IDENTIFICATION SECONDARY METABOLITES FROM CALLUS *Piper retrofractum* Vahl

Fahrauk Faramayuda[*], Jaka Permana[*], Akhirul Kahfi Syam[*], Elfahmi[**]
[*]*Division of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Jenderal Achmad Yani, Cimahi, Indonesia, ramayuda.f@gmail.com, jakapermana04@gmail.com, akhirulkahfisyam@gmail.com*
[**]*School of Pharmacy Institut Teknologi Bandung, Bandung, West Java, Indonesia, elfahmi@gmail.com*

Email Correspondence : ramayuda.f@gmail.com

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Abstract: Javanese chili (*Piper retrofractum* Vahl) is a traditional medicinal plant originating from Indonesia and has many pharmacological activities, one of which is often used as a base for aphrodisiac herbal medicine. The population of *P. retrofractum* is limited, so it is necessary to design secondary metabolite production and propagation efforts using plant tissue culture techniques. The materials used in this study were explants of *P. retrofractum* leaves that were induced in Murashige and Skoog (MS) media and the ratio of growth regulators 2.4-dichlorophenoxyacetis acid (2,4-D): Benzyl Amino Purine (BAP) 0.5: 0.5. The results showed that the callus of *P. retrofractum* was formed in the growth regulator 2.4D: BAP (0.5: 0.5). TLC and spectrophotometry identified the secondary metabolite content of callus. Secondary metabolite analysis using the thin layer chromatography (TLC) method using the mobile phase ethyl acetate: n-hexane (7: 3) showed a terpenoid compound indicated by purple spots on the visual appearance after spraying 10% spotting vanillin. Identification using infrared spectrophotometry shows functional groups -CH, C = O, C = C, -CH_2, and -CH_3, characteristic of terpenoid compounds. Based on TLC data and spectrophotometry, callus *P. retrofractum* is thought to contain terpenoid compounds. This study's results are expected to be the basis for developing secondary metabolite production in *P. retrofractum* with cell suspension culture and *P. retrofractum* propagation by *in vitro* culture.
Keywords: *P. retrofractum* Vahl, Plant tissue culture, Callus, Identification of secondary metabolites

Abstrak: Cabai jawa (*Piper retrofractum* Vahl) merupakan tanaman obat tradisional yang berasal dari Indonesia dan banyak memiliki aktivitas farmakologis salah satunya sering digunakan sebagai bahan dasar jamu afrodisiaka. Populasi tanaman cabai jawa terbatas maka perlu dirancang upaya produksi metabolit sekunder dan perbanyakan tanaman cabai jawa salah satunya menggunakan teknik kultur jaringan tanaman. Bahan yang digunakan pada penelitian ini adalah eksplan daun tanaman cabai jawa yang diinduksi pada media Murashige and Skoog (MS) dan perbandingan zat pengatur tumbuh 2.4-dichlorophenoxyacetis acid (2,4-D) : benzyl amino purine (BAP) 0,5 : 0,5. Hasil penelitian menunjukkan bahwa kalus cabai Jawa terbentuk dalam zat pengatur tumbuh 2,4D: BAP (0,5: 0,5). Kandungan metabolit sekunder dari kalus diidentifikasi dengan KLT dan spektrofotometri. Analisis metabolit sekunder menggunakan metode kromatografi lapis tipis (KLT) dengan fasa gerak etil asetat: n-heksana (7: 3) menunjukkan adanya senyawa terpenoid yang ditunjukkan adanya bercak ungu pada penampakan visual setelah disemprotkan vanilin 10%. Hasil Identifikasi menggunakan spektrofotometri *UV-V*is menunjukkan isolat mempunyai panjang gelombang

maksimum 272,6 nm. Identifikasi menggunakan spektrofotometri inframerah menunjukkan adanya gugus fungsi -CH, C = O, C = C, -CH_2, dan -CH_3 yang merupakan ciri khas senyawa terpenoid. Berdasarkan data KLT dan spektrofotometri kalus cabai jawa diduga mengandung senyawa terpenoid. Hasil penelitian ini diharapkan dapat menjadi dasar pemgembangan produksi metabolit sekunder dalam tanaman cabai jawa dengan kultur suspensi sel dan perbanyakan tanaman cabai jawa dengan kultur *in vitro*.

Kata kunci: *P. retrofractum* Vahl, Kultur jaringan tanaman, Kalus, Identifikasi metabolit sekunder.

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Introduction

P. retrofractum Vahl. It is one of the native plants of Indonesia with a high benefit value. The fruit of the *P. retrofractum* plant is one of Indonesia's flagship at this time which has been included as one of the components in a phytopharmaceutical formula, namely natural medicines which have been proven through preclinical and clinical trials and have been approved by the National Agency of Drug and Food Control Indonesia. The spread of *P. retrofractum* plants can be found in Southeast Asia, such as Malaysia and Thailand.

P. retrofractum is an annual plant that grows on climbing poles and has a large round stem shape, 5-7 cm in diameter. The length of the main stem segment is 2.93-9.82 cm, and stem color varies from black, brown to blackish brown. *P. retrofractum* also has quite some fruit branches, with a round shape and green, dark green (Haryudin & Rostiana., 2015). According to research conducted by Tang in 2019, there are new compounds contained in the *P. retrofractum* plant, which are two amide compounds; (E) -N-cinnamoyl-2-methoxypiperidine and (R)-1-(2-oxopyrrolidine-3-yl)-5,6-dihydropyridine-2 (1H) -one, four amide compounds glucoside; retrofractosides AD, and two phenylpropanoid glycoside compounds; retrofractosides E and F (Tang *et al*., 2019; Muharini *et al*., 2015; Jadid *et al*., 2018; Musthapa *et al*., 2018).

P. retrofractum as a mixture of herbal medicine to overcome hyperuricemia rats. *P. retrofractum*, as a plant, has an analgesic effect, is mixed with celery plants and leaves in the form of herbal concoctions (Fitriani *et al*., 2018). Hepatoprotective activity test against acute hepatotoxicity of paracetamol induction using ethanol extract of roots and fruit of *P. retrofractum* (Mahaldar *et al*., 2019). *P. retrofractum* extracted and made into three fractions, methanol extract, ethyl acetate extract, and n-hexane extract, and then made in several variations of concentration with a standard comparison of ascorbic acid. Methanol, ethyl acetate, and n-hexane extract of *P. retrofractum* es showed potential antioxidant activity dependent on concentration (Jadid *et al*., 2017). *P.*

retrofractum bioactive compounds extracted with methanol as a solvent have a better potential for pathogenic microorganisms (Panphut *et al.*, 2020). The results of the study, a group of animal tests giving ethanol extract of *P. retrofractum* has a high phagocytic constant value indicating activity as an immunostimulant, higher compared to the phytopharmaceutical immunostimulant group. It also increases peripheral blood neutrophil levels, which play an essential role in the process of phagocytosis in the body (Roseno *et al*., 2019). In Japan, it is used as a cooking spice (Takahashi *et al*., 2017). Other studies have reported the potential of *P. retrofractum* as anti-photoaging effects (Yun *et al*., 2018), antitubercular activity (Amad *et al*., 2017), antiproliferation activity (Hasan *et al*., 2016), larvicidal activity (Wiwattanawanichakun *et al*., 2018), and cytotoxic activity (Amad f., 2017).

Several previous studies have carried out tissue culture methods on *P. retrofractum* plants. Like the research conducted by Faramayuda in 2016, The results obtained in the study mentioned 2,4-D: BAP (0.5 ppm: 0.5 ppm) as the optimum concentration in inducing callus *P. retrofractum (*Faramayuda *et al*., 2016*).* The fastest callus induction time, *P. retrofractum*, was 11.5 days with an NAA concentration of 0.5 mg/L and BAP 0.5 mg/L. The best fresh weight of callus is 70.6 mg, the best dry weight is 18 mg, and the resulting callus is white and has a compact texture. Based on these two studies, it can be concluded that the concentration of added auxin influences callus induction and cytokine groups will stimulate cell growth and division so that when combined properly, it will produce optimal growth (Junairiah *et al.*, 2018).

Several studies on the *P. retrofractum* plant's tissue culture have not identified secondary metabolites callus. This study's results can be the basis for developing secondary metabolite production in *P. retrofractum* with cell suspension culture and the propagation of *P. retrofractum* using plant tissue culture techniques.

Material and Methods

Chemicals and reagents

2,4-dichlorophenoxyacetis acid (2,4-D) and agar phytagel were purchased from Sigma (St. Louis, MO, USA). Methanol, ethanol, ethyl acetate, toluene, hexane were purchased from Merck (Jakarta, Indonesia).

Instrumentation

Laminar airflow cabinet (Holten, UK), autoclave (PBI international Italy), ovens (Memert, Germany), rotary evaporators (Heidolp, Germany), pH meter (Mettler Toledo, Hong Kong), balance sheet (Mettler Toledo, Hong Kong), analytical scales (Shimadzu, Japan).

Collection of plants

The leaves of *P. retrofractum* were collected from the Manoko experimental garden, West Bandung, Indonesia. The plants were taxonomically identified at the School of Life Science and Technology, Institut Teknologi Bandung. *P. retrofractum* Vahl Leaf Tissue Culture.

***P. retrofactum* leaf crude drugs extraction**

The extract of *P. retrofractum* leaves is carried out by the cold extraction method, namely by maceration using multilevel solvents including n-hexane, ethyl acetate, and ethanol.40 grams of dry crude drugs are put into the macerator and added 200 mL of n-hexane solvent until wholly immersed. Then left for 24 hours. Then the filtrate was filtered, the residue was macerated with 200 ml of ethyl acetate and treated as n-hexane. The resulting filtrate was macerated with 200 ml of ethanol and filtered.

Phytochemical screening of crude drugs and leaf of *P. retrofractum* extract (Anonymous, 2017)

Alkaloid Identification

One gram of crude drugs powder was alkalized with dilute ammonia, then crushed in a mortar, then added chloroform to form 2 layers. Then take part of the ammonia layer, put the filtrate into a test tube then add 2 N HCl. The mixture is shaken vigorously until two layers are formed. The acid layer is separated then divided into three parts. The first part is used as a blank. The second part is dripped with 2-3 drops of Mayer reagent and then observed whether white-green sediment is present. The third part is dripped with 2-3 drops of Dragendorff's reagent, then observed whether or not a yellow-yellow to brick-red sediment is present.

Flavonoid Identification

One gram of crude drugs powder is heated with water over a water bath, then filtered. The filtrate is put into a test tube, then added magnesium powder and 2N hydrochloric acid. The mixture is heated on a water bath, then filtered. The filtrate is put into the test tube, and then amyl alcohol is added. The mixture is then shaken vigorously and allowed to separate. The formation of a yellow indicates the presence of flavonoids to red color on the amyl alcohol layer.

Tannin Identification

Crude drugs as much as 1 gram heated with water, then transferred into a test tube, then filtered into two parts of the filtrate. Filtrate 1 is dropped (5 drops) of 1% gelatin reagent solution. The formation of a white precipitate indicates the presence of tannin compounds. Filtrate 2 dripped with the steady solution. The presence of pink sediment indicates tannin compounds.

Polyphenol Identification

One gram of crude drugs powder is heated with water over a water bath, then filtered. Part of the filtrate is added 2-3 drops of iron (III) chloride solution. The formation of a green-blue-black color indicates the presence of phenolics.

Saponin Identification

One gram of crude drugs powder is heated with water over a water bath, put into a test tube, then shaken vigorously for 10 seconds, then added one drop of 2 N HCl. A positive test is shown by forming a stable foam as high as 1 cm for not less than 10 minutes.

Steroid and Triterpenoid Identification

One gram of crude drugs powder is crushed with n-hexane, then filtered. The filtrate is then evaporated on the evaporator plate to dry. The Liebermann-Bourchard reagent was dropped on the residue. The formation of purple color indicates that the crude drugs contains compounds of the triterpenoid group. In contrast, if it is formed, the green-blue color indicates a steroid group compound.

Monoterpenoid and Sesquiterpenoid Identification

One gram of crude drugs powder is crushed with n-hexane, then filtered. The filtrate obtained is evaporated in an evaporator plate until dry. The residue is dropped 10% vanillin reagent in concentrated sulfuric acid. The formation of iridescent indicates the presence of monoterpenoids and sesquiterpenoids.

Quinone Identification

One gram of crude drugs powder is heated with water over a water bath, then filtered. The filtrate is added 2-3 drops of KOH solution. The formation of a yellow to red color indicates the presence of a quinone group compound.

Sterilization of room and Tools

The culture room is cleaned by spraying the workplace surface in a liquid / Laminar airflow (LAF) with 70% alcohol. The tools that will be used are washed thoroughly using detergent and then dried. After being dry wrapped in paper (except culture bottles), sterilized in an autoclave at 121^{o}C for 15 minutes (Faramayuda et al., 2020).

Sterilization of Culture Media and Tool

The media and distilled water used were first sterilized in an autoclave, and tools for planting such as a culture bottle, scalpel, petri dish with an autoclave at 121^{o}C for 15 minutes (Faramayuda et al., 2020).

Media

The MS medium was carried out by weighing several MS used, then put into a measuring flask and added distilled water to the limit.

Characterization of *P. retrofractum* Vahl Plants

Observations of crude drug characterization include macroscopic, microscopic observations, and phytochemical screening (Anonymous, 2017)

Addition of Growth hormone to MS Media

The growth hormone used in this study was 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) with a ratio of 0,5: 0,5 ppm (Faramayuda et al., 2016). Growth hormones used in this study include 2,4-D, and BAP. MS media solution that has been mixed correctly, the acidity is measured using a pH meter. If the acidity of the media obtained less than 5.4, then into the media solution are added a few drops of NaOH solution with a concentration of 1 N, and if the media solution has acidity more than 5.8, then into the media solution is added 1 N HCl solution a few drops so that the acidity of the solution can be fulfilled (Elangomathavan et al., 2017)

Heating is done until the media solution is boiling. Media solution that has been heated, put into a culture bottle, and then covered with aluminium foil. The filled bottle was sterilized by autoclaving for 15 minutes at 121^{o}C. Autoclaved media is stored in a cool place for a while before the media is used for planting. This storage aims to determine whether there is contamination in the culture medium before being used to plant explants (Ali et al., 2017).

Sterilization of *P. retrofractum* Vahl Leaf Explants

P. retrofractum Vahl leaves were washed with running water, soaked in 70% alcohol solution for 1 minute, then soaked in tween mixture three drops of soaking in hypochlorite, 2% for 5 minutes. To clean the plant's inner tissue, then rinse it in sterile water for 1 minute in 3 parts of sterile water (Faramayuda et al., 2016).

***P. retrofractum* Vahl Culture**

In the planting of *P. retrofractum* Vahl, the leaves that are used are the tops of plants. The explant planting was carried out by taking plants that had previously been sterilized with tweezers and then placed on Petri dishes. The tops of the plants were cut using a scalpel. The mouth of the bottle containing the media is heated first with bunsen to prevent contamination. Then the *P. retrofractum* explants were planted in the media, and the bottle caps were heated again before being closed using aluminium foil to prevent contamination. After that, bottles to the growth regulator were added to the media and the date of planting. The scalpel and tweezers are always heated before use and soaked in 70% alcohol (Razali et al., 2017).

Preparation of *P. retrofractum* Callus Extract from Tissue Culture

P. retrofractum Vahl callus extract was carried out using the cold extraction method, namely maceration with sonification using multilevel solvents including n-hexane, ethyl acetate, and ethanol. Several dried calluses were added

to the vial added with n-hexane solvents until they were submerged. Then it was for 20 minutes to accelerate the extraction process., The residue is macerated with ethyl acetate until it is submerged and treated as n-hexane. The resulting filtrate was macerated with ethanol until submerged and filtered.

Analysis and isolation of secondary metabolites Analysis of secondary metabolites *P. retrofractum* callus using thin layer chromatography (TLC)

The extract obtained in each fraction was concentrated in a water bath until it became a thick extract and dissolved in each of the appropriate solvents. Then the solution was applied to the silica gel plate GF254. The mobile phase used was ethyl acetate: n-hexane (7:3) (Anonymous, 2017). The mobile phase is a saturated chamber for 30 minutes, then the silica plate that has been sprayed on each extract is eluted to the limit specified in the silica plate. TLC extract of *P. retrofractum* Vahl leaves (wild type) compared to *P. retrofractum* Vahl leaves from*in vitro* culture.

Isolation of secondary metabolites *P. retrofractum* callus using TLC-Preparative

Silica pulp was made by mixing 25 grams of silica gel 60 F254 with 50 mL of aqua dest in a ratio (1:2) to erlenmeyer and shaken vigorously (90 seconds) until homogeneous. Silica slurry is then poured and flattened on the glass, then allowed to stand for 24 hours, and heated in an oven at 106 ºC for 30-60 minutes before use.

Mobil Phase used according to the optimization results on extracts, namely ethyl acetate and n-hexane (7:3) were put into the chamber and saturated for 60 minutes, then spotted the n-hexane extract in a silica plate in a row to form a ribbon, as the initial line of development (1-2 cm from the lower end). In the elution stage, the plate containing the sample solution is inserted into the chamber containing the mobile phase that has been saturated and awaited until the elution process reaches the plate boundary. (1-2 cm from the top), remove, and wait dry. Then look at UV 365 and 254 nm (Febriani et al., 2017).

Two-dimensional Thin Layer chromatography

The results of TLC-Preparative were scraped and dissolved in n-hexane, allowed to stand for 24 hours. Then the scrapings are filtered. The filtrate is evaporated and analyzed. In the first elution, the isolate was eluted using the mobile phase ethyl acetate: n-hexane (7:3), obtained by a single spot with Rf: 0.78, bright blue fluorescence at UV 365 nm. Then the second elution was carried out using a different polarity mobile phase using ethyl acetate (Febriani *et al*., 2017).

Characterization of isolates

Identification of the isolated was carried out using a Uv-Visible spectrophotometry instrument to determine the wavelength and Infrared spectroscopy to determine the functional group (Febriani et al., 2017).

Uptake of isolates on UV-Vis Spectrophotometry

The sample was dissolved in a solvent that did not provide absorption in UV-Vis spectrophotometry, for the solvent used was methanol pa. Identification uses the UV-Vis Spectrophotometry instrument to determine the wavelength of the sample exposed to light so that the sample groups are known.

Methods for determining functional groups with Infrared Spectrophotometry

The samples were dried first, then placed in KBr pellets, mixed samples with KBr with a sample concentration of 0.1 - 2.0 % by weight of the mixture, then crushed in a mortar and pressed. The tool used is FTIR (Fourier Transform Infra-Red).

Results and Discussion

Characterization of *P. retrofractum* Vahl Plants

Plant determination conducted at the Taxonomy Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, showed that the sample was *P. retrofractum* Vahl. The results of macroscopic observations show that *P. retrofractum* is compound fruit, elongated to cylindrical and brown-red (figure 1) (Anonymous, 2017).

Figure 1. Macroscopic of *P. retrofractum* Vahl

Phytochemical screening was carried out on the crude drug, ethanol extract, ethyl acetate, and n-hexane of *P. retrofractum* leaves. The crude drug identified polyphenol alkaloid compounds, flavonoids, tannins, quinones, saponins, monoterpene-sesquiterpenes steroid-triterpenoids. In the ethyl acetate and n-hexane extracts, there were no detected alkaloids, polyphenols, tannins, quinones, and saponins. The ethanol extract did not identify any tannin compounds (Table 1).

Table 1. Crude drug phytochemical screening and leaf extract of *P. retrofractum* Vahl

Phytochemical Screening	Crude Drug	Ethanol Extract	Ethyl Acetate Extract	n-Hexane Extract
Alkaloids	+	+	-	-
Polyphenols	+	+	-	-
Flavonoids	+	+	+	+
Tannin	+	-	-	-
Quinone	+	+	-	-
Saponins	+	+	-	-
Monotepen-sesquiterpenes	+	+	+	+
Steroids-Triterpenoids	+	+	+	+

The results of microscopic observations on the crude drug of *P. retrofractum* leaves showed the presence of anomocytic type stomata (Figure 2).

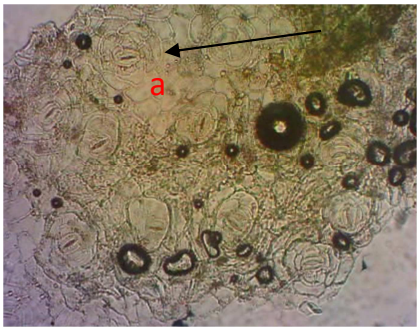


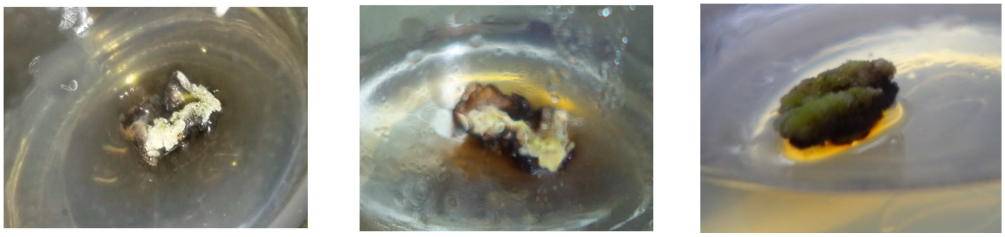
Figure 2. *P. retrofractum* Vahl plant microscopic analysis. (a) anomocytic type stomata

P. retrofractum Vahl Leaf Tissue Culture

Previous studies have reported that which induces callus by adding auxin and cytokine at various concentrations accompanied by an analysis of secondary metabolite content found in the callus. The results obtained in the study mentioned 2,4-D: BAP (0.5 ppm: 0.5 ppm) as the optimum concentration in inducing *P. retrofractum* callus, and based on analysis using Thin Layer Chromatography (TLC) showed *P. retrofractum* callus chromatogram patterns contains steroid compounds, triterpenoids, and flavonoids (Faramayuda *et al*., 2016). This study is a follow-up study from Faramayuda in 2016. The optimal media in *P. retrofractum* callus induction was reported, while this study further identified the secondary metabolite content of *P. retrofractum* callus.

In this study, a media consisting of the composition growth regulating Substances was made by previous research using a growth regulator 2,4-D: BAP 0.5 ppm: 0.5 ppm. Callus growth results can be seen in Figure 3. The growth of *P. retrofractum* callus was observed on the seventh and 28th days the callus had reached its maximum growth (Figure 3). Several factors influence the response

given by growth regulator. The combination of the growth regulator is of particular concern. Each plant that comes from different organs and species will require a different growth regulator. growth regulator plays a vital role in controlling biological processes in plant tissues. In this study, two combinations of growth regulator BAP were used: cytokinins functioning in cell division and 2,4-D, auxin hormones that function to induce cell enlargement and root growth. The development of culture is determined by the balanced interaction of the growth hormone produced in the plant itself. Because each explant has an endogenous growth regulator, but in the development and growth in vitro, exogenous hormones must still be added.



(a) (b) (c)

Figure 3. *P. retrofractum* Vahl Callus tissue culture results. With a comparison of growth gegulating substances 2.4-D: BAP (0.5 ppm : 0.5 ppm). Callus growth on day 7 (a), callus growth on day 14 (b), and callus growth on day 28 (c).

Other studies conducted by Junairiah in 2018) also using variations in the plant growth regulator concentration. Data obtained in the study included callus induction time, percentage of explants forming callus, fresh callus weight, dry callus weight, and callus color and texture. The fastest callus induction time was 11.5 days with the NAA plant growth regulator concentration of 0.5 mg/L and BAP 0.5 mg/L. The best fresh weight of callus is 70.6 mg, the best dry weight is 18 mg, and the resulting callus is white and has a compact texture (Junairiah *et al*., 2018).

Analysis and isolation of secondary metabolites

Analysis of secondary metabolites using Thin Layer Chromatography (TLC)

After extraction of *the P.retrofractum* callus, each extract based on its polarity was analyzed using chromatography. The chromatography used is thin layer chromatography with the same mobile phase solvent. The mobile phase solvent used is ethyl acetate: n-hexane with a ratio of 7: 3. The TLC profile results were compared with the TLC profile of *P.retrofractum* leaf extract without tissue culture. The one produced between callus and *P. retrofractum* leaves without tissue culture gave a different TLC pattern. Thin-layer chromatography callus and extract of *P. retrofractum* plants can be seen in the figure 4.

TLC profiling of *P. retrofractum* callus

The results of the TLC are sprayed with the appearance of specific spots. The spot appearance used was 10% Vanillin SO_4, then heating 10 minutes in an oven to evaporate the hydrogen component in silica so that organic compounds could be seen and reacted to the maximum. The plate sprayed with a 10% vanillin spotting appearance gives a reaction by giving a purple colour to the visual appearance (Figure 5). This suggests that the compounds contained in the plates are steroids - triterpenoids or monoterpenes - sesquiterpenes. The next stage is the isolation of secondary metabolites with the TLC-Preparative method.

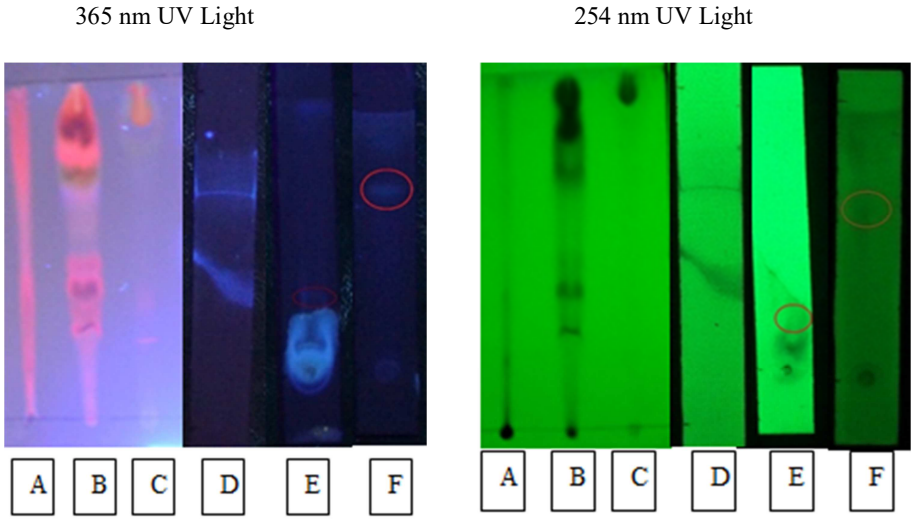


Figure 4. Thin-layer chromatography callus and extract of *P. retrofractum* plants.

Description :
Stationary phase : silica gel plate GF_{254}
Mobil phase : ethyl acetate: n-hexane (7: 3)
A : Wild type *P. retrofractum* ethanol extract
B : Wild type *P. retrofractum* ethyl acetate extract
C : Wild type *P. retrofractum* hexane extract
D : Ethanol extract of *P. retrofractum* callus culture 2,4-D: BAP (0.5 ppm: 0.5 ppm)
E : Ethyl acetate extract of *P. retrofractum* callus culture 2,4-D: BAP (0.5 ppm: 0.5 ppm)
F : n-hexane extract of *P. retrofractum* callus 2,4-D culture: BAP (0.5 ppm: 0.5 ppm)

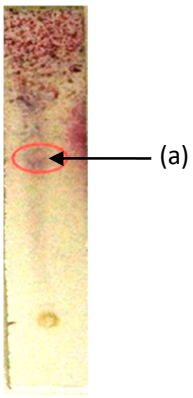


Figure 5. n-Hexane extract TLC profiling of *P. retrofractum* callus 2,4-D culture: BAP (0.5 ppm: 0.5 ppm) after spraying 10 % vanillin sulfate. purple spot at Rf 0.72 (a)

Isolation of Secondary metabolites using TLC-Preparative (TLC-P)

Further analysis is carried out by isolating the compound, mostly in n-hexane extract from the *P. retrofractum* callus because the resulting TLC profile forms a right spot with Rf: 0.75 has bright blue fluorescence under 365 nm UV light and dampens 254 nm UV lamp. Thin Layer Chromatography Preparative is done to isolate and multiply the production of compounds with the principle of separation and the application of compounds to silica gel. Preparative thin layer chromatography measuring 20 x 20 cm with a solution along with the plate and looking like a ribbon when eluted. The mobile phase used ethyl acetate: n-hexane (7: 3) in the large chamber that has been saturated first (± 60 minutes). The results can show a band with Rf: 0.76 which produces blue fluorescence on a 365 nm UV lamp, giving damping at UV 254 nm. The result of TLC-P tape is scraped, dissolved in n-hexane solvent and let stand for 24 hours before being filtered (Figure 6).

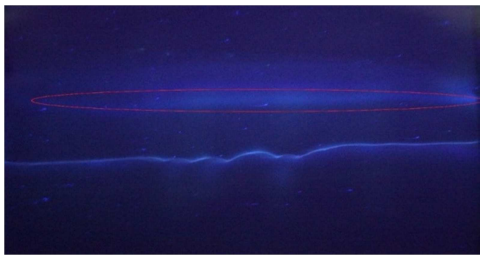


Figure 6. n-hexane extract of *P. retrofractum* callus culture Thin layer chromatography preparative profile. TLC profiling at 365 nm

Isolates obtained from TLC-P scrapings were carried out in 2-dimensional TLC stage. The purpose of 2-dimensional TLC was to determine the purity of the isolates' results by removing spots in 2 different directions and using eluents that were different from polarity. In the first elution using the same mobile phase as the previous TLC stage using ethyl acetate: n-hexane (7:3), there are a single

visible spot with Rf: 0.78, bright blue fluorescence at 365 nm UV lamp and spotlight on UV lamps 254 nm (Figure 7). A second elution was then carried out using a different polarity mobile phase using ethyl acetate, obtained by a single visible spot with Rf: 0.24, bright blue fluorescence at 365 nm UV lamp, and spot muffle on a 254 nm UV lamp (Figure 7). From the results of 2-dimensional TLC, isolates can be concluded as one spot.

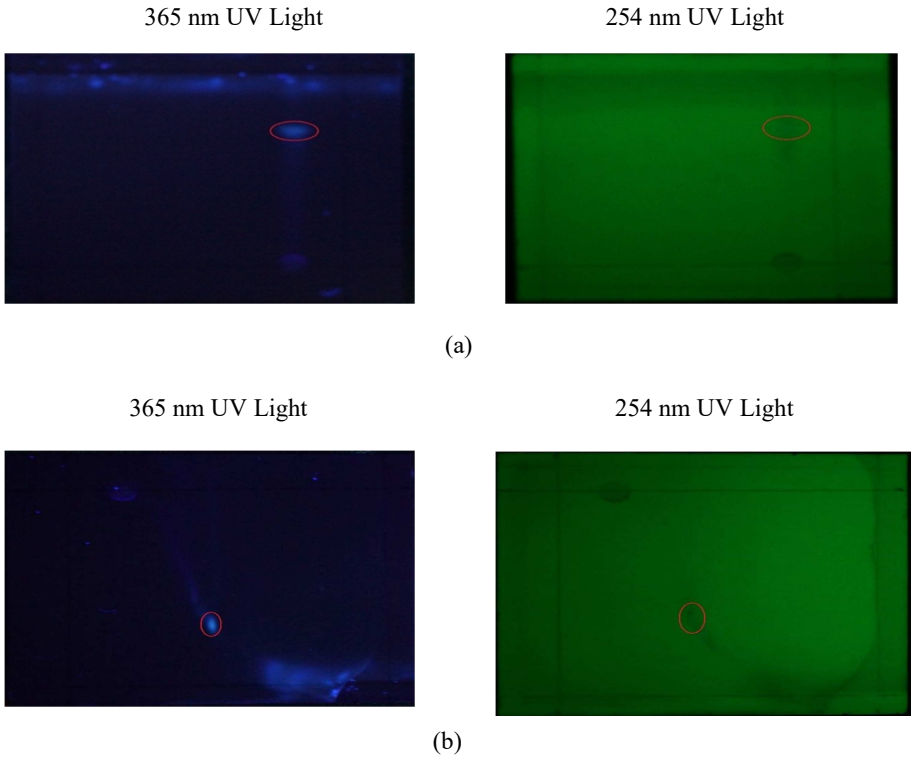


Figure 7. Two-dimensional thin layer chromatography isolate of n-hexane extract of *P. retrofractum* callus culture. first elusion direction with mobile phase ethyl acetate: n-hexane (7: 3) (a) and second elusion direction with mobile phase ethyl acetate: n-hexane (3: 7)

Identification of isolate structures

Identification of isolates using visible UV spectrometry instruments at a 200 - 400 nm wavelength using methanol pa solvents. The examination results showed that the isolate had a wavelength of 272.6 nm and 226.8 nm (Figure 8). The uptake shows the presence of chromophore groups in isolates. Advanced identification using infrared spectrometry, this analysis aims to see the functional groups of a compound in the range 400-4000 cm^{-1}. The spectrum that appears is the transition between different vibrational energies when emitted by the infrared detector. The results that appear are the results of a wave number graph that continuously changes along a narrow area of the electromagnetic spectrum versus transmittance-percent (T%). Identification using infrared spectrophotometry showed the presence of –CH bonds at 2953,02–2852,72 cm^{-1}, C=O bonds at

1743.65 - 1735.93 cm^{-1}, C=C at 1460.11 cm^{-1}, -CH_2 and -CH_3 at 1377.17 cm^{-1}, C–O at 1184.29 - 1165.00 cm^{-1} (Figure 9). Several previous studies also reported the presence of terpenoid compounds in *P. retrofractum*. The compounds belonging to the terpenoid group in *P. retrofractum* are eugenol, β-caryophillen, 3-caren, dlimonen, kubenol, and zingiberene (Jamal et al., 2013). The functional groups CH_2 and -CH_3 at 1377.17 cm^{-1} and C = C are characteristic of terpenoid compounds (Su et al., 2008).

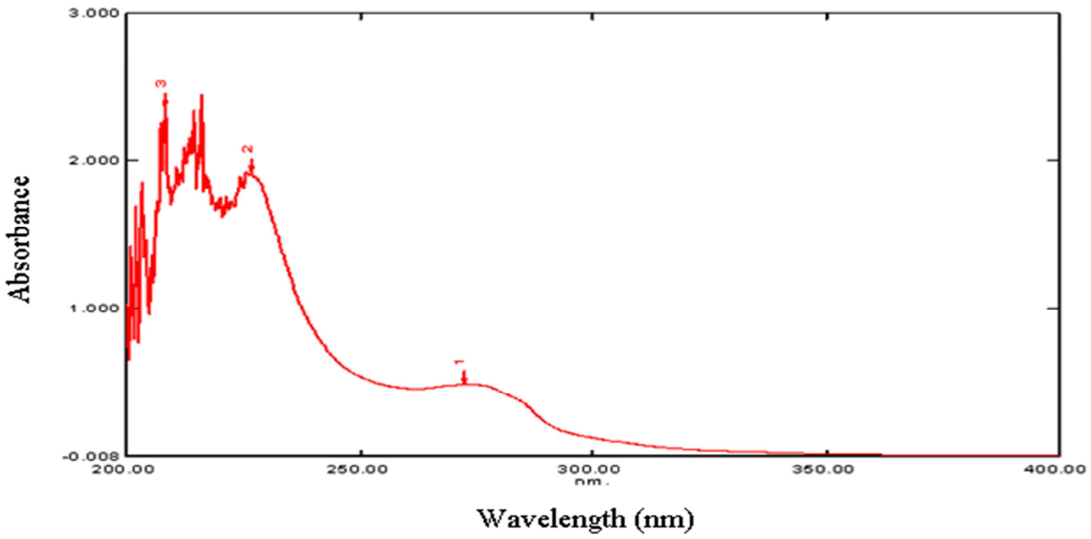


Figure 8. Spectrophotometry Uv-Visible spectrum isolate of n-hexane extract of *P. retrofractum* callus culture

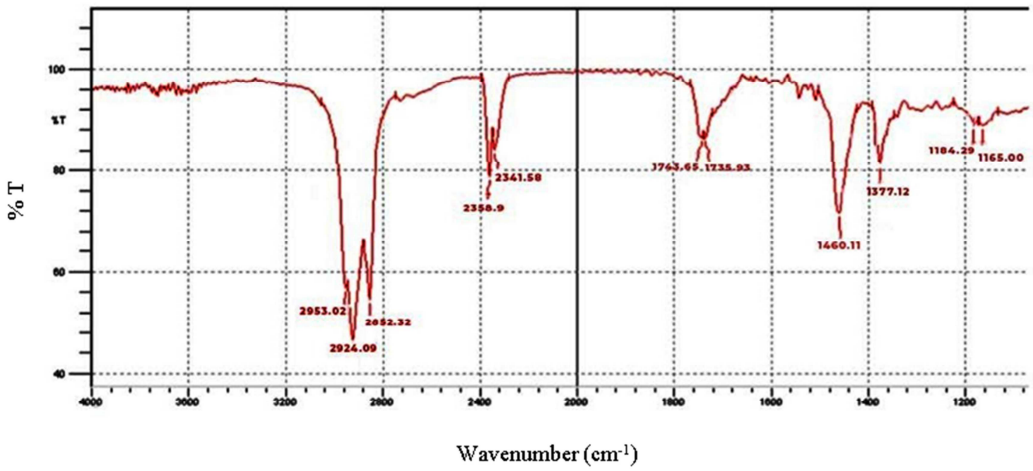


Figure 9. Infrared spectrometry spectrum isolate of n-hexane extract of *P. retrofractum* callus culture

There have not been many studies on plant tissue culture on *P. retrofractum*, especially the identification of secondary metabolites from the callus. This study's results can be the basis for the development of the active compound of *P. retrofractum,* especially steroids and triterpenoids. Its pharmacological activity as an aphrodisiac of *P. retrofractum* is inseparable from the steroid content in the plant. The development stage carried out is cell suspension culture. Several studies have reported cell suspension culture's success in producing secondary metabolites, including callus suspension culture of *O. aristatus* on liquid cell proliferation medium (MS + 1 mg / L 2,4-D and 1 mg / L NAA) with 30 g / L elicitor. Sucrose Increases cell biomass and rosmarinic acid levels (Bordbar *et al*., 2015). *O. aristatus* shoot culture on MS medium + 3% w / v sucrose + 2.2% w / v gel + 0.1 Myo-inositol and yeast extract elicitor 5.0 mg / L can induce shoot growth and increase phenolic levels (Razali *et al*., 2017).

Conclusion

P. retrofractum callus formed well on MS medium added with growth regulator 2,4-D: BAP (0.5 : 0.5 ppm) and was thought to contain terpenoid compounds. Subsequent studies directed the production of active compounds in *P. retrofractum* by cell suspension culture and plant propagation efforts by *in vitro* culture

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