Cabe Jawa

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IDENTIFICATION SECONDARY METABOLITES FROM CALLUS *Piper retrofractum* Vahl

Fahrauk Faramayuda [(1*)], Elfahmi [(2)], Jaka Permana [(1)]

[(1)] Division of Pharmaceutical Biology, Faculty of Pharmacy, Jenderal Achmad Yani University
[(2)] School of Pharmacy Institut Teknologi Bandung (ITB)

corresponding author: ramayuda.f@gmail.com

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ABSTRACT

Callus on *Piper retrofractum* is formed in the growth regulatory Substance 2.4D: BAP (0.5: 0.5). Secondary metabolite analysis using thin-layer chromatography (TLC) method using ethyl acetate: n-hexane (7: 3), a spot appeared on the extract yield in Piper retrofractum tissue culture method whereas in plant extract raw material is not visible. Based on this, further, identification was made to find out secondary metabolite from plant tissue culture. Separation of secondary metabolites was done by Preparative Thin Layer Chromatography and was identified using spectrophotometry UV-Vis and Infrared Spectrophotometry. The identification results indicate the presence of terpene compounds shown by purple stains on the visual appearance after sprayed spotting vanillin 10% in H2SO4. The isolate wavelengths of 272.6 nm and 226.8 nm indicate the presence of chromophore groups. Identification using infrared spectrophotometry showed –CH bonds at wavelengths of 2953,02–2852,72 cm^{-1}, C=O bonds at wavelengths of 1743.65 - 1735.93 cm^{-1}, C=C at wavelengths 1460.11 cm^{-1}, -CH2 and -CH3 at wavelengths 1377.17 cm^{-1}, C–O at wavelengths 1184.29 - 1165.00 cm^{-1}. The isolates were known as terpenoid compounds from the result of identification using spectrophotometry UV-Vis, IR and vanillin 10 % in H2SO4.

Keywords: Piper retrofractum Vahl, Plant tissue culture, TLC, Identification, Terpenoid

Introduction

Piper retrofractum Vahl has long been known as a widely used plant in herbs and medicine traditional. *Piper retrofractum* Vahl a chemical content that is spicy piperine, cavisin, palmitate acid, acid tetrahydropiperic, 1- ndensylenyl-3,4-ethylenedioxybenzene, piperidine, essential oil, isobutane-trans-2-trans-4-dynamide, and sesamin [(1)]. *Piper retrofractum* Vahl is included in the Piperaceae family[(2)], another name for *Piper retrofractum* Vahl in the Sumatra and Javanese regions is Javanese chilli while in England Javanese long pepper [(3,4)]. *Piper retrofractum* Vahl is included in perennials, irregular branching, climbing, twisting, or creeping with sticky roots, can reach 10 m in length [(5)]. *Piper retrofractum* Vahl grows on fertile and loose soil, 1- 600 meters above sea level with temperatures of 20-30 ° C, rainfall ranges from 1,200-3,000 mm / year [(6)].

Figure 1. *Piper retrofractum* Vahl Plant

Clinical test the effect of extract *Piper retrofractum* Vahl for the androgenic effect on humans has been carried out. Extracts of *Piper retrofractum* Vahl at a dose of 100 mg/day were of nature as androgenic phytopharmaceutical, increasing hormone levels blood testosterone and libido in hypogonadal men and are safe [(7)]. The methanol extract of *Piper retrofractum* Vahl fruit has a hepatoprotective effect [(8)]. *Piper retrofractum* Vahl leaves contain essential oils which have antibacterial activity [(9)]. The gastroprotective activity was also shown by an acetone-water extract from *Piper retrofractum* Vahl fruit [(10)]. Madurese herbalists use Javanese chilli, which has been dried as the main component in some herbs, such as herbal medicine, herbal medicine for stomach aches and headaches [(11)]. The use of *Piper retrofractum* Vahl in the form of steeping is relatively safe because it includes the type of raw material plants of the Food and Drug Supervisory Agency and is classified as an efficacious aphrodisiac plant [(13)]. Therefore, it is necessary to preserve and increase levels of *Piper retrofractum* Vahl secondary metabolites with tissue culture techniques, where plant tissue culture is a method or technique of isolating tissue, organs, cells, and plant protoplasts, making explants and growing them in aseptic growth media so that The explant can grow and develop, organogenesis and can be a perfect plant. The

success of plant tissue culture is influenced by several factors, including sterilization, selection of explant materials, environmental factors such as pH, light and temperature, and the content of growth regulators in culture media [14]. The advantages of procuring seeds through tissue culture include obtaining a large number of superior and uniform plant materials, besides being able to obtain sterile cultures so that they can be used as materials for further propagation [15]. In the pharmaceutical field, tissue culture techniques are very beneficial because they can produce secondary metabolites for large amounts of drugs and in a short time [16].

Growth regulators on media in tissue culture must contain growth-regulating substances that can affect morphogenesis in tissue and organ cell cultures. The plant used in this study is *Piper retrofractum* Vahl and using Murashige and Skoog media. Based on the study of Riska Sigit Ramelan in 2016 [17]. In the case of *Piper retrofractum* Vahl plants from tissue culture with a comparison of 2.4-D: BAP 0.5ppm: 0.5ppm Growth Regulating Substances, there were differences in Thin Layer Chromatography between *Piper retrofractum* Vahl explants and *Piper retrofractum* Vahl leaves without tissue culture uses the mobile phase n-hexane: ethyl acetate (3:7) and toluene: ethyl acetate (1: 1). TLC results from *Piper retrofractum* Vahl callus with tissue culture techniques were thought to contain steroid compounds, triterpenoids, and flavonoids.

MATERIAL AND METHODS

In this research, several initial stages were carried out, including sterilization of space and equipment, sterilization of distilled water and culture media added to the growth regulating agent, namely 2,4-D: BAP with a ratio of 0.5: 0.5. ppm in murashige and Skoog (MS) media, explant sterilization, explant planting on culture media, and explant development observation. Preparation of *Piper retrofractum* Vahl ingredients includes determination, collection, processing of ingredients into raw material, making extracts and phytochemical screening of raw material and extracts. *Piper retrofractum* Vahl leaf explants that have been planted in the media are taken, and weighing is done to determine the weight of callus. A fresh callus is dried and made into extract with multilevel extraction method using sonification maceration. The resulting extract was analyzed and compared the secondary metabolite content between *Piper retrofractum* Vahl leaves from culture and non-culture using Thin Layer Chromatography (TLC). Secondary metabolites of *Piper retrofractum* Vahl leaves were isolated from tissue culture using Preparative Thin Layer Chromatography (TLC-P) as a purification medium. The isolate bands contained in TLC-P were identified qualitatively using UV-Vis spectrophotometry and infrared spectrophotometry.

Piper retrofractum Vahl Leaf Tissue Culture

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 laundry soap and then dried. After being dry wrapped in paper (except culture bottles), sterilized in an autoclave at 121°C for 15 minutes. Tools such as tweezers and scalpel can be re-sterilized by heating over methylated flamboyant after dipping in 96% alcohol before planting.

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°C for 15 minutes.

Media

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

Addition of Growth hormone to MS Media

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. Growth hormones used in this study include Kinetin, 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.

The compactor is added after the pH in the media solution is as expected. 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°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.

Sterilization of *Piper retrofractum* Vahl Leaf Explants

Piper 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 which is 2% for 5 minutes. To clean the plant's inner tissue, then rinse it in sterile aqua dest for 1 minute in 3 parts of sterile aqua dest.

***Piper retrofractum* Vahl Culture**

In the planting of *Piper retrofractum* Vahl**,** 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 Javan chilli 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 ZPT added to the media and the date of planting. The scalpel and tweezers are always heated before use and soaked in 70% alcohol.\

Preparation of Javanese Chilli Callus Extract from Tissue Culture

The manufacture of *Piper retrofractum* Vahl callus extract was carried out using 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 filtrate is filtered, the residue is remunerated with ethyl acetate until it is submerged and treated as n-hexane. The resulting filtrate was remunerated with ethanol until submerged and filtered.

Analysis and isolation of secondary metabolites Analysis of secondary metabolites 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). 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. Profile of TLC extract of *Piper retrofractum* Vahl leaves that grow in their original habitat compared to the extract of *Piper retrofractum* Vahl leaves from tissue culture.

Isolate Secondary metabolites 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, then closed using plastic 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 ℃ 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 fraction 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.

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, and spot muffle on UV 254 nm. Then the second elution was carried out using a different polarity mobile phase using ethyl acetate.

Identification of isolate structures

Identification of the isolated structure was carried out using Uv-Vis spectrophotometry instrument to determine wavelength and infrared to determine the functional group.

Uptake of isolates on Uv-Vis Spectrophotometry

The sample was dissolved in a solvent which 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 so that no air was trapped in the mixture. The tool used is FTIR (Fourier Transform Infra-Red).

RESULTS AND DISCUSSION

Piper retrofractum Vahl Leaf Tissue Culture

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 2.

Several factors influence the response given by Growth Regulator. The combination of Growth Regulator is of particular concern. Each plant that comes from different organs and species will require a different Growth Regulator[18]. 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, which are 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 of plants in vitro, exogenous hormones must still be added.

Piper retrofractum Vahl leaves that have been sterilized are planted in the media. In growing *Piper retrofractum* Vahl explants, cultivation was carried out, and the callus was dried and weighed. The dried callus is mashed and extracted to analyze the compounds in the *Piper retrofractum* Vahl callus qualitatively by thin-layer chromatography. Extraction is carried out in a vial by multilevel maceration with sonification because the leaves' weight results from minimal tissue culture, which is 455 mg. Maceration uses multilevel solvents. Namely, n-hexane to attract non-polar compounds, then it is remunerated with ethyl acetate to attract more semi-polar compounds and finally using ethanol solvents to attract more polar compounds. This method was chosen to anticipate the presence of damaged and thermolabile secondary metabolites.

Analysis and isolation of secondary metabolites

Analysis of secondary metabolites using Thin Layer Chromatography (TLC)

After extraction of *the Piper retrofractum* Vahl 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 *Piper retrofractum* Vahl leaf extract without tissue culture, and the one produced between callus and *Piper retrofractum* Vahl leaves without tissue culture gave a different TLC pattern. Further analysis is carried out by isolating the compound, mostly in n-hexane extract from the *Piper retrofractum* Vahl 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. Result of Thin layer chromatography callus and extract of *Piper retrofractum* Vahl plants can be seen in the figure. 3.

Isolate Secondary metabolites using TLC-Preparative

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 4). 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.

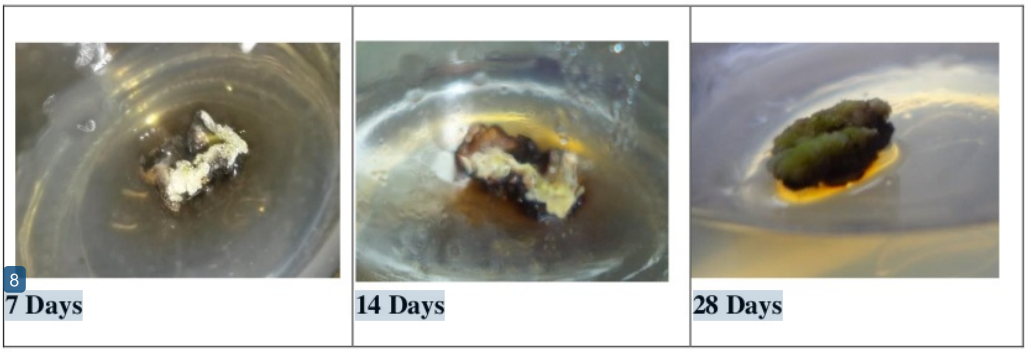


Figure 2. *Piper retrofractum* Vahl Callus tissue culture results. With a comparison of Growth Regulating Substances 2.4-D: BAP (0.5ppm: 0.5ppm)

365 nm Uv Light 254 nm Uv light

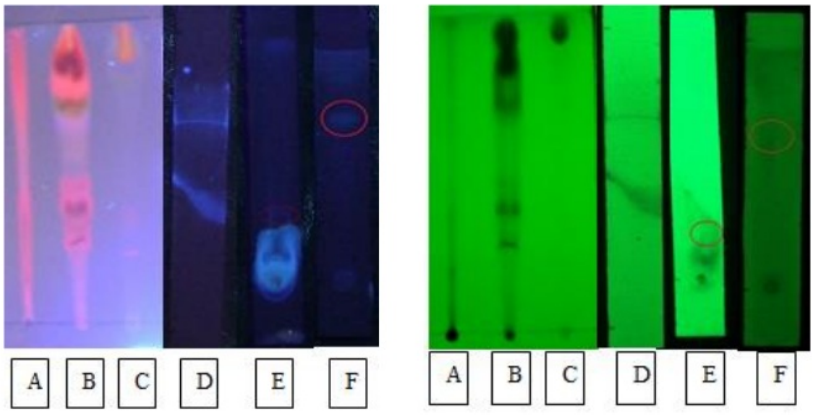


Figure 3. Thin-layer chromatography callus and extract of *Piper retrofractum* Vahl plants.

Stationary phase: silica gel plate GF_{254}
Mobil phase : ethyl acetate: n-hexane (7: 3)

A: Non-culture *Piper retrofractum* Vahl ethanol extract

B: Non-culture *Piper retrofractum* Vahl ethyl acetate extract

C: Non-culture *Piper retrofractum* Vahl hexane extract

D: Ethanol extract of *Piper retrofractum* Vahl callus culture 2,4-D: BAP (0.5 ppm: 0.5 ppm)

E: Ethyl acetate extract of *Piper retrofractum* Vahl callus culture 2,4-D: BAP (0.5 ppm: 0.5 ppm)

F: n-hexane extract of *Piper retrofractum* Vahl callus 2,4-D culture: BAP (0.5 ppm: 0.5 ppm)

Figure 4. n-hexane extract of *Piper retrofractum* Vahl callus 2,4-D culture: BAP (0.5 ppm: 0.5 ppm) after spraying 10 % vanillin sulfate

Isolate Secondary metabolites using TLC-Preparative (TLC-P)

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 and not looking at the tape's visual appearance. The result of KLTP tape is scraped, dissolved in n-hexane solvent and let stand for 24 hours before being filtered (Figure 5).

Two-dimensional Thin Layer chromatography

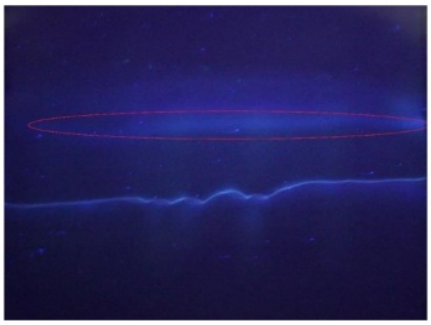
Isolates obtained from TLC-P scrapings were carried out in 2-dimensional TLC stage. The purpose of 2-dimensional TLC was to determine the purity of the isolates' results by removing spots in 2 different directions and using eluents that were different from polarity. Isolates were said to be sure if the visible spot was single. In the first elution using the same mobile phase as the previous TLC stage using ethyl acetate: n-hexane (7: 3), we got a single visible spot with Rf: 0.78, bright blue fluorescence at 365 nm UV lamp and spotlight on UV lamps 254 nm (Figure 6). 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.

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-per cent (T%). Identification using infrared spectrophotometry showed the presence of –CH bonds at wavelengths of 2953,02–2852,72 cm^{-1}, C=O bonds at wavelengths of 1743.65 - 1735.93 cm^{-1}, C=C at wavelengths 1460.11 cm^{-1}, -CH2 and -CH3 at wavelengths 1377.17 cm^{-1}, C–O at wavelengths 1184.29 - 1165.00 cm^{-1} (Figure 9).

365 nm Uv Light



254 nm Uv light

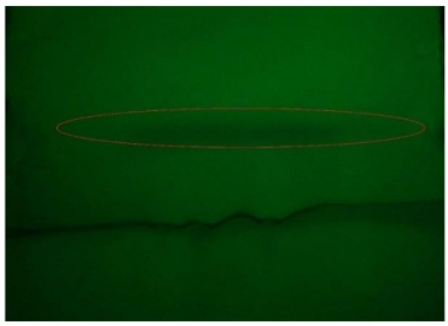


Figure 5. n-hexane extract of *Piper retrofractum* Vahl callus culture Thin Layer Chromatography Preparative Profile

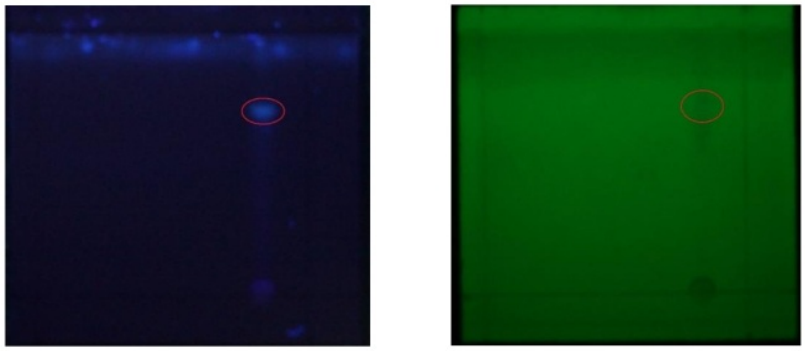


Figure 6. Two-dimensional Thin Layer chromatography isolate of n-hexane extract of *Piper retrofractum* Vahl callus culture (first elusion direction)

Mobile phase: ethyl acetate: n-hexane (7: 3)

Stationary phase: silica gel F254

365 nm Uv Light 254 nm Uv light

Figure 7. Two-dimensional Thin Layer chromatography isolate of n-hexane extract of *Piper retrofractum* Vahl callus culture (second elusion direction)

Mobile phase: ethyl acetate: n-hexane (7: 3)

Stationary phase: silica gel F254

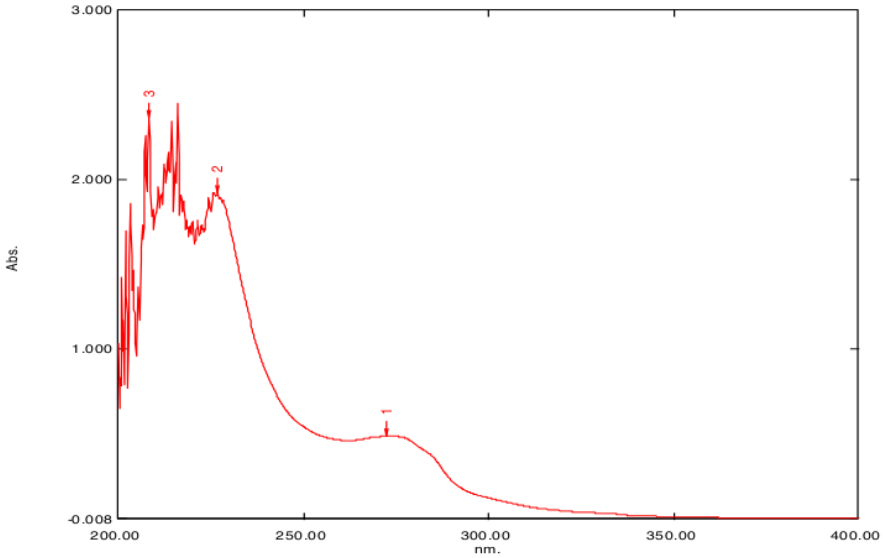


Figure 8. Spectrophotometry Uv-Visible Spectrum isolate of n-hexane extract of *Piper retrofractum* Vahl callus culture

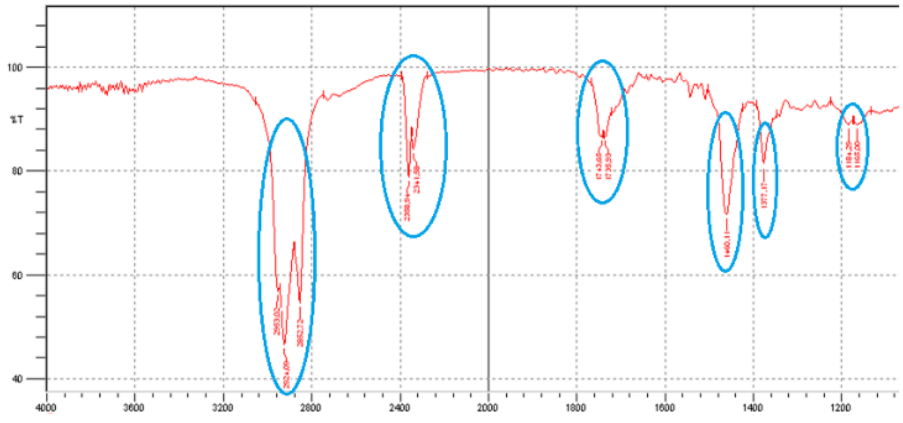


Figure 9. Infrared spectrometry spectrum isolate of n-hexane extract of *Piper retrofractum* Vahl callus culture

Conclusion

Identification using TLC, UV spectrophotometry and IR suspected isolates are terpenoids. Seen from the Thin Layer Chromatography profile shows a positive reaction using the 10% Vanillin blotches in H_2SO_4, infrared spectra, which refers to group C = C shown in wave numbers 1460.11 cm^{-1}.

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