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# Rapid Screening for Cytotoxicity and Group Identification of Secondary Metabolites in Methanol Extracts from Four Sponge Species Found in Kapoposang Island, Spermonde Archipelago, Indonesia

**Ajuk Sapar[1,2*], Alfian Noor[1], Nunuk Hariani Soekamto[1] and Ahyar Ahmad[1]**
[1] Chemistry Department, Hasanuddin University, Macassar 90245
[2] Chemistry Department, Tanjungpura University, Pontianak 78124

## Abstract
Four sponge samples collected from Kapoposang Island, Spermonde Archipelago have been investigated to estimate their cytotoxicity and existence of secondary metabolite groups in their methanol extracts at a preliminary level. The species were *Niphates* sp., *Biemna* sp., *Liosina paradoxa, Haliclona (Reniera) fascigera*. Rapid screening of small scale extracts for cytotoxicity was performed using a Brine Shrimp Lethality Test and the results were expressed as 50% lethal concentration (LC50). Results of the test for all extract are the followings: 391.56 ppm, 1,730 ppm, 456.04 ppm and 478.63 ppm respectively. Only the extract from *Biemna* sp. was inactive while the others, particularly the extract from *Niphates* sp. had potent cytotoxic agents. Identification of molecules using specific reagents like Lieberman-Burchard, Salkowski, Mayer, Wagner and Dragendorff showed that methanol extracts contained steroid, terpenoid and alkaloid compounds. Identification of group compounds was supported by a Fourier transform infrared spectroscopy (FTIR) instrument and reference shows that these extracts contain alkaloid, steroid and terpenoid compounds.

Key words: sponge, screening, cytotoxicity, secondary metabolite, Spermonde

## Introduction

Sponges are marine animals that have been known as a source of bioactive compounds which can be used as a medicine. It is very interesting to study sponges that live as a filter feeder in competition with other species in a coral reef ecosystem. Their bioactive compounds have effects other organisms and threaten other sponges. This is a phenomenon called allelopathy mediated by allelochemicals. This phenomenon is demonstrated by *Plakortis halichondroides* sponge overgrowing the coral *Agaricia lamarcki* by forming a zone on the coral tissue by inducing necrosis of coral cells (Sebastian and Pawlik, 2000). Similarly, sponge *Dysidea* sp. can induce necrosis of the sponge *Cacospongia* sp. (Thacker *et al*., 1998). Secondary metabolites that cause necrosis of other species have been widely reported in *Biemna triraphis Niphates* sp. sponge.

Two metabolites exhibiting a moderate cytotoxicity have been isolated in *Niphates* sp. sponge from the New Caledonian. The metabolites were polyoxygenated acetylenic acids, nepheliosyne B and nepheliosyne A (Nathalie Legrave, *et al*., 2013). A steroid compound, 5α, 8α-Epidioxy-24(*S*)-ethylcholest-6-en-3$\beta$-ol was successfully isolated from *Biemna triraphis* Topsent collected at Andavadoaka near Toliary (west coast of Madagascar-Indian Ocean) (Julia *et al*., 2008). Excretion of secondary metabolites from the sponge is influenced by the environment and it is assumed that the different environments induce different secondary metabolites from the same species of sponge. Based on this assumption, we have studied the secondary metabolites from four species of sponges, *Niphates* sp., *Biemna* sp., *Liosina paradoxa*, *Haliclona* (Reniera) *fascigera* from Kapoposang Island. This is a preliminary study to explore the bioactive secondary metabolites in sponges from Kapoposang Island.

## Material and methods

### Tools and material

Fourier transform infrared spectroscopy (FTIR) (IRPrestige-21 Shimadzu), an analytic balance (Mettler

*Corresponding author: e-mail ajuk_chem@yahoo.com


**Fig. 1. Sampling location in Kapoposang Island.**

AE 100), and a rotary evaporator (Heidolph 4000) were used. Reagents: Spectrophotometric grade of methanol, chloroform, and sulfuric acid were used. Lieberman-Burchard reagent, Salkowski reagent, Mayer reagent, Wagner reagent, and Dragendorff reagent were purchased.

## Sampling and identification of species

Sponges of *Niphates* sp., *Biemna* sp., *Liosina paradoxa*, and *Haliclona (Reniera) fascigera* were collected by hand (scuba) from various depth (6-9 m) in Kapoposang Island (Fig. 1), Spermonde Archipelago, South of Sulawesi, Indonesia on 15 August 2012. Sponge taxonomy was determined in Research Center for Oceanography LIPI Jakarta and all specimens has been deposited in Research Center for Oceanography (RCO) LIPI Jakarta, Indonesia 14430. Vouchers code for *Niphates* sp., *Biemna* sp., *Liosina paradoxa, Haliclona (Reniera) fascigera* were SPV 01/10/13, SPV 02/10/13, SPV 03/10/13, and SPV 04/10/13, respectively. The species data like taxonomy, morphology, consistency, skeleton and specula are described below.

The morphology of *Niphates* sp. is a flabelliform with conulose surfaces at the upper side (Fig. 2A). The body colour is pale brown in a dry state. Its consistency is spongy and the skeleton are nultispicular tracts that consist of spicules-oxeas (110- 150 μm), no microscleres (Fig. 2B and C).

**Fig. 2A. Morphology Niphates sp..**

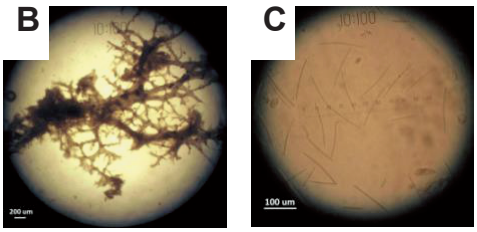


**Fig. 2B, C. Skeleton (B) and specule (C) of Niphates sp..**

The morphology of *Biemna* sp. is massive and cushion-shaped as shown in Fig. 3A. The body colour is brown in a dry state. Its consistency is tough and the skeleton consist of spicules Styles (310-350 μm), sigmas (60-70 μm), raphides (110-150 μm) like in Fig. 3B and C.

**Fig. 3A. Morphology Biemna sp..**

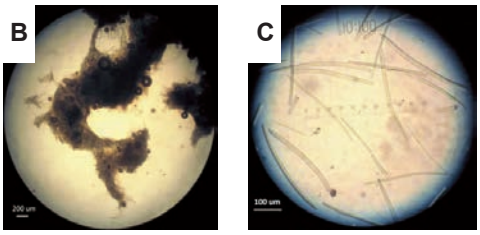


**Fig. 3B, C. Skeleton (B) and specule (C) of Biemna sp..**

The morphology of *Liosina paradoxa* is massive, tube-shape with muddy surfaces as shown in Fig. 4A and its consistency is firm. The skeleton of *Liosina paradoxa* is loose bundles with foreign materials that consist of oxeote (600-700 μm) spicules as shown in Fig. 4A and B.

**Fig. 4A. Morphology of *Liosina paradoxa*.**

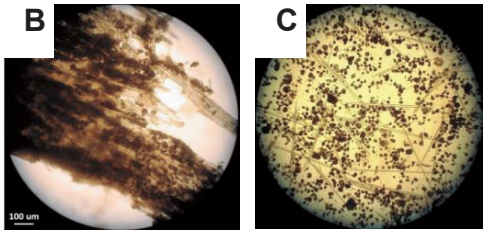


**Fig. 4B, C. Skeleton (B) and specule (C) of *Liosina paradoxa*.**

The morphology of *Haliclona (Reniera) fascigera* is long-tube shaped (Fig. 5A) and its consistency is sot and fragile. The skeleton *Haliclona (Reniera) fascigera* are unispicular tracts, isotropic and consist of oxeas (60-80 um) spicule in Fig. 5 (A) and (B), respectively.

**Fig. 5A. Morphology of *Haliclona (Reniera) fascigera.***

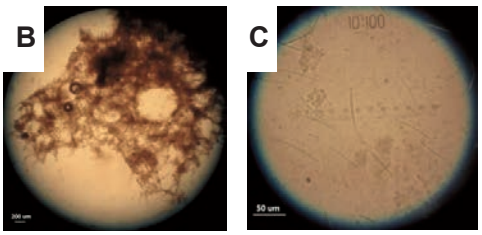


**Fig. 5B, C. Skeleton (B) and specule (C) of *Haliclona (Reniera) fascigera.***

## Small scale extraction

Samples of *Niphates* sp., *Biemna* sp., *Liosina paradoxa*, *Haliclona (Reniera) fascigera,* whose amounts were 41 g, 47 g, 39 g and 45 g respectively were extracted by methanol. Extracted solutions were filtered and evaporated using an evaporator to obtain 52.2 mg, 46.2 mg, 67.2 mg and 45.4 mg methanol extracts respectively. All extracts were examined in a bioactivity test and group compounds were identified by specific reagents and FTIR.

## Bioactivity Test

Secondary metabolites are often toxic to shrimp larvae *Artemia salina*. Lethality tests to shrimp *Artemia salina* larvae performed to get the data of toxicity LC50 of each sample. The method has been developed for the natural products to monitor cytotoxic natural compounds (Meyer *et al*. 1982; McLaughlin and Rogers, 1998). In this method, the sample was prepared in a concentration of 1000, 100 and 10 ppm in triplets and emulsified with DMSO. The solution was tested against 10 larvae of *Aremia salina*. The mortality of the larvae of *Aremia salina* was analyzed using Probit Analysis.

## Identification of secondary metabolite groups

Specific tests of each methanol extract were carried out on the sponge *Agelas nakamurai* Hoshino. The test using specific reagent as follows: Lieberman-Burchard (LB) reagent for steroids, Salkowski reagent for terpe-

noids, Wagner regent, Mayer reagent, and Dragendorff reagent for alkaloids. Meanwhile, analysis with FTIR was carried out to support the prediction of secondary metabolites groups in each specific test.

## Result and discussion

### Specific test and cytotoxicity

The LC50 value of each extract of *Niphates* sp., *Biemna* sp., *Liosina paradoxa, Haliclona (Reniera) fascigera* were 391.56 ppm, 1,730 ppm, 456.04 ppm and 478.63 ppm respectively. Only the methanol extract of *Biemna* sp. was not cytotoxic, while the others, were cytotoxic. Particularly the methanol extract of *Niphates* sp. was most toxic. Specific tests to estimate the groups of secondary metabolites in methanol extracts showed that methanol extracts contain the compounds of steroids, terpenoids and alkaloids (Table 1).

### FTIR Analysis

FTIR analyses were conducted for methanol extracts of *Niphates* sp., *Biemna* sp., *Liosina paradoxa*, *Haliclona (Reniera) fascigera*. It should be noted that the current analyses can provide limited information and prediction concerning the functional groups of secondary metabolites.

### FTIR analysis of *Niphates* sp. methanol extract

Generally, FTIR Spectrum patterns of the methanol extract of *Niphates* sp., as shown in Fig. 6, were dominated by several broad band absorption, indicating band absorption is overlapped. Appearance of 3421.72 $cm^{-1}$ was assigned to be a typical stretching band of OH, supported by a primary or secondary bending band of OH observed at 1242.16 $cm^{-1}$. A bending band of CO (medium) was

**Table 1. Results of specific test group of secondary metabolites.**

| Sample of methanol extract | Reagent / response strength | | | | | Group compounds |
|---|---|---|---|---|---|---|
| | LB | Salkowsky | Meyer | Wagner | Dragendorff | |
| *Niphates* sp. | Green (+++) | Red-brown (+) | Yellow-white (+) | Brown (+++) | Red (++) | Terpen, steroid, alkaloid |
| *Biemna* sp. | Green (+) | Red-pink (++) | Yellow clear (-) | Brown (+++) | Red (+++) | Terpen, steroid, alkaloid |
| *Liosina paradoxa*, | Green (+++) | Red-pink (++) | Yellow clear (-) | Brown (++) | Red (+) | Terpen, steroid, alkaloid |
| *Haliclona (Reniera) fascigera* | Green (++) | Red-pink (+++) | Yellow clear (-) | Brown (+++) | Red (++) | Terpen, steroid, alkaloid |

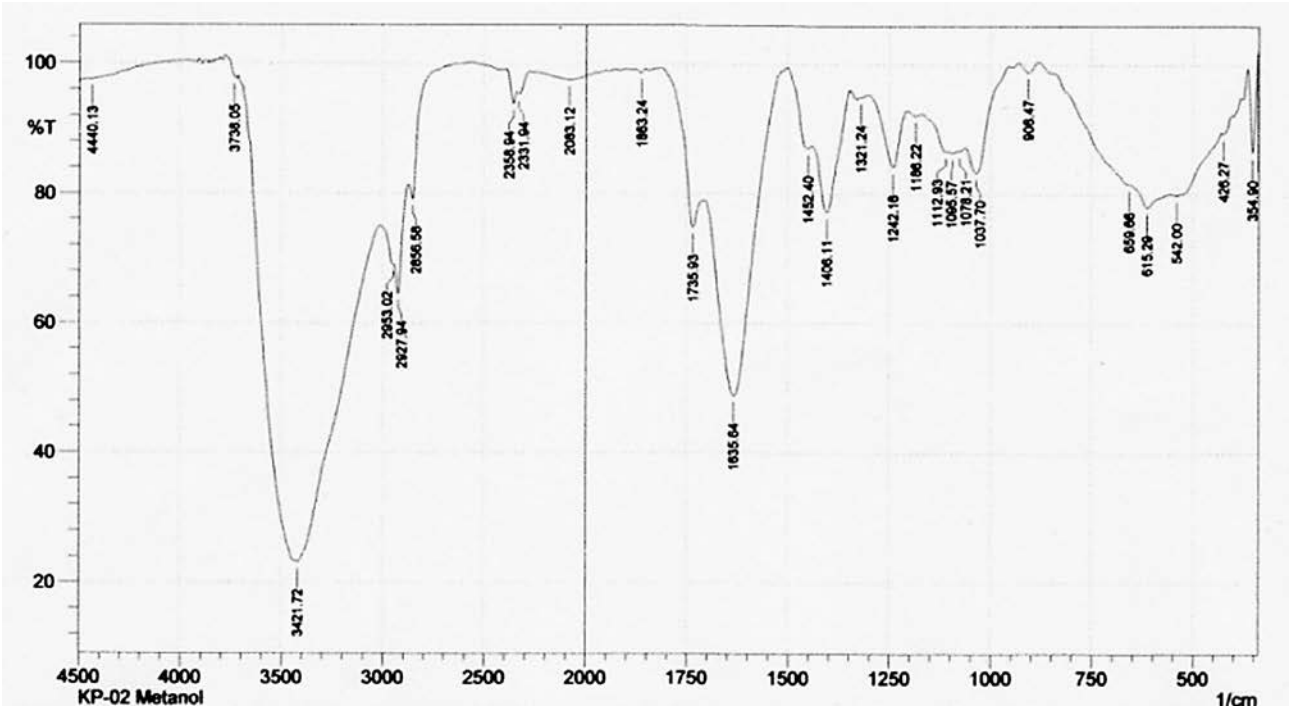


**Fig. 6. FTIR spectrum for *Niphates* sp. extract.**

observed at 1037.70 $cm^{-1}$, and bands absorption at 2953.02 $cm^{-1}$, 2927.94 $cm^{-1}$ and 2856.56 $cm^{-1}$ were estimated to be a C-H stretching of $CH_3$ and $CH_2$ groups. A shoulder observed at about 3190 $cm^{-1}$ could be estimated as band absorption of =C-H olefin, supported by appearance of 1635.64 $cm^{-1}$ for a C=C stretching band. Stretching bands of $CH_3$ and $CH_2$ groups were supported by their bending absorption at 1406.11 $cm^{-1}$and 1452.40 $cm^{-1}$, and a methyne band. Band absorption at 1735.93 $cm^{-1}$ were estimated as a C=O stretching even though medium band but supported by overtone (weak) at 3738.05 $cm^{-1}$. Sharp bands absorption at 615.29 $cm^{-1}$ and 659.66 $cm^{-1}$ could be estimated as *cis* CH out of plane olefin or stretching CBr for aliphatic bromo compounds. Broad bands observed at 3421.72 $cm^{-1}$ and 1635.64 $cm^{-1}$ could be assigned as overlapping band absorption of OH stretching, NH stretching, and the bending.

## FTIR analysis of *Biemna* sp methanol extract

FTIR spectrum patterns of the methanol extract of *Biemna* sp., as shown in Fig. 7, were similar to those of *Niphates* sp. A broad band at 3421.72 $cm^{-1}$ is assigned as a typical stretching band of OH, supported by a weak primary or secondary bending band of OH at 1219.01 $cm^{-1}$, a bending band CO (medium) at 1095.57 $cm^{-1}$, and 1047.35 $cm^{-1}$. Aliphatic bands absorption of CH stretching of $CH_3$ and $CH_2$ groups typically appeared at 2954.95 $cm^{-1}$, 2924.09 $cm^{-1}$, and 2852.72 $cm^{-1}$. Bands absorption at 1465.90 $cm^{-1}$, 1456.90 $cm^{-1}$, and 1406.11 $cm^{-1}$ arose from typical bending absorption of $CH_3$ and $CH_2$ groups in a similar manner to bending absorption of CH at 1336.60 $cm^{-1}$. A small shoulder was observed at about 3190 $cm^{-1}$, which could be estimated to be band absorption of =C-H olefin, supported by a stretching band of C=C at 1635.64 $cm^{-1}$. Weak band absorption at 1714.72 $cm^{-1}$ could be estimated as C=O stretching. Bands absorption overlapped at 617.22 $cm^{-1}$ and 659.66 $cm^{-1}$ could be estimated as *cis* CH out of plane olefin or stretching C-Br for aliphatic bromo compounds. Band absorption at 719.45 $cm^{-1}$ was expected to be overlapped by several bands absorption, which is typical for rocking bending methylene groups. Bands absorption at 1558.40 $cm^{-1}$ and 1635.64 $cm^{-1}$ were estimated as an N-H bending.

## FTIR Analysis of *Liosina paradoxa* methanol extract

FTIR spectrum patterns of the methanol extraxt of *Liosina paradoxa*, as shown in Fig. 8, were similar to those of *Biemna* sp. It was also dominated by several broad band sorption.

## FTIR Analysis of *Haliclona (Reniera) fascigera* methanol extract

Bands absorption patterns of the methanol extract of *Haliclona (Reniera) fascigera*, as shown in Fig. 9, were

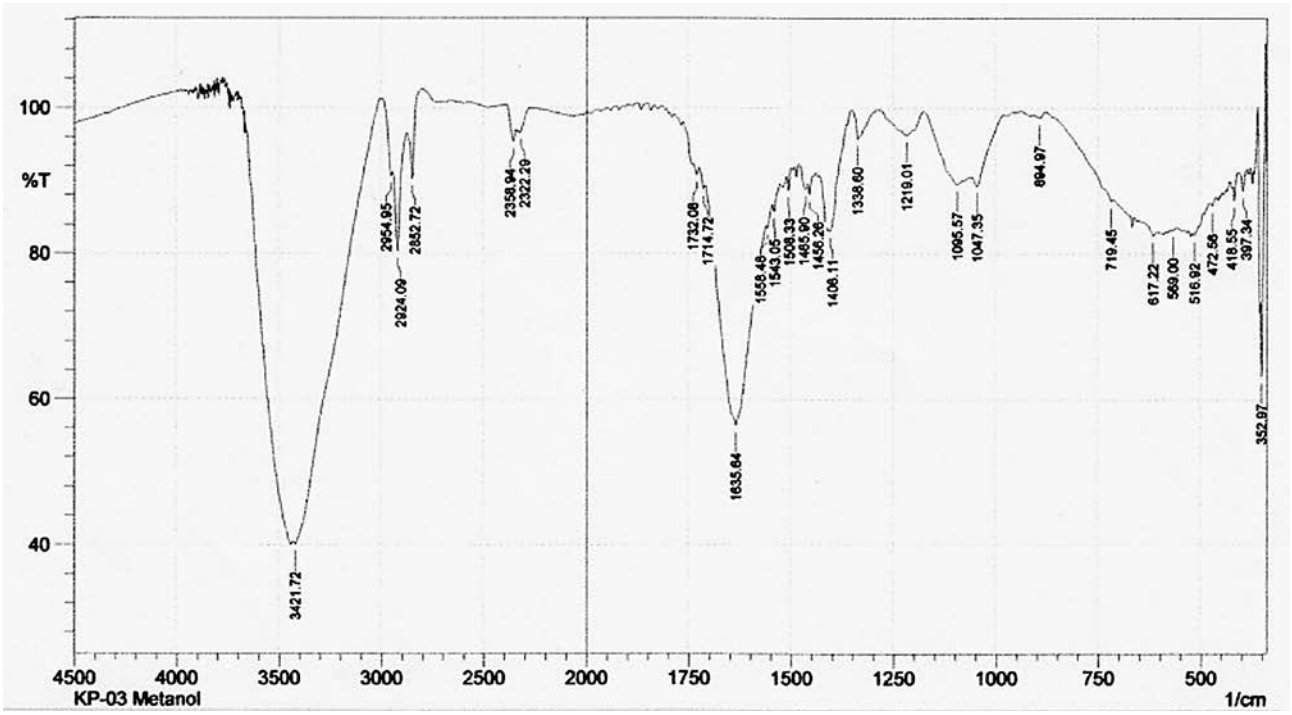


**Fig. 7. FTIR analysis of *Biemna* sp methanol extract.**

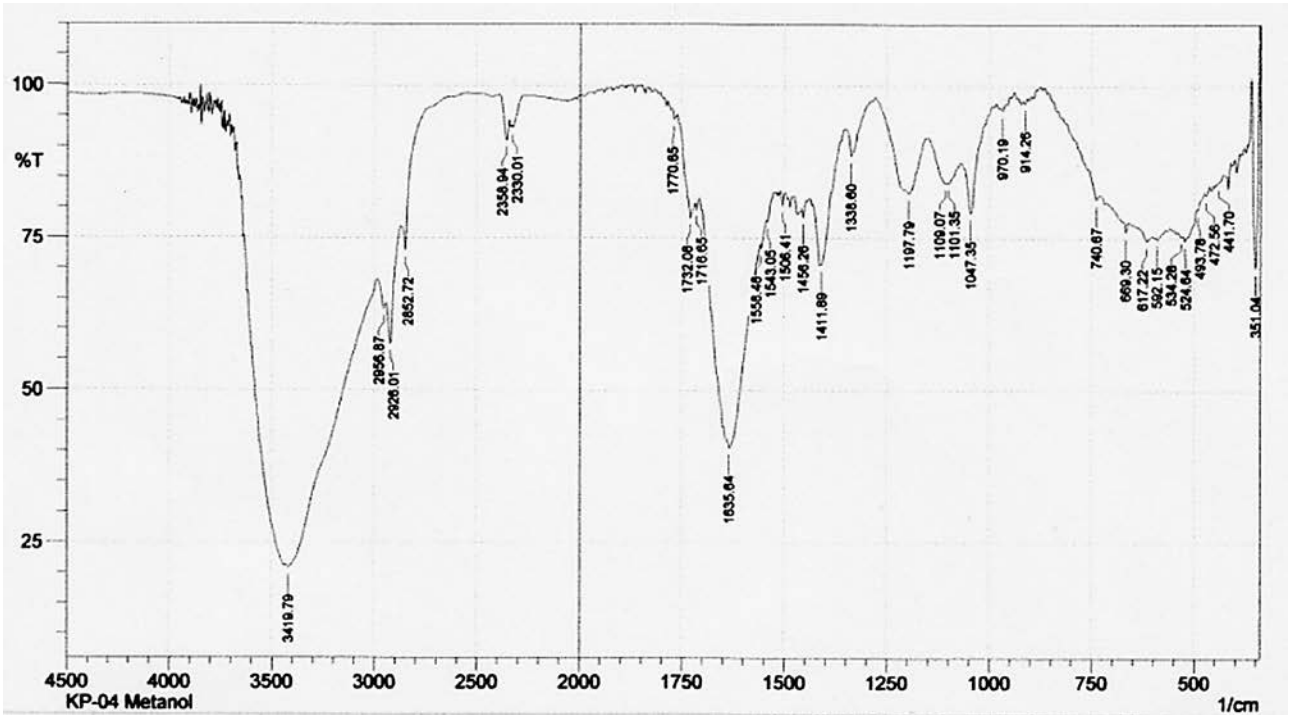


**Fig. 8. FTIR Analysis of *Liosina paradoxa* methanol extract**

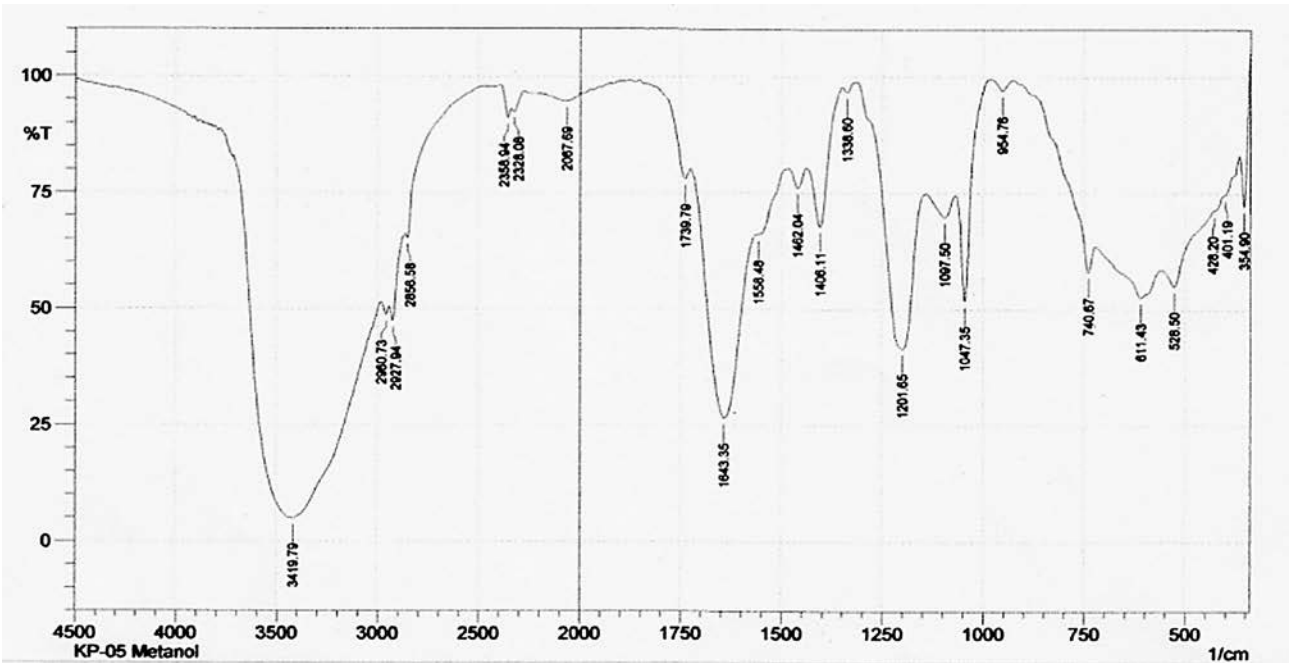


**Fig. 9. FTIR Analysis of *Haliclona (Reniera) fascigera* methanol extract**

simpler than those in Figs 6-8. A broad band at 3419.79 $cm^{-1}$ was assigned to be a typical stretching band of OH, supported by a primary or secondary bending band of OH at 1201.65 $cm^{-1}$ and bending band CO (strong) at 1047.35 $cm^{-1}$. Bands absorption at 2990.73 $cm^{-1}$, 2927.94 $cm^{-1}$, and 2856.56 $cm^{-1}$ were estimated as a stretching C-H of $CH_3$ and $CH_2$ groups, and shoulders observed at 3200 $cm^{-1}$ and 3090 $cm^{-1}$ could be estimated as bands absorption of =C-H olefin and ≡C-H, supported by stretching bands at 1643.36 $cm^{-1}$ and at 2067.69 $cm^{-1}$ for C=C and C≡C, respectively. Band absorption at 1739.79 $cm^{-1}$ was assigned to be a stretching band of C=O, supported by overtone (weak) near 3700 $cm^{-1}$. Sharp band absorption at 611.43 $cm^{-1}$ could be estimated as *cis* C-H out of plane olefin or stretching C-Br for aliphatic bromo compounds. Band absorption at 740.6 $cm^{-1}$ was estimated

as rocking band $CH_2$ or stretching band C-Cl. Broad bands absorption at 3419.79 $cm^{-1}$ and 1558.48 $cm^{-1}$ could be estimated by overlapping of band absorption for OH stretching and either NH or $NH_2$ stretching and bending.

## Conclusion

All extracts identified contain alkaloid, steroid and terpenoid. Typical band sorption stretching CH aliphatic, CH olefin, OH, C-O, C=C, bending $CH_3$ and $CH_2$ appear in all extract. Its mean in all extracts contain aliphatic, olefinic and alcohol compounds. Typical band sorption NH for alkaloid also appears in all extracts. The strength of these bands sorption varied from weak to medium, overlapping with other bands. *Niphates* sp., *Liosina paradoxa*, *Haliclona (Reniera) fascigera* were cytotoxic.

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