Studies of Biological Properties of Major Bioactive Compounds from the Stems of *Paederia foetida*. Linn

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Oleh :
Aryandi Ramadhan
166100100111011

PROGRAM PASCASARJANA
FAKULTAS TEKNOLOGI PERTANIAN
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NIM : 166100100111011
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學號　：M10618032

所提之論文 ：雞屎藤莖部化學成分及生物活性之研究

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國立屏東科技大學/生物科技系
特聘教授

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摘要

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論文摘要內容：

Paederia foetida，俗稱雞屎藤，為茜草科植物。傳統上，它用於治療腸腫大、腸炎、胃腸脹氣、胃萎縮、風濕病、鼻炎、糞血症、疼痛、胃痛和牙痛。在現代藥理學研究中，據文獻指出 *P. foetida* 的萃取物具有抗菌、止瀉、抗發炎、抗痙攣、驅蟲、鎮咳和保肝活性。本研究目的為分離與鑑定 *P. foetida* 萃取物之化學成分，及探討其主要的藥理活性，並將具生物活性之化合物進行抗菌活性分析。*P. foetida* 之甲醇粗萃物依序經由乙酸乙酯 (EtOAc) 及正丁醇 (*n*-BuOH) 進行分配萃取。將 EtOAc 層萃取物進一步分離純化，得到十個化合物，包含兩種固醇類、四種蒽醌類、兩種香豆素及兩種芳香類。化合物的的結構則是由核磁共振光譜 (Nuclear Magnetic Resonance，NMR) 及質譜 (Mass Spectrometry, MS) 來鑑定，分別為 β-sitosterol **(S1)**、stigmastan-3-one **(S2)**、morindaparvin A **(An1)**、1,3-dihydroxy-2-methoxyanthraquinone

(An2)、alizarin **(An3)**、digiferrol **(An4)**、scopoletin **(C1)**、fraxidin **(C2)**、ferulic acid **(A1)** 與 vanillic acid **(A2)**。

P. foetida 之分配萃取物及 EtOAc 層分液之抗菌活性將使用瓊脂擴散法分析，而刃天青還原試驗則用來做最小抑菌濃度 (minimum inhibitory concentration, MIC) 的確效。結果顯示 EtOAc 層比 *n*-BuOH 層及水層具有較顯著的抑制大腸桿菌 (11 mm) 及葡萄球菌 (11.5 mm)，並發現 1,3-dihydroxy-2-methoxyanthraquinone **(An2)**、digiferrol **(An4)** 和 vanillic acid **(A2)** 具葡萄球菌之抑制活性，其 MIC 為 4.68-100 μg/mL。*P. foetida* 甲醇粗萃物的 EtOAc 層富含蒽醌類化合物，為抗菌的活性成分，因此其萃取物及分液具有抗菌感染的潛力。

關鍵字：雞屎藤 (*Paederia foetida* Linn)、分離、純化、光譜分析、生物活性

Abstract

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The Contents of Abstract in This Thesis:

Paederia foetida Linn locally known as skunk vine or Chinese fever vine from Rubiaceae Family. Traditionally, it is used to treat enteromegaly, enteritis, flatulence, gastromegaly, rheumatism, rhinitis, copraemia, sore, stomachache and toothache. In modern pharmacological studies, the extract of *P. foetida* has been reported to possess antimicrobial, antidiarrheal, anti-inflammatory, antispasmodic, anthelminthic, antitussive, and hepatoprotective activities. In this study, *P. foetida* extract was investigated for bioactive constituents and their main pharmacological properties. The research purpose was to isolate and identify the chemical constituents of the stem of *P. foetida* and evaluate their antibacterial activities. The crude methanol extract was sequentially partitioned with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The EtOAc layer was further isolated and purified to obtain ten compounds including two steroids, four anthraquinones, two coumarins, and two aromatics. The compounds were identified using

Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) analysis. The ten compounds were identified as β-sitosterol **(S1)**, stigmastan-3-one **(S2)**, morindaparvin A **(An1)**, 1,3-dihydroxy-2-methoxyanthraquinone **(An2)**, alizarin **(An3)**, digiferrol **(An4)**, scopoletin **(C1)**, fraxidin **(C2)**, ferulic acid **(A1)**, and vanillic acid **(A2)**. The antibacterial activity of the partitions and fractions were evaluated using agar well diffusion assay. The resazurin assay was used to determine the minimum inhibitory concentration (MIC). The result of agar diffusion assay showed that the EtOAc layer has significant inhibition against *Escherichia coli* (11 mm) and *Staphylococcus aureus* (11.5 mm) than the *n*-BuOH and water layer. *S. aureus* was very susceptible to, 1,3-dihydroxy-2-methoxyanthraquinone **(An2)**, digiferrol **(An4)**, and vanillic acid **(A2)** with MIC values ranging from 4.68-100 µg/mL. The EtOAc layer of methanol crude extract contains mainly anthraquinones as a dominant bioactive compound. The extract and fractions of *P. foetida* could be considered as prospective candidates for the prevention of bacterial infections.

Keywords: *Paederia foetida* Linn, isolation, purification, spectroscopic analysis, bioactive properties

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1. INTRODUCTION

1.1 Background

Over the past decade, natural products have gained importance in the health system. Although modern medicine is advanced in most parts of the world. Many developing countries still use traditional medicine to meet their primary health needs, which relies mostly on medicinal plants. Even though modern medicine may exist side-by-side with such traditional practice, natural products have often retained its status for historical and cultural reasons. Herbal medicines have come from numerous source materials including microorganisms, marine organisms, and plants.

Paederia foetida L. locally known as skunk vine or Chinese fever vine from Rubiaceae Family. The species name foetida is Latin for "stinky" or "foul smelling". The name was derived from the odor of carbon disulfide from the leaves. Various traditional treatments have been linked to this plant. It is used to treat enteromegaly, enteritis, flatulence, gastromegaly, rheumatism, rhinitis, copraemia, pain, abdominal pain and toothache (Afroz et al., 2006).

Previous phytochemical studies of the aerial parts of *P. foetida* from Borgohain et al. (2017) showed it contains iridoid glycosides, paederolone, paederone, paederoside, and asperuloside. In addition, Soni et al. (2013) reported that the plant also contains carotene, vitamin C, keto-alcohol, alkaloid, sitosterol, friedelin, campesterol, ursolic acid, hentriacontane, hentriacontanol, vitamin C, ceryl alcohol, palmitic acid, terpenoids, and methyl mercaptan.

Soni et al. (2013) reported that fresh and dried plant extracts of *P. foetida* showed their ability to the reactive oxygen species as free radicals using β-carotene bleaching and the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) method. The percentage of

antioxidant activity for fresh and dried plant extract samples was between 58 and 80%. According to Upadhyaya (2013), antioxidant phytochemicals have a strong protective effect against cancer and cardiovascular disease. Furthermore, the leaf extract of this plant possesses antihyperglycemic. Other reported biological activities of the extract of *P. foetida* are an antimicrobial, antidiarrheal, anti-inflammatory, antispasmodic, anthelminthic, antitussive, and hepatoprotective activity (Macwan, 2010).

Interestingly, even though *P. foetida* is known as herbal medicine, it is still rare, threatened in the wild, and needs further investigation. Although there is some information on the biological activity of *P. foetida* in literature, information is still lacking on the biological activity of the chemical constituents of the plant. Thus, the lack of studies on phytochemical compounds of this plant became the background of this study.

1.2 Research Objective

This research purpose was to isolate and identify the chemical constituent of the stem of *P. foetida*. Then, evaluate and compare the antibacterial activities of crude extract, fractions and chemical constituent of *P. foetida* with known antibacterial agents.

1.3 Future Impact

Hopefully, *P. foetida* as a new source of herbal medicine could trigger and improve the medicinal system for human and could be developed not only in laboratory scale but also in pharmaceutical industrial level.

2. LITERATURE REVIEW

2.1 Herbal Medicine

Plants and their secondary metabolite constituents have a long history of utilization in cutting-edge medication and in certain frameworks of conventional medication and are the sources of imperative drugs. Utilization of natural products in developed countries has extended strongly within the last half of the twentieth century. They utilize the plants which contain chemical constituents in any of its portion like root, stem, leaf, fruit, and seed. They are classified as traditional herbal medication in the event that they create a definite curing physiological reaction within the treatment of different disease in humans and animals (Hamayun et al., 2006).

In some cases, the active principles of plant-derived products have been isolated and characterized, and their working mechanisms are understood. Many people have the perception that most herbal products found on the market provide inaccurate information. This is due to the complexity of the preparation and processing of herbal plants. Even though they are not pure compounds. They believe that the combination of several active components in herbal plants has a very beneficial effect (Ajazuddin and Saraf, 2012).

2.2 *Paederia foetida*. Linn

Paederia foetida or Skunk vines are locally prominent plant species in the east and south Asia. The plant was first presented to the USDA field station near Brooksville in 1897. In 1916, it was reported as an invasive weed in the region. The plant was known as a weed that spread on the peninsula of Florida in 1933. In 1977, it was considered a weed that had economic value. In 1993, it was included in the Florida Exotic Pest Plants Invasive Species as Category I, which was defined

as "a species that are invasive and disrupts the native plant communities in Florida". It was also included in the Florida Noxious Weed List (5B-57,007 F.A.C.) in 1999, this plant is illegal to own, move or release in Florida. (Langeland et al., 2000).

P. foetida was classified following from Macwan (2010)

Kingdom	: Plantae
Subkingdom	: Tracheobionta
Superdivison	: Spermatophyte
Division	: Magnoliophyte
Class	: Magnoliopsida
Subclass	: Asteridae
Order	: Rubiales
Family	: Rubiaceae
Genus	: Paederia L.
Species	: *Paederia foetida L.*

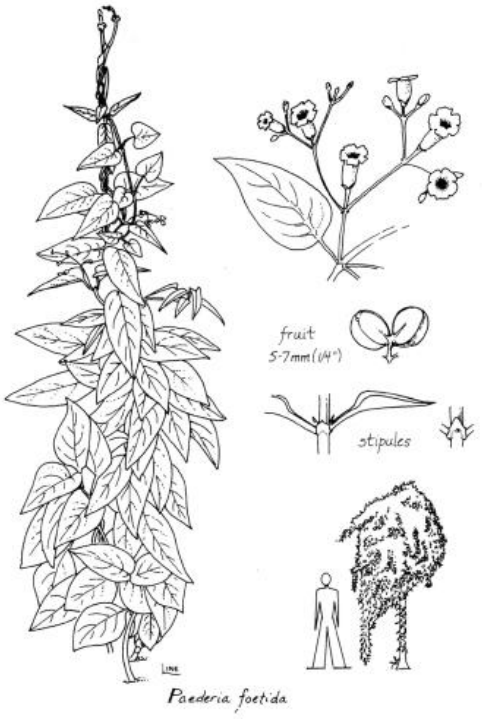

Figure 1. *P. foetida* L. drawn by (Langeland et al., 2000)

According to Morshed et al. (2012a) describes plants. *P. foetida* is a climber plant, growing between shrubs, hairy or smooth slender branches. The height of the lower stem to the tip of the stem can reach 7 m (23ft) or more, propagating on the tree or fence. The leaves and stems have an unpleasant odor, especially when it crashed. The flowers are small, have grayish or light purple-pink color, in wide or long curved clusters, "leafy", and terminals or on axis. The corolla is very hairy, tubular with 5 lobes usually spreading. Fruits are brown shiny, round capsules, up to 0.7 cm (0.3 inches) wide, with round black seeds.

2.3 Chemical Constituents

A variety of chemical constituents are found in *P. foetida* such as iridoids (Shukla et al., 1976), saponins, tannins, phenols, flavonoids, cardiac glycoside, and alkaloids (Upadhyaya, 2013). The chemical constituents of *P. foetida* are shown in Table 1.

Table 1. Chemical constituents of *P. foetida* Linn

Compounds	References
7-hydroxy-6-methoxy-2H-1-benzopyran- 2-one **(1)**	Zhuang et al. (2013)
Ethyl (E)-3-(4-methoxyphenyl) prop-2-enoate **(2)**	Uddin et al. (2013)
β-Sitosterol **(3)**	Tripathi and Dasgupta (1974)
Stigmasterol **(4)** Lupeol **(5)** Ellagic Acid **(6)** Epifriedelinol **(7)** Friedelin **(8)** Scandosides **(9)** Asperuloside **(10)** Paederoside **(11)**	Soni et al. (2013)
Catechin **(12)**	Dasgupta and De (2007)
Ursolic acid **(13)** Lupenol **(14)**	Borgohain et al. (2017)

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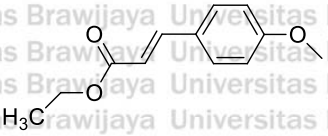

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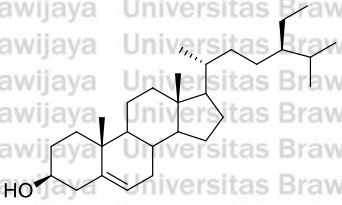

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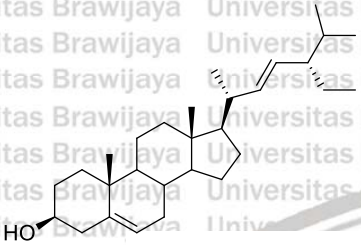

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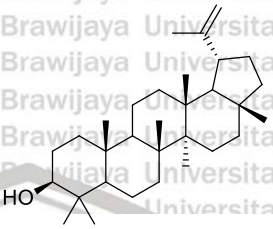

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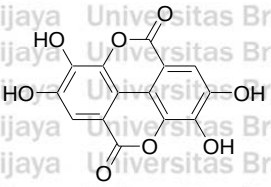

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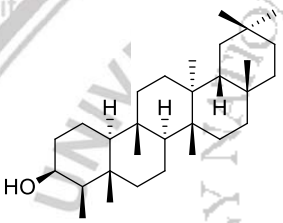

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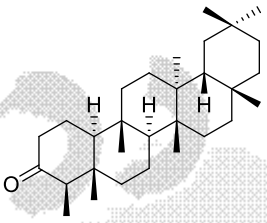

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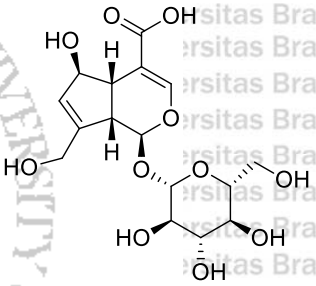

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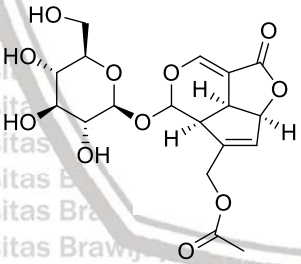

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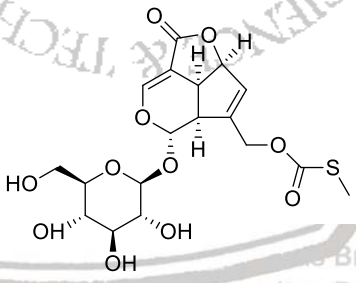

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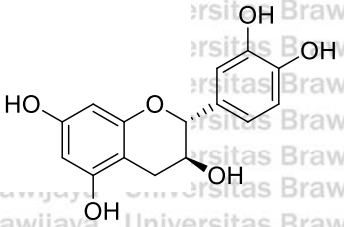

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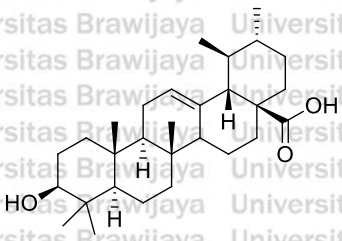

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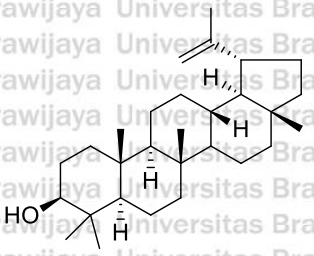

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2.4 Pharmacological uses

In folklore, *P. foetida* is used to treat enteromegaly, enteritis, flatulence, gastromegaly, rheumatism, rhinitis, sapramia, sore, stomachache, and toothache. Previous studies back up the pharmacological basis for ethnomedical use of the plant. The antidiarrheal activity (Afroz et al., 2006), the anti-inflammatory activity from butanol fraction of a methanol extract (BMEL) (De et al., 1994), antidiabetic and antithrombolytic from methanolic extract (Morshed et al., 2012a), antioxidant and antimicrobial activity from ethanolic extract (Upadhyaya, 2013), antihyperlipidemic and antihyperglycemic from leaf extract (Kumar et al., 2014) have been reported. Ethanolic extract *P. foetida* is also reported to be an aphrodisiac and stimulate sexual behavior (Soni et al., 2012).

2.4.1. Antidiarrheal activity

Afroz et al. (2006) investigated the effect of *P. foetida* ethanol extract as a remedy for diarrhea. The activity was indicated using castor oil and magnesium sulfate-induced diarrhea models in mice. The extract significantly increased the latent period of diarrhea in the models. The effect of extract particularly reduced the purging index (PI) value in a dose-dependent manner in magnesium sulfate-induced diarrhea. Generally, the ethanol extract of *P. foetida* reduced the gastrointestinal motility with barium sulfate milk both in 15 and 30 min time interval. Another result, the extract significantly decreased the cisplatin-induced gastrointestinal motility at both time intervals. The extract also enhanced the morphine-induced reduction of motility. These results indicate that ethanolic extract of *P. foetida* possess antidiarrheal activity by inhibiting intestinal motility and confirm it can be used in traditional medicine as a remedy for diarrhea.

2.4.2. Anti-inflammatory activity

The butanol extract of methanol extract (BMEL) of *P. foetida* leaves was investigated for anti-inflammatory activity by De et al. (1994). The fraction showed significant inhibition of granulation tissue formation on cotton implanted in mice. The fraction significantly decreased aspartate liver transaminase activity and affected the adrenal body weight and ascorbic acid content, thus ruling out stimulation of the adrenal-pituitary axis. BMEL also inhibits the increase of orosomucoid serum levels in mice, which suggests the possibility of disease-modifying anti-rheumatic activity. The results show that the plant is capable to treat inflammatory disorders.

2.4.3. Antidiabetic and antithrombolytic activities

Morshed et al. (2012a) investigated the antidiabetic and antithrombolytic properties of methanolic extracts of the whole plant of *P. foetida*. Methanolic extracts of the whole plant of *P. foetida* has moderate antidiabetic activity. It decreased the blood glucose levels by 30% respectively, while the positive control (glibenclamide, 2 mg/kg) decreased blood glucose level by 70%. 100 µl extract of *P. foetida* exhibited the highest thrombolytic activity with clot lysis value of 23.82% whereas standard streptokinase (positive control) and water (negative control) demonstrated clot lysis value of 45.85% and 2.81%, respectively.

2.4.4. Antimicrobial and cytotoxic activities

Morshed et al. (2012b) reported the antibacterial activities from several fractions of methanolic extracts of the whole plants of *P. foetida*. *n*-hexane, chloroform, and ethyl acetate were tested against several pathogenic bacteria using ‘disc diffusion method’. The methanolic extract indicated no antimicrobial activity but the other

fractions showed moderate to less activity against some organisms as compared to the standard antibiotic Kanamycin. Then cytotoxicity was tested using brine shrimp lethality bioassay. The LC_{50} result indicated that methanolic extract has significant cytotoxicity.

2.4.5. Antihyperlipidemic and antihyperglycemic activities

Kumar et al. (2014) explained that the leaf extract of *P. foetida* shows remarkable antihyperglycemic activity due to the possibility of a sustainability effect involving the mechanism of the pancreas. As long as the antihyperlipidemic activity is given it may reduce the level of lipid profile in the blood and reduce blockages in the heart. The antioxidant activity of the extract was in the form of inhibition of lipid peroxidation and increased superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). It has been confirmed that *P. foetida* leaf extract has the potential to act as an antidiabetic, antihyperlipidemic and antioxidant.

2.5 Purification and Identification Methods

When isolating active compounds from dried stem *P. foetida*, extraction is the most important stage in the process. The extraction process is divided into several sub-steps. It includes maceration, partition, column chromatography, TLC, separation using HPLC and identification by spectral method (NMR).

2.5.1 Maceration

Maceration is the popular extraction method used with unstable, stable or thermal products. For small-scale extraction, maceration generally consists of several steps. The first step is to reduce plant material into small particles. Secondly, suitable solvents are added to the plant material and leave it to stand for at least 24 hours. Third, the

liquid is compressed as well as the solids remaining from the extraction process are pressed to recover a large amount of clogged solution. The tension and release of the released liquid are separated from the dirt through filtration. Occasional stirring is carried out in maceration to optimize the extraction process in two ways; (a) increase diffusion, (b) release concentrated solution from the surface of the sample and add new solvents for more extraction yield (Azmir et al., 2013).

2.5.2 Partition

Partitions are a technique for separating solutions based on their polarity properties in different liquids or solvents. This is based on the process of transferring solutes from one liquid phase to another liquid phase in accordance with the solubility properties. When the solution is shaken in a separating funnel with two immiscible solvents, the solution will separate itself between two the solvents.

In alcoholic extract (MeOH or EtOH) from plant materials contain various types of polar compounds to moderately polar. Many compounds that cannot dissolve individually but can be extracted with MeOH or EtOH. Usually, the concentrated extract of MeOH and the volume is reduced to an appropriate level so that can be easily partitioned with a separating funnel. Then the concentrated extract is extracted continuously at the same volume using non-polar solvents, n-hexane, for example, three times or more, to obtain fractions containing non-polar compounds. This process also called "defatting". MeOH and *n*-hexane are not completely mixed, they can be mixed only to a certain extent. Sometimes, a small amount of water is added to MeOH to get a 95% methanol solution and get the same two layers of partition volume (Otsuka, 2005).

2.5.3 Column Chromatography

Column chromatography is a purification technique that use glass columns filled with adsorbents (i.e., silica gel, alumina, or C18 phase reversal) in large samples or mixed products to produce large quantities (milligrams to grams) as well. As with thin layer chromatography (TLC), the adsorbent used varies based on the type of separation carried out. It is very useful to analyze the mixture by thin layer chromatography before using large-scale column chromatography. For example, to determine the mobile phase suitable for column chromatography, TLC plates can be used to confirm. After compliance, silica gel must be used as an adsorbent for column chromatography and the TLC plate mobile phase can be used for the purification process (Meyers, 2001).

Before performing column chromatography, the glass column must be packed with an appropriate adsorbent and solvent. There are two methods for packing columns: wet packing and dry packing. Wet packing is a commonly used technique and is preferred for silica gel columns. While dry packing is less desirable because sometimes the formation of air bubbles is difficult to remove. When this happens, the column must be repackaged. However, when the absorbent is alumina, the method that suitable for use is dry packing. Because if it is packed by wet packing, alumina will be slurry-shaped which is difficult to pour. (Meyers, 2001).

Usually, to separate polar substances, the stationary phase suitable for use is silica gel, because the surface is covered by a hydrated hydroxyl group, which is very polar. It will also interact with polar dipoles. The charge field associated with hydroxyl groups hydrated on silica gel can also produce induced dipoles in solutes by polarization, and thus silica will retain polarized compounds. The difference in interaction between dissolved compounds and hydrated hydroxyl

groups in silica gel can occur if more nonpolar solvents are used as the mobile phase. (Scott, 1983).

2.5.4 Thin Layer Chromatography (TLC)

Thin layer chromatography is a technique used to separate non-volatile mixtures. TLC is carried out using sheets of glass, plastic or aluminum foil, which are covered with a thin layer of adsorbent, usually in the form of silica gel, cellulose or aluminum oxide (alumina). The adsorbent layer is called the stationary phase. TLC can be used to guarantee the existence of a compound. Then, examine it using UV light. Identification of the presence of mixed compounds must also be adjusted to the R_f value (Preethi et al., 2017). According to Poole (2003), thin layer chromatography is a very simple method of compound characteristic detection. Because the sample is in the chromatogram, thin layer chromatography is the most suitable technique for surveying sample properties.

Different samples are usually applied to the stationary phase before being contacted by the mobile phase that later migrates past it in a particular direction. The motion of the mobile phase in the stationary phase is called the development step. After development, the mobile phase will evaporate and detection is carried out in a stationary phase. detectors used to record plot responses to separation distances are called densitogram (Spangenberg et al., 2011).

2.5.5 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a type of liquid chromatography in which the sample must be changed in liquid conditions. HPLC is used to detect a specific compound on the solution. In HPLC and liquid chromatography, where the liquid sample will interact with the solid or stationary phase and the second liquid (mobile)

phase. The difference in polarity interactions can help the separation process to get specific compounds.

High-performance liquid chromatography is basically another form of column chromatography that has modern performance. Solvents entering through a column other than gravity are forced into high pressure up to 400 atmospheres. This process is a lot faster than ordinary column chromatography.

HPLC also includes a method of separating mixed samples in shorter periods of time, using a very small particle of samples, having a small column diameter, having very high liquid pressures and producing several peaks based on the retention times of each compound. Some factors that influence HPLC performance are: internal column diameter, column size, high sensitivity, higher pump pressure, higher separation, sample particle size, the polarity of the sample, solvent, and the last factor is temperature.

The advantages of using HPLC are that it only requires a small sample that has been able to identify with high accuracy and precision and is not destroying the sample during operation compared to GC (Kupiec, 2004).

2.5.6 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance spectroscopy is a spectroscopic technique that utilizes the energy transition from processing atomic nuclei in static magnetic fields and alternating (rotating) magnetic field resonances. This required instrument consists of a direct current or permanent magnet as a supplier of static fields, radio frequency generators for alternating field suppliers, probes that can hold the sample in a relatively precise position in these two fields, the radio receiver to detect the signal produced by the hydrogen nucleus in the sample and recorders to make permanent recordings in the form of spectrum. The static homogeneity of the field must be given a fine band and structure that shows the chemical environment in which each hydrogen nucleus is located. The spectrum that shows this fine structure is called a high-resolution spectrum (as opposed to a wide line spectrum where chemical environmental features are obscured by broad line widths). When a sample containing hydrogen is placed in between a static magnetic field, each hydrogen nucleus will resonate at a frequency determined by the magnetic field. The regulatory process in this field is determined by an electronic system. Thus, various chemical environments that exist in the molecule will produce a precession frequency spectrum that will show the chemical properties of various parts of the molecule. Then the last is to interpret this frequency spectrum into a chemical structure.

NMR spectrum appears in the form of a series of vertical peaks or signals distributed along the x-axis of the spectrum. Each of these signals corresponds to the atoms in the observed molecule. then the position of each signal in the spectrum provides information about the number of atomic molecules in the surrounding environment that produce signals (Chamberlain, 1974).

3. MATERIALS AND METHODS

3.1. Time and Place

This research was conducted for fifteen months from September 2017 until December 2018 in the Laboratory of Natural Products at National Pingtung University of Science and Technology in Pingtung County, Taiwan.

3.2. Plant material

The initial dry weight of the air-dried stem of *P. foetida* was 22.28 kg. The plant was collected from Pingtung County, Taiwan.

3.3. Solvents and reagents

1. Methanol, *n*-BuOH, ethyl acetate (EtOAc), dichloromethane, acetone, and isopropanol were purchased from American Tedia Company
2. *d*-Chloroform, d_6-acetone, d_6-dimethylsulfoxide (DMSO), d_4-methanol were purchased from Acros Organics Company.
3. Column Chromatography used silica gel (63-200 mesh, Merck)
4. Thin Layer Chromatography (TLC) used Kieselgel 60 F254 (0.2 mm, Merck)
5. H_2SO_4 (10% v/v in H_2O)
6. Luria Bertani Broth used acumedia Lab, NEOGEN.
7. Agar used Cyrusbioscience.
8. Mueller Hinton Broth used Mast, Mast Group.
9. Resazurin sodium salt used Acros Organics Company
10. *Escherichia coli* (MTCC 1302) was from IMTECH, India
11. *Staphylococcus aureus* (MTCC 737) was from IMTECH, India
12. *Bacillus subtilis* (NK-1) was isolated from Natto
13. *Salmonella enterica* (ATCC 14028) was from ATCC, America

3.4. Instruments

1. Nuclear Magnetic Resonance Spectrometer (NMR) used Varian Mercury Plus 400 MHz NMR. Tetramethylsilane (TMS) was used as a standard for chemical shifts (δppm); singlet (s), doublet (d), triplet (t), the quartet (q), broad (br) and multiplet (m).
2. The mass spectrometer (MS) used Bruker Daltonics APEX 30e Spectrometer, Finnigan MAT 95S Mass Spectrometer, Finnigan MAT 95 XL High-Resolution Mass Spectrometer21
3. Ultraviolet Spectrometer (UV) used Shimadzu UV-1700 Pharma Spec UV -Visible Spectrometer
4. Infrared Spectrometer (IR) used Bruker Vector 22 Ft-IR Spectrometer
5. Polarimetry used Optical Activity AA-10R Automatic Spectrometer
6. Melting Point (MP) used Fargo Melting Point Apparatus MP-2D
7. Ultrasonic Cleaner used Ultrasonic Cleaner DC-400
8. High-Performance Liquid Chromatography (HPLC) Pump used Hitachi L-7110, Waters R401 Differential Refractometer Detector, and Bischoff differential Refractive Index Detector RI8120. The column used Thermo hypersil-keystone BETASIL silica-100 (5µm, 250 × 10mm)
9. Petri Dishes used Jetbiofil

3.5. The First Research Stage

The Figure below, show the first research stage of extraction and isolation of bioactive compounds from the air-dried stem of *P. foetida*.

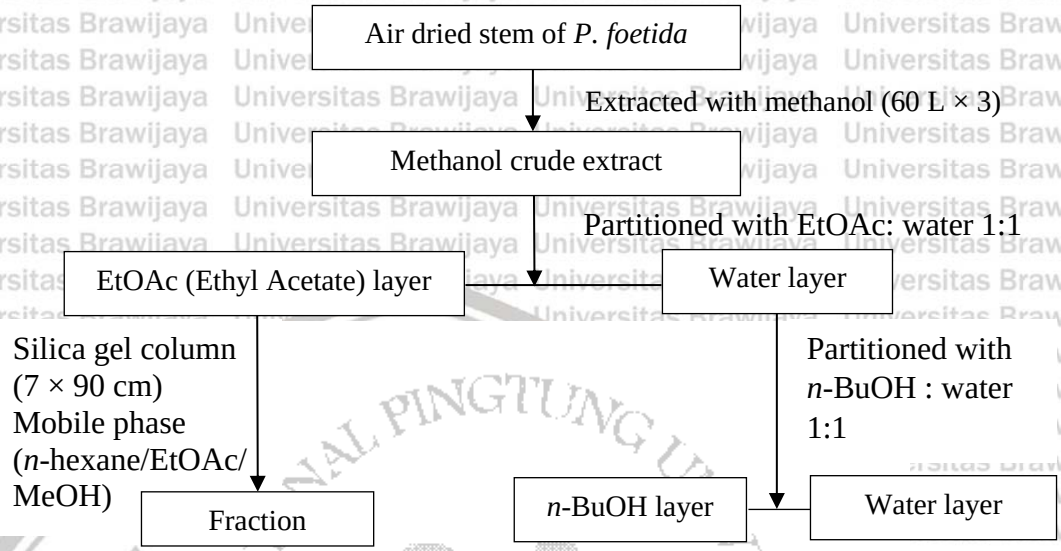


Figure 2. The First Research Stage

3.5.1. Sample Preparation

After collected, samples were sliced into a small size and then dried. The dry samples were packed into barrels then weighed before it is macerated by methanol.

3.5.2. Maceration

The plant material was macerated with methanol for 7 days. After maceration, the resultant solution was evaporated using rotary evaporation at 50 ^{o}C and 55 rpm. After evaporation, the crude methanol extract was prepared for partition step.

3.5.3. Partition

The crude methanol extract was partitioned sequentially to obtain various layers by polarity differences. The crude methanol extract was suspended in water and partitioned sequentially using ethyl acetate and

n-butanol. Partition was conducted using a separatory funnel. A part of ethyl acetate or *n*-butanol was added with a part of the water as a solvent comparison. The water residue of ethyl acetate partition process was used for the further step to obtain n-butanol layer. This process took 30 minutes to obtain *n*-butanol layer after the water residue was partitioned with *n*-butanol. Each step was repeated 6 times. Each layer was concentrated using rotary evaporator at 50°C to obtain concentrate residue (brown color) and used for further bioactivity analysis.

3.5.4. Isolation using Column Chromatography

Compounds of ethyl acetate layer were isolated using column chromatography. The ethyl acetate layer was subjected on silica gel (230-400 mesh, E. Merck) with column size 7 x 90 cm. Hexane and ethyl acetate solvent mixtures were used as an eluent by increasing the polarity and yielded fractions. The mobile phase used for column chromatography was *n*-hexane → hexane/ethyl acetate → hexane/ethyl acetate/methanol → ethyl acetate → ethyl acetate/methanol → methanol. Thereafter, fractions were collected in Erlenmeyer flask (1000 mL) then concentrated using rotary evaporator and transferred to a scintillation vial. The column was washed with methanol and 10 % water were used to eluate the high polarity sample. Finally, each fraction from column chromatography was confirmed by Thin Layer Chromatography (TLC).

3.5.5. Thin Layer Chromatography (TLC)

Thin layer chromatography is a separation method to identify compounds present in a given mixture. Dried sample on vials was dissolved in dichloromethane (DCM) then pointed into TLC paper. Furthermore, samples were developed with a suitable mobile phase.

Compound separation was detected using UV light at 254 nm for short wavelength and 365 for long wavelength.

3.5.6. Separation using High-Performance Liquid Chromatography (HPLC)

The preparative HPLC equipment was used Hitachi L-7110 LaChro Elite (Hitachi Inc., Japan) chromatography system, equipped with waters R401 Differential Refractometer Detector or Bischoff Differential Refractive Index Detector RI 8120. The HPLC column was semi-preparative thermo hypersilkeyston BETASIL silica 100 (5µm, 250 x 10 mm) column. The injection volume was 2 ml at a flow rate of 1-2 ml/min. The HPLC results were collected in a vial. Spots and bands appearances were confirmed by TLC. Pure compounds show in a single spot of TLC and the structure was confirmed by NMR analysis.

3.5.7. Identification by Nuclear Magnetic Resonance (NMR)

^{1}H NMR and ^{13}C NMR spectra were measured using Varian Mercury plus 400 MHz, NMR. TMS (tetramethylsilane) was used as internal standard, δ chemical shifts ‘s’ as single peak (singlet), ‘d’ as double peaks (doublet), ‘t’ for three peaks (triplet), ‘q’ for quadrupole peaks (quartet), ‘br’ stands for broad peak (broad), and ‘m’ represents the multiple peaks (multiplet).

Hydrogen or carbon nuclear test was conducted by dissolving samples in a deuterated solvent of chloroform-d, kept the sample in 5 mm NMR tube, determined by NMR which yield the peaks as electromagnetic wave absorption signal. Chemical shift is expressed in parts per million (ppm) unit relative to TMS as an internal standard.

3.5.8. Purification chemical constituent of *Paederia foetida* stem

3.5.8.1. β-sitosterol (Compound S1)

Compound **S1** was obtained from column chromatography of ethyl acetate layer which is fraction 10. After that, it was purified with *n*-hexane then recrystallized. Compound **S1** also identified the purity using TLC preparative before examined into NMR. It showed the purple spot on TLC paper by mobile phase dichloromethane/ethyl acetate, 1000:1 (v/v).

3.5.8.2. Morindaparvin A (Compound An1)

Compound **An1** was purified from fraction 15 using column chromatography with hexane → MeOH as eluent. It was purified with hexane then recrystallized. Compound **An1** showed as a yellow spot on TLC with dichloromethane/ethyl acetate eluent (1000:1, v/v).

3.5.8.3. Digiferrol (Compound An4)

Compound **An4** was purified from fraction 17 using column chromatography with hexane → MeOH as eluent. The compound was purified by dichloromethane/acetone subsequently recrystallized. Pure compound **An4** showed orange spot on preparative TLC with hexane/acetone eluent (5:1, v/v).

3.5.8.4. Stigmastan-3-one (Compound S2)

Compound **S2** was obtained from fraction 13. Fraction 13 was purified over normal-phase HPLC with gradient elution of dichloromethane/ethyl acetate (5000:1-1000:1, v/v), subsequently recrystallized. Pure compound **S2** was showed pink spot by hexane /acetone, 5:1 (v/v) on TLC.

3.5.8.5. 1,3-dihydroxy-2-methoxyanthraquinone (Compound An2)

Compound **An2** was purified from fraction 15 by normal-phase HPLC with gradient elution of dichloromethane/ethyl acetate (500:1-125:1, v/v) subsequently recrystallized. The pure compound **An2** was shown as a yellow spot on TLC using hexane/acetone (6:1, v/v).

3.5.8.6. Alizarin (Compound An3)

Compound **An3** was purified from fraction 22. Fraction 22 was subjected to normal-phase column chromatography silica gel with dichloromethane/MeOH eluent, (10.000:1, v/v) to give 6 subfractions. Thereafter, compound **An3** was purified by dichloromethane/acetone from subfraction 3. Pure compound **An3** showed yellow spot on TLC using hexane/acetone (6:1, v/v).

3.5.8.7. Scopoletin (Compound C1)

Compound **C1** was obtained from subfraction 4 of fraction 22. Subfraction 4 was purified by dichloromethane/acetone eluent subsequently recrystallized to give compound **C1**. The pure compound **C1** showed light-yellow spot-on TLC using eluent Hexane/Acetone (3:1, v/v).

3.5.8.8. Fraxidin (Compound C2)

Compound **C2** was purified from subfraction 5 of fraction 22, subfraction 5 was subjected over normal-phase column chromatography silica gel hexane/acetone eluent (25:1, v/v) to give 2 subfractions. Thereafter, subfraction 1 was purified by dichloromethane/acetone subsequently recrystallized to give pure compound **C2**. Compound **C2** was shown light green spot on TLC using eluent hexane/ethyl acetate eluent (1:1, v/v).

3.5.8.9. Vanillic acid (Compound A2)

Compound **A2** was obtained from subfraction 2 by purification dichloromethane/acetone/ethyl acetate subsequently recrystallized. Pure compound **A2** showed red spot on TLC using hexane/ethyl acetate (1:1, v/v).

3.5.8.10. Ferulic acid (Compound A1)

Compound **A1** was obtained from subfraction 6 of fraction 22. It was subsequently fractionated over normal-phase column chromatography silica gel with hexane/acetone eluent (25:1, v/v) to give 3 subfractions. Thereafter, subfraction 3 was purified by Dichloromethane/Ethyl acetate to give compound **A1**. Pure compound **A1** showed a light pink spot on TLC using dichloromethane/ethyl acetate (8:1, v/v).

3.6. Antibacterial assay for the second research stage

3.6.1. Well diffusion assay

The antibacterial of crude extract and fractions was tested using agar well diffusion method based on Revathi et al. (2017). The human pathogens bacteria, such as *Staphylococcus aureus* (MTCC 737) *and Escherichia coli* (MTCC 1302) were selected. *S. aureus* and *E. coli* were obtained from IMTECH, India.

The bacteria strains were inoculated from the stock into Mueller Hinton Agar for 12 hours. After 12 hours, the single colony was inoculated into Luria broth. Then, the 12 hours bacterial culture (10^8 CFU/mL) was continuously inoculated into the agar medium and spread over on the plates. The well was made using 8 mm sterile pipette tip. Then, the well was filled 100 µL (2 mg/mL) of crude extract and fractions. Streptomycin was used as a positive control and DMSO as a negative control. The culture plates were incubated at 37 ^{o}C for 24

hours. The clear zone of inhibition around the well was measured in mm.

3.6.2. Minimum Inhibitory Concentration

The minimum inhibitory concentration was determined using a microdilution technique with a resazurin as an indicator based on Sarker et al. (2007). Four pathogens bacterial were tested. *Bacillus subtilis* (NK-1, isolated from Natto), and *Staphylococcus aureus* (MTCC 737) were used for gram-positive while *Escherichia coli* (MTCC 1302) and *Salmonella enterica* (ATCC 14028) for gram negative.

The bacterial suspension was prepared after 12 hours incubation in Luria broth. Turbidity was adjusted approximately 1 x 10^6 colony-forming units (CFU/mL). The 96 well plates were prepared under aseptic conditions. The samples for pure compound were dissolved with 100 µL DMSO to give the concentration 10 mg/mL. The working solutions of each compound were prepared by means of serial dilutions from 600 µg/mL to 4.68 µg/mL. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use 50 µL the test material. The resazurin indicator solution was mixed with the broth ratio 1:7.5. Finally, 10 µL of bacterial suspension was added to each well (1 x 10^6 CFU/mL). The plates were placed in an incubator set at 37 ^oC for 6-12 h. The color change was observed visually. Any color change from purple to pink was recorded as positive. The lowest concentration at which color change occurred was considered as the MIC value.

4. RESULTS AND DISCUSSION

4.1. Extraction, Partition, and Isolation

After the first stage of the experiment which included extraction, partition and isolation twenty-seven fractions were obtained as shown in Figure 3 below.

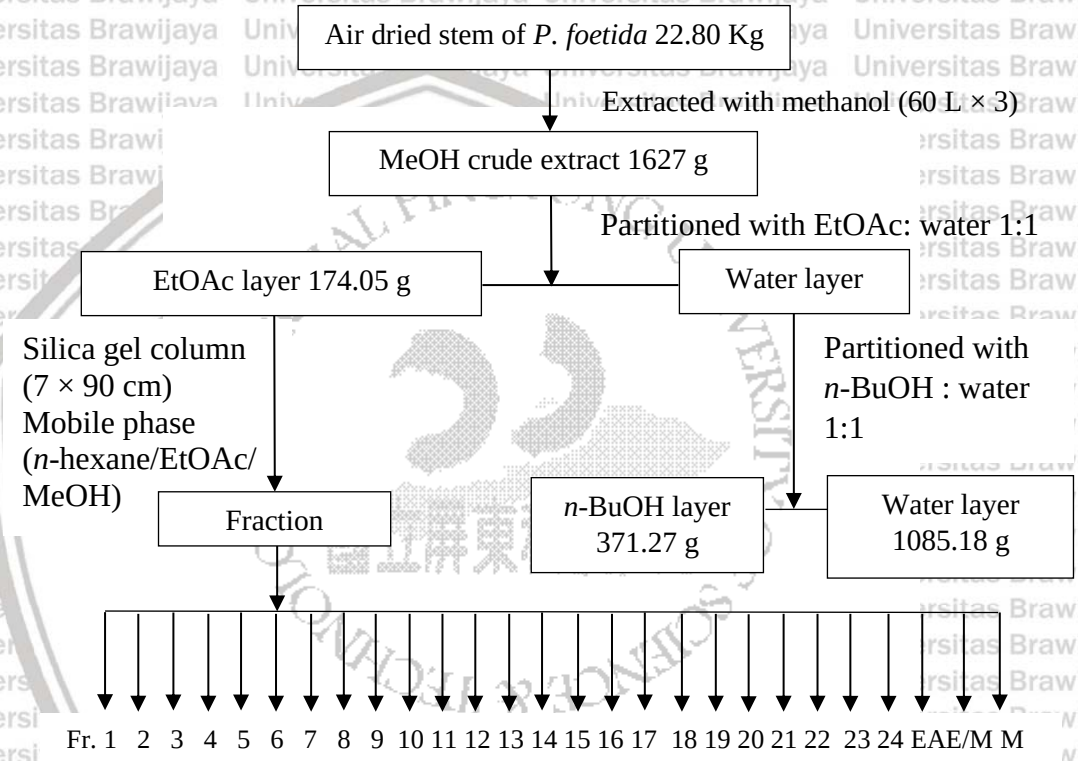


Figure 3. The Result of The First Research Stage

The weight of partition layers EtOAc, *n*-BuOH, water, and MeOH crude extract obtained are displayed in Table 2 below.

Table 2. The weights and extraction percentages of *P. foetida* Linn

	Weight (g)	Extraction Percentage (%)
EtOAc Layer	174.05	0.76
n-BuOH Layer	321.27	1.41
Water Layer	1085.18	4.76
MeOH Crude Extract	1627	7.13
Air-dried sample	22800	

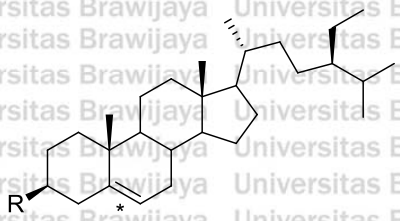
4.2. Chemical constituents of *Paederia foetida* stem

Ten compounds were obtained from the EtOAc layer. There are two steroids, four anthraquinones, two coumarins, and two aromatics, shown in Table 3 and Figure 5. The compounds were identified by comparing the Nuclear Magnetic Resonance and mass spectrophotometric data with previous studies.

Table 3. Chemical constituents of the stem of *P. foetida* Linn.

Compounds	Reference
Compound **S1**	Chang et al. (2000)
Compound **S2**	Luo et al. (2009)
Compound **An1**	de Oliveira Figueiredo et al. (2014)
Compound **An2**	Simoneau and Paul (1986)
Compound **An3**	Dhananjeyan et al. (2005), Berger et al. (1980)
Compound **An4**	Imre and Ersoy (1978), Permana et al. (1999)
Compound **C1**	Siddiqui et al. (2007), Terra et al. (2013)
Compound **C2**	Yasuda et al. (2006)
Compound **A1**	Wang et al. (2015)
Compound **A2**	Phan Duc et al. (2016)

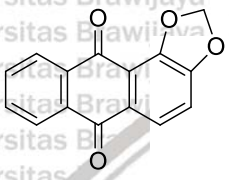
Steroids

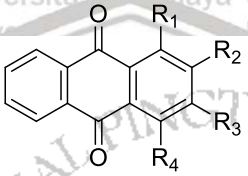


S1: R = OH

S2: R = O; * = Single bond

Anthraquinones

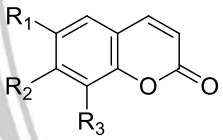




An1

	R_1	R_2	R_3	R_4
An2	OH	OCH_3	OH	H
An3	OH	OH	H	H
An4	OH	CH_2OH	H	OH

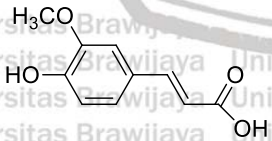
Coumarins



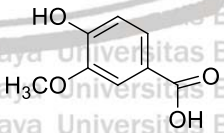
C1: $R_1 = OCH_3$; $R_2 = OH$; $R_3 = H$

C2: $R_1 = OCH_3$; $R_2 = OCH_3$; $R_3 = OH$

Aromatics



A1



A2

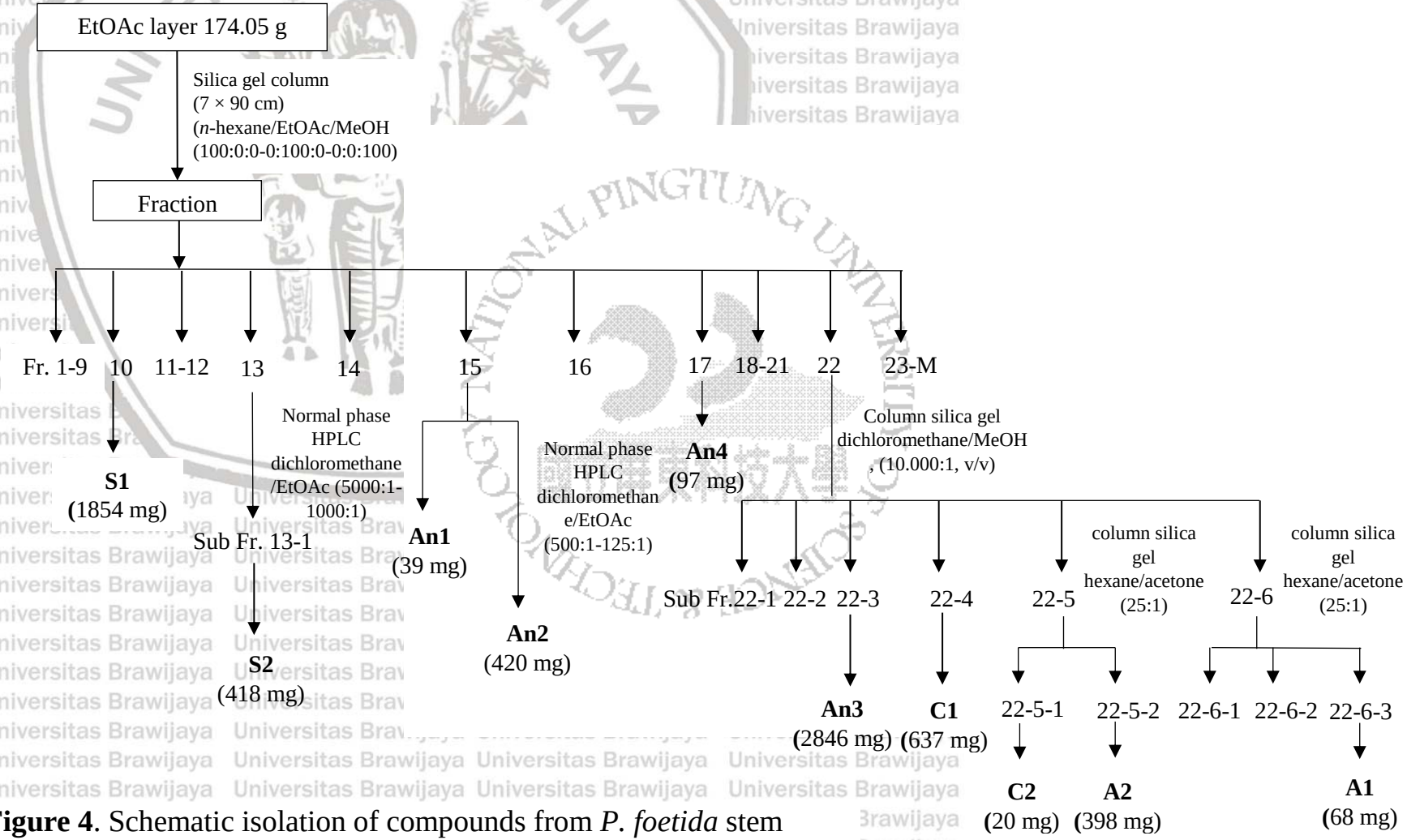


Figure 4. Schematic isolation of compounds from *P. foetida* stem

4.2.1. Steroid

4.2.1.1. Compound S1

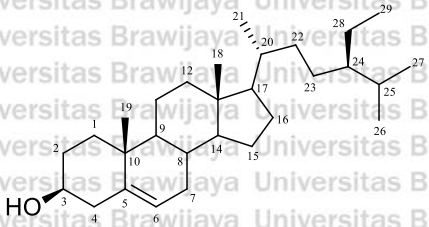


Figure 5. Structure of compound **S1** with carbon number.

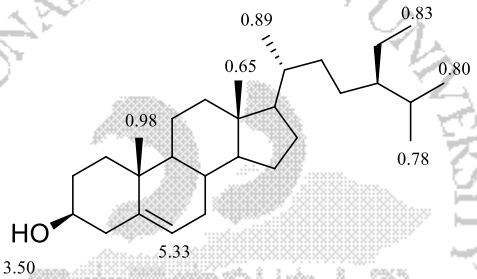
Compound **S1** ($C_{29}H_{50}O$) was isolated from column chromatographic over silica gel as white needles with a melting point 138-140 ^{o}C. The structure of compound **S1** is shown in Figure 5.

The ^{1}H NMR spectrum of compound **S1** in Table 4 and Figure 6 exhibited for six methyl signals (3H for each) at δ_H 0.65 (s, H-18), 0.78 (d, *J* = 6.8 Hz, H-26), 0.80 (d, *J* = 7.2 Hz, H-27), 0.83 (t, *J* = 7.2 Hz, H-29), 0.89 (d, *J* = 6.4 Hz, H-21) and 0.98 (s, H-19). The multiplet signal at δ_H 3.51 assigned to H-3 is a characteristic of the hydroxymethine group. The broad signal δ_H 5.33 represent an endocyclic double bond.

The EI-MS spectrum in Figure 7 showed the molecular ion *m/z* 414 $[M]^{+\cdot}$. The molecular ion and ^{1}H NMR spectral data of the compound **S1** are corresponding with Chang et al. (2000) as β-Sitosterol.

Table 4. ^{1}H NMR data for compound **S1** (400 MHz, $CDCl_3$)

Position	δ_H (*J* in Hz) (ppm)	δ_H (*J* in Hz) (ppm) by Chang et al. (2000)
3	3.51 m	3.53 m
6	5.33 br s	5.36 br s
18	0.65 s	0.68 s
19	0.98 s	1.01 s
21	0.89 d (6.4)	0.92 d (6.4)
26	0.78 d (6.8)	0.81 d (6.8)
27	0.80 d (7.2)	0.84 d (6.8)
29	0.83 t (7.2)	0.86 t (7.0)



^{1}H NMR spectral data for compound **S1**

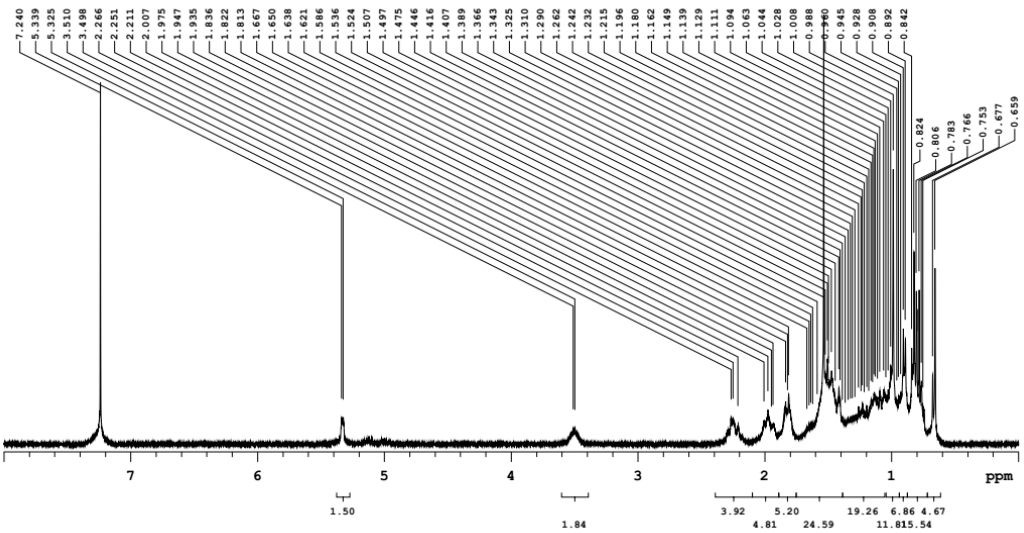


Figure 6. ^{1}H NMR spectrum of compound **S1** (400 MHz, $CDCl_3$).

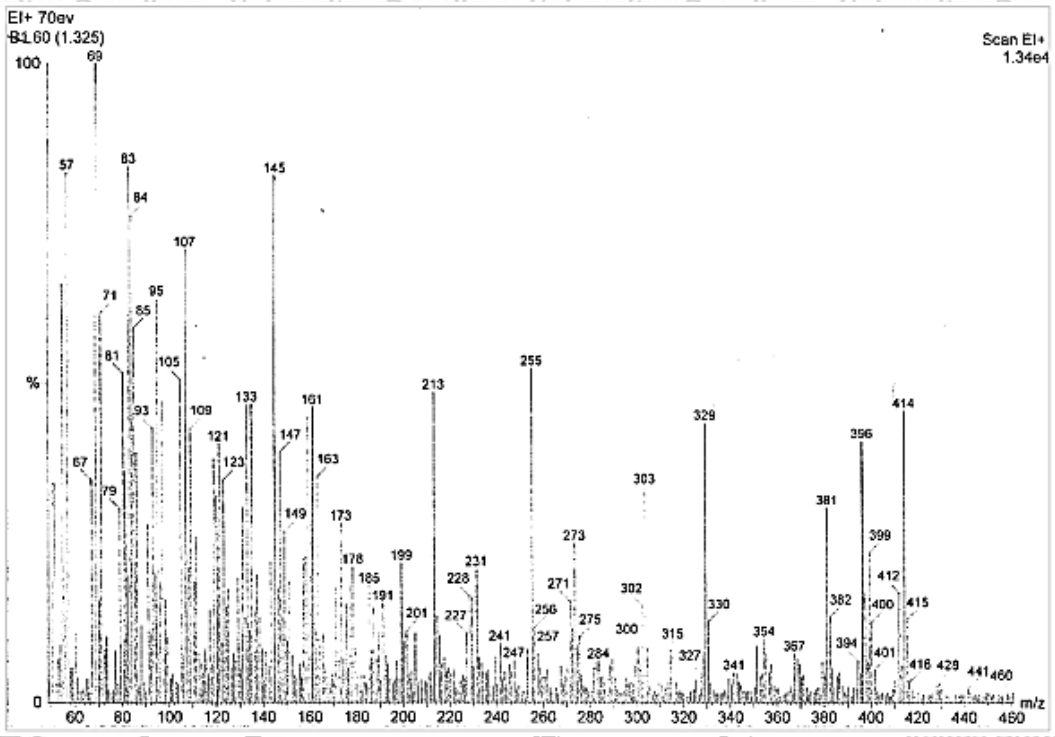


Figure 7. EI-Mass spectrum of compound **S1**

4.2.1.2. Compound S2

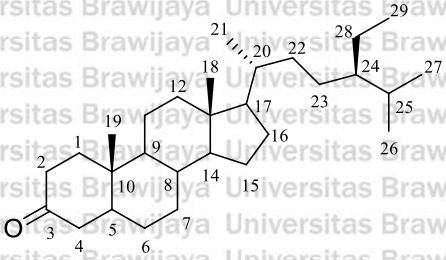


Figure 8. Structure of compound **S2** with a carbon number

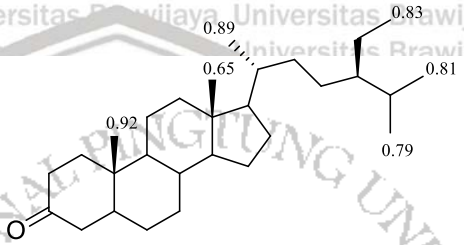
Compound **S2** ($C_{29}H_{50}O$) with melting point 157-160 ^{o}C was isolated as a white needle. The structure is similar to compound **S1**, the hydroxyl group in carbon three is substituted by ketone. In addition, compound **S2** does not have an endocyclic double bond on carbon position number six. The structure of compound **S2** is shown in Figure 8.

The ^{1}H NMR spectral data of compound **S2** as shown in Table 5 and Figure 8 showed six methyl signals at δ_H 0.65 (s, H-18), 0.79 (d, *J* = 6.8 Hz, H-26), 0.81 (d, *J* = 7.6 Hz, H-27), 0.83 (t, *J* = 7.6, Hz, H-29), 0.89 (d, *J* = 6.8 Hz, H-21), 0.92 (s, H-19).

The EI-MS spectrum in Figure 10 showed the molecular ion *m/z* 414.4 $[M]^{+\cdot}$ Its molecular ion and the ^{1}H NMR spectral data for compound **S2** is corresponding with Luo et al. (2009) as stigmastan-3-one.

Table 5. ^{1}H NMR data for compound **S2** (400 MHz, $CDCl_3$).

Position	δ_H (*J* in Hz) (ppm)	δ_H (*J* in Hz) (ppm) by Luo et al. (2009)
18	0.65 s	0.70 s
19	0.92 s	1.03 s
21	0.89 d (6.8)	0.93 d (6.5)
26	0.79 d (6.8)	0.86 d (7.2)
27	0.81 d (7.6)	0.84 d (6.8)
29	0.83 t (7.6)	0.87 t (7.6)



^{1}H NMR spectral data for compound **S2**

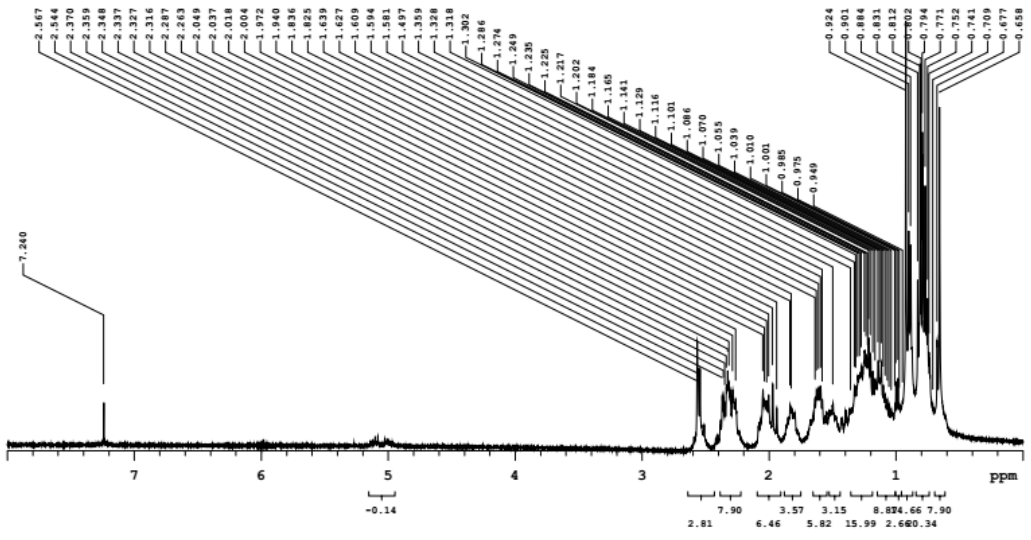


Figure 9. ^{1}H NMR spectrum of compound **S2** (400 MHz, $CDCl_3$).

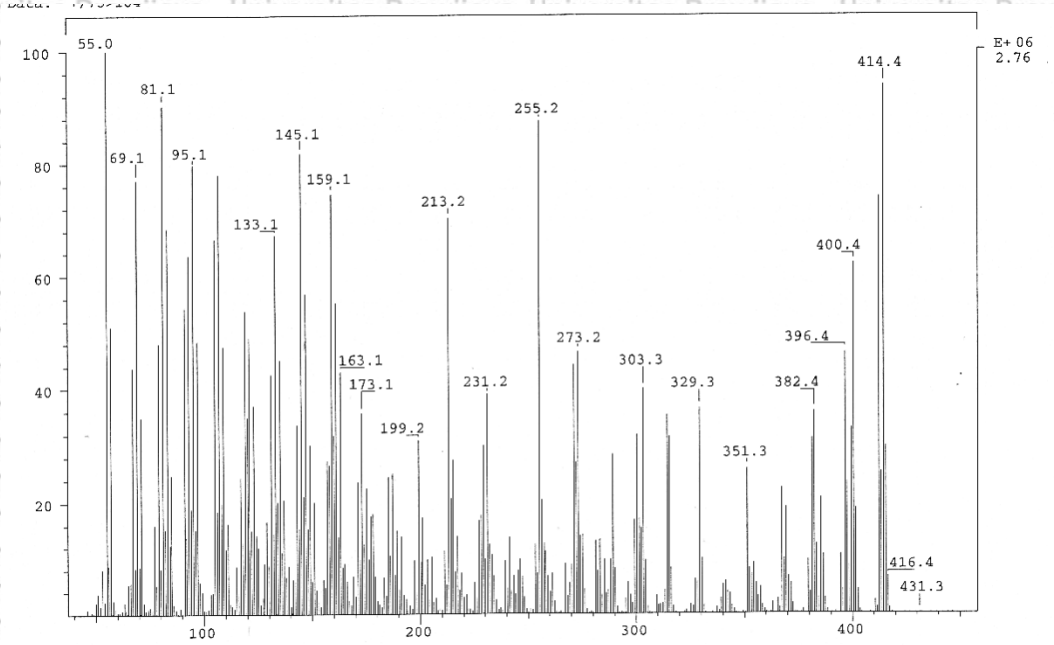


Figure 10. EI-MS Spectrum of compound **S2**

4.2.2. Anthraquinone

4.2.2.1. Compound An1

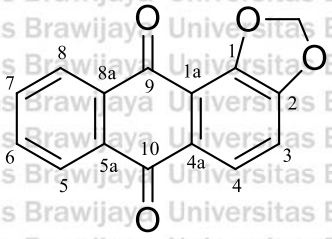


Figure 11. Structure of compound **An1**

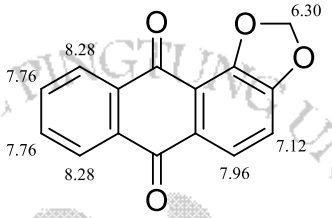
Compound **An1** was isolated from column chromatographic over silica gel as a yellow powder with a melting point 255-257 ºC. The structure of compound **An1** is shown in Figure 11.

The ^{1}H NMR spectrum compound **An1** in Figure 12 and Table 6 showed the presence of two aromatic ortho coupled protons at δ_H 7.12 and 7.96 (1H, J = 8.0 Hz). Four aromatic protons signals appeared as multiplets at δ_H 7.76 (2H, m, H-6, and H-7) and 8.28 (2H, m, H-5, and H-8). A singlet signal at δ_H 6.30 (2H, s) revealed the presence of a methylenedioxy group.

The EI-MS spectrum of compound **An1** in Figure 13 showed the molecular ion *m/z* 252.1 $[M]^+$ which is corresponding to the molecular formula $C_{15}H_8O_4$. On the basis of the spectral data of this compound, compound **An1** was identified as 1,2-methylenedioxyanthraquinone or Morindaparvin A and its spectral data proved similar to that reported by de Oliveira Figueiredo et al. (2014).

Table 6. ^{1}H NMR data for compound **An1** (400 MHz, $CDCl_3$)

Position	δ_H (*J* in Hz) (ppm)	δ_H (*J* in Hz) (ppm) by de Oliveira Figueiredo et al. (2014)
3	7.12 d (8.0)	7.13 d (8.0)
4	7.96 d (8.0)	7.96 d (8.0)
6 and 7	7.76 m	7.76 m
5 and 8	8.28 m	8.28 m
1,2-OCH_2	6.30 s	6.29 s



^{1}H NMR spectral data for compound **An1**

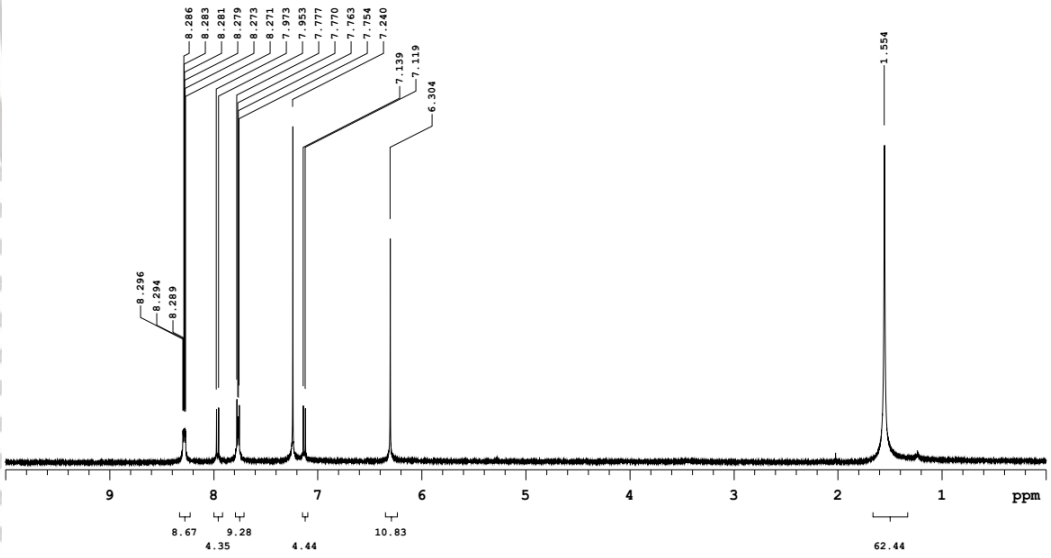


Figure 12. ^{1}H NMR spectrum of compound **An1** (400 MHz, $CDCl_3$).

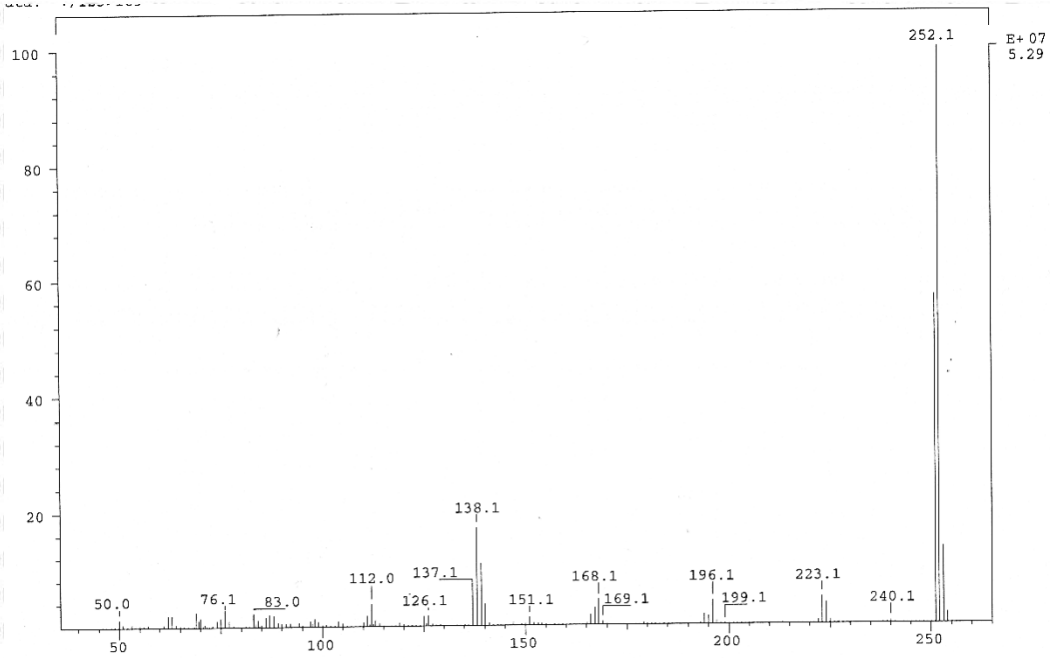


Figure 13. EI-MS spectrum of compound **An1**

4.2.2.2. Compound An2

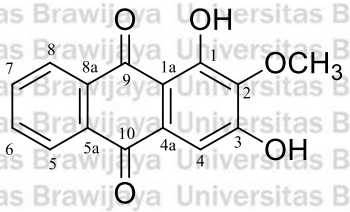


Figure 14. Structure of compound **An2** with a carbon number

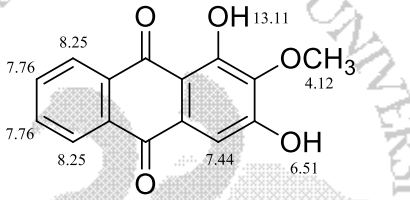
Compound **An2** ($C_{15}H_{10}O_5$) was isolated from normal phase HPLC as a yellow crystal with a melting point 218-220 ^oC. The structure of compound **An2** is shown in Figure 14.

The ^1H NMR spectral data of compound **An2** in Figure 15 and Table 7 exhibited a single peak at δ_H 4.12 (3H) indicating the presence of a methoxy group. Two signals at δ_H 6.51 (1H, s, H-3) and 13.11 (1H, s, H-1) proved the existence of two hydroxyl groups at the C ring. The other single peak showed at δ_H 7.44 (1H) as H-4. The appearance of two multiplets at δ_H 7.76 (2H, H-6 and H-7) and 8.25 (2H, H-5 and H-8) in the A-ring displayed a characteristic of the A_2B_2 type.

EI-MS spectrum of this compound revealed the molecular ion *m/z* 270 $[M]^+$ in Figure 16. The compound **An2** was identified as 1,3-dihydroxy-2-methoxyanthraquinone by comparison of spectroscopic data with reported data by Simoneau and Paul (1986).

Table 7. ^{1}H NMR data for compound **An2**, (400 MHz, $CDCl_3$)

Position	δ_H (*J* in Hz) (ppm)	δ_H (*J* in Hz) (ppm) by Simoneau and Paul (1986).
4	7.44 s	7.47 s
5	8.25 m	8.22 m
6	7.76 m	7.74 m
7	7.76 m	7.74 m
8	8.25 m	8.22 m
1-OH	13.11 s	13.14 s
2-OCH_3	4.12 s	4.15 s
3-OH	6.51 s	6.49 s



^{1}H NMR spectral data for compound **An2**

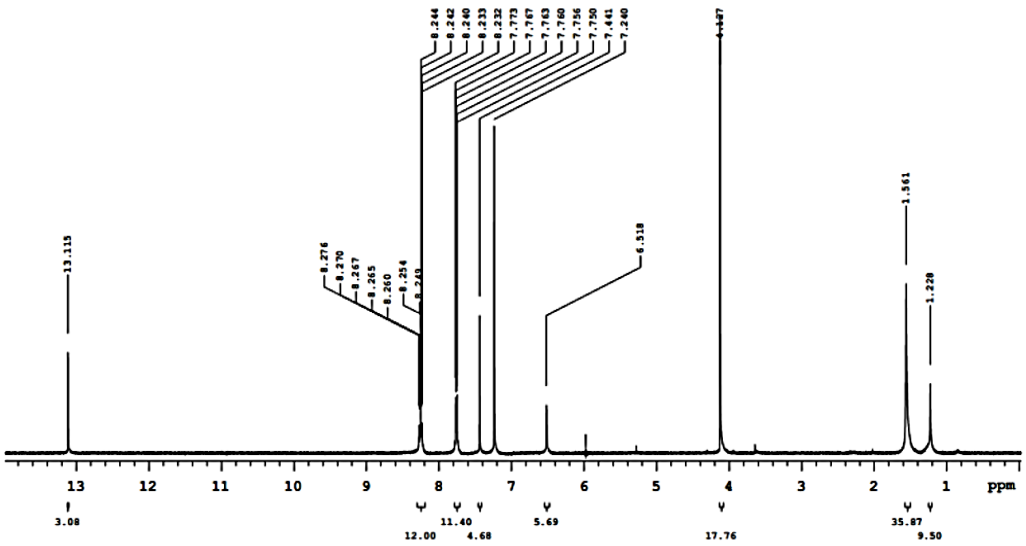


Figure 15. ^{1}H NMR spectrum of compound **An2** (400 MHz, $CDCl_3$).

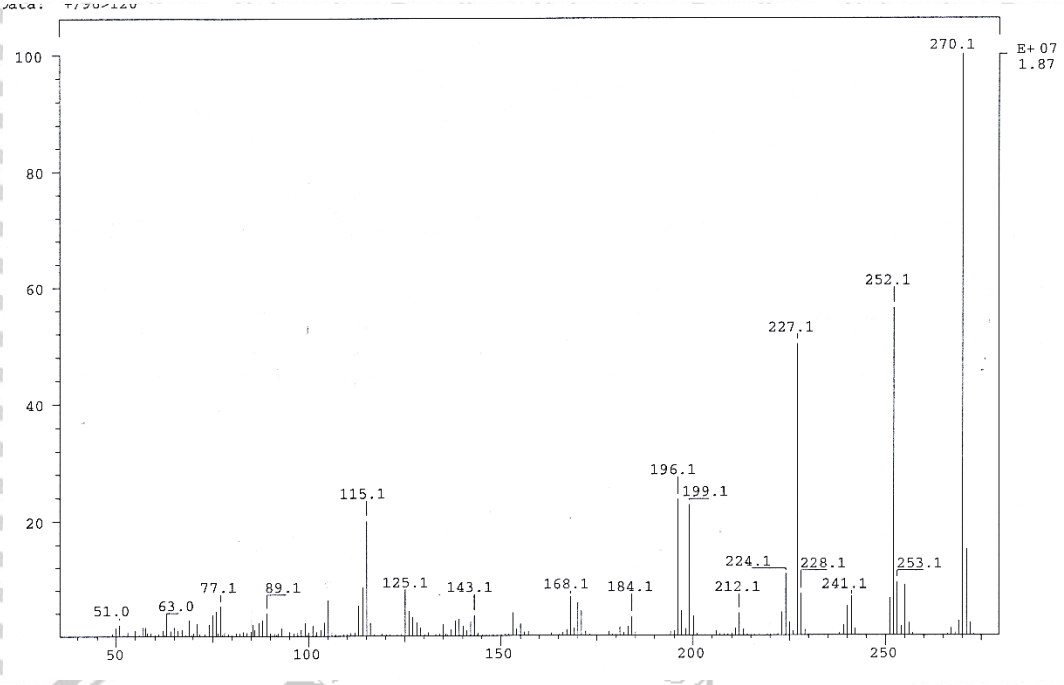


Figure 16. EI-MS spectrum of compound **An2**

4.2.2.3. Compound An3

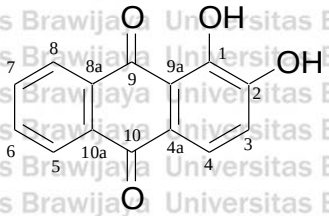


Figure 17. Structure of **An3** with a carbon number

Compound **An3** ($C_{14}H_8O_4$) was obtained as an orange powder with a melting point range 186 – 189 ^oC. Compound **An3** was isolated by column chromatography from fraction 22. The structure of compound **An3** is shown in Figure 17.

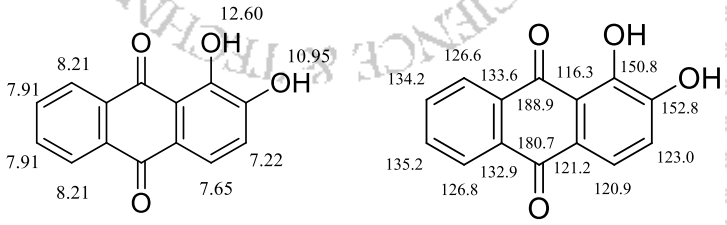
The ^1H NMR spectral data of compound **An3** in Figure 19 and Table 8 revealed the presence of two aromatic ortho coupled protons at δ_H 7.22 (1H, d, J = 8.4 Hz, H-3) and 7.65 (1H, d, J = 8.4 Hz, H-4). Four proton signals (5,6,7,8) correlated at δ_H 7.91 (H-6 and H-7) and 8.21 (H-5 and H-8) in the A-ring which revealed an A_2B_2 system. Two hydroxyl proton signals appeared at δ_H 10.95 (1H, s, 2-OH) and 12.60 (1H, s, 1-OH) in the C-ring.

The ^{13}C NMR and DEPT spectral data (Figure 18 and Table 8) showed the presence of six methines at δ_C 135.2, 134.2, 126.8, 126.6, 121.2, 120.9, eight quaternary carbons at δ_C 133.6, 132.9, 123.9 116.3, with ketone groups at δ_C 188.9 and 180.7, and carbon hydroxyl bonds at δ_C 152.8 and 150.8.

The EI-MS spectrum showed a molecular ion signal at *m/z* 240 $[M]^+$ (Figure 20) corresponds to the molecular formula $C_{14}H_8O_4$. The compound **An3** was identified as alizarin by comparison of spectroscopic data with reported data from Dhananjeyan et al. (2005) and Berger et al. (1980).

Table 8. ^{1}H and ^{13}C NMR spectral data for compound **An3**, (400 MHz, DMSO-d_6)

Position	δ_H (*J* in Hz) (ppm)	δ_C (ppm)	δ_H (*J* in Hz) (ppm) by Dhananjeyan et al. (2005)	δ_C (ppm) by Berger et al. (1980)
1		150.8		150.7
2		152.8		152.7
3	7.22 d (8.4)	123.9	7.27 d (8.5)	125.8
4	7.65 d (8.4)	120.9	7.70 d (8.5)	121.0
5	8.21 m	126.8	8.23 m	126.6
6	7.91 m	135.2	7.95 m	134.9
7	7.91 m	134.2	7.95 m	133.9
8	8.21 m	126.6	8.23 m	126.3
9		188.9		188.6
10		180.7		180.3
4a		121.2		123.7
8a		133.6		133.4
9a		116.3		116.1
10a		132.9		132.7
1-OH	12.60 s			
2-OH	10.95 s			



^{1}H and ^{13}C NMR spectral data for compound **An3**

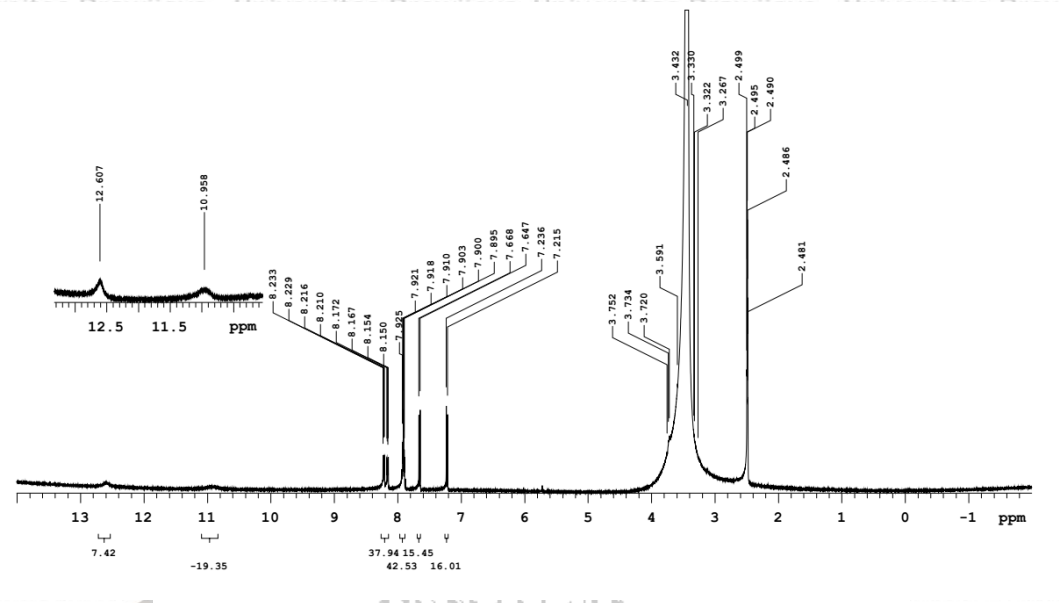


Figure 19. ^{1}H NMR spectrum of compound **An3** (400 MHz, DMSO-d_6)

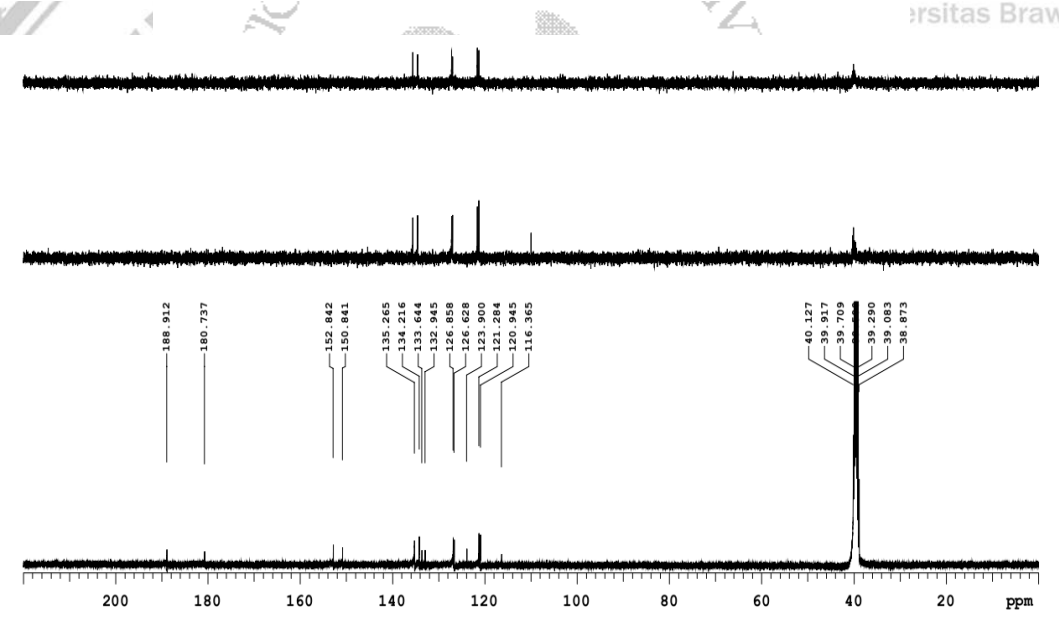


Figure 18. ^{13}C and DEPT NMR spectra of compound **An3** (100 MHz, DMSO-d_6)

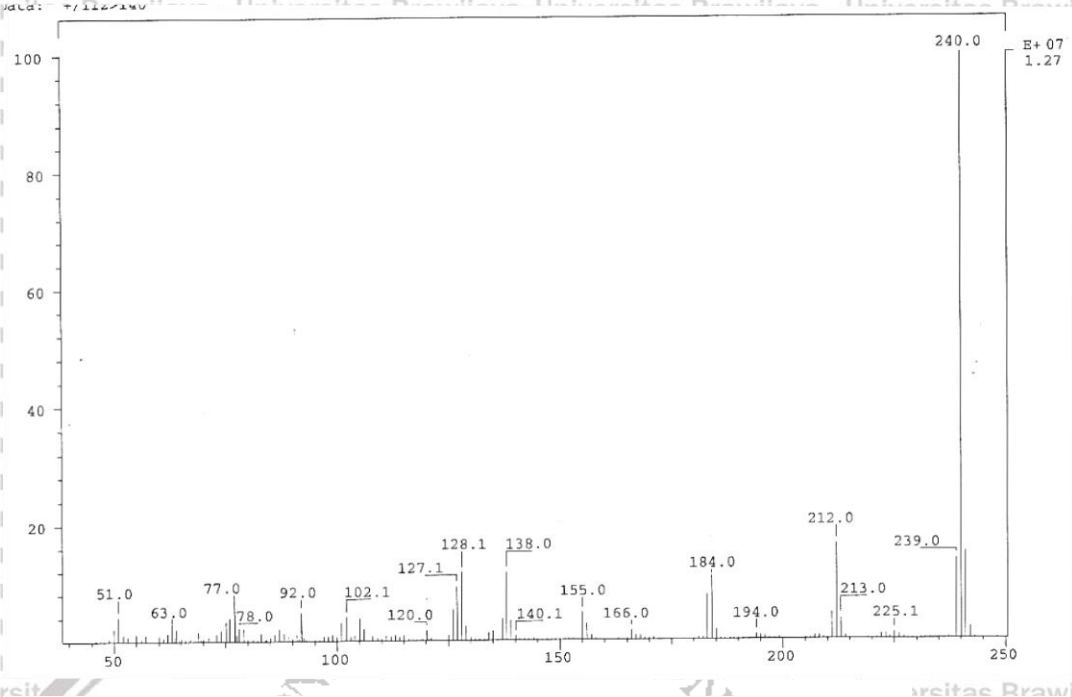


Figure 20. EI-MS spectrum of compound **An3**.

4.2.2.4. Compound An4

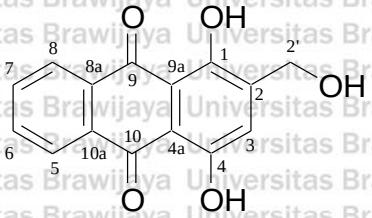


Figure 21. Structure of compound **An4** with a carbon number

Compound **An4** ($C_{15}H_{10}O_{15}$) was isolated as a red powder from fraction 17. Compound **An4** has a melting point range 208-209 ^{o}C and the structure of compound **An4** is shown in Figure 21.

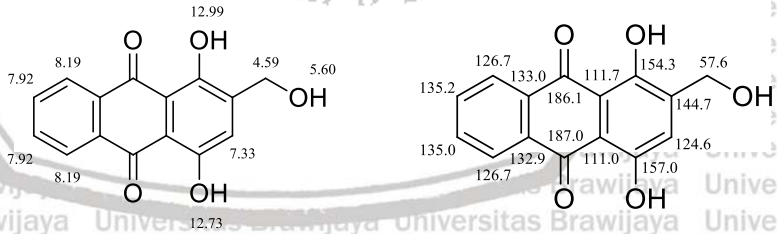
The ^{1}H NMR spectral data of compound **An4** in Figure 23 and Table 9 exhibited three hydroxyl proton signals at δ_H 12.99 (1H, s, 1-OH), 12.73 (1H, s, 4-OH), and 5.60 (1H, t, J = 5.2 Hz, 2'-OH). The spectrum further displayed a two-proton signal at δ_H 4.59 (2H, d, J = 2.2 Hz, 2-CH_2). An aromatic proton signal appeared at δ_H 7.33 (1H, d, J = 2.2 Hz, H-3). The remaining aromatic signals are typical for a 6 or 7 substituted A-ring, a pair of multiplets were displayed at δ_H 7.93 (2H, H-6 and H-7) and 8.19 (2H, H-5 and H-8).

The ^{13}C NMR and DEPT spectral data (Figure 22 and Table 9) displayed five methines at δ_C 135.22, 135.05, 126.76, 126.69, 124.67, nine quaternary carbons, at δ_C 157.09, 154.33, 144.74, 133.06, 132.90, 111.77 and 111.00 with two ketone groups at δ_C 187.04 and 186.19. The methylene appeared at δ_C 57.65.

The molecular ion was showed at *m/z* 270.0 $[M]^+$ (Figure 24) which correspond to the proposed structure. The compound **An4** was identified as digiferrol by comparison of its spectroscopic data with reported data from Imre and Ersoy (1978), and Permana et al. (1999).

Table 9. ^{1}H and ^{13}C NMR spectral data for compound **An4** (400 MHz, DMSO-d_6)

Position	δ_H (*J* in Hz) (ppm)	δ_C (ppm)	δ_H (*J* in Hz) (ppm) by Imre and Ersoy (1978)	δ_C (ppm) by Permana et al. (1999)
1		154.3		154.2
2		144.7		144.6
3	7.33 d (2.2)	124.6	7.43 s	124.5
4		157.0		156.9
5	8.19 m	126.7	8.27 m	126.5
6	7.92 m	135.0	7.97 m	134.9
7	7.92 m	135.2	7.97 m	135.0
8	8.19 m	126.7	8.27 m	126.6
9		186.1		186.1
10		187.0		186.9
4a		111.0		110.9
8a		133.0		132.9
9a		111.7		111.7
10a		132.9		132.8
1-OH	12.99 s		13.09 s	
4-OH	12.73 s		12.84 s	
2-$\underline{CH_2}$	4.59 d (2.2)	57.6	4.67 d (2.8)	57.5
2-$\underline{OH}$	5.60 t (5.2)		5.53 s	



^{1}H and ^{13}C NMR spectral data for compound **An4**

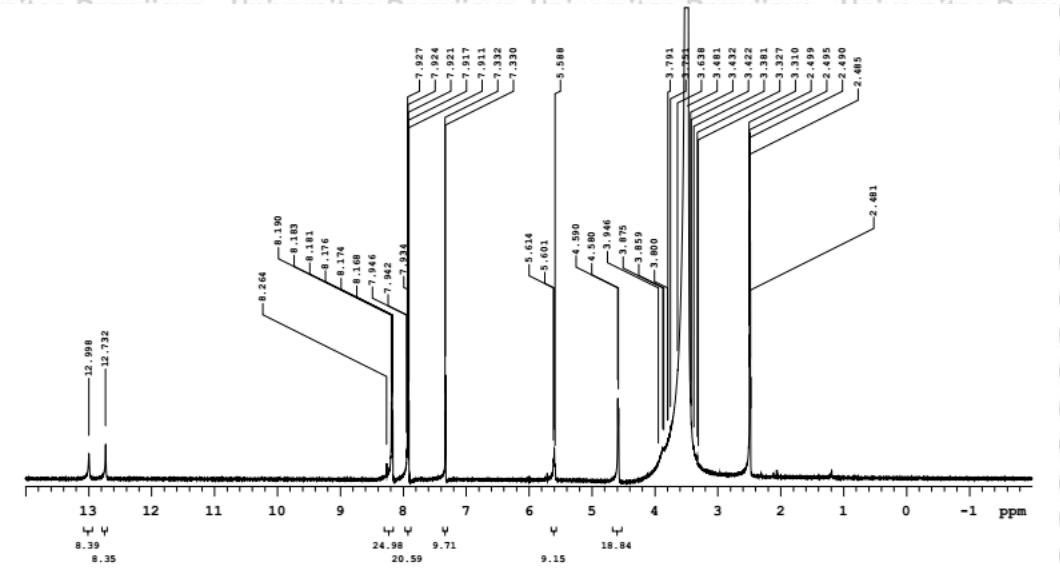


Figure 23. ^{1}H NMR spectrum of compound **An4** (400 MHz, DMSO-d_6)

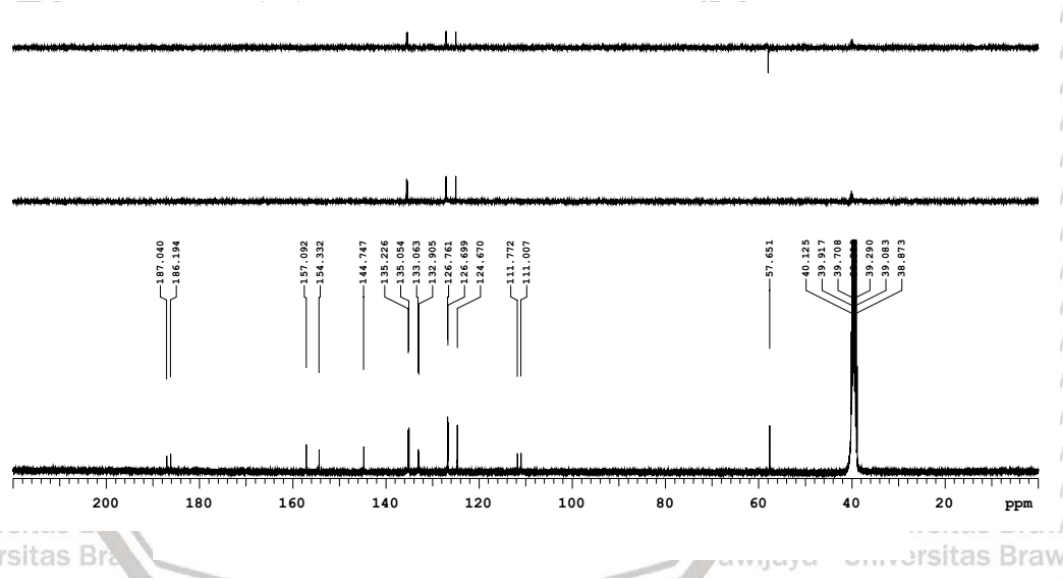


Figure 22. ^{13}C and DEPT NMR spectra of compound **An4** (100 MHz, DMSO-d_6)

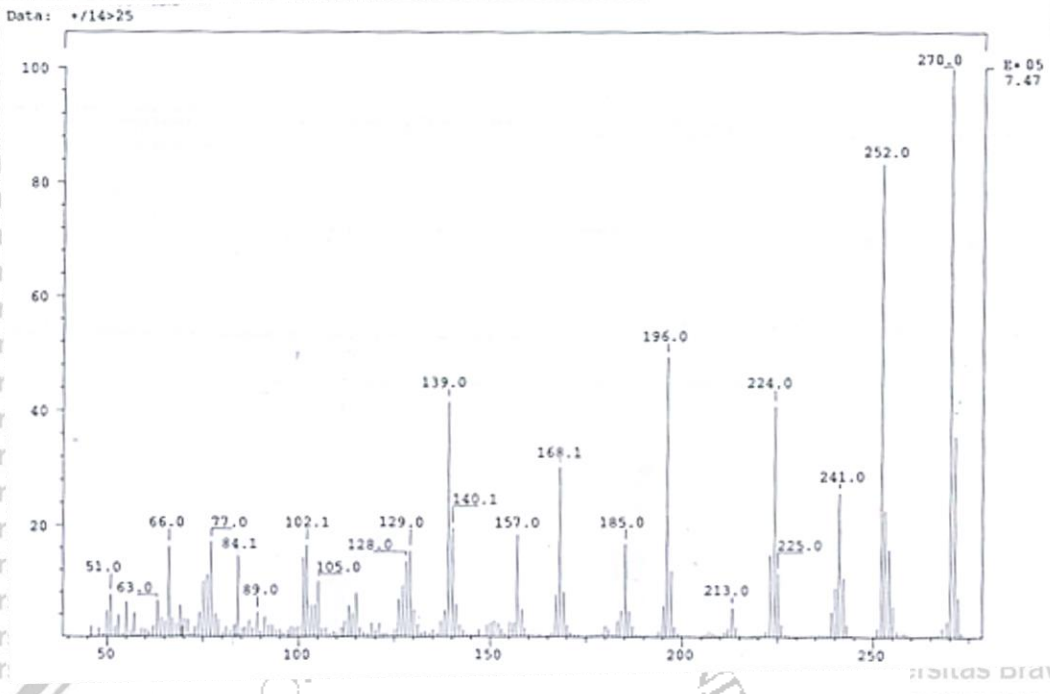


Figure 24. EI-MS spectrum of compound **An4**

4.2.3. Coumarin

4.2.3.1. Compound C1

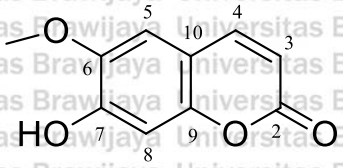


Figure 25. Structure of compound **C1** with a carbon number

Compound **C1** ($C_{14}H_8O_4$) was obtained as a colorless needle with a melting point 204 °C. The structure of compound **C1** is shown in Figure 25.

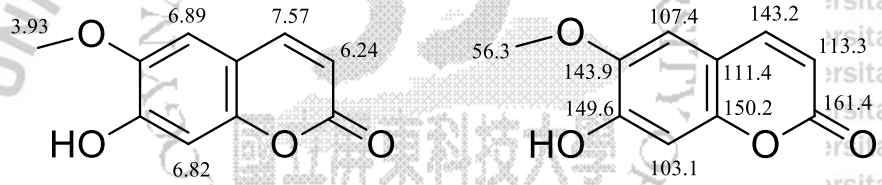
The ^{1}H-NMR spectrum in Figure 26 and Table 10 for compound **C1** showed the presence of a methoxy group as a singlet at δ_H 3.93. The spectrum further displayed two doublets at δ_H 6.24 (1H, d, J = 9.2 Hz H-3) and 7.57 (1H, d, J = 9.2 Hz, H-4). The remaining signals appeared at δ_H 6.82 (1H, s, H-8) and 6.89 (1H, s, H-5).

The ^{13}C NMR and DEPT spectral data (Figure 27 and Table 10) revealed the existence of four methines at δ_C 143.2, 113.3, 107.4, and 103.1. Five quaternary carbons showed at δ_C 161.4, 150.2, 149.6, 143.9, and 111.4. The methoxy group presenced at δ_C 56.3.

The EI-MS spectrum of compound **C1** revealed a molecular ion at *m/z* 192.1 $[M]^+$ shown in Figure 28. The compound **C1** was identified as scopoletin by comparison of its spectroscopic data with reported data from Siddiqui et al. (2007) and Terra et al. (2013).

Table 10. ^{1}H and ^{13}C NMR spectral data for compound **C1**, (400 MHz, DMSO-d_6)

Position	**δ_H (*J* in Hz) (ppm)**	**δ_C (ppm)**	**δ_H (*J* in Hz) (ppm)** by Siddiqui et al. (2007)	**δ_C (ppm)** by Terra et al. (2013)
2		161.4		161.4
3	6.24 d (9.2)	113.3	6.26 d (9.4)	113.4
4	7.57 d (9.2)	143.2	7.58 d (9.4)	143.3
5	6.89 s	107.4	6.89 s	107.5
6		143.9		144.0
7		149.6		149.7
8	6.82 s	103.1	6.82 s	103.2
9		150.2		150.0
10		111.4		111.5
6-OCH_3	3.93 s	56.3	3.93 s	56.4



^{1}H and ^{13}C NMR spectral data for compound **C1**

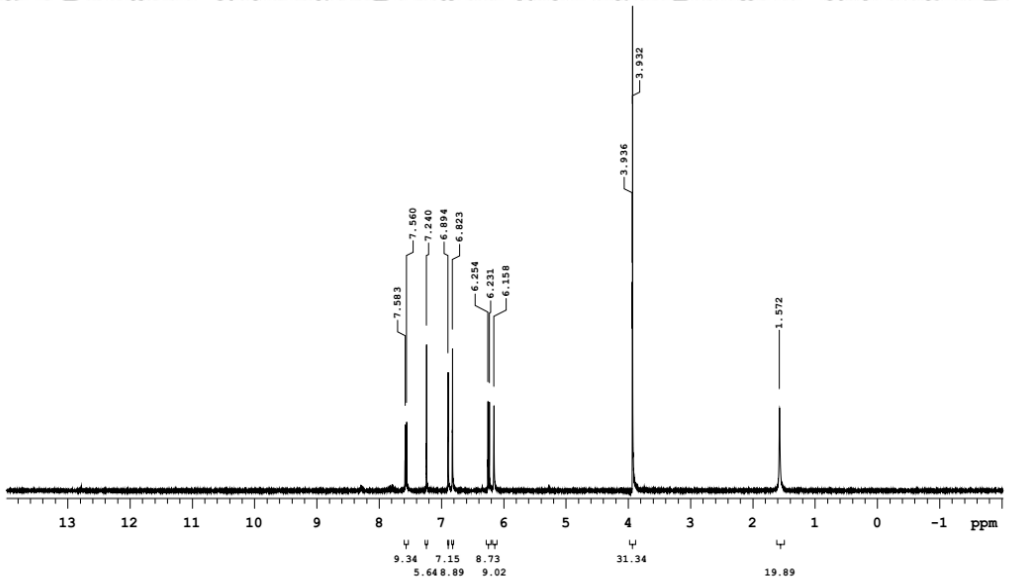


Figure 26. ^{1}H NMR spectrum of compound **C1** (400 MHz, $CDCl_3$)

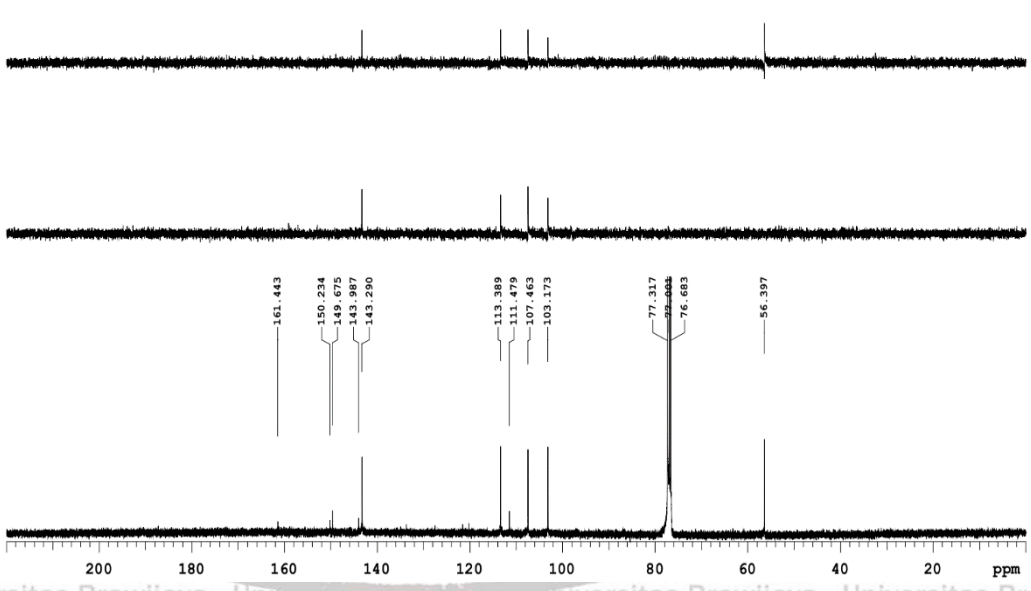


Figure 27. ^{13}C and DEPT NMR spectra of compound **C1** (100 MHz, $CDCl_3$)

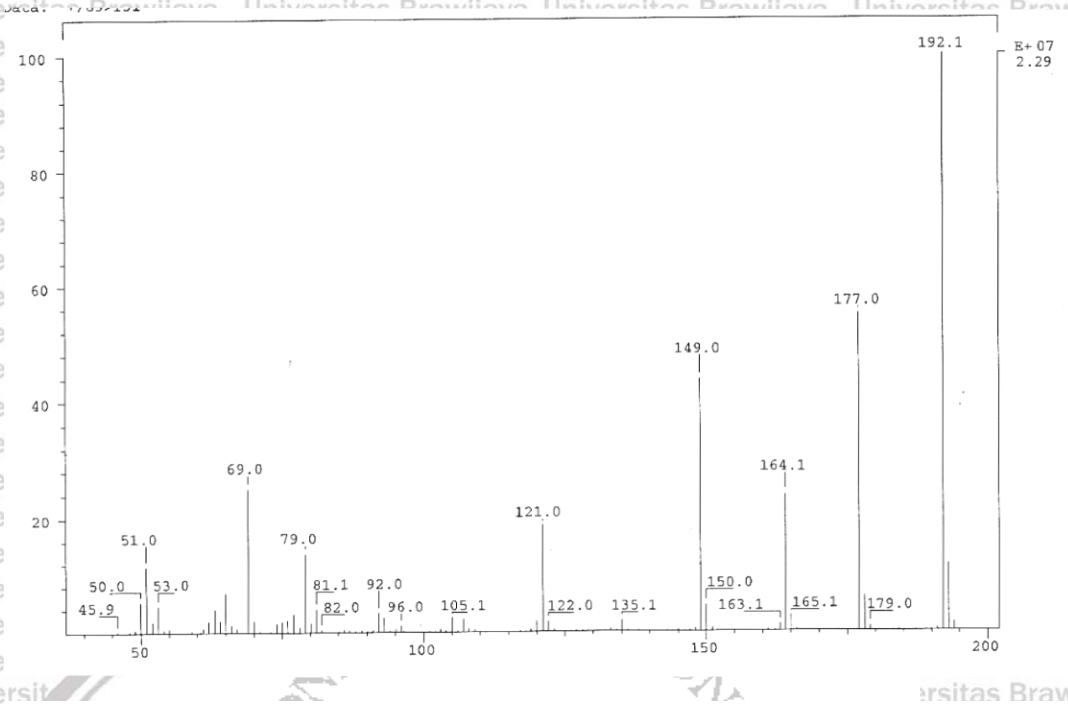


Figure 28. EI-MS spectrum of compound **C1**

4.2.3.2. Compound C2

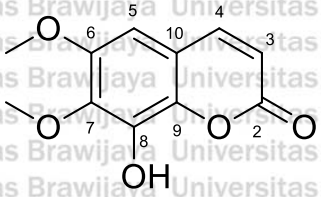


Figure 29. Structure of compound **C2** with a carbon number

Compound **C2** ($C_{11}H_{10}O_5$) was isolated as brown needles with melting point range 172 - 174 ^oC. The structure of compound **C2** is shown in Figure 29.

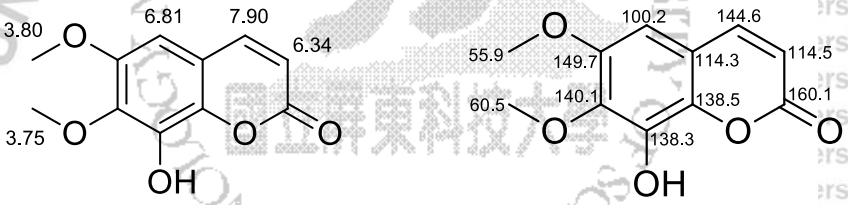
The ^1H NMR spectrum for compound **C2** in Figure 30 and Table 11 displayed two methoxy signals at δ_H 3.80 (3H, s) and δ_H 3.75 (3H, s). A pair of doublets appeared at δ_H 6.34 (1H, J = 9.6 Hz) and δ_H 7.90 (1H, J = 9.6 Hz) and were assigned to H-3 and H-4 respectively. The spectrum also showed singlet signal at δ_H 6.81 (1H) assignable to H-5.

The ^{13}C NMR and DEPT spectral data (Figure 31 and Table 11) showed a pair of methoxy groups at δ_C 55.9 and 60.5. The ketone group appearance at δ 160.1, then the three methines showed at δ_C 114.3 (CH, C-3), 144.6 (CH, C4), and 100 (CH, C-5). The six-carbon quarternaries showed at δ_C 160.1 (C-2), 149.7 (C-6), 140.1 (C-7), 138.3 (C-8), 138.5 (C-9), and 114.5 (C-10).

According to Yasuda et al. (2006), compound **C2** has a molecular ion *m/z* 222.05 $[M]^+$ that corresponds to the molecular formula $C_{11}H_{10}O_5$. The ^1H and ^{13}C spectrum of compound **C2** were identified as fraxidin by spectroscopic data from Table 11 and compared the data from Yasuda et al. (2006).

Table 11. ^{1}H and ^{13}C NMR spectral data for compound **C2**, (400 MHz, DMSO-d_6)

Position	δ_H (*J* in Hz) (ppm)	δc (ppm)	δ_H (*J* in Hz) (ppm) by Yasuda et al. (2006)	δc (ppm) by Yasuda et al. (2006)
2		160.1		160.0
3	6.34 d (9.6)	114.3	6.37 d (9.6)	114.2
4	7.90 d (9.6)	144.6	7.93 d (9.6)	144.6
5	6.81 s	100.2	6.83 s	100.0
6		149.7		149.6
7		140.1		140.0
8		138.3		138.3
9		138.5		138.4
10		114.5		114.4
6-OCH_3	3.80 s	55.9	3.82 s	55.8
7-OCH_3	3.75 s	60.5	3.77 s	60.4



^{1}H and ^{13}C NMR spectral data for compound **C2**

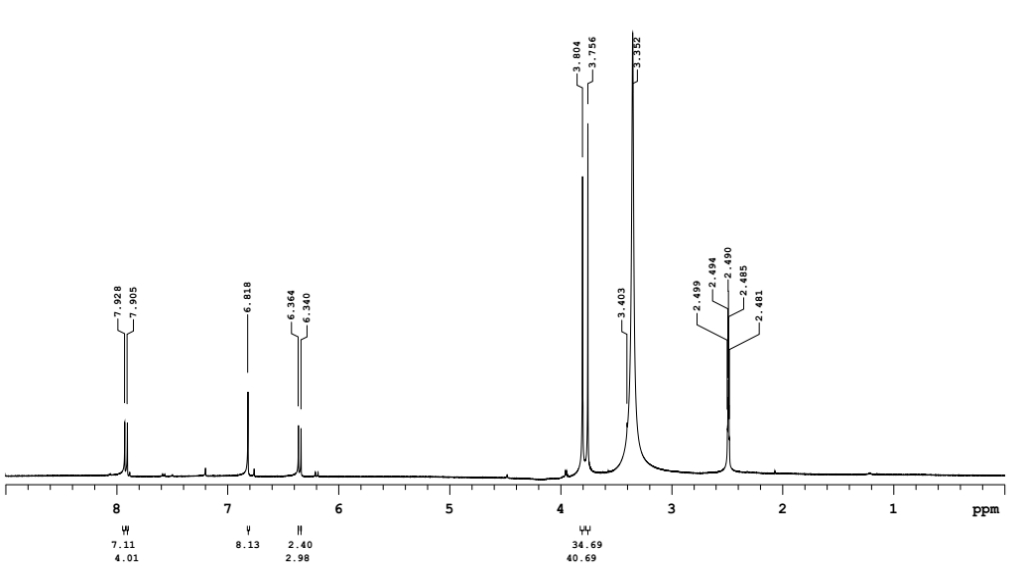


Figure 30. ^{1}H NMR spectrum of compound **C2** (400 MHz, DMSO-d_6)

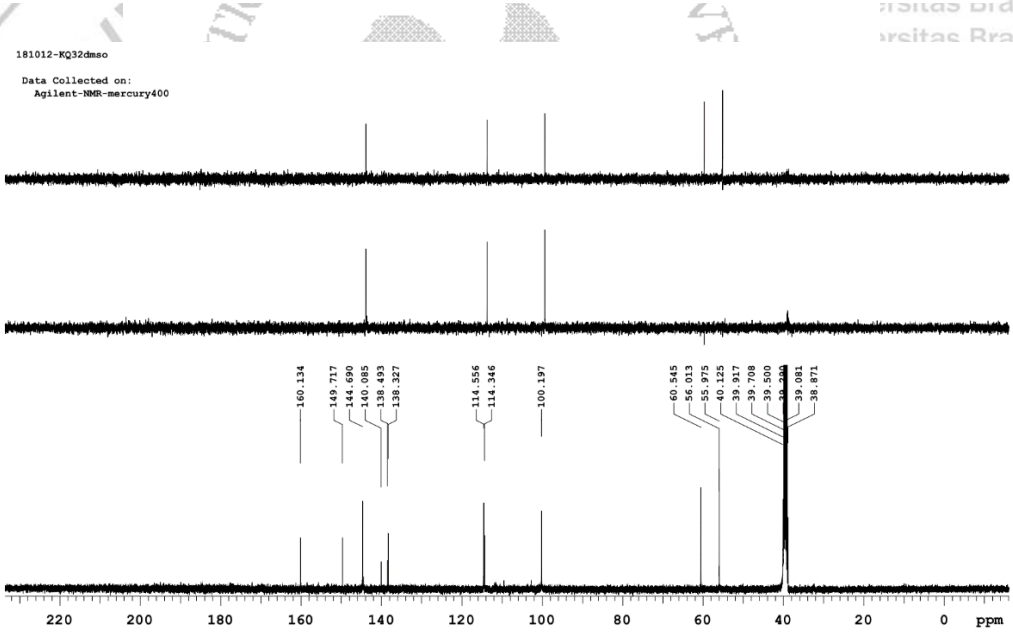


Figure 31. ^{13}C and DEPT NMR spectra of compound **C2** (400 MHz, DMSO-d_6)

4.2.4. Aromatic

4.2.4.1. Compound A1

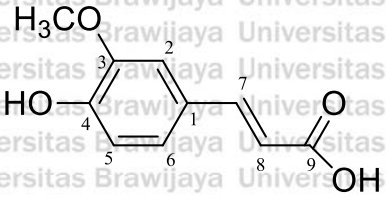


Figure 32. Structure of compound **A1** with a carbon number

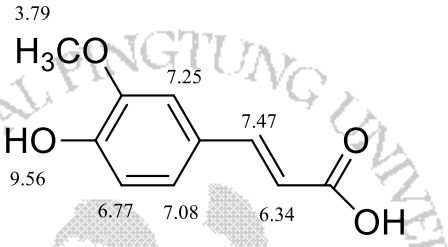
Compound **A1** was purified as a white powder from fraction 22 with a melting point 174 ^{o}C. The structure of compound **A1** is shown in Figure 32.

The ^{1}H NMR spectrum (Figure 33, Table 12), showed three proton signals at δ_H 6.77 (1H, d, J = 8.0 Hz, H-5), 7.08 (1H, dd, J = 1.6 and 8.0 Hz, H-6) and δ 7.25 (1H, d, J = 1.6 Hz, H-2) which display a characteristic of a trisubstituted aromatic ring. Two trans ethenyl protons signals appeared at δ_H 6.34 (H-8) and δ 7.47 (H-7) with a coupling constant of 16 Hz. The spectrum also revealed a methoxy signal at δ_H 3.79. The hydroxyl group signal appeared at δ_H 9.56.

The EI-MS spectrum exhibited molecular ion peak at *m/z* 194.19 $[M]^+$ (Figure 34) which correspond to the molecular formula $C_{10}H_{10}O_4$. The spectral data of compound **A1** is corresponding with that from Wang et al. (2015) for ferulic acid.

Table 12. ^{1}H NMR data for compound **A1**, (400 MHz, DMSO-d_6)

Position	δ_H (*J* in Hz) (ppm)	δ_H (*J* in Hz) (ppm) by Wang et al. (2015)
2	7.25 d (1.6)	7.27 d (2.1)
5	6.77 d (8.0)	6.79 d (8.1)
6	7.08 dd (1.6 and 8.0)	7.08 dd (1.8 and 8.0)
7	7.47 d (16)	7.50 d (15.8)
8	6.34 d (16)	6.38 d (15.8)
3-OCH_3	3.79 s	3.80 s
4-OH	9.56 s	



^{1}H NMR spectral data for compound **A1**

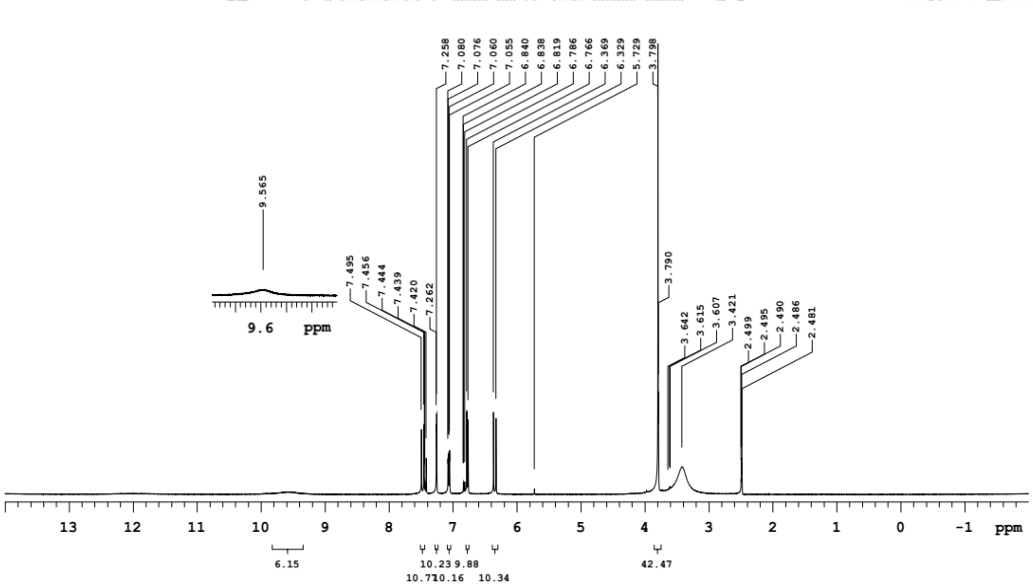


Figure 33. ^{1}H NMR spectrum of compound **A1** (400 MHz, DMSO-d_6)

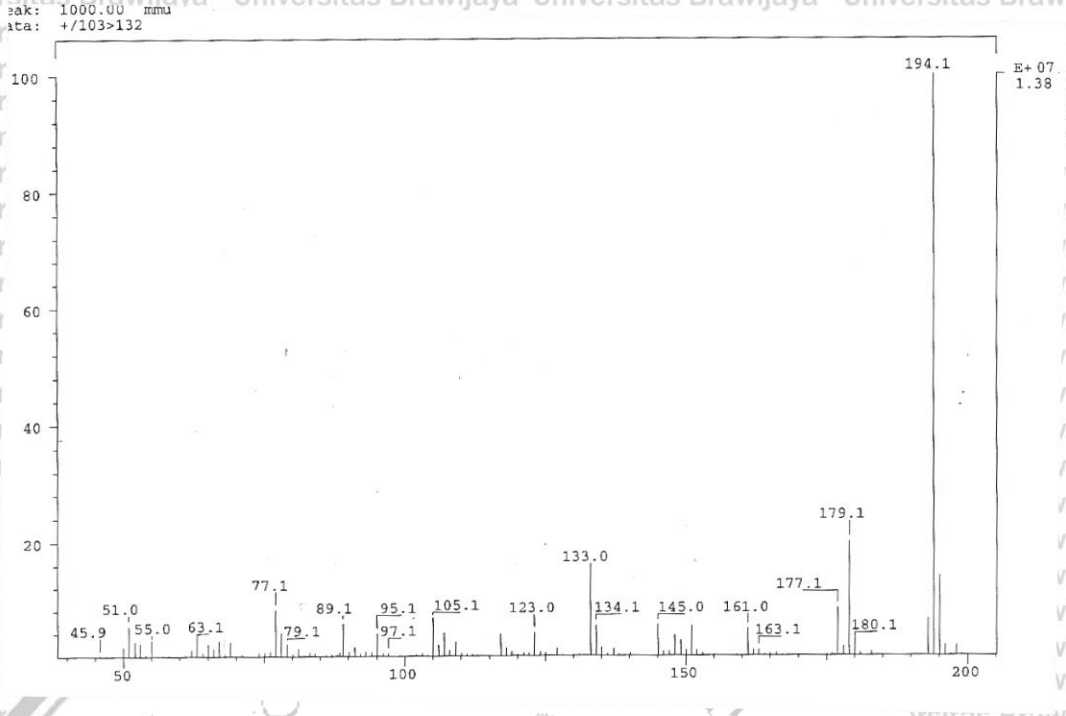


Figure 34. EI-MS spectrum of compound **A1**

4.2.4.2. Compound A2

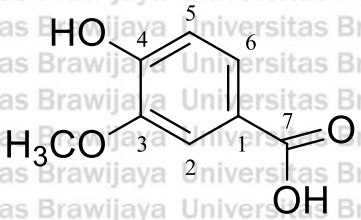


Figure 35. Structure of compound **A2** with a carbon number

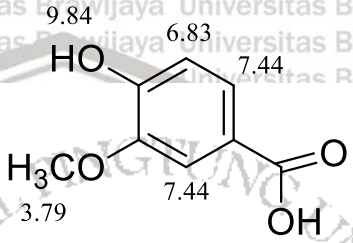
Compound **A2** ($C_8H_8O_4$) was isolated as a brown needle with melting point 210-213 ^oC. The structure of compound **A2** is shown in Figure 35.

The ^1H NMR spectrum of compound **A2** (Figure 36, Table 13) showed a singlet at δ_H 3.79 which indicated the presence of an oxygenated methyl group. Furthermore, the spectrum displayed a multiplet and doublet signals at δ 7.44-7.41 (2H, H-2 and H-6) and 6.83 (1H, H-5) respectively, which indicated a characteristic of an ABX system. In addition, a hydroxyl group signal appeared at δ 9.84 and was assigned to 4-OH.

The molecular formula of compound **A2** was $C_8H_8O_4$ that corresponds to the molecular ion signal at *m/z* 168 $[M]^+$ from the EI-MS in Figure 37. The compound was identified as vanillic acid by comparing its spectral data with the data from Phan Duc et al. (2016).

Table 13. ^{1}H NMR data for compound **A2**, (400 MHz, DMSO-d_6)

Position	**δ_H (*J* in Hz) (ppm)**	**δ_H (*J* in Hz) (ppm)** by Phan Duc et al. (2016)
2	7.44 m	7.44 m
5	6.83 d (8.4)	6.83 d (9.0)
6	7.44 m	7.44 m
3-OCH_3	3.79 s	3.55 s
4-OH	9.84 s	



^{1}H NMR spectral data for compound **A2**

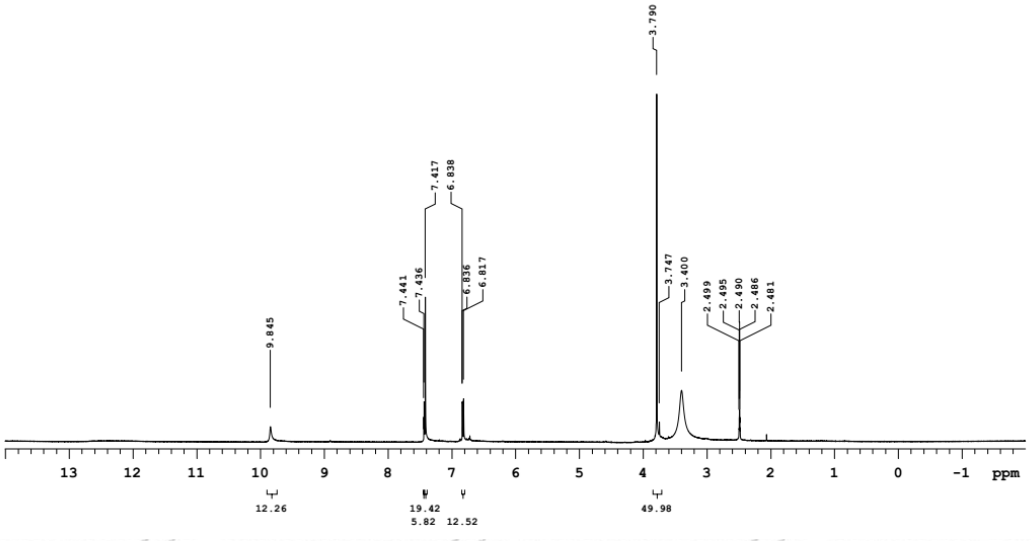


Figure 36. ^{1}H NMR spectrum of compound **A2** (400 MHz, DMSO-d_6)

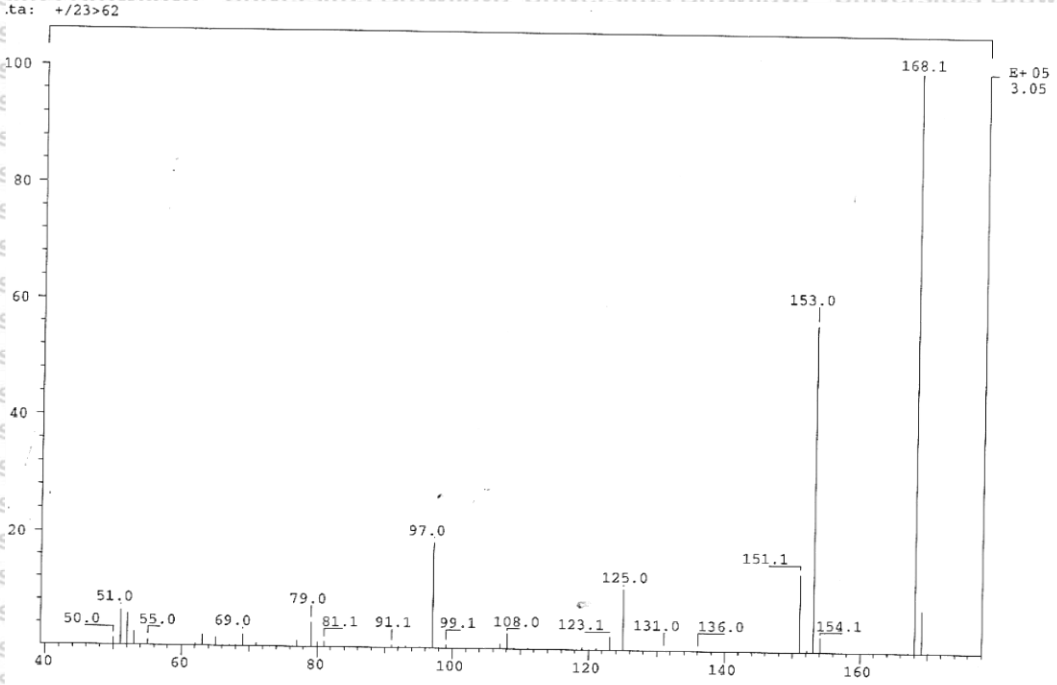


Figure 37. EI-MS spectrum of compound **A2**

4.3. The yield of the chemical constituent

The yield of the chemical constituents obtained from the crude methanol extract is displayed in Table 14. The methanol crude extract contained steroids, anthraquinones, coumarins, and aromatics.

Table 14. Weights of isolated compounds

Compound	Weight (mg)
S1	1854
S2	418
An1	39
An2	420
An3	2846
An4	97
C1	637
C2	20
A1	68
A2	398

The results indicated that the ethyl acetate layer from the crude methanol extract contains the dominant anthraquinone compound as evidenced by the compound **An3**, it has the highest yield, obtained from the fraction 22.

According to Duval et al. (2016) and Diaz-Muñoz et al. (2018), many anthraquinones can be found in wide range of species, especially in the families' Rubiaceae, Polygonaceae, and Rhamnaceae. Anthraquinones are a group of compounds abundant in the universe of natural substances. They are classified as quinones, and their derivatives are the largest group of natural quinones. Benzoquinones and naphthoquinones are also part of this group. Anthraquinones constitute the largest group of natural pigments, with approximately 700 compounds described. About 200 of these compounds were isolated from plants, while the rest were isolated from lichens and fungi. They are found in all plant parts: roots, rhizomes, fruits, and flowers.

Most of these compounds are derived from the basic structure 9,10-anthracenedione, a tricyclic aromatic organic compound.

Anthraquinones also has a broad range of bioactivities such as anticancer, anti-inflammatory, immunosuppressive, antimicrobial, diuretic, cathartic, laxative, vasorelaxant, antioxidant and phytoestrogen activities. Furthermore, the presence of steroids, aromatics, and coumarins support the extent of biological activity by the extract of the plant in various studies. Jalhan (2017) and Sova (2012) discovered that coumarins and cinnamic acids exhibit a wide range of pharmacological activities, which includes anti-diabetic, anti-viral, anti-microbial, anticancer, antioxidant, antiparasitic, anti-helminthic, antiproliferative, anti-inflammatory and antihypertensive activities. In addition, steroids were reported to possess antioxidant and antiviral (Parvez et al., 2018).

4.4. Antibacterial activities

4.4.1. Agar well diffusion assay

In this study, the crude extract, partition samples, fractions, and compounds isolated were tested the antibacterial activity against human pathogens following bio-guided fractionation. The results are displayed in Figure 38 and Figure 39.

The crude extract was very effective in the inhibition of bacteria growth against *E. coli* (9.5 mm) and *S. aureus* (10.5 mm). In particular, partition samples showed selective activity. The ethyl acetate layer showed the highest activity against bacteria strains with an inhibition zone of 11 mm and 11.5 mm against *E. coli* and *S. aureus* respectively. Whereas, BuOH and water layer did not show activity against *E. coli* and *S. aureus*. The negative control, DMSO did not show any inhibition zone against all the test strains. The positive control, streptomycin,

showed high inhibition zone 20.5 mm against *E. coli* and *S. aureus* respectively.

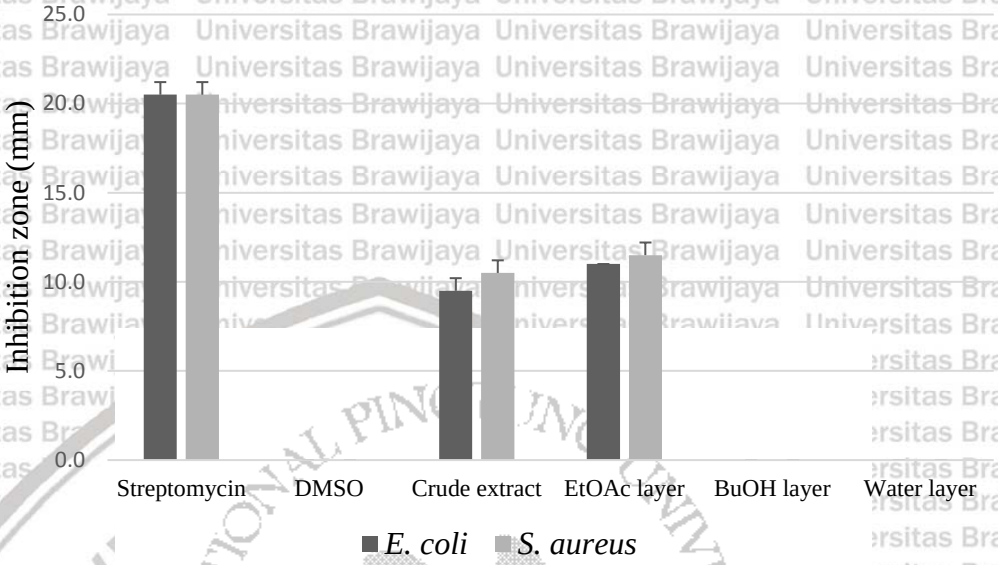


Figure 38. The antibacterial activity of crude extract and partitions (2 mg/mL) against *E. coli* and *S. aureus* with streptomycin as control

Meanwhile, most of the fractions showed the antibacterial activity against test strains as shown in Figure 39 and Appendix 2. The high polar fractions (such as Fraction 15 – 26) where most of the compounds isolated from, showed the activity against test strains. The Fraction 21 displayed the highest inhibition zone (15.7 mm for *E. coli*; 16.7 mm for *S. aureus*), then followed by fraction 18 (14.5 mm) against *E. coli*, and fraction 14 (15.3 mm) against *S. aureus*. However, several low polar fractions showed selective activity. Fraction 1 and 5 were active on one of two test strains (*S. aureus* and *E. coli* respectively). Rath and Padhy (2015) reported that the ethyl acetate, ethanol, and methanol extract of *P. foetida* leaf were highly effective at inhibiting eight enteropathogens (*Enterobacter aerogenes, Escherichia coli, K. pneumoniae, S. paratyphi, S. typhi, Shigella dysenteriae, S. sonnei,* and *V. cholerae*).

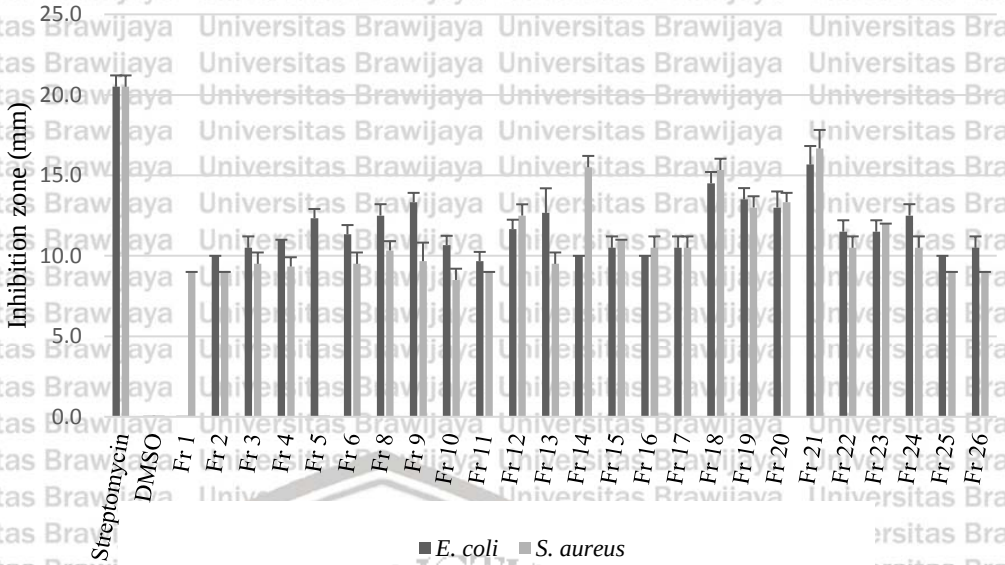


Figure 39. Antibacterial activity of the fractions (2 mg/mL) against *E. coli* and *S. aureus*.

4.4.2. Minimum Inhibitory Concentration (MIC) using resazurin assay

The MIC was determined using a microdilution technique with resazurin as an indicator of cell growth. Seven compounds isolated from the EtOAc layer which include four anthraquinones, one coumarin, and two aromatics were observed the minimum inhibition concentration against pathogenic bacteria and the results are shown in Table 15.

Table 15. MIC determination using the resazurin assay

Compound	MIC µg/ mL			
	E. coli	***B. subtilis***	***S. aureus***	***Salmonella enterica***
Streptomycin	15	15	1.875	15
Compound **An1**	600	300	>600	300
Compound **An2**	300	150	18.75	300
Compound **An3**	150	150	150	300
Compound **An4**	150	150	9.37	300
Compound **C1**	150	300	>600	150
Compound **A1**	300	300	150	150
Compound **A2**	300	150	75	150

The results exhibit that Gram-positive bacteria are selectively inhibited by compounds isolated from the stem of *P. foetida*. Seven compounds isolated from fractions possessed antibacterial activity against four human pathogens and three of compounds had MIC values less than 100 µL/mL. Regarding the MIC value, most of the test strains showed similar susceptibility to the compounds. Except for the *S. aureus* strains, was more susceptible to streptomycin as control and compound **An2**, **An4,** and **A2**. According to Comini et al. (2011), plant compounds are classified as "antimicrobial", if they have MIC values in the range of 100-1000 µg/mL and others of a higher magnitude than those of typical antibiotics (0.01-10 µg/mL). Plants compounds having MICs equal or lower to 100 µg/mL are considered good antimicrobial agents.

The previous study from Lu et al. (2011), the antibacterial activity of the anthraquinone derivatives might be related to the type of substituent groups on the molecular structure. The presence of the polar functional group (carboxyl, hydroxyl, and hydroxymethyl) can increase antibacterial activity. Although compound **An2** and **An4** have the same amount of polar functional groups, the methoxy in compound **An2** might weaken their antibacterial activity. This suggestion also might affect in compound **A2** which has methoxy group in its aromatic ring. Lu et al. (2011) also reported that anthraquinone derivative such as emodin can increase membrane permeabilization and caused leakage of intracellular contents. Their study also has demonstrated that anthraquinone derivatives could inhibit macromolecular synthesis in cells.

In addition from Pretto Juliana et al. (2004), the pattern of chemical selectivity to against Gram-positive bacteria is not restricted to compounds from plants but is a general phenomenon observed among most antibiotics.

However, the low antibacterial activity of isolated compounds against Gram-negative bacteria also can be explained by Pretto Juliana et al. (2004). The outer membrane of Gram-negative bacteria is known to present a barrier to the penetration of antibiotic agents. The periplasmic space contains several enzymes which are able to break down foreign molecules introduced from outside. Several Gram-negative organisms also display high-level resistance to a range of antimicrobial agents and support a role for the outer membrane and active efflux as a barrier to antibiotics.

5. CONCLUSION

The crude methanol extract of *P. foetida* has been investigated for antibacterial properties. In this study, ten bioactive compounds including four anthraquinones, two coumarins, two aromatics, and two steroids were isolated from the ethyl acetate layer of the crude extract. The isolated compounds were identified as morindaparvin A, 1,3-dihydroxy-2-methoxyanthraquinone, alizarin, digiferrol, scopoletin, ferulic acid, vanillic acid, fraxidin, β-sitosterol, and stigmastan-3-one.

The result of the research indicates that the extract of *P. foetida* has antibacterial activity against the tested *E. coli* and *S. aureus*. This is supported by the significant antibacterial effect of the fractions. In addition, these results indicate that *S. aureus* is more susceptible to 1,3-dihydroxy-2-methoxyanthraquinone, digiferrol, and vanillic acid.

Nevertheless, the compounds still need to continue investigation such as cytotoxicity before they can be developed into new medical agents.

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Appendix

Appendix 1. Summary of the chemical constituents' spectral data

β-Sitosterol **(S1)**

White needles; mp 136 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.77 (3H, d, *J* = 6.8 Hz, CH_3-26), 0.80 (3H, d, *J* = 7.2 Hz, CH_3-27), 0.83 (3H, t, *J* = 7.2 Hz, CH_3-29), 0.89 (3H, d, *J* = 6.4 Hz, CH_3-21), 0.98 (3H, s, CH_3-19), 3.50 (1H, m, CH-3), 5.33 (1H, br s, H-6). EI-MS (70 eV) *m/z* (%): 412 $[M]^+$ (100), 396 (81), 381 (70), 303 (72), 213 (72), 145 (83), 69 (100).

Stigmastan-3-one **(S2)**

White needles; mp 157-159 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.65 (3H, s, CH_3-18), 0.92 (3H, s, CH_3-19), 0.89 (3H, d, *J* = 6.8 Hz, CH_3-21), 0.79 (3H, d, *J* = 6.8 Hz, CH_3-26), 0.81 (3H, d, *J* = 7.6 Hz, CH_3-27), 0.83 (1H, t, *J* = 7.6 Hz, CH_3-29). EI-MS (70 eV) *m/z* (%): 414 (94), 400 (62), 396 (46), 382 (36), 351 (26), 329 (36), 303 (40), 273 (46), 255 (88), 231 (38), 213 (70), 199 (30), 173 (36), 163 (42), 159 (74), 145 (82), 133 (68), 95 (80), 81 (88), 55 (100).

Morindaparvin A **(An1)**

Yellow powder; mp 255-257 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.30 (2H, s, CH_2), 7.12 (1H, d, *J* = 8.0 Hz, H-3), 7.76 (2H, m , H-6,7), 7.96 (1H, d, *J* = 8.0 Hz, H-4), 8.28 (2H, m, H-5,8). EI-MS (70 eV) *m/z* (%): 252 $[M]^+$ (100), 223 (8), 196 (4), 168 (6), 138 (18), 112 (4), 83 (2), 76 (5).

1,3-dihydroxy-2-methoxyanthraquinones **(An2)**

Yellow crystal; mp 218-220 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.12 (3H, s, 2-OCH_3), 6.51 (1H, s, 3-OH), 7.76 (2H, m , H-6,7), 7.44 (1H,

s, H-4), 8.25 (2H, m, H-5,8), 13.11 (1H, s, 1-OH). EI-MS (70 eV) *m/z* (%): 270 $[M]^+$ (100), 252 (58), 227 (52), 199 (22), 196 (24), 115 (20), 77 (6).

Alizarin **(An3)**

Orange powder; mp 279-283 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.22 (1H, d, *J* = 8.4 Hz, H-3), 7.65 (1H, d, *J* = 8.4 Hz, H-4), 7.91 (2H, m, H-6,7), 8.21 (2H, m, H-5,8), 10.95 (1H, s, 2-OH), 12.60 (1H, s, 1-OH). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 150.8 (C-1), 152.8 (C-2), 123.0 (C-3), 120.9 (CH-4), 126.8 (CH-5), 135.2 (CH-6), 134.2 (CH-7), 126.6 (CH-8), 188.9 (C-9), 180.7 (C-10), 121.2 (CH-4a), 133.6 (C-8a), 116.3 (C-9a), 132.9 (C-10a). EI-MS (70 eV) *m/z* (%): 240 (100), 239 (16), 121 (18), 184 (12), 138 (12), 128 (12), 127 (10), 77 (8), 51 (4).

Digiferrol **(An4)**

Red powder; mp 200-202 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.33 (1H, d, *J* = 2.2 Hz, H-3), 8.19 (2H, m, H-5,8), 7.92 (2H, m, H-6,7), 12.99 (1H, s, 1-OH), 12.73 (1H, s, 2-OH), 4.59 (2H, d, *J* = 2.2 Hz, 2-CH_2), 5.60 (1H, t, *J* = 5.2 Hz, 2-OH,). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 154.3 (C-1), 144.7 (C-2), 124.6 (C-3), 126.7 (C-5), 135.0 (C-6), 135.2 (C-7), 126.7 (C-8), 186.1 (C-9), 187.0 (C-10), 111.0 (C-4a), 133.0 (C-8a), 111.7 (C-9a), 132.9 (111,7), 157.0 (C-4), 57.6 (2'-CH_2). EI-MS (70 eV) *m/z* (%): 270 (100), 252 (84), 241 (26), 224 (40), 196 (50), 185 (18), 168 (30), 139 (42), 77 (18), 66 (16), 51 (8).

Scopoletin **(C1)**

Colorless needle; mp 206-207 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.24 (1H, d, *J* = 9.2 Hz, H-3), 7.57 (1H, d, *J* = 9.2 Hz, H-4), 6.89 (1H, s, H-5), 6.82 (1H, s, H-8), 3.93 (1H, s, 6-OCH_3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 161.4 (C-2), 113.3 (CH-3), 143.2 (CH-4), 107.4 (CH-5),

143.9 (C-6), 149.6 (C-7), 103.1 (CH-8), 150.2 (C-9), 111.4 (C-10), 56.3 (OCH_3). EI-MS (70 eV) *m/z* (%): 192 (100), 177 (56), 164 (24), 149 (44), 121 (20), 79 (14), 69 (26), 51 (12).

Fraxidin **(C2)**

Brown needle/crystal; mp 196-197 ^oC; ^1H NMR (400 MHz, DMSO-d_6) 6.34 (1H, d, *J* = 9.6 Hz, H-3), 7.90 (1H, d, *J* =9.6 Hz, H-4), 6.81 (1H, s, H-5), 3.80 (1H, s, 6-OCH_3), 3.75 (1H, s, 7-OCH_3). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 160.1 (C-2), 114.3 (CH-3), 144.6 (CH-4), 100.2 (CH-5), 149.7 (C-6), 140.1 (C-7), 138.3 (C-8), 138.5 (C-9), 114.5 (C-10), 55.9 (6-OCH_3), 60.5 (7-OCH_3).

Ferulic acid **(A1)**

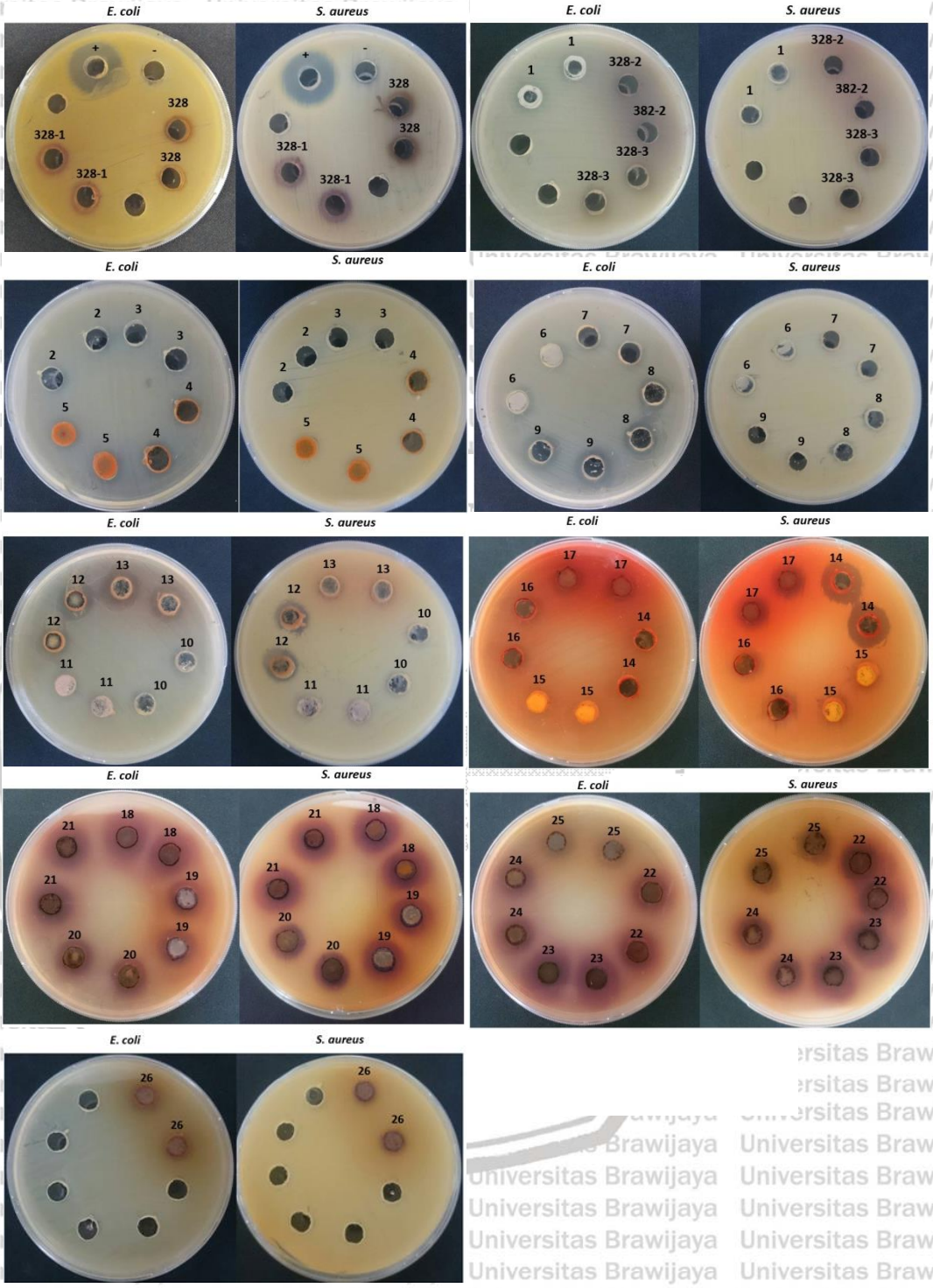
White powder; mp 168-172 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ: 7.25 (1H, d, *J* = 1.6 Hz, H-2), 6.77 (1H, d, *J* = 8.0 Hz, H-5), 7.08 (1H, dd, *J* = 1.6 and 8.0 Hz, H-6,), 7.47 (1H, d, *J* = 16 Hz, H-7), 6.34 (1H, d, *J* = 16 Hz, H-8), 3.79 (1H, s, 3-OCH_3), 9.56 (1H, s, 4-OH). EI-MS (70 eV) *m/z* (%): 194 (100), 179 (20), 177 (8), 133 (18), 77 (8), 51 (6).

Vanilic acid **(A2)**

Brown needle/crystal; mp 210-213 ^oC; ^1H NMR (400 MHz, DMSO-d_6) δ: 7.44 (2H, m, H-2 and 6), 6.83 (1H, d, *J* = 8.4 Hz, H-5), 3.79 (1H, s, 3-OCH_3), 9.84 (1H, s, 4-OH). EI-MS (70 eV) *m/z* (%): 168 (100), 153 (56), 151 (14), 125 (12), 97 (18), 79 (4), 51 (6).

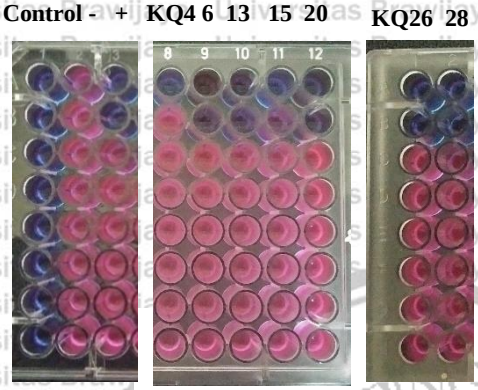
Appendix 2. The result of agar well diffusion assay

Crude extract / Fractions	*E. coli*	*S. aureus*
Streptomycin	20.5	20.5
DMSO	0.0	0.0
Crude extract	9.5	10.5
EtOAc layer	11.0	11.5
BuOH layer	0.0	0.0
Water layer	0.0	0.0
Fr 1	0.0	9.0
Fr 2	10.0	9.0
Fr 3	10.5	9.5
Fr 4	11.0	9.3
Fr 5	12.3	0.0
Fr 6	11.3	9.5
Fr 7	11.3	10.3
Fr 8	12.5	10.3
Fr 9	13.3	9.7
Fr 10	10.7	8.5
Fr 11	9.7	9.0
Fr 12	11.7	12.5
Fr 13	12.7	9.5
Fr 14	10.0	15.5
Fr 15	10.5	11.0
Fr 16	10.0	10.5
Fr 17	10.5	10.5
Fr 18	14.5	15.3
Fr 19	13.5	13.0
Fr 20	13.0	13.3
Fr 21	15.7	16.7
Fr 22	11.5	10.5
Fr 23	11.5	12.0
Fr 24	12.5	10.5
Fr 25 (EA)	10.0	9.0
Fr 26 (E/M)	10.5	9.0



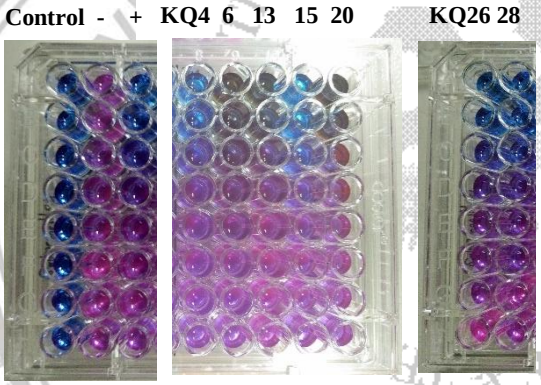
Appendix 3. The result of MIC with the resazurin assay utilizing 96 microtitre-plate

E. coli



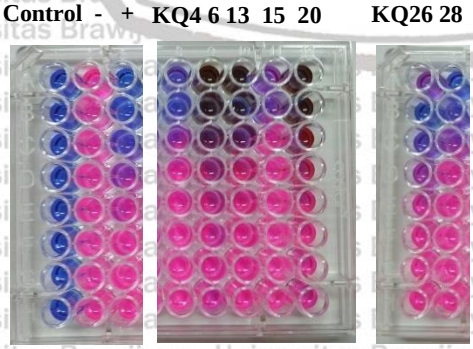
Concentration :
Control + : 60 – 0.468 µg/mL
Compounds: 600 – 4.68 µg/mL

Salmonella enterica



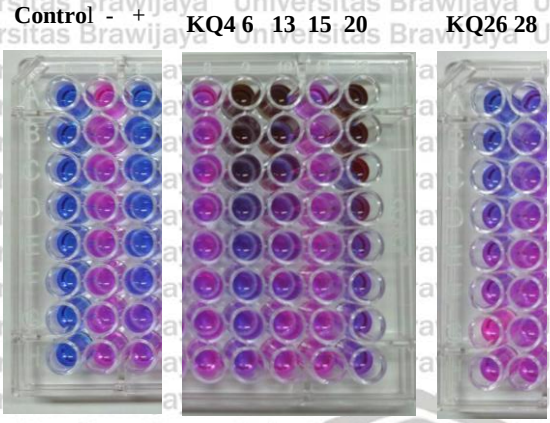
Concentration :
Control + : 60 – 0.468 µg/mL
Compounds: 600 – 4.68 µg/mL

B. subtilis



Concentration :
Control + : 60 – 0.468 µg/mL
Compounds: 600 – 4.68 µg/mL

S. aureus



Concentration :
Control + : 60 – 0.468 µg/mL
Compounds: 600 – 4.68 µg/mL

Concentration :
Control + : 3.75 – 0.0146 µg/mL
Compounds: 37.5 – 0.146 µg/mL

KQ4: Compound **An1**
KQ6: Compound **An2**
KQ13: Compound **An3**
KQ15: Compound **C1**
KQ20: Compound **An4**
KQ26: Compound **A2**
KQ28: Compound **A1**

Biosketch of Author

Personal Information

Name:	Aryandi Ramadhan
Date of Birth:	1st March 1993
Place of Birth:	Surabaya, Indonesia
Nationality:	Indonesia
Gender:	Male
Email:	Aryandir93@gmail.com

Education Background

2016-2018:	Master of Science Degree in Biological Science and Technology. Major in Natural Products Chemistry. National Pingtung University of Science and Technology. Master of Science Degree in the Faculty of Agricultural Technology. Major in Food Science. Brawijaya University.
2011-2015:	Bachelor Science Degree in the Faculty of Fisheries Marine Science. Major in Fisheries product Technology. Brawijaya University.