tissue Culture, Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University; and in laboratory of Chemistry and Biology, Faculty of Agriculture, Siliwangi University. GC-MS Chromatography analysis was performed in Dopping Laboratory, DKI Jakarta.

Callus culture of soybean

The solidified MS medium containing B5 vitamin and gelrite 2 g L^{-1} was used for callus induction. The medium was added with 3% sucrose and growth regulator of 2,4-D 5 mg/l + NAA 5 mg/l (I1 treatment) and 2,4-D 40 mg/l (I2 treatment). Pods were surface-sterilized by immersion for 1 min in 70% ethanol and 15 min in 1% solution of sodium hypochloride and then rinsed three times in sterile water. In a laminar flow hood, immature cotyledons were aseptically removed from the pods and the end containing the embryonic axis was cut off and discarded. After the seed coats were removed, the two cotyledons were separated and the abaxial side was placed on the media. The pH of the media was adjusted to 5.6-5.8 and autoclaved at 121 ^oC for 15 min. Four cotyledons of each genotypes were placed on each flask and incubated under white fluorescent light of 1,500 lux at 24 h photoperiod at 24 ± 3 ^oC. The 5 weeks-old somatic embryo clusters of globular stage or embryogenic callus were harvested and proliferated in the same media. The experiment was set up in paired and unpaired test with two treatments (I1 and I2) and different replications (18 times for I1, and 12 times for I2). The observed variables were callus growth condition, callus texture and colour, callus diameter, and fresh weight of callus. The collected data (treatment means) was analysed using T test at a probability level of 0.05.

Regeneration and propagation of bacteria

The tested bacteria (*B. substilis, E. coli*) were propagated in sterilized agar media with obliqued position, then incubated at 37° C, over night. These bacteria from agar media were inoculated aseptically to the flask containing sterilized NB (Nutrient Broth) medium, and put in the shaker for 24 hours and 37° C.

Extraction of antibacterial compounds

Callus extraction for each treatment were performed in chain based on the difference of organic solvent polarities. The

used organic solvents were: cold methanol, ethyl acetate, chloroform and hexane. 1 g of soybean callus was mashed by mortar/blender in volume of 10 ml H_2O in order to obtain the callus suspension. The suspension, then centrifuged at 5000 rpm for 10 min. Afterwards, it mixed with cold methanol (1:1), and stored refrigerator at 10° C for 24 hours. From storage, the supernatant was recentrifuged at 5000 rpm for 10 min to result the second supernatant and the pellet. This supernatant (named : methanol fraction) was then evaporated at 40° C to be prepared for antibacterial activities test. This methanol extract was also used in further extraction process (extraction methods I and II) as shown in Fig. 1.

Test of antibacterial activities

100 µl of bacterial inoculum (optical density/OD *B. substilis* and *E. coli* respectively 0.87 and 0.89) was poured into the vessels/petridishs and mixed with 12 ml of NA (Nutrient Agar after melting at 45° C), and allowed to solidified at room temperature. Paper discs (Whatman No. 1 filter paper, diameter of 0.5 cm) was put on the surface of this agar media, and dropped with 20 µl test extract in determined concentration. Antibiotic standard disc (20 µl amoxicillin 0.05 mg/ml) was also put on the surface of media. The whole of culture vessels were incubated at 37° C for 24 hours. The finding of transparent zone diameter around paper discs indicated the inhibition zone of bacterial growth or the presence of antibacterial activities in the callus extract and to be compared to antibiotic standard. The transparent zone diameter was the average from two replicates.

GC-MS for analysis of extract components

The resulted active extract that have highest antibacterial activity was analysed by GC-MS (GC-MSD) in order to identify the antibacterial compunds obtained. This extract was evaporated to dryness and reconstituted in to 2 ml hexane, and then subjected to GC-MS analysis. Chromatographic separation was carried out with instrument GC-MS-Hewlet Packard (HP) tipe 6890 series (GC) and tipe 5972 (MSD) instrument with Ultra 2 column (17 m×200 µm x 0,11 µm) and pressure 4.29 psi constant flow. The oven temperature was 50° C; inlet temperature and pressure respectively were 250° C and 3.82 psi with split model. Mass spectra was taken at 70 eV; the detector was MSD. Helium was used as carrier gas with flow 0.5 ml/min and electronic pressure control on. Samples were dissolved in hexane and injected automatically (HP 6890 Injector, volume of 1 µl).

RESULTS AND DISCUSSION

Effect of 2,4-D and NAA to callus growth

(1) Callus growth condition, callus colour and texture

The different growth condition of callus was exhibited by the two induction media (MS + 2,4-D 40 mg/l and MS + 2,4-D 5 mg/l + NAA 5 mg/l) (Table 1). Callus induction for the two treatments of media generally started at 3 weeks after culture. Callus colour of MS + 2,4-D 40 mg/l was predominantly green and yellowish colours. It was different with callus colours in MS + 2,4-D 5 mg/l + NAA 5 mg/l which the brownish or mixed with yellow and white colours founded. The faster growing of callus exhibited after 5 weeks old. Callus growing in MS + 2,4-D 40 mg/l was tend to be slower compared to MS + 2,4-D 5 mg/l + NAA 5 mg/l.

From these results, it was indicated that 2,4-D solely or combined with other auxins (NAA) can induce and trigger growth of callus at proper concentrations. Khanam, et al. (2000) reported that callus induction of Duboisia myoporoides was obtained in

 ISSN 2477-3891

medium containing 2,4-D alone or varied 2.4-D + cytokinins. Callus formation in explant tissues will involve the development of random and spreadly cell division; the cells is still undiffererntiated cells and it undergoes loosing of organized cell structures (Thorpe, 1980; Wagley, et al., 1987). 2,4-D and NAA are the two PGR that frequently used in plant tissue culture to induce cell division as well as on callus formation; the auxins + cytokinins (such as: kinetin, BA) can be incorporated to the media to form callus tissue on the explant (Wetter dan Constabel, 1991).

Table 1. Growth condition of callus, callus colour and texture on the treatment of induction media

Callus induction media	Replications	Callus colour	Callus texture	Callus growth condition
MS+2,4-D40 mg/l (I1)	1	pc	ak	++
	2	hkc	ak	++
	3	pc	ak	++
	4	pc	ak	++
	5	pc	ak	+
	6	pc	kk	++
	7	pc	kk	+
	8	hkc	kk	++
	9	pc	kk	+
	10	kc	kk	+
	11	pc	kk	+
	12	pc	kk	+
	13	pc	kk	+
	14	pc	kk	+
	15	hkc	kk	+
	16	hkc	kk	++
	17	pc	kk	+
	18	pc	kk	++
MS+2,4-D5mg/l+NAA5mg/l (I2)	1	kh	ak	++
	2	kh	ak	++
	3	kph	re	+++
	4	kh	kk	++
	5	kh	ak	++
	6	kh	ak	++
	7	kh	re	+++
	8	kph	kk	++

Note : c = brown/brownish, h = green, k = yellow, p = white. +, ++, +++ = respectively less, adequet, and well growth.

Besides the growing callus was faster, callus of MS + 2,4-D 5 mg/l + NAA 5 mg/l also proceed better consistency with bright colour indicating the friable callus was formed. The browning callus (pc, hkc) founded in media 2,4-D 40 mg/l presumably because of the exceeded concentration of 2,4-D 40 mg/l used, although in solely addition. This condition stopped developing callus and eventually caused the death of callus cells. Wiedenfeld, et al (1997) revealed that the suplementing of 2 mg/l 2,4-D and the varied cytokinins into MS medium caused callus browning, the calli turned brown then black and eventually died.

The presence of greenish callus in this experiment was parallel with the result obtained by Khanam, et al. (2000) that the colour of Duboisia myoporoides callus induced by combining 10 μM BA with 1 μM NAA or 0,1 μM 2.4-D turned green at one weeks old. The green callus indicate the occurrence of stimulating chlorophyl formation in callus cells.

(2) Callus diameter and fresh weight

In this experiment, among the induction media used (MS + 2,4-D 40 mg/l and MS + 2,4-D 5 mg/l + NAA 5 mg/l) callus diameter and fresh weight were significantly different at 5 weeks after culture (Table 2).

Tabel 2. Effect of induction media on callus diameter and fresh weight after 5 weeks of culture.

Callus induction media	Callus diameter (cm)[1)]	Weight of fresh callus (g)[2)]
I1 (MS + 2,4-D 40 mg/l)	0,64	0,092
I2 (MS+2,4-D 5 mg/l+NAA 5 mg/l)	1,66 *	1,244 *

Note : * significantly different according to T test (paired and unpaired test) at $\alpha = 0.05$. [1)] and [2)] respectively from 18 times of I1 replications and 8 times of I2 replications.

Callus diameter and fresh weight of callus resulted in MS + 2,4-D 5 mg/l + NAA 5 mg/l (I1) were more higher than that

in MS + 2,4-D 40 mg/l (I1). The exceeded concentration of 2.4-D used may be the caused from which the inhibited growth of callus brought on the lower result of MS + 2,4-D 40 mg/l. In the high concentration of 2.4-D characterized by browning callus, the enlargement and elongation of callus cells were inhibited or even halted. This condition effected the decreased callus diameter and callus cell masses obtained. Wiedenfeld, *et al*. (1997) revealed the bright callus is friable callus with long cells, whereas the black ones is compact but there is no cell differentiation observed. The friable callus mostly founded on media of MS + 2,4-D 5 mg/l + NAA 5 mg/l (I2) showed that cell elongation require further develops of callus, such as with the incraesing of callus diameter or callus cell masses.

3.2. Extraction of active fraction

The harvested callus from media of MS + 2,4-D 40 mg/l was 1.66 g, whereas in MS + 2,4-D 5 mg/l + NAA 5 mg/l was 9.95 g. These callus, then extracted to derive fraction/active extract having antibacterial activitiy. The chained process of extraction using several organic solvent was as follows:

a) Fresh callus+H_2O resulted (centrifuging at 5000 rpm, 10 min) derived supernatant and the pellet. This pellet was unused because it presumably did not contain antibacterial compounds due to bringing it into the supernatant.
b) Cold methanol+resulted supernatant from each induction media derived second supernatant and the pellet. This pellet was also unused. The second supernatant (named by mathanol fraction) was used in further extraction (extraction method I and II).
c) In extraction method I, the addition of acetone solvent into methanol fraction (1;1) resulted the methanol+acetone fraction. It mixed with hexane solvent (1:1) derived 2 layers : top layer was methanol fraction (transparent colour) and the bottom ones was the hexane fraction (yellowist colour).
d) In extraction method II, the methanol fraction was mixed only with hexane solvent (without dissolved by acetone) derived directly 2 layers as well as in method 2 (methanol fraction of top layer and hexane fraction of bottom layer).

The molecular weight (MW) of hexane is more higher than MW of methanol caused hexane layer was in the bottom. 3 ml of each evaporated fraction from extraction method I and II was dissolved by H_2O to be used in test of antibacterial potencies (antibacterial activities) using *Escherichia coli* dan *Bacillus substilis*.

Test of antibacterial activities

The investigated antibacterial activity (inhibition zone of diameter) of each active fraction of extraction method I and II against two bacteria (Gram negative bacteria of *E. coli* and Gram positive bacteria of *B. substilis*) were presented in Table 3 and 4.

From callus of MS + 2,4-D 40 mg/l, methanol+acetone fraction of extraction method I exhibited antibacterial activities against *E. coli* (0.95 mm, Fig. 1)and *B. substilis* (1.90 mm) showed the antibacterial bioactive compounds were removed to polar (methanol)+semipolar (acetone) solvents. Hexane fraction obtained from mixing hexane into methanol+acetone fraction exhibited inhibition of *E. coli* (0.60 mm) and of *B. substilis* (1.57 mm) antibacterial activity revealed the antibacterial bioactive compounds were then removed to non polar solvents of hexane (although for whole it have not yet removed to hexane fraction as indicated by the existed bacterial inhibition

in methanol fraction) (fig. 1). The inhibition zone was also exhibited by hexane fraction at method I in same callus sources (MS + 2,4-D 40 mg/l), that were 1.10 mm of *E. coli* and 1.15 mm of *B. substilis*

Table 3. Inhibition zone of *Escherichia coli* and *Bacillus substilis* from callus extract planted on media of MS + 2,4-D 40 mg/l

Callus extracts/active fractions	Inhibtion zona of diameter (mm)			
	Escherichia coli		*Bacillus substilis*	
	Control[1)]	Fractions[2)]	Control[3)]	Fractions[4)]
Methanol+acetone fraction of Method I	0.90	0.95	2.05	1.90
Methanol fractions of Method I	1.10	0.70	0.95	1.40
Hexane fraction of Method I	0.62	0.60	2.10	1.57
Methanol fraction of Method II	0.70	0.80	1.30	1.30
Hexane fraction of Method II	0.95	1.10	1.30	1.15

Note: [1)] [2)] [3)] [4)] each from two replications.

Table 4. Inhibition zone of *Escherichia coli* and *Bacillus substilis* from callus extract planted on media of MS + 2,4-D 5 mg/l + NAA 5 mg/l

Callus extract/active fraction	Inhibition zona of diameter (mm)			
	Escherichia coli		*Bacillus substilis*	
	Control[1)]	Fractions[2)]	Control[3)]	Fractions[4)]
Methanol+acetone fraction of Method I	3.40	1.70	2.25	0.20
Methanol fractions of Method I	2.45	0.90	2.85	0.25
Hexane fraction of Method I	3.10	1.15	6.85	2.25
Methanol fraction of Method II	1.85	0.15	5.25	1.25
Hexane fraction of Method II	4.00	1.60	7.05	5.10

Note: [1)] [2)] [3)] [4)] each from two replications.

From callus of MS + 2,4-D 5 mg/l + NAA 5 mg/l of extraction method I, the methanol+acetone fraction also showed the antibacterial activities against *E. coli* (1.70 mm) and *B. substilis* (0.20 mm). In further extraction, the inhibition of *E.coli* was 0.9 mm of methanol fraction (top layer) and *B. substilis* was 1.15 mm of hexane fraction (bottom layer); whereas for *B. substilis*, the inhibition zone was 0.25 mm of methanol fraction and 2.25 mm of hexane fraction. In extraction methods II (directly suplemented by hexane) of same source of callus (MS + 2,4-D 5 mg/l + NAA 5 mg/l), the resulted hexane and methanol fractions also gave the antibacterial activities against *E. coli* (respectively 0.15 mm and 1.60 mm) and *B. substillis* (respectively 1.25 mm and 5.10 mm) (Fig 2.).

The presence of bacterial inhibition on hexane fraction of the two sources of callus (MS + 2,4-D 40 mg/l and MS + 2,4-D 5 mg/l + NAA 5 mg/l) indicated, although the extraction without acetone solvent (directly mixed by hexane), the actively antibacterial compounds were remain moved to hexane solvent. This situation was proved by the transparent colour excisted in methanol fraction of top layer with almost no inhibition zone formed. In conclusion, the antibacterial bioactive compounds from this soybean callus extract were more preferable to dissolve to non polar solvent (hexane) than to polar solvent (methanol).

The inhibition of *B. substilis* (5.10 mm) from hexane fraction of MS + 2,4-D 5 mg/l + NAA 5 mg/l was the highest compared to other fractions tested (Table 3 and 4). This case showed, after the methanol extraction formed, hexane compound was the proper solvent to be used to further extract antibacterial compounds from this soybean callus.

Based on the used induction media, hexane fraction of MS + 2,4-D 5 mg/l + NAA 5 mg/l exhibited the two bacterial inhibition more greater than that of MS + 2,4-D 40 mg/l. The media of induction callus in this experiment influenced the production and accumulation of secondary metabolits as well as antibacterial compounds from plant cells or tissues. Husin *et al*. (2002) revealed the content of alkaloid compounds in the culturs can be increased through media optimation. The media optimation can be done by the modification of media komponen including plant growth regulators.

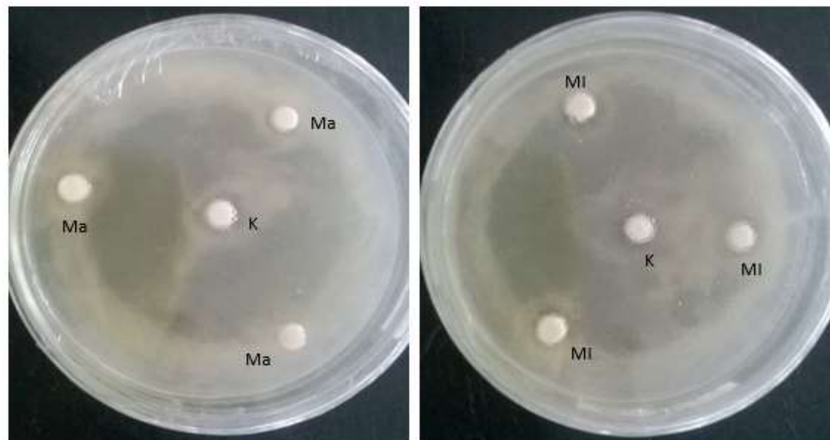

Figure 1. Antibacterial activities (inhibition zone) against *E. coli* from methanol+acetone fraction (Ma) and methanol fraction of extraction method I and callus source of MS + 2,4-D 40 mg/l. K = Antibiotic control of amoxicillin 100 µg/µl.

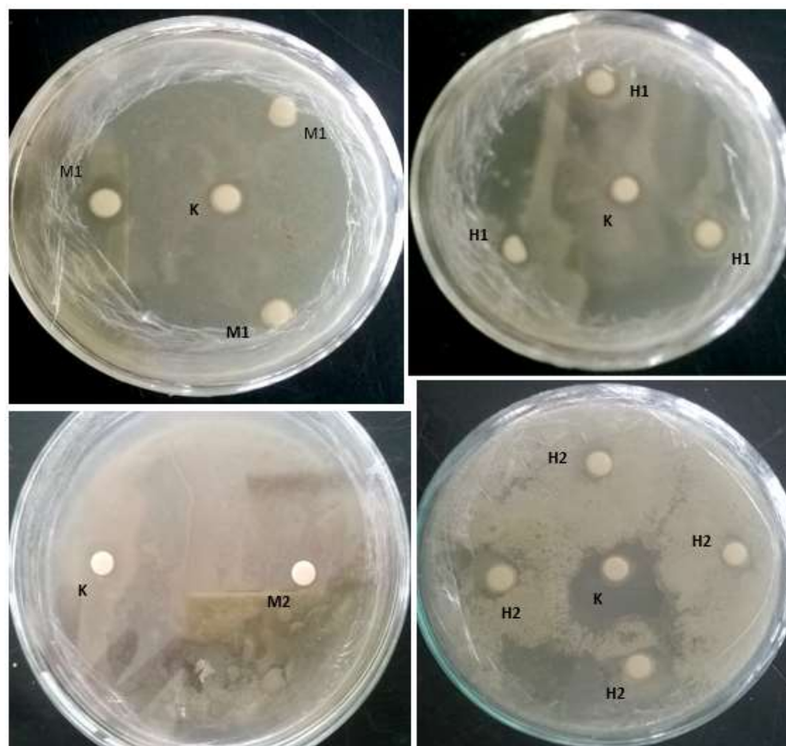

Figure 2. Antibacterial activities (inhibition zone) against *B. substilis* from methanol fraction (M) and hexane fraction (H) of extraction method II and callus source of MS + 2,4-D 40 mg/l and MS + 2,4-D 5 mg/l + NAA 5 mg/l. K = Antibiotic control of amoxicillin 100 µg/µl.

ISSN 2477-3891

The inhibition of *B. substilis* of hexane fraction either from callus of MS + 2,4-D 40 mg/l or MS + 2,4-D 5 mg/l + NAA 5 mg/l was more greater than that of *E.coli* (Fig. 3). This result may be caused by the difference of cell wall structures of the two bacteria tested. Cell wall structure of *E. coli* (Gram negative bacteria) is more complexed compared to that of *B. substilis* (Gram positive bacteria) effecting antibacterial compounds is not easy to move or penetrate to the bacteria cell targets. According to Pelczar and Chan (1988) the complexed cell structure of Gram negative bacteria is composed by three layers, that are lipoprotein (outer layer), lipopolysaccharide (middle layers) and peptidoglycans (inner layer). Tegos and Hamblin (2006) also stated that Gram negative bacteria have, apart from the inner membrane, and in difference with Gram-positive bacteria, an outer membrane which is an additional impenetrable barrier for antibacterial compounds and the place of action for many multidrug resistance systems). In the present study, the susceptibility of among the bacteria tested to bioactive compounds probably was different as showed by Didry *et al*. (1998) and Krolicka *et al*. (2009) that Gram-positive bacteria such as *Streptococcus* and *Enterococcus* are usually susceptible to active compounds of plants extracts. The deleterious effect of peptidoglycan synthesis by antibacterial compounds (such as : penicillin and β lactam) is also presumably the reason of the greater antibacterial activities against *B. substilis*.

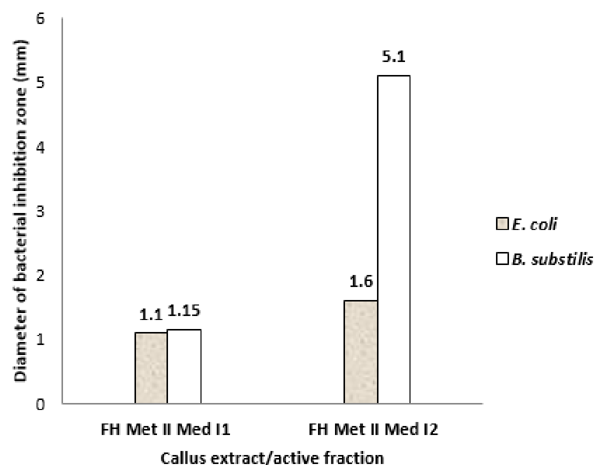

Figure 3. Inhibition diameter of *E. coli* and *B. substilis*

Figure 3. Inhibition diameter of *E. coli* and *B. substilis* from hexane fraction of extraction method II and callus source of MS + 2,4-D 40 mg/l and MS + 2,4-D 5 mg/l + NAA 5 mg/l.

Note : FH Met II Med I1 = hexane fraction of extraction method II with callus sources of MS + 2,4-D 40 mg/l.

FH Met II Med I2 = hexane fraction of extraction method II with callus sources of media MS + 2,4-D 5 mg/l + NAA 5 mg/l.

Identification of antibacterial compounds using GC-MS

Gas chromatography and mass spectroscopy (GC-MS) analyses was carried out using the sample of hexane fraction of extraction method II either from callus of MS + 2,4-D 40 mg/l or from callus of MS + 2,4-D 5 mg/l + NAA 5 mg/l. From GC-MS chromatography, the presence of antibacterial compounds were detected and identified as shown in Table 5. GC-MS spectrogram of hexane fraction of extraction method II and callus source of MS + 2,4-D 40 mg/l and MS + 2,4-D 5 mg/l + NAA 5 mg/l presented Fig. 4 and 5. These result revealed that the founded antibacterial compounds were varied in type and the amount of compounds, it may be was due to the effect of induction media in this study. Plant growth regulator that

incorporated in culture medium could probably control or influence the formation and the content of secondary metabolits in plants. Ferreira *et al*. (1992) reported that callus of *Euphorbia characias* growing on media containing 2.4-D showed the increasing β-cytosterol content during stage of exponencial growth.

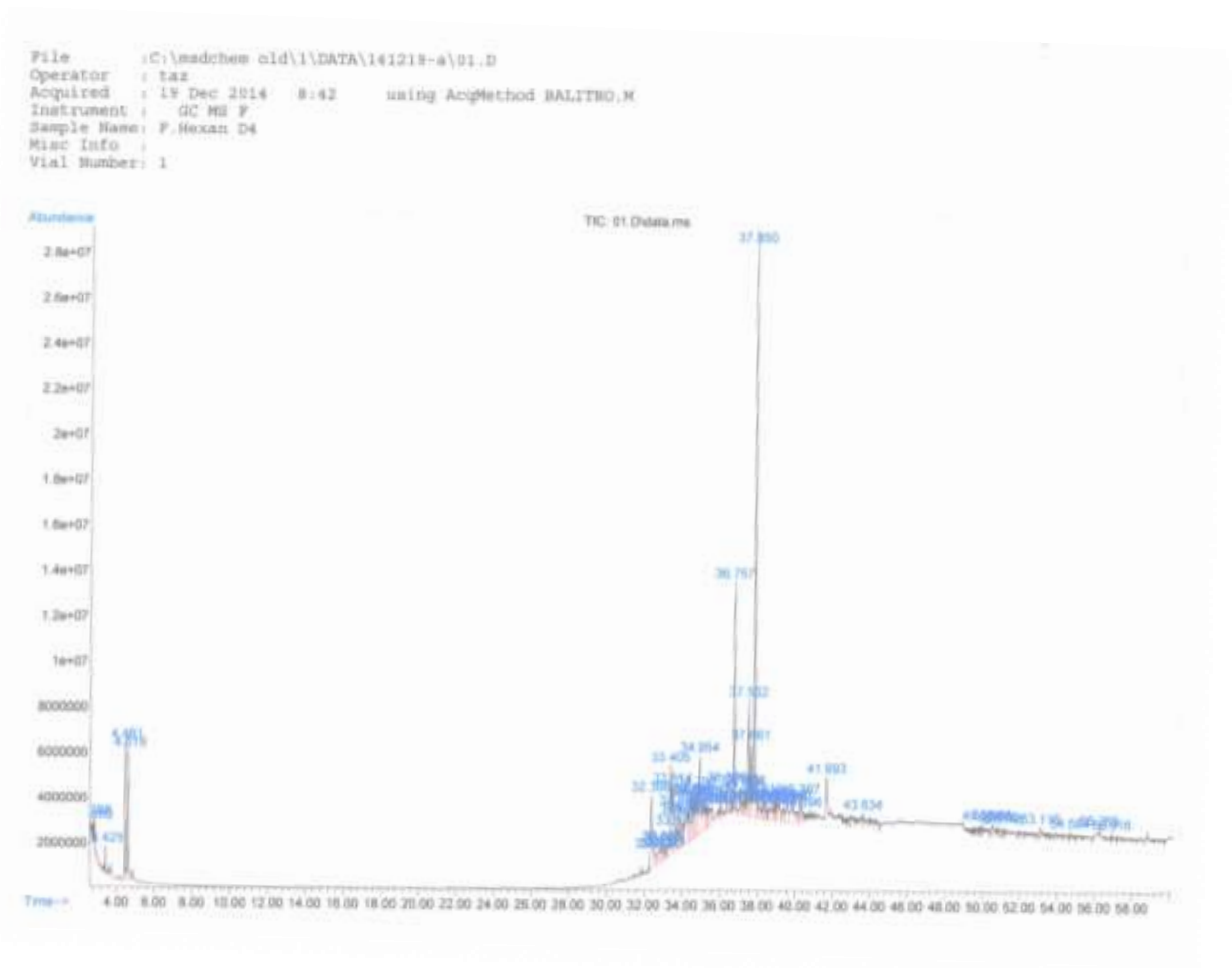

Figure 4. GC-MS spectrogram of hexane fraction from extraction method II and callus source of MS + 2,4-D 40 mg/l.

Oleic acid is primer metabolits synthesized in primer metabolism pathway, whereas beta-sitosterol, cholesta-3,5-dien-7-one, and phenol are secondary metabolites synthesized through the specific pathway after primer metabolism. Mariska (2013) stated secondary metabolites compounds produced only in low concentration and not continously produced for plant defends, and it have not important role in primer metabolism.

 ISSN 2477-3891

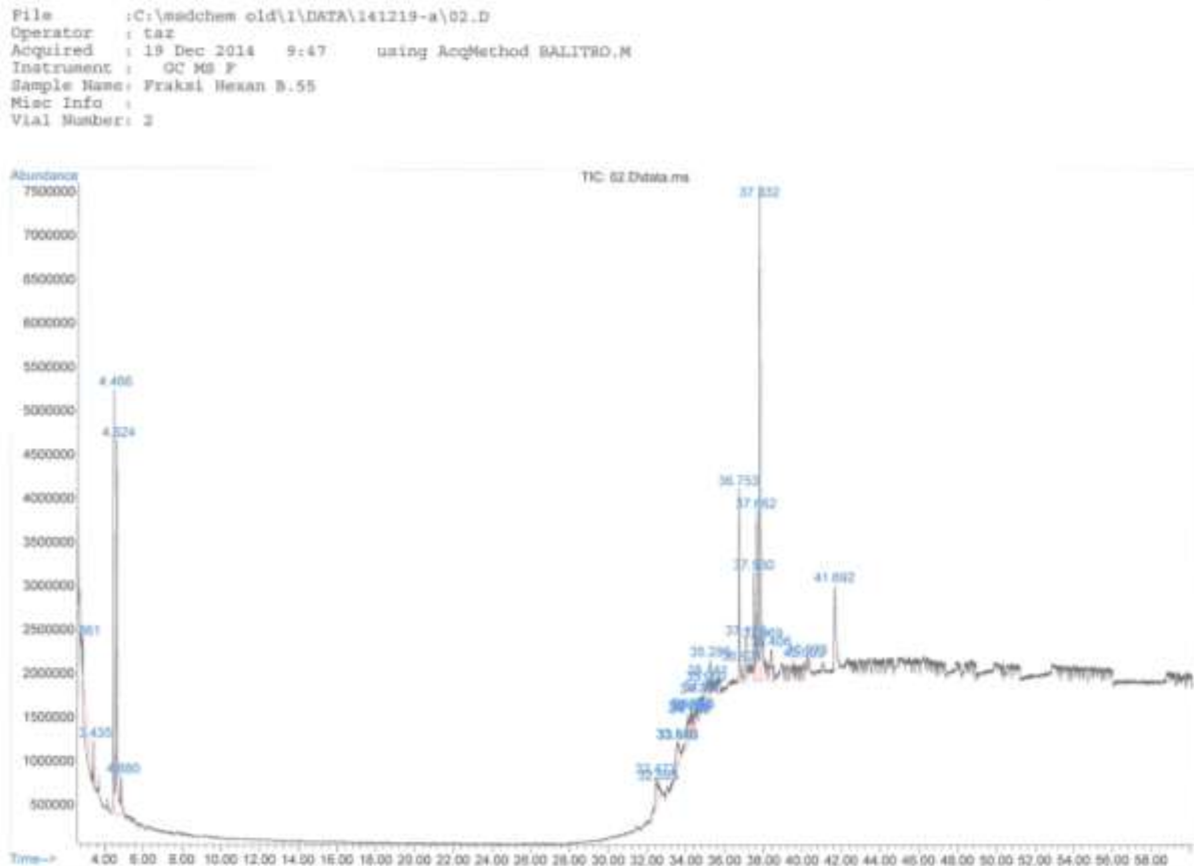

Figure 5. GC-MS spectrogram of hexane fraction from extraction method II and callus source of MS + 2,4-D 5 mg/l + NAA 5 mg/l.

Table 5. Antibacterial bioactive compounds identified by GC-MS on callus sources of MS + 2,4-D 40 mg/l (I1) dan MS + 2,4-D 5 mg/l + NAA 5 mg/l (I2).

Induction media	RT	Antibacterial compounds (and references)	Molecular formula	Area %
I1	34.685	***Beta-sitosterol*** (Duke, 2002)	$C_{29}H_{50}O$	1.66
	41.690	***Cholesta-3,5-dien-7-one*** (Silverio et al., 2007)	$C_{27}H_{42}O$	2.10
	54.681	***Phenol*** (Duke, 2002)	C_6H_5OH	0.24
I2	32.595	**Asam oleat** (Sun et al., 2003; Seidel dan Taylor, 2004)	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	0.87
	41.690	***Cholesta-3,5-dien-7-one*** (Silverio et al., 2007)	$C_{27}H_{42}O$	7.35

CONCLUSION

MS + 2,4-D 5 mg/l + NAA 5 mg/l was the better media for inducing callus soybean with immature cotyledone explants. Hexane fraction from callus of MS + 2,4-D 5 mg/l + NAA 5 mg/l exhibited higher antibacterial activity compared to that of MS + 2,4-D 40 mg/l and other fractions. GC-MS analyses to hexane fraction showed the identified antibacterial compounds, that were : *beta-sitosterol*, *cholesta-3,5-dien-7-one* dan *phenol* (from

callus source of MS + 2,4-D 40 mg/l), and oleic acid and *cholesta-3,5-dien-7-one* (from callus source of MS + 2,4-D 40 mg/l). Antibacterial activities of hexane fraction against *Bacillus substilis* was more higher than that of *Escherichia coli*.

From our findings of the antibacterial compounds in this study, the suggestions may be proposed such as the further research for purification and isolation of antibacterial compouds resulted.

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ACKNOWLEDGEMENT

This study was supported by the Ministry of National Education of Indonesia, Directorate General of Higher Education via DIPA Siliwangi University, contract number (Research Grant): 135/UN.58.09/PL/2014.