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Submission date: 30-May-2019 11:03PM (UTC-0700)
Submission ID: 1138233769
File name: jpsr09041714.pdf (1.83M)
Word count: 3764
Character count: 20637

Laode Rijai et al /J. Pharm. Sci. & Res. Vol. 9(4), 2017, 420-428

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Secondary Metabolites Compounds Profile by LC-MS/MS and Chemical Methods from Kolowe Stem Bark (*Chydenanthus excelsus*)

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Abstract

Secondary metabolites's profiles from stem bark of Kolowe (*C. excelsus*) are steroidal saponin and glycoside's flavonoids, which consists of five kinds of steroidal saponins and eight types of flavonoids glycoside's compounds. Both these classes of compounds in the chemical identification and analysis on the results by LC-MS/MS. Pharmaceutical potential that has been revealed from a mixture of secondary metabolites compounds such are cytotoxic, antioxidants and antimicrobials activity. Information of cytotoxic activity during the test results with bio-indicators of the third instar larvae of *A. salina*, antioxidant activity with DPPH radical compounds test indicators, while the antimicrobial is a result of screening against 19 types of microbes (bacteria and fungi). Other potential methanol crude extracts from stem bark of Kolowe (*C. excelsus*) is the yield that is 8.97%, which is extracted by cold maceration method using methanol 96%.

Keywords;*Stem bark of Kolowe (C. excelsus);Secondary metabolites profile, pharmaceuticals potency.*

INTRODUCTION

Study of pharmaceutical potential kolowe plant (*C. excelsus*) continues to be done because it has the traditional benefits that are interesting to study as a fish poison, the first plant is an endangered species in the world. Studies have been reported typed of compound of the content of seeds [4],[5]. The profile of chemical compounds from the leaves to the molecular weight [6], pharmaceutical potential of seed and leaves extracts [5], [6]. Pharmaceutical potentials of seeds and leaves are reported to antioxidant activity, cytotoxic, and antimicrobial. The study results were reported on the leaves are still limited in the analysis of secondary metabolites's content in the extract is methanol and ethyl acetate fraction as well as the testing of biological activity is confined to the antioxidant, cytotoxic and antimicrobial [6]. Isolation saponins and flavonoids glycosides suspected in methanol and ethyl acetate extract fraction of the leaves are underway and will be reported right away if that have found good and reliable. Usefulness of the data content of secondary metabolites to the type of compound is for the purposes as the study the mechanism of action of these compounds as an active compound antioxidant and cytotoxic or other biological activities. The analysis of secondary metabolites's content in the extract is methanol and ethyl acetate fraction as well as the testing of biological activity is confined to the antioxidant, Cytotoxic and antimicrobial. Assessment of the working mechanism of molecular compounds with methods of abortion performed at least insilico against a number of receptors associated with biological activity possessed. In addition, if the content of secondary metabolites kolowe whole plant parts (seeds, fruit peel, leaves, flowers, bark, stems, and roots) have been revealed to the type of compound, useful in the preparation of plant Chemotaxonomic kolowe as a rare species. Similarly, study of potential pharmaceutical will remain expanded apart as an antioxidant, cytotoxic, antimicrobial to all parts through the plant. This article reports secondary metabolites profiles and some biological activity of the bark, which has not been reported. Profile of secondary metabolites disclosed chemistry and analytical LC-MS/ MS, while the biological activity as a potential pharmacy reported antioxidant activity against DPPH radical compounds, cytotoxic against *A. salina* larvae as well as screening of antimicrobial against 19 microbial bacteria and fungi. Biological activities as potential pharmaceutical plants reported were similar to those reported with its leaf extract. [6]. The similarity of the traditional use of fruit seeds, leaves, and bark is one reason the assessment of parameters of the same activities, but then will continue the study of other parameters of potential pharmaceutical ingredients. Kolowe (*C. excelsus*) including plants around the world [2], However, still abundant in Kamaru Buton islands, Indonesia; because the plant seeds used by the community as a catcher fish into the sea so that the public tends to preserve it. These plants are fruiting, continuous and fruitful have much is a potential, so that the community continues to preserve it. The following plants Kolowe (*C. excelsus*) from Kamaru, Buton islands, Indonesia has also been reported earlier in the article about the seeds and leaves.

Figure 1. Kolowe plants at Kamaru, Buton, Southeast Sulawesi, Indonesia

MATERIALS AND METHOD

The research material used was 96% methanol as a solvent, silica gel GF60, reagent for identification of secondary metabolites, DPPH test indicators and eggs of *A. salina*. Furthermore, the material under study is bark kolowe (*C. excelsus*). The research variables are profiles of secondary metabolites content to the sample based on the reaction reagents and spectral characteristics with LC-MS / MS, and the variable potency of pharmaceutical extracts, antioxidant activity, cytotoxic, antimicrobial and stability. The research activities are carried out are (1) taking the bark of plants of locations: the islands of Buton, Indonesia as a sample (2) the processing of the sample to obtain a dry powder of bark kolowe (3) extraction (4) identification of groups of secondary metabolites (5) The profile analyzes the compound secondary metabolites content of the samples using LC-MS / MS (6) test the antioxidant activity, cytotoxic, and antimicrobials, as well as testing the stability of both activities on the terms at the storage time.

Plant materials

Samples bark of Kolowe (*C. excelsus*) taken from Kamaru, islands Buton, Southeast Sulawesi, Indonesia in August 2015, along with samples of leaves that had been reported previously. The bark is taken from twigs or branches of trees, which are considered not interfere with the survival of the tree. Twig that has been cut from the mother tree was washed with running water (rivers around the site) and then the bark is peeled as a sample. Furthermore, the bark of the chopped up the vast size of 2-4 cm and dried in direct sunlight and continued with oven until the moisture content reaches about 5-10%. Samples were dried barks are crushed and ready for extraction. Dry powder bark of kolowe obtained was 3.84 kg.

Extraction

Cold maceration extraction method using 96% methanol. Dry powder samples of 3.84 kg Kolowe leaves are soaked with 12 L of methanol for 72 hours, to complete. The solution was evaporated solvent extract obtained by using Rotary Evaporator, and extracts obtained 344.55 g. About 300 g of extract was mixed with 500 g of silica gel GF60 then be included in a Buchner funnel as column chromatography. The crude mixture of methanol extracts eluted with 96% methanol until all the methanol extracts clearer without the various impurities. The extract is ready for screening chemical and bioactivity test.

Profile Analysis of Secondary Metabolites Compounds

Analysis of secondary metabolites is done with two approaches, chemical methods (use of reagents) and spectrum analysis of compounds by LC-MS/MS. Class of secondary metabolites analyzed are flavonoids, steroids, triterpenes, phenolics, tannins, carbohydrates, and saponins. The grouping of secondary metabolites based upon the content of seeds and leaves that had been reported previously, for the third usefulness of these organs is the same that as a fish poison. Identification triterpenes and steroids as analytical approach allegations of saponin triterpene or steroid saponin.

1. Secondary Metabolites Screening

Identification of secondary metabolites by chemical methods performed on crude extract's extraction with cold maceration followed by the column chromatography to obtain extracts that are free from impurities. Reagents for testing of each class of secondary metabolites extract added until the solution that has been prepared and observed physical changes (color) happen. The color change is caused by the chemical reaction between the reagent with chromophore active secondary metabolites or reactive centers of secondary metabolites were identified [8].

2. Secondary metabolites analysis by LC-MS/MS

LC-MS/MS instrument used is UPLC- QToF-MS/MS System (Waters), software *MassLynk versi 4.1. UPLC Acquity SDS* (*Waters*) with *Acquity UPLC BEH C-18 1,7 um*, 2,1 x 50 mm column ; *flow rate* 0,3 mL/min; size *injection* 5 uL, temperature 40 °C; eluent : water and *0,1 % formic acid (A)*; and mixed *acetonitrile and 0,1 % formic acid (B)*, eluted with gradient system.

Pharmaceutical potential assay

Potential pharmaceutical in question is its antioxidant activity, cytotoxic, and antimicrobial. These three biological activities is very useful as pharmaceuticals, particularly related to drug and functional food products. Antioxidant test used as an indicator of DPPH radical and for the cytotoxic, tests are the third instar larvae of *A. salina*. The second parameter such as activity until the IC_{50} values for antioxidant and LC_{50} for cytotoxic, and stability. Stability test the antioxidant activity and cytotoxic based upon the time that the storage time at room temperature. Furthermore, for a screening test only antimicrobials against 19 types of microbes, as did the seeds and leaves that have been reported previously.

1. Cytotoxic assay

Bioindicator for cytotoxic tests are the third instar larvae of *A. salina*. Total larvae of each repeat testing are 10 selected based on criteria agile or active move by the larvae of *A. salina*. The extract was found for cytotoxic testing 3 ppm; 5 ppm; 7 ppm; 9 ppm; and 11 ppm.

2. Antioxidant assay

Indicators to test the antioxidant are compound DPPH radical solution with a concentration of 40 ppm with the solvent methanol. Absorbance measurement instruments are UV-VIS spectrometer. The concentration during the test that was found was 1 ppm; 3 ppm; 5 ppm; 7 ppm; and 9 ppm.

3. Antimicrobe assay

Types of microbes that are used together with testing for seed extract and its leaf has been previously reported. Types of microbes are shown Table 1.

4. Cytotoxic and antioxidant stability assay

The stability of the biological activity of a material becomes very important because it is associated with the planned commercial use. The designs of the cytotoxic and antioxidant stability test are shown in Table 2.

Table 1. Types of microbes for antimicrobial activity screening bark of kolowe extract (*C. excelsus*)

NO	Microbe	Information
1	*Salmonella thyphosa,*	Bacteria
2	*Clostrididum tetani,*	Bacteria
3	*Pneumonia carinii*	Fungi
4	*Candida albycan*	Fungi
5	*Neisseria gonorrchoeae*	Bacteria
6	*Treponema pallidum*	Bacteria
7	*Coxiella burnetii*	Bacteria
8	*Clostridium tetani*	Bacteria
9	*Clostridium botulinum*	Bacteria
10	*Bacillus anthracis*	Bacteria
11	*Propionibacterium acnes*	Bacteria
12	*Vibrio parahaemolyticus*	Bacteria
13	*Streptococcus mutans*	Bacteria
14	*Agrobacterium tumefaciens*	Bacteria
15	*Streptococcus pneumonia*	Bacteria
16	*Pseudomonas solanacrearum*	Bacteria
17	*Chlamydia trachomatis*	Bacteria
18	*Treponema pallidum pertenue*	Bacteria
19	*Staphylococcus aureus*	Bacteria

RESULT AND DISCUSSION

The results reported from the bark kolowe (*C. excelsus*) are (1) profiles of secondary metabolites's content of bark and (2) the potential of pharmaceutical extracts. Profile of secondary metabolites based upon the identification of chemical and spectral analysis of LC-MS / MS, while the pharmaceutical reported potential antioxidant activity, cytotoxic and antimicrobial extract.

Secondary metabolites profile of kolowe stembark (*C. excelsus*)

Profiles of secondary metabolites that are reported are the results of qualitative screening by chemical methods and molecular weight profile analysis results with LC-MS / MS. Type compound structurally cannot be reported so that the isolation of compounds to study structure elucidation will be continued.

1. Metabolites secondary profile by chemical method

Class of secondary metabolites bark of Kolowe (*C. excelsus*) which is chemically identified phenolics, flavonoids, saponins, triterpene, carbohydrates and steroids. Identified phenolic group illustrates that the phenol group is a flavanoid compounds, because tannins, which are polyphenol undetected. Metabolites are predominantly followed flavonoids saponins. In Stem bark of Kolowe (*C. excelsus*) also detected the existence of carbohydrates, which describes that such compounds are glycosides, including flavanoid glycosides. Furthermore, saponins detected alleged steroid saponin as dominant identified in the extract are believed to be the aglycone of saponins. Therefore, based upon the chemical method, the dominant compound profiles on bark extract Kolowe (*C. excelsus*) is a flavonoid glycoside and steroidal saponins. Both classes of compounds are very potent in the pharmaceutical field because it has multiple biological activities [3]. Because of the presence of two classes of compounds then bark at Kolowe (*C. excelsus*) has a very good potential pharmacy. Another is the potential yield of the methanol extract of which is based upon the extraction process of cold maceration using methanol obtained 96% yield of crude extract of 8.97%. The yield is quite high due to the general recovery of plant secondary metabolites bark quite low if extraction by cold maceration method [7]. The results from the identification of secondary metabolites bark ofKolowe (*C. excelsus*) by chemical methods is shown in Table 3.

2. Metabolites secondary analysis by LC-MS/MS

Analysis of secondary metabolites's profile bark of Kolowe (*C. excelsus*) followed by LC-MS / MS for a bit to make sure. The results of the analysis instrument LC-MS / MS has been able to unravel the molecular weight of up to fragmentation so that the number of compounds that have been found can be known. However, LC-MS/MS cannot be sure until the molecular structure of compounds. The result from the analysis of LC-MS/MS was found 13 types of compounds shown in Table 4. The results from the analysis of LC-MS/MS in accordance with the method of screening chemical. Certainty screening results with LC-MS/ MS is more correct than the chemical method that has been identified five saponins alleged steroid saponin, and eight flavanoid compounds are also suspected as flavanoid glycosides. The spectrum of LC-MS/MS of each of these compounds is shown in Figure 2

Pharmaceuticals potency of kolowe stem bark (*C. excelsus*)

Hostettman and Marston (1995) state that the class of saponins have biological activity as a potential pharmaceutical to 24 types. Nevertheless, the potential for pharmaceutical bark kolowe (*C. excelsus*) reported only antioxidant, cytotoxic and antimicrobial, although it has been shown to contain saponin.

Table 2. Cytotoxic stability assay and antioxidant from bark of kolowe extract (*C. excelsus*)

Storage time (Mounth)	Date and replication			Information
1st s/d 30th Sep 2016	30th Sept. – 5th Okt 2016			4 times with 12 replication
	I	II	III	
30th Sept s/d 30th Okt. 2016	30th Okt. – 3rd Nov 2016			
	I	II	III	
30th Okt s/d 29th Nov 2016	29th Nov –3rd Des 2016			
	I	II	III	
29th Nov s/d 30th Dec 2016	30th April – 3rd May 2016			

Table 3. Profile of secondary metabolites from the stem bark of Kolowe (*C. excelsus*), identification by chemical methods

No	Class of secondary metabolites	result
1	Phenolic	+++
2	Flavanoid	++++
3	Triterpene	+
4	Steroid	++
5	Saponin	+++
6	Tanin	-
7	Carbohidrat	+++

1. Cytotoxic potency

Criteria cytotoxic a material if it has toxicity LC_{50} values <30 ppm against larvae of *A. salina* [7]. Product biological activities of natural materials are generally less stable, so their use is generally performed on fresh ingredients to get the desired effect. Because they were testing the stability of the biological activity of natural ingredients, products become very important. The toxicity of the methanol extracts from the bark of plants Kolowe (*C. excelsus*) is LC_{50} = 7.68 ppm and is expressed as a cytotoxic potential based upon these criteria. The stability of the cytotoxic activity was also performed testing with storage up to 16 weeks old. Cytotoxic stability test results are shown in Table 5. The results from these tests show that the cytotoxic activity of the methanol extracts from the bark of plants Kolowe (*C. excelsus*) is very stable even though the first four weeks dropped cytotoxic activity value, but until 16 weeks cytotoxic have the same relative to the fourth week. Decrease in LC_{50} values that occurred in the fourth week still remains within the category of cytotoxic because LC_{50} <30 ppm. Thus the methanol extracts from the bark of plants Kolowe (*C. excelsus*) has stable potential cytotoxic.

2. Antioxidant potency

Criteria powerful antioxidant of a substance if it had IC_{50} values on the range 50> IC_{50} <100 ppm and powerful if 0 > IC_{50} < 50 ppm (Block in Delgado and Remers, 1991). However, the biological activities as the product of natural materials are generally less stable, especially antioxidants that are more reactive than compounds in general. Because of the use of natural materials is generally carried out in the form of fresh ingredients to get the desired effect. The antioxidant activity of methanol extracts from the bark of plants Kolowe (*C. excelsus*) also conducted stability tests as well as cytotoxic activity has been described. The antioxidant activity of methanol extracts from the bark of plants Kolowe (*C. excelsus*) is very strong that IC_{50} = 5.48 ppm. The stability of the antioxidant activity has also been carried out as well as cytotoxic namely testing with a storage time of up to 16 weeks. The results of stability testing of antioxidant shown in Table 6.

Antioxidant extracts of the leaves Kolowe quite stable at room temperature with storage time of up to 16 weeks or four months. During storage changes IC50 values were significant after four weeks, namely from IC_{50} = 5.48 ppm to 10.22 ppm. However, at 8th to 16th week IC_{50} values become stable. Thus the antioxidant activity of methanol extracts from the bark of plants Kolowe (*C. excelsus*) is very strong and stable. These are potential pharmaceutical natural ingredients that are very useful. Levels of radicals in the cells will continue to increase as a result of exposure to pollutants into the environment, as well as an unhealthy lifestyle such as smoking has become triggers an increase as the number of radicals in cells. Radicals in cells that increasing potential to be a chain reaction with a variety of metabolites in cells, including DNA, which ultimately lead to degenerative diseases, including errors of metabolism. Therefore, the need for exogenous antioxidants becomes very important today, and plant kolowe (*C. excelsus*) is one of them.

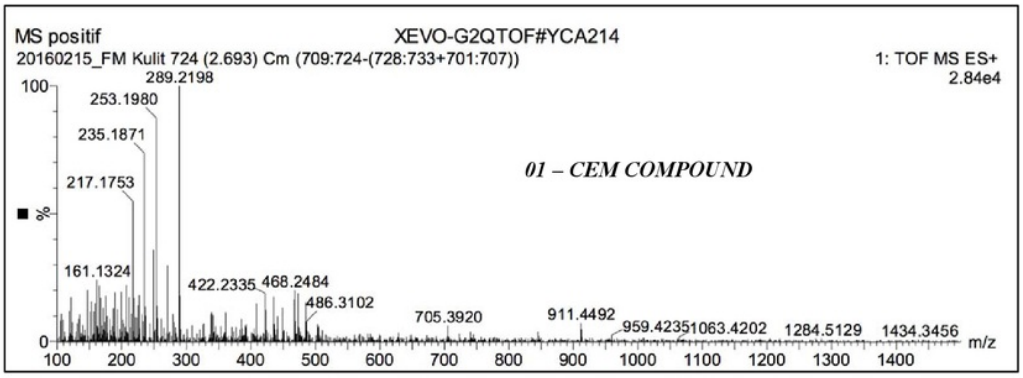
Antimicrobe activity

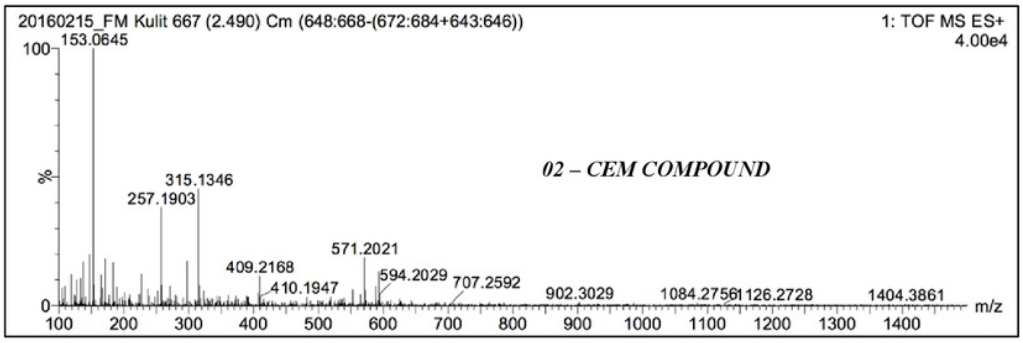
Antimicrobial Test methanol extracts from the bark of plants Kolowe (*C. excelsus*) that has just been done on the leaf extracts that is only a screening against 19 types of microbes comprising gram-positive bacteria, gram-negative, and mushrooms. The test results of the methanol extract entirely positive that can kill or inhibit the growth of microbes. The test results from antimicrobial methanol extracts from the bark of plants Kolowe (*C. excelsus*) shown in Table 7, which shows that the methanol extracts from the bark of plants Kolowe (*C. excelsus*) also has antimicrobial activity rather strong exception to some microbes such as *Bacillus anthracis, Streptococcus pneumoniae, Coxiella burnetii, and Candida albicans.*

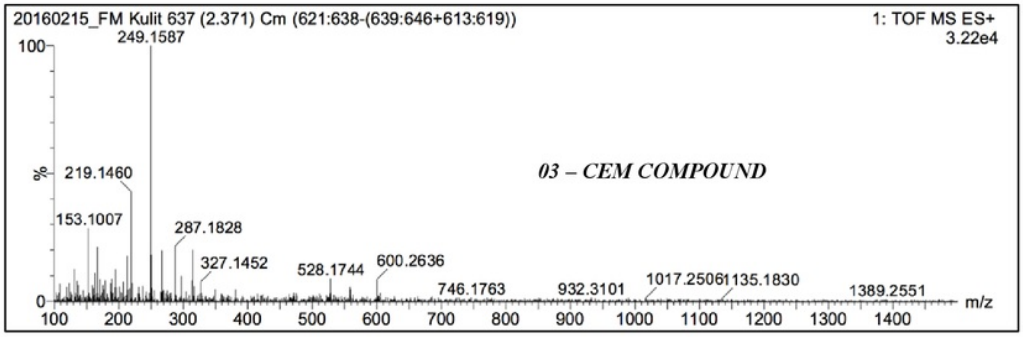
Table 4. Profile of secondary metabolites *Crude Extracts Methanol* (CEM) stem bark of Kolowe (*C. excelsus*) based on LC-MS/MS analysis

Compound code	Retention Time	(M + H$^+$) *m/z*	Molecul Weight (MW)	Prediction
01-CEM	2,693	289	288	Glycoside Flavanoid
02-CEM	2,490	571	570	Saponin steroid
03-CEM	2,371	249	248	Flavanoid or Glycoside Flavanoid
04-CEM	0,458	365	364	Glycoside Flavanoid
05-CEM	4,551	371	370	Glycoside Flavanoid
06-CEM	3,191	668	667	Saponin steroid
07-CEM	2,851	778	777	Saponin triterpene or saponin steroid
08-CEM	2,729	310	309	Glycoside Flavanoid
09-CEM	6,151	926	925	Saponin triterpene/Steroid
10-CEM	5,968	429	428	Glycoside Flavanoid
11-CEM	5,639	291	290	Glycoside Flavanoid
12-CEM	4,880	705	704	Saponin triterpene/steroid
13-CEM	7,810	720	719	Saponin triterpene/steroid

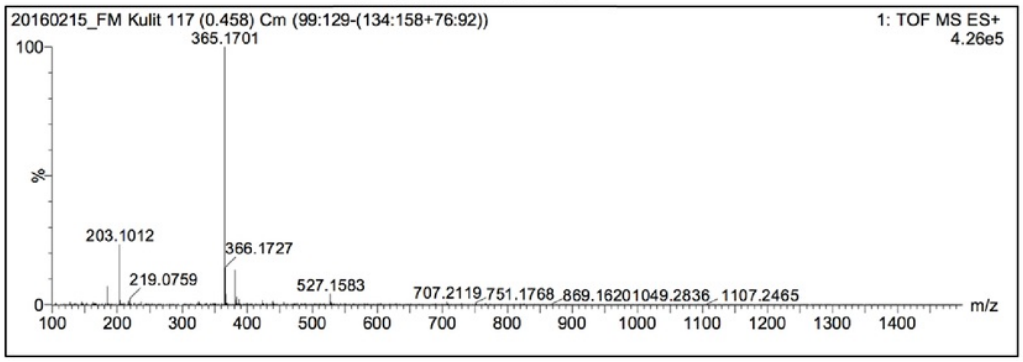
information: CEM = Crude Extracts of Methanol

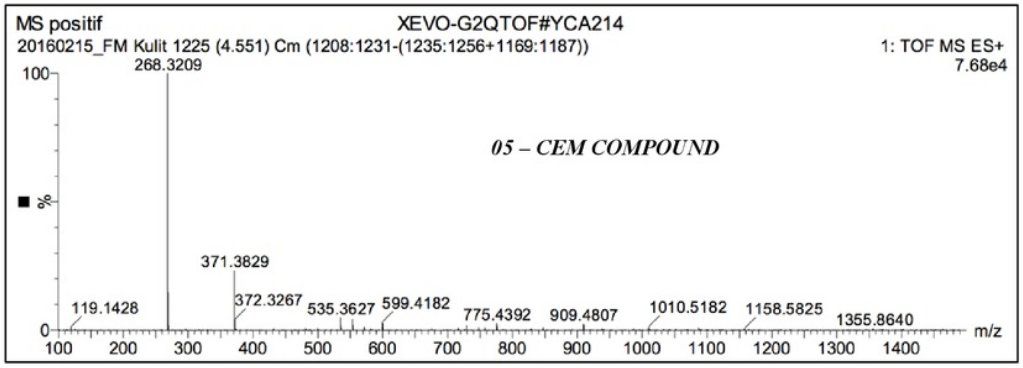






04 – CEM COMPOUND





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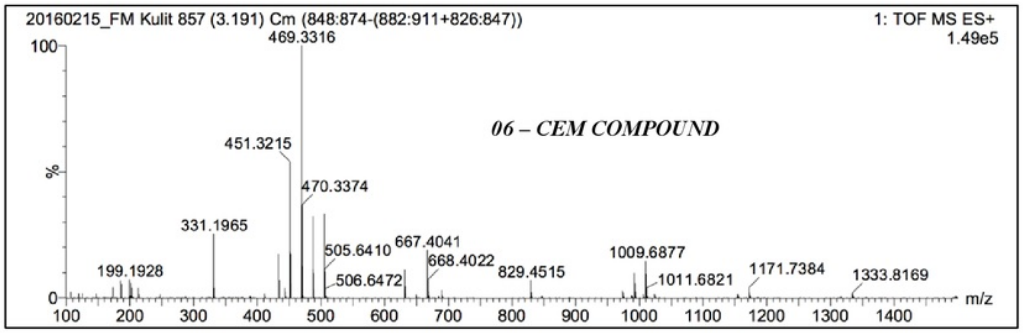

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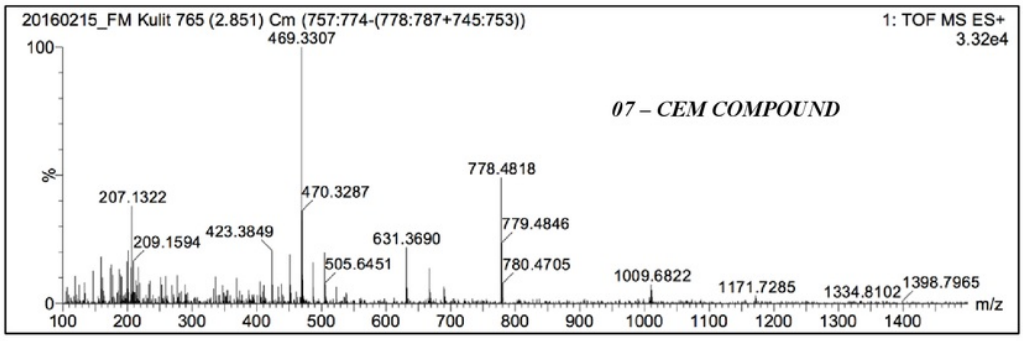

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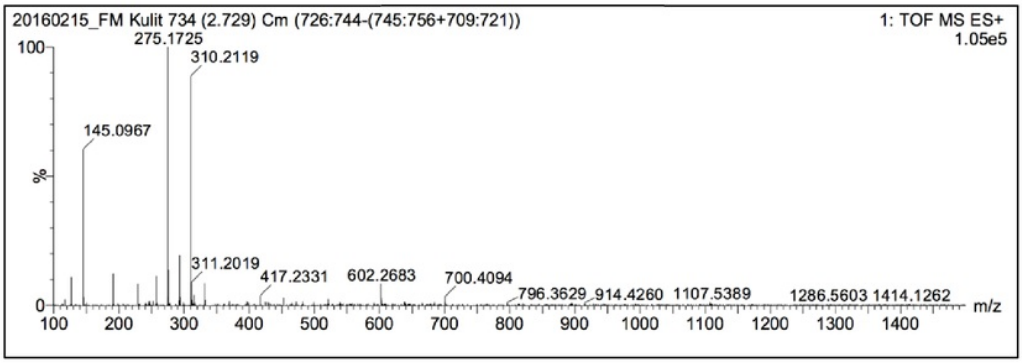
08 – CEM COMPOUND



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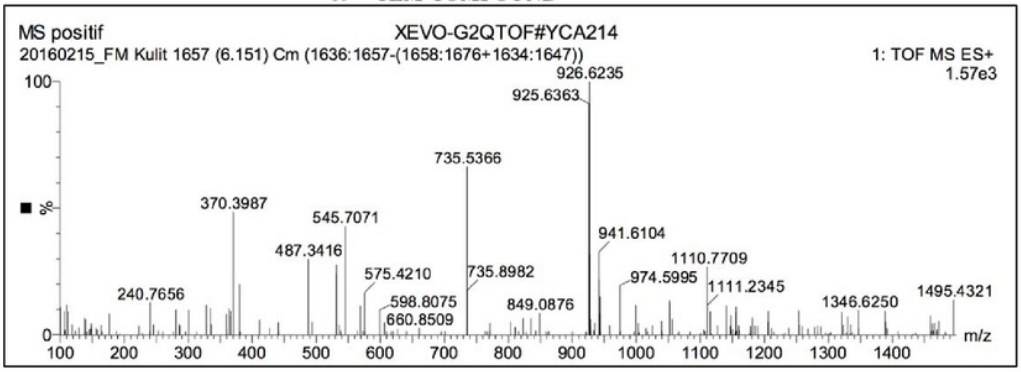

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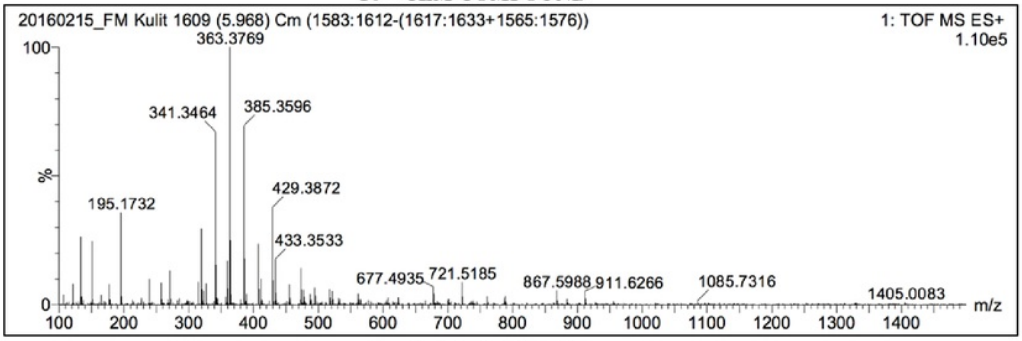

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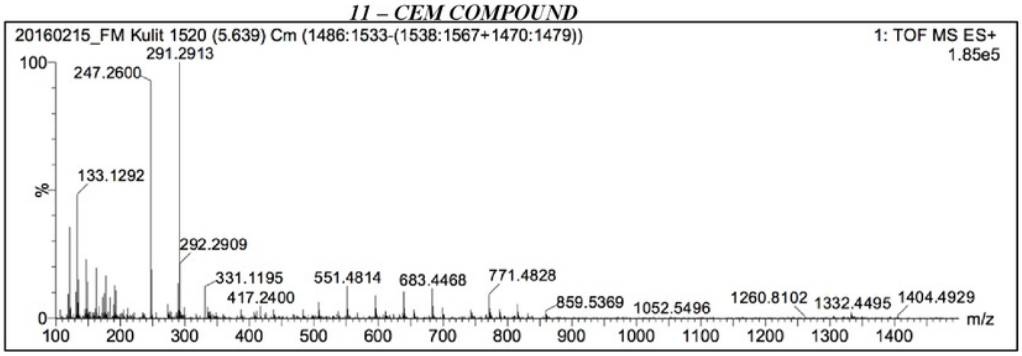

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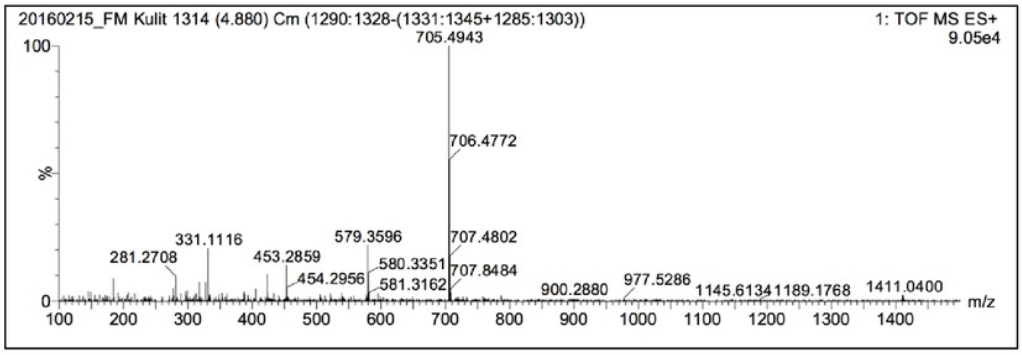

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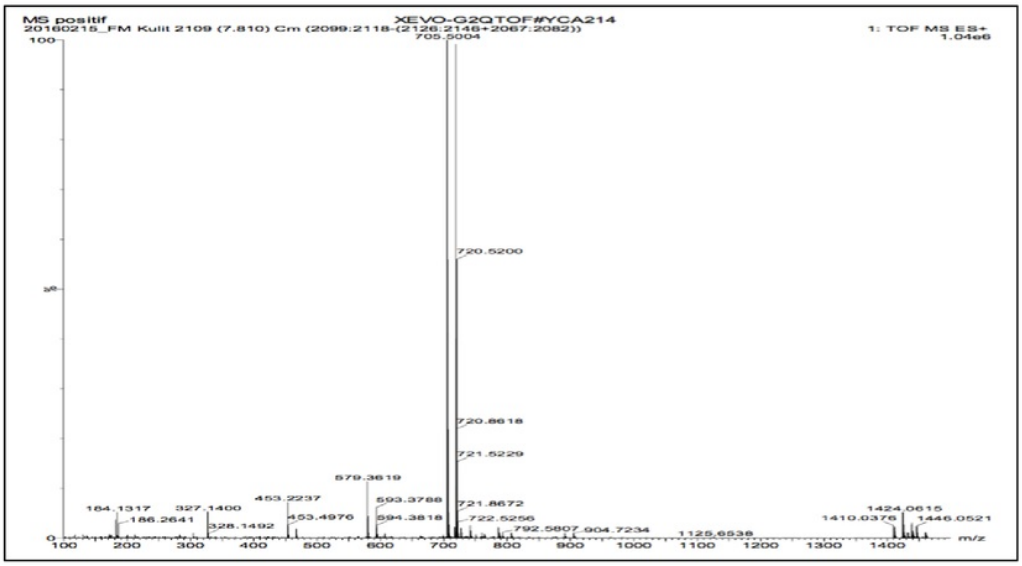

Figure 2. LC-MS/MS spektrum some compounds contains in bark of Kolowe (C. excelsus) are compounds 01 - CEM to 13 - CEM.

CONCLUSION

Profile of secondary metabolites content to the bark of plants Kolowe (*C. excelsus*), the most dominant by screening with chemical and instrumental analysis methods LC-MS / MS are steroid saponin and flavonoid glycosides are five types as triterpene saponins and eight types as flavanoid glycosides.

Potential pharmaceutical kolowe bark extracts (*C. excelsus*) are a cytotoxic material with LC_{50} = 7.68 ppm and an antioxidant with IC_{50} = 5.48 ppm and both activities are very stable at room temperature storage. Other pharmaceutical potentials is to have the yield and antimicrobial. The yield is high enough methanol crude extracts obtained by using methanol is 8.97%. Besides the methanol extract also be antimicrobial against 19 types of microbes.

ACKNOWLEDMENT

Thanks to the entire staff of Laboratorium riset dan pengembangan Kefarmasian FARMAKA TROPIS, Dean of the Faculty of Pharmacy, Mulawarman University who helped research activities. Moreover, it also conveyed gratitude to the Head of the Laboratory of Biotechnology BPPT, Jakarta, which has helped in the analysis of LC-MS / MS.

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