Antioxidant and Cytotoxic Properties of Soft Coral *Nepthea* sp.

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Abstract

Soft coral Nepthea sp. grows in the seas of South-East Sulawesi, Indonesia. However, information on the chemical and pharmaceutical aspects of this genus is still limited. Therefore, this research aims to explore the chemical contents and biological activities of Nepthea sp. The sample was collected from the waters of Saponda Island by SCUBA diving. It was extracted by ethyl acetate and fractionated using vacuum liquid chromatography. The chemical content was analyzed by phytochemical screening, LC-MS/MS analysis, Total Phenolics Content and Total Flavonoids Contents. Antioxidant potency was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). Cytotoxicity property was analyzed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. The result showed that the fractionation of Nepthea ethylacetate extracts produced six fractions (A-F). Fractions A and B contain non-polar compounds. Based on LC-MS/MS data, the non-polar compounds in Fraction A and B include achillin, atractylenolide II, buthyl isobuthyl phthalate, rengyolester, 2a-acetoxycostic acid, ocotillol acetate, petasitolone and some unidentified compounds that are $C_{33}H_{58}O_4$, $C_{15}H_{21}NO$, $C_{21}H_{33}NO$, and $C_{16}H_{20}O_4$. In general, the antioxidant and cytotoxic properties of all samples are in the weak category, however, when examined for each sample, the antioxidant properties of fraction B is slightly better than fraction A based on the IC_{50} value of DPPH and ABTS. Cytotoxicity of Fraction A is better than Fraction B against Breast Cancer cell lines MCF-7. The non-polar fraction of Nepthea sp. can be developed as raw material for the discovery of new compounds, antioxidant and anticancer agents, especially breast cancer.

Keywords: *Nepthea* sp., non-polar fraction, antioxidant, cytotoxic, South East Sulawesi

Introduction

Southeast Sulawesi is an archipelagic province, has 651 islands, 74.25% of its territory is the ocean (Bangwilsultra, 2016). One of the important and abundant marine natural resources is soft coral. In Waworaha Waters, Konawe, with a monitoring area of 700 m2, eight types of soft coral were found (Wanda *et al*., 2018). The chemical content and efficacy in the pharmaceutical field have not been reported yet. Even though there are many new compounds from soft corals that have very interesting properties, such as sarcophine from *S. glaucum* growing in the Red Sea is active as a modulator of glycine receptors in the nervous system (Saleh *et al*., 2020), *S. solidum* produces diterpene compounds (Zhu *et al*., 2015), and several terpenoid compounds are also obtained from *Lobophytum crasum* and oxygenated steroids from *S. pauciplicatum*, can increase the immune system to fight infection of cancer patients (Florean *et al*., 2020).

Nepthea sp. (Nephtheidae) is one of the soft corals found in Waworaha, It is also considered widespread in the Indo-Pacific region (Seah *et al*., 2015), Mauritius and Rodrigues Island (Jahajeeah *et al*., 2021), and the Brazilian Coast (Almeida *et al*., 2014). Some compounds reported from *Nepthea* that are tetraprenyl-benzoquinone derivative from *Nepthea* sp. and a guaiane-based sesquiterpene from *N. chabroli* (Almeida *et al*., 2014), nephtoacetal

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https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.27.1.29-36
Received : 12-12-2021
Accepted : 25-01-2022

from *Nepthea* sp. is active against HeLa cell lines (Zhang *et al*., 2013), erectasteroids A-H from *N. erecta* are cytotoxic against P-388 and HT-29 cell lines (Cheng *et al*., 2007), nebrosteroids A-H from N. chabroli have potential as anti-inflammation (Huang *et al*., 2008). The larger scope is the family level, Neptheidae, which has 20 genera, including *Nepthea*. Several secondary metabolites that have been identified from this family are sesquiterpenes. diterpenes, steroils, and quinones (Almeida *et al*., 2014). Some of them are paramlemnolin isolated from *Paralemnalia thyrsoides* (Huang *et al*., 2005), linardosenenes A-C and lineolimnenes A-D from *Litophyton nigrum* which active against some human cancer cell lines (Yang *et al*., 2020), and linardosenenes D-G from *L. nigrum* were active towards BRD4 (Yang *et al*., 2021). *Dendronepthtthya rebeola* produced acetoxycapnelle-2D,8E,13-triacetoxycapnell-9(12)-ene-10D-ol (Grote *et al*., 2007), flavalin E identified from *Lemnia flava* (Su *et al*., 2011) and sclerosteroid J isolated from *Scleronephthya gracillimum* (Fang *et al*., 2013).

In continuing our study on chemical and pharmaceutical aspects of marine natural resources, soft coral especially *Nepthea* sp. was chosen as the research sample. Before research on soft corals, we had worked with sponges and through the process of isolation and structure determination, we obtained a new compound from *Chlatria* sp, namely Clathruohate, and has been published in MJAS, 2018 (Sahidin *et al*., 2018), and chemical screening of some sponges (Sahidin *et al*., 2020). Biological activities of sponges include antihyperlipidemic (Wahyuni *et al*., 2019), Anti-Inflammatory (Fristiohady *et al*., 2019), antioxidant properties and acute toxicity (Fristiohady *et al*., 2020).

Based on the literature review (searching) in Science Direct and Springer Link, no information has been found regarding the study of chemical and pharmaceutical aspects of *Nepthea* sp. from South East Sulawesi (Indonesia). Soft corals from Indonesia whose chemical and pharmaceutical aspects have been reported include *Sinularia depresa* from the South China Sea which produces sinulasterol A-C which plays a role in cancer prevention through anti-inflammatory effects (Yang *et al*., 2020) and ethyl acetate and butanol extracts of *Lobophytum* sp. from Selayar, South Sulawesi, is active as an anti-bacterial and antioxidant (Putra *et al*., 2016). Meanwhile, the study of soft corals from Southeast Sulawesi is still in the mapping stage (Pedoja *et al*., 2018). This article explains the study of antioxidant and toxicity against breast cancer cells lines (MCF-7) as well as the study of the chemical content using LC-MS/MS of the nonpolar fraction of soft coral *Nepthea* sp. that grows in Southeast Sulawesi.

Materials and Methods

General procedures

A Waters Acquity UPLC I–Class was used in conjunction with a Xevo G2–X2 Quadrupole Time-of-Flight (QToF) mass spectrometer to perform the LC-MS/MS study. Methanol, ethyl acetate, and n-hexane are among the chemicals used, and the aquades are of analytical quality.Thin-layer chromatography plate: Kieselgel 60 F_{254} 0,25 mm (Merck), silica gel 60 GF_{254} p.a (Merck®), silica 60 G (Merck®), cerium sulphate ($CeSO_4$) (Merck®), ascorbic acid (Merck®), gallic acid (Merck®), quercetin (Merck®), doxorubicin (Merck®), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino - bis-3-ethylbenzthiazoline-6-sulphonicacid) Merck®), and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Merck®).

Nepthea sp. collection

The sample was taken on the reef slopes of the Saponda Islands in Indonesia's Province of Southeast Sulawesi. SCUBA diving at a depth of 4-10 m was used to collect the sample. The material was collected and put in separate ice containers before being returned to the laboratory for further analysis.

Extraction and fractionation

Fresh *Nepthea* sp. (3 kg) samples were chopped into small pieces and extracted in ethyl acetate (3x10 L, 24 h each time) at room temperature. Each dried soft coral extract was collected under reduced pressure and stored at 4 °C for future analysis in an amber bottle. The ethyl acetate extract (160 g) was fractionated using Vacuum Liquid Chromatography (VLC) with silica gel as an adsorbent and a combination of *n*-hexane: ethyl acetate (100:0 up to 0:100%) and methanol 100 per cent as eluent, yielding six fractions (A-F).

Phytochemical screening

The presence of alkaloids, flavonoids, tannins, terpenoids, steroids, and saponins in the selected sponges was determined using phytochemical screening methods (Harborne, 1973).

Total Phenolics Content (TPC) and Total Flavonoids Content (TFC)

The TPC of the samples were determined using the Folin and Ciocalteu reagent, following Singleton and Rossi's method with minor adjustments and the total flavonoids of the samples were determined using Chang *et al*'s (Chandra *et al*., 2014). The non-polar fraction of the soft coral *Nepthea* sp. was prepared for LC-MS/MS analysis using the standard

operating procedure of this instrument (Sahidin *et al*., 2020). Antioxidant assay of the samples was assessed using the DPPH radical (Sahidin *et al*., 2020). The modified Moniruzzaman method was used for ABTS Assay (Wahyuni *et al*., 2021). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess cytotoxicity in MCF-7 cells in vitro (Asasutjarit *et al*., 2021).

Result and Discussion

Vacuum Liquid Chromatography with silica gel as an adsorbent and a solvent mixture of n-hexane: ethyl acetate (100%:0 % up to 0%:100%) followed by methanol was used to fractionate ethyl acetate extract, to give six fractions (A-F). Fraction A and B have interesting TLC chromatogram patterns after being developed by *n*-hexane : ethyl acetate=8:2 as eluent (Figure 1.), and also have sufficient weight for further experiments such as biological activity tests or isolation of nonpolar compounds, 35.5 g of A and B= 4.3 g, respectively (Tabel 1.).

Table 1 shows that the ethyl acetate extract contains saponins, tannins, alkaloids and terpenoids, while fractions A and B only contain terpenoids. Because of fractions, A and B are the non-polar fraction, so that the compounds in the two fractions are non-polar. This phenomenon can also be observed from the Retardation factor (Rf) values of fractions A and B which are higher than the other fractions and also become the place where the top (non-polar) part of the ethyl acetate extract (T) TLC chromatogram is concentrated. The absence of polar compounds such as flavonoids, saponins, tannins, alkaloids and other phenolics in Fraction A and B can be seen from the value of TPC and TFC, where TPC and TFC of fractions A and B are 0.0 mgGAE.g Ex^{-1} and 0.0 mgQE. g Ex^{-1} respectively. This means that the polar portion of the ethyl acetate extract is stored in the other fraction. The presence of non-polar compounds from the non-polar fraction of *Nepthea* sp. is relevant with the compounds previously found from *Nepthea* sp. (Coll *et al*., 1985).

An in-depth study of the compounds of the non-polar fraction of *Nepthea* sp. ethyl acetate extract can be clearly explained based on the data from the LC-MS/MS analysis in Table 2 and Figure 2. Ethyl acetate extract has ten compounds and five of them has been identified, Fraction A has five compounds and seven compounds of Fraction B. Generally, all of the identified compounds are terpenoids consisting of monoterpenes (compound 3), sesquiterpenes (1,2,4,5,6,7,8,10) and steroids (9), which matches the previous invention (Almeida *et al*., 2014; Florean *et al*., 2020; Saleh *et al*., 2020).

An interesting phenomenon is the presence of nitrogenous compounds in the non-polar fraction. Usually, nitrogenous compounds in secondary metabolites are alkaloids which are semi-polar up to polar compounds. It is presumed that the alkaloids are found in the form of free nitrogen and not in the form of nitrogen salts. The use of this fractionation method can describe the diversity of compounds in the non-polar fraction of *Nepthea* sp., it will also make it easier to carry out the next step, namely the isolation and elucidation of non-polar compounds from *Nepthea* sp.

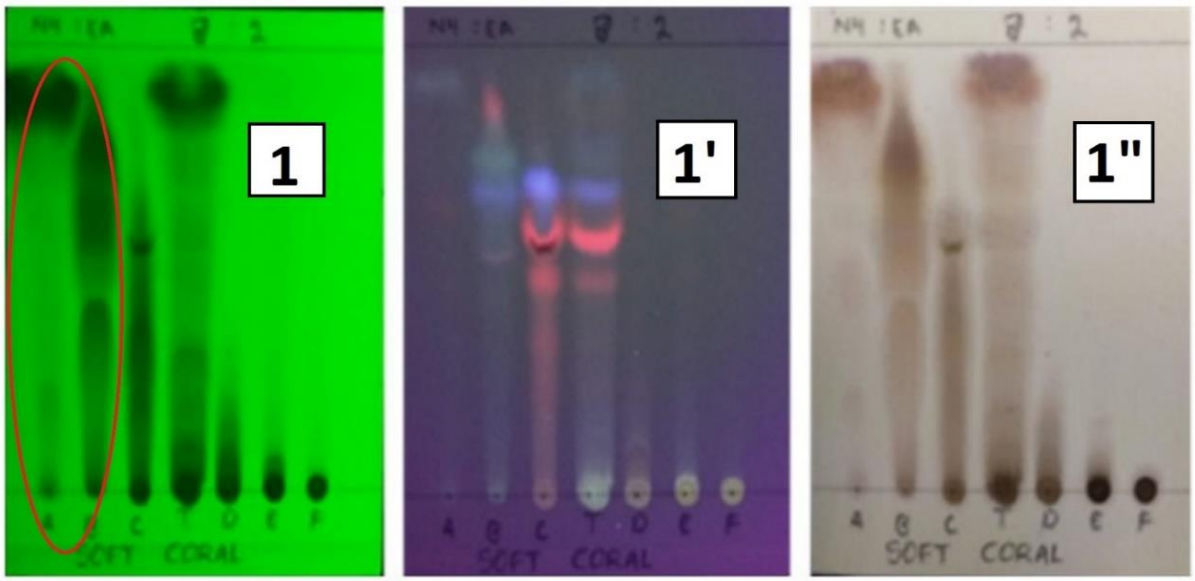

Figure 1. TLC Chromatogram of ethylacetate extract (T) and the fraction A-F
Note: **1 =** at λ 254 nm (short); **1'** = λ 366 nm (long); **1"** = $CeSO_4$ + heat

The biological activity of all samples will be clearer and more interesting when presented in Table 3 and graphical form in Figure 3. Antioxidant activity is represented by the IC_{50} value of DPPH and ABTS. Cytotoxicity of samples was evaluated against breast cancer cell lines MCF-7.

Based on the IC_{50} value of DPPH and ABTS of all samples in the Table 3, the IC_{50} value of all samples is >150 ppm (mgL^{-1}) or in other words the antioxidant properties of all samples are in the weak category (Tristantini *et al.,* 2016). The study for each sample shows that fractions A and B have antioxidant potential better than ethyl acetate extract. Although the ethyl acetate extract contains phenolic and flavonoid compounds which are indicated by the TPC and TFC values as listed in Table 3, the diversity and solubility of the compounds, as well as the mole fraction of non-TPC and non-TFC compounds, greatly affect the antioxidant power of the ethyl acetate extract. Furthermore, the antioxidant properties of fraction B were slightly better than fraction A, both based on the IC_{50} DPPH and ABTS values. This fact is supported by differences in compound content. Fraction B has compounds that have been identified LC-MS/MS consisting of 1,9 and 10 while fraction A contains 6,7 and 8. Compounds 1,9 and 10 contain hydroxyl units, so it is easier to donor hydrogen radicals than compounds 6,7 and 8. In other words, the compounds in Fraction B are easier to donate proton radicals or are easier to neutralize radicals or have stronger antioxidant properties.

Table 1. Weight, chemical profiles and biological activities of the samples

	Ethyl acetate macerate	Fraction	
		A	B
Weight (g)	160.0	35.2	4.3
Flavonoids	-	-	-
Saponins	+	-	-
Tannins/ Phenolics	+	-	-
Alkaloids	+	-	-
Terpenoids	+	+	+
TPC ($mgGAE.g\ Ex^{-1}$)	23.9± 0.87	0	0
TFC ($mgQE.\ g\ Ex^{-1}$)	2.35± 0.56	0	0

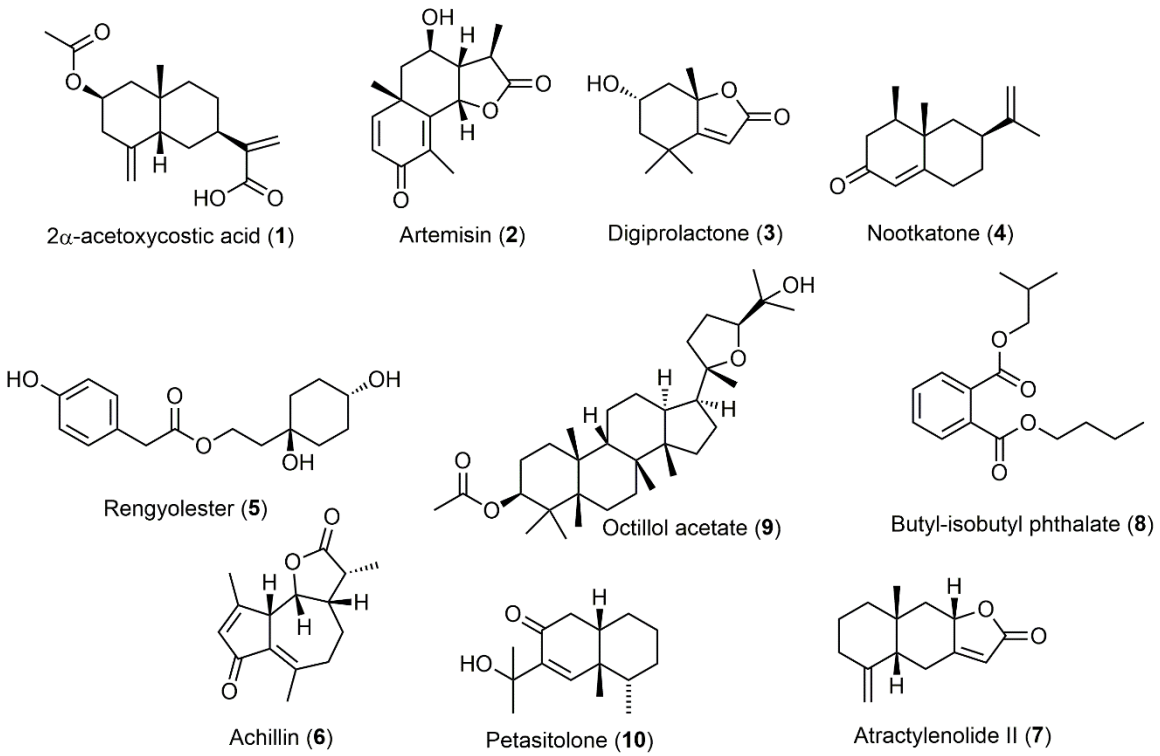

Figure 2. Identified compounds of ethyl acetate extract, fraction A and B

Table 2. Chemical profile of ethyl acetate extract, Fraction A and B based on LC-MS/MS data

. g Ex^{-1}	. g Ex^{-1}					
	. g Ex^{-1}	Ethylacetate Extract	Rt (min)	Fraction A	Rt (min)	Fraction B
. g Ex^{-1}	. g Ex^{-1}	2a-Acetoxycostic acid		-	10.44	2a-Acetoxycostic acid
. g Ex^{-1}	. g Ex^{-1}	Artemisinin		-		-
. g Ex^{-1}	. g Ex^{-1}	Digiprolactone		-		-
. g Ex^{-1}	. g Ex^{-1}	Nootkatone		-		-
. g Ex^{-1}	. g Ex^{-1}	Rengyolester	10.21	Rengyolester	7.91	rengyolester
. g Ex^{-1}	. g Ex^{-1}	-	10.16	Achillin		-
. g Ex^{-1}	. g Ex^{-1}	-	10.45	Atractylenolide II		-
. g Ex^{-1}	. g Ex^{-1}	-	10.35	Buthyl isobuthyl phthalate		-
. g Ex^{-1}	. g Ex^{-1}	-		-	12.61	Ocotillol acetate
. g Ex^{-1}	. g Ex^{-1}			-	6.64	Petasitolone
. g Ex^{-1}	. g Ex^{-1}	Candidate Mass $C_{15}H_{21}NO$		-	10.37	$C_{15}H_{21}NO$
. g Ex^{-1}	. g Ex^{-1}	Candidate Mass $C_{21}H_{33}NO_2$		-	10.74	$C_{21}H_{33}NO$
. g Ex^{-1}	. g Ex^{-1}	Candidate Mass $C_{16}H_{22}O_6$		-		-
. g Ex^{-1}	. g Ex^{-1}	Candidate Mass $C_{15}H_{23}NO_3$		-		-
. g Ex^{-1}	. g Ex^{-1}	Candidate Mass $C_{54}H_{78}O_9$		-		-
. g Ex^{-1}	. g Ex^{-1}	-	11.96	$C_{33}H_{58}O_4$		-
. g Ex^{-1}	. g Ex^{-1}	-		-	10.24	$C_{16}H_{20}O_4$

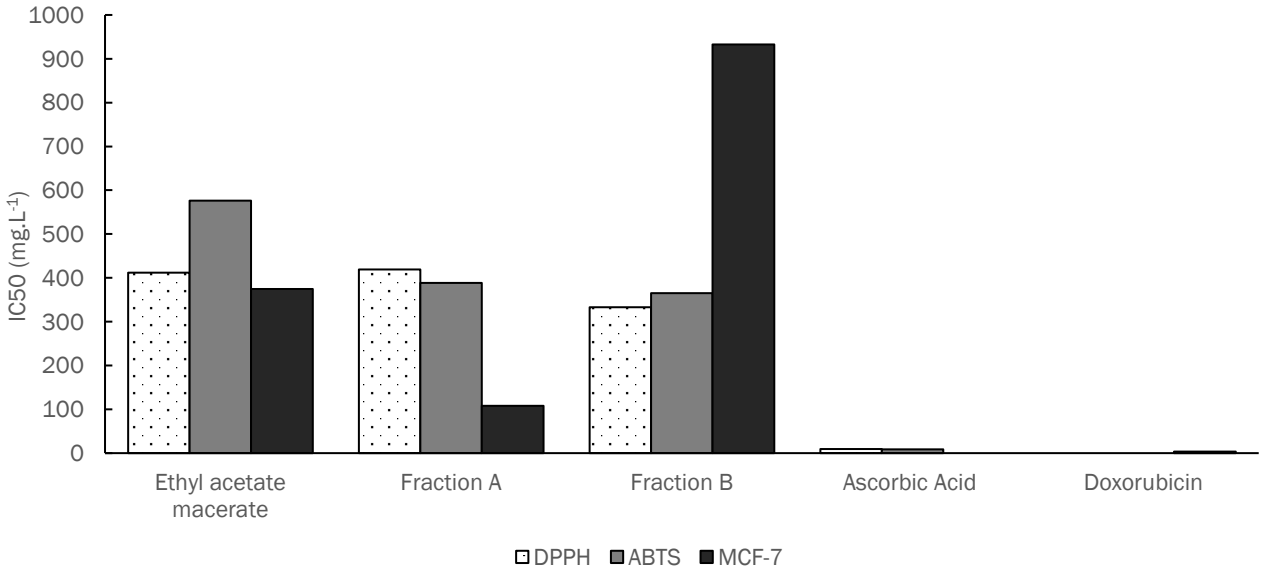

Figure 3. Biological activities of the samples

Table 3. Antioxidant and cytotoxic properties of the samples

	Ethyl acetate macerate	Fraction		Standard (positive control)	
		A	B	Ascorbic Acid	Doxorubicin
DPPH (IC_{50} in $mg.L^{-1}$)	412.01± 5.78	491.41± 4.22	333.26± 2.41	9.58± 0.57	
ABTS (IC_{50} in $mg.L^{-1}$)	576.30± 3.62	388.76± 2.64	364.87± 2.74	8.84± 0.69	
MCF-7 (IC_{50} in $mg.L^{-1}$)	374.97± 31.80	107.81± 6.40	932.96± 58.63		3.40± 0.25

Ethylacetate extract and Fraction A have cytotoxic potential against breast cancer cells lines (MCF-7), although their activities are weaker than that positive control. Based on the level of cytotoxicity (Weerapreeyakul *et al.*, 2012) that the extract is categorized as very active if it has IC_{50} <10 $mg.mL^{-1}$, active (10-100 $mg.mL^{-1}$), moderately active (100-500 $mg.mL^{-1}$), then ethylacetate extract and fraction A

were categorized as moderately active. For the samples, the cytotoxic properties of Fraction A had better cytotoxic properties than the ethylacetate extract and Fraction B. Furthermore, the ethyl acetate extract was stronger than the fraction B. This phenomenon is still quite difficult to explain. However, when looking at the structure of the standard compound (positive control) in this study, namely doxorubicin, and the active compound used in the treatment of breast cancer (tamoxifen) (Thayyib *et al.*, 2020), both compounds are alkaloids. Doxorubicin and tamoxifen have structural formulas $C_{27}H_{29}NO_{11}$ and $C_{26}H_{29}NO$, respectively.

Conclusion

The non-polar fraction of *Nepthea* sp. ethyl acetate extract has interesting potential for both the diversity of compounds and their biological activities. The compounds in the non-polar fraction based on LC-MS/MS data include achillin, atractylenolide II, buthyl isobuthyl phthalate, rengyolester, 2a-acetoxycostic acid, ocotillol acetate, petasitolone and some unidentified compounds. Furthermore, the non-polar fraction can be further developed as raw material for the discovery of new compounds, antioxidant and anticancer agents, especially breast cancer.

Acknowledgement

The Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia supported the project with a research grant under the scheme of the Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2021, contract No. 21/UN29.20 /PG/2021.

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