Chemical Profile, Antioxidant and Cytotoxic Potencies and Docking Study of Semipolar Fraction of *Nepthea* sp.

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Abstract. The aims of research is to explore pharmaceutical and chemicals aspects of *Nepthea* sp. The methods include fractionation of ethylacetate extract (EAE) by vacuum liquid chromatrography (VLC). The chemical content was evaluated by LC-MS/MS analysis, phytochemical screening, Total Phenolics Content (TPC) and Total Flavonoids Contents (TFC). Antioxidant and cytotoxic potencies were evaluated by DPPH/ABTS methods and MTT assays and also docking study. Fractionation of EAE produced six fractions (1-6). Fraction 3 and 4 were semi-polar compounds, which contained terpenoids, phenolics and alkaloids. According to LC-MS/MS, the identified semi-polar compounds consist of 3-acetyl-3,4-dihydro-5,6-dimethoxy-2(1)H-benzopyrone and oxyphyllenone B from Fraction 3 and 1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propanetriol, digiprolactone, petasitolone, δ-humulene, rengyolester, and piperolactam-C9:1(8E) for Fraction 4. The antioxidant potency of fraction 4 was better than Fraction 3. Based on IC_{50} value, Fraction 3 is active category and Fraction 4 is moderately active against MCF-7 cell lines. At the molecular level, rengyolester and piperolactam-C9:1(8E) revealed potential antioxidant activity by inhibiting XO, which was correlated with antiradical in vitro results. Meanwhile, the anticancer activity of oxyphyllenone B and piperolactam-C9:1(8E) demonstrated a higher affinity for inhibiting ERα than other compounds. As conclusion, semipolar fractions of EAE have good potential as an antioxidant and cytotoxic agent against MCF-7 cell lines.

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1. Introduction

Soft coral, particularly *Nepthea* sp., was selected as the research sample in the course of our ongoing study into the pharmacological and chemical aspects of marine resources. Before studying soft corals, we investigated some sponges. By isolation and structure elucidation, we discovered Clathruohate as a new compound from *Chlatri*a sp. and published in MJAS, 2018 [1]. In addition, chemical screening of various sponges have been reported [2]. The biological properties of some species of sponges have been studied including antihyperlipidemia [3], Anti-Inflammation [4], acute toxicity and antioxidant potency [5].

As an archipelagic province, Southeast Sulawesi has 651 islands, and the ocean covers 74.25% of its land area [6]. Soft coral is one of the valuable and plentiful marine natural resources. With a monitoring area of 700 m2 in Waworaha Waters, Konawe, eight different kinds of soft coral were discovered [7]. However, the pharmaceutical and chemical aspects have not yet reported.

In other locations, some soft corals have reported pharmaceutical and chemicals sides. In the red sea, *Sarcophagus glaucum* produces sarcophine, which acts as a modulator of glycine receptors in the neurological system, among other novel chemicals with highly fascinating features [8]. Diterpene compounds are produced by *S. solidum* as well as numerous terpenoid compounds from Lobophytum crasum and oxygenated steroids from *S. pauciplicatum*. These substances can boost cancer patients' immune systems to help them fight infection [9]. One of the soft corals found at Waworaha is *Nepthea* sp. (Nephtheidae). It is also thought to be common in the Indo-Pacific region [10], Mauritius and Rodrigues Island [11], and the Brazilian Coast [12]. Nephtoacetal from *Nepthea* sp. is active against HeLa cell lines [13], *N. erecta* produced erectasteroids A–H which are cytotoxic towards P-388 and HT-29 cell lines [14], and nebrosteroids A–H from *N. chabroli* have potential as anti-inflammatory substances, according to some compounds reported from *Nepthea* [15]. The Neptheidae family, which has 20 genera and includes *Nepthea*, has a wider scope. Sesquiterpenes are among the secondary metabolites from this class that have been found. steroils, quinones, and diterpenes [12]. Among them are paramlemnolin, which was isolated from *Paralemnalia thyrsoides* [16], linardosenenes A–C and lineolimnenes A–D from *Litophyton nigrum*, both of which are active against some human cancer cell lines [17], and linardosenenes D–G from *L. nigrum* [18]. Acetoxycapnelle-2D,8E,13-triacetoxycapnell-9(12)-ene-10D-ol was produced by *Dendronepthtthya rebeola* [19], flavalin E isolated from *Lemnia flava* [20] and sclerosteroid J identified from *Scleronephthya gracillimum* [21].

There is no information on the research of the pharmacological and chemicals features of *Nepthea* sp. from South East Sulawesi. According to the literature of Indonesian soft corals, ethylacetate and butanol extracts of Selayar's *Lobophytum* sp., South Sulawesi, are active as an antioxidant and anti-bacterial [22], and *Sinularia depresa* of the South China Sea produces sinulasterol A, B and C, which contributes to cancer prevention through anti-inflammatory effects [23]. The research on Southeast Sulawesi's soft coral is still in the plotting phase at the moment [24]. In this article, the chemical composition of the semipolar fraction of *Neptha*e sp., which is found in Southeast

Sulawesi, as well as the investigation of antioxidant and toxicity potencies towards breast cancer cells (MCF-7) and also docking study are both discussed.

2. Material and Methods

2.1 General Procedures.

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

2.2 *Nepthea* sp. Collection

The sample was collected form the Saponda Islands, Province of Southeast Sulawesi, Indonesia's which used SCUBA diving at a depth of 4-10 m. The sponge was saved and put in separate ice containers before analysing in the laboratory. Expert staff (Baru Sadarun, Ph.D) from the Faculty of Fisheries and Marine Science of Universitas Halu Oleo performed the sample identification, and the specimen was deposited in the Faculty of Pharmacy of Universitas Halu Oleo.

2.3 Sample Extraction and Fractionation

The sample (*Nepthea* sp., 3 kg) was chopped into small pieces and extracted in ethylacetate (3 x 10 L, 24 hours each time) at temperature's room. Each dried sample extract was gathred under reduced pressure and stored at 4°C for further analysis in an amber bottle. The ethylacetate extract was fractionated using VLC (Vacuum Liquid Chromatography) with silica gel as an adsorben and a mixture of n-hexane:ethylacetate (100:0 up to 0:100%) and methanol 100 percent as eluen, yielding six fractions (A-F).

2.4 Phytochemical screening

The phytochemical screening methods [25] was used for checking the presence of alkaloids, flavonoids, tannins, terpenoids, steroids, and saponins of the sample.

2.5 Total phenolics content (TPC) and total flavanoids content (TFC)

The TPC of *Nepthea* sp were evaluated by the Folin and Ciocalteu procedure, following Singleton and Rossi's method with minor modification and the total flavonoids of the samples were decided using Chang et al [26].

2.6 LC-MS/MS Analysis

he fraction 3 and 4 of *Nepthea* sp. ware prepared for LC-MS/MS analysis using standard operational procedure of this device [2].

2.7 DPPH and ABTS Assays

According to a prior study, the antioxidant potency of the samples was judged using the DPPH radical [2] and ABTS test used the modified Moniruzzaman Method [27].

2.8 MTT Assay

Cytotoxicity in MCF-7 cells in vitro potency was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test [28].

2.9 Molecular Docking Simulation

We selected the 3D structure of the estrogen receptor alpha (ERα) (PDB CODE: 3ERT) and xanthine oxidase (XO) (PDB CODE: 3NRZ) from the RCSB website [29,30]. These structures were selected based on 4-hydroxytamoxifen and hypoxanthine as native ligands. The receptors were prepared by removing unbounded ligands and water molecules. Lastly, the protonation and the Kollman charges were applied to the receptors with the help AutoDockTool 1.5.6 [31]. The structure of identified compounds in LC-MS/MS data was collected from the PubChem database. All compounds were arranged in the same software by inserting hydrogen atoms and Gasteiger charges. The final step was to arrange the each compound's bond to rotate freely [32]. The molecular docking simulations were performed with iDock software with default settings [33]. The process of redocking of the ligand's native to the ERα and XO was a method to authenticate the docking protocol. Valid protocols are given by a root mean square deviation (RMSD) value below 2 Å. The binding pocket was fitted by monitoring the 4-hydroxytamoxifen and hypoxanthine at coordinates in each receptor with a grid area of 35 x 35 x 35 Å for ERα and 25 x 25 x 25 Å for XO. The binding energies of total molecules were summarized, and their interactions were evaluated with Discovery Studio Visualizer.

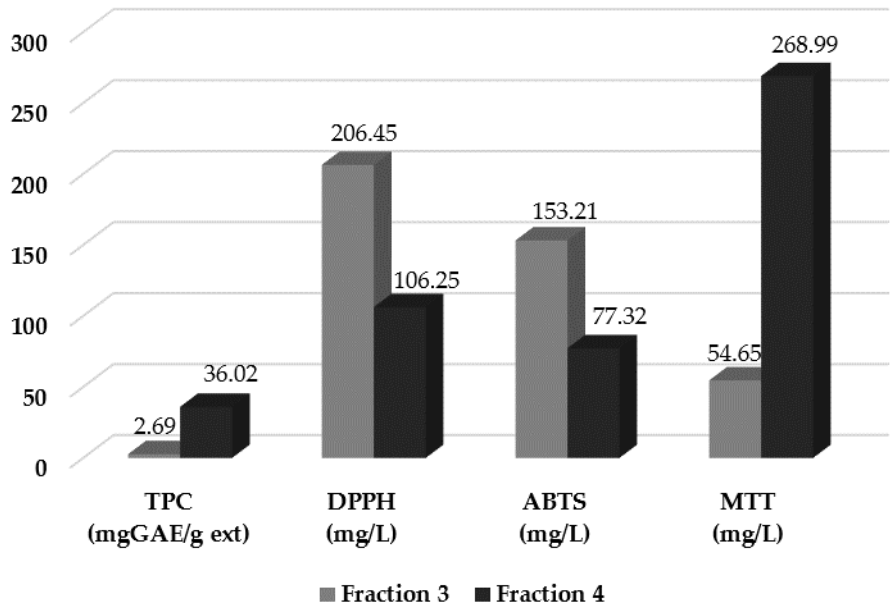
3. Results and Discussion

The VLC with silica gel as an stationare phase and an eluent mixture of n-hexane: ethylacetate (100% : 0 % up to 0%:100%) followed by methanol were used to fractionate EAE, to give six fractions (1-6). Fraction 1-2 are non-polar fraction, 3-4 are semipolar fraction and 5-6 are polar fraction. Fraction 3-4 have interesting biological activities, fraction 3 is active anticancer towards MCF-7cells lines and Fraction 4 is active anti-

oxidant using DPPH and ABTS methods [29]. The detail data is displayed in Table 1 and Figure 1.

Table 1. The TPC and biological activities of Fraction 3 and 4

Method	Fraction 3		Fraction 4	
	Regression Linear	Value	Regression Linear	Value
TPC (mgGAE/g ext)	y = 0.0063x + 0.1284 R^2 = 0.9885	2.69 ± 1.74	y = 0.0063x + 0.1284 R^2 = 0.9885	36.02 ± 1.15
DPPH (IC_{50} in *mg/L*)	y = 0.1972x + 9.2886 R^2 = 0.9023	206.45 ± 1.36	y = 0.2927x + 18.902 R^2 = 0.9985	106.25 ± 1.02
ABTS (IC_{50} in *mg/L*)	y = 0.195x + 20.123 R^2 = 0.9645	153.21 ± 1.54	y = 0.6203x + 2.0355 R^2 = 0.9476	77.32 ± 1.62
MTT (IC_{50} in *mg/L*)	y = 0.2657x + 35.48 R^2 = 0.9531	54.65 ± 1.30	y = 0.1983x - 3.3418 R^2 = 0.9614	268.99 ± 1.27



Figu.1. Comparison TPC and biological activities of Fraction 3 and 4

TPC of Fraction 4 is greater than TPC of Fraction 3, the impact on the antioxidant potential of Fraction 4 is stronger than Fraction 3, but inversely proportional to its cytotoxic properties. The cytotoxic properties of EAE against MCF-7 breast cancer cells, Fraction 3 showed better potential than the potential of Fraction 4. The difference in potency, both antioxidant and cytotoxic, was powerfully influenced by the chemicals of Fractions 3 and 4. Based on the results of phytochemical screening in Table 2, Fraction 3

and Fraction 4 have the same type of compound, different in amount or concentration. Both fractions contain terpenoids, saponins, tannins/phenolics and alkaloids.

Table 2. Phytochemical screening of fractions 3 and 4 of *Nepthea* sp.

Sample (s)	Weight (g)	Chemical Contents				
		terpenoids	saponins	Tannins/ phenolics	Flavonoids	Alkaloids
Fraction 3	5.9 (3.5 %)	++	++	++	-	++
Fraction 4	10,7 (6.7 %)	++	+	+++	-	+

A more detailed study on the chemical content of Fraction 3 and Fraction 4 of *Nepthea* sp was carried out using the LC-MS/MS instrument. Two of eight major compounds of Fraction 3 have been identified namely 3-acetyl-3,4-dihydro5,6-dimethoxy-2(1)H-benzopyrone and oxyphyllenone B. The unidentified compounds consist of $C_{15}H_{21}NO$, $C_{21}H_{33}NO_2$, $C_{45}H_{84}NO_{14}$, $C_{15}H_{23}NO_3$, $C_{15}H_{21}NO_3$ and $C_{15}H_{21}NO_2$. Fraction 4 has nine major compounds consist of six identified compounds and three unidentified compounds. The identified compounds namely 1-(4-Hydroxy-3-methoxy)-phenyl-1,2,3-propanetriol, digiprolactone, petasitolone, δ-humulene, rengyolester and piperolactam-C9:1 (8E), and unidentified compounds including $C_{15}H_{21}NO$, $C_{21}H_{33}NO_2$ and $C_{28}H_{42}O_4$. Decision making on the names of identified compounds was based on MS/MS data consisting of Retention time, Observed [M+H]/ [M+Na] (m/z), Experimental Neutral Mass (Da), Theoretical Neutral Mass (Da) and MS^n Fragmentation as listed in Table 3.

Table 3. Compounds of Fraction 3 and Fraction 4 based on LC-MS/MS data

Sample	No	Rt (min)	Observed [M+H]/ [M+Na] (m/z)	Experimental Neutral Mass (Da)	Theoretical Neutral Mass (Da)	MS^n Fragmentation	Component Name
Fraction 3	1	9.74	251.0893	250.08412	250.08412	197.08; 137.05	3-Acetyl-3,4-dihydro5,6-dimethoxy-2(1)H-benzopyrone [34]
	2	10.23	233.1142	210.12559	210.12559	193.13; 165.09	Oxyphyllenone B [35, 36]
Fraction 4	3	7.34	237.0732	214.08412	214.08412	137.06	1-(4-Hydroxy-3-methoxy)-phenyl-1,2,3-propanetriol [37]
	4	8.04	197.1168	196.10994	196.10994	179.11; 135.12	Digiprolactone [38]
	5	9.60	237.1848	236.17763	236.17763	219.17; 201.16	Petasitolone [39]
	6	10.03	203.1790	202.17215	202.17215	173.08; 119.08	δ-Humulen [40]
	7	10.24	295.1539	294.14672	294.14672	277.14; 217.12	Rengyolester [41]

	8	10.32	352.1906	329.19909	329.19909	232.17, 217.12	Piperolactam-C9:1 (8E)[42]

The certainty of the names and structures of the compounds listed in Table 1 was determined by comparing the fragmentation patterns of these compounds with the literature as shown in Figures 2 and 3.

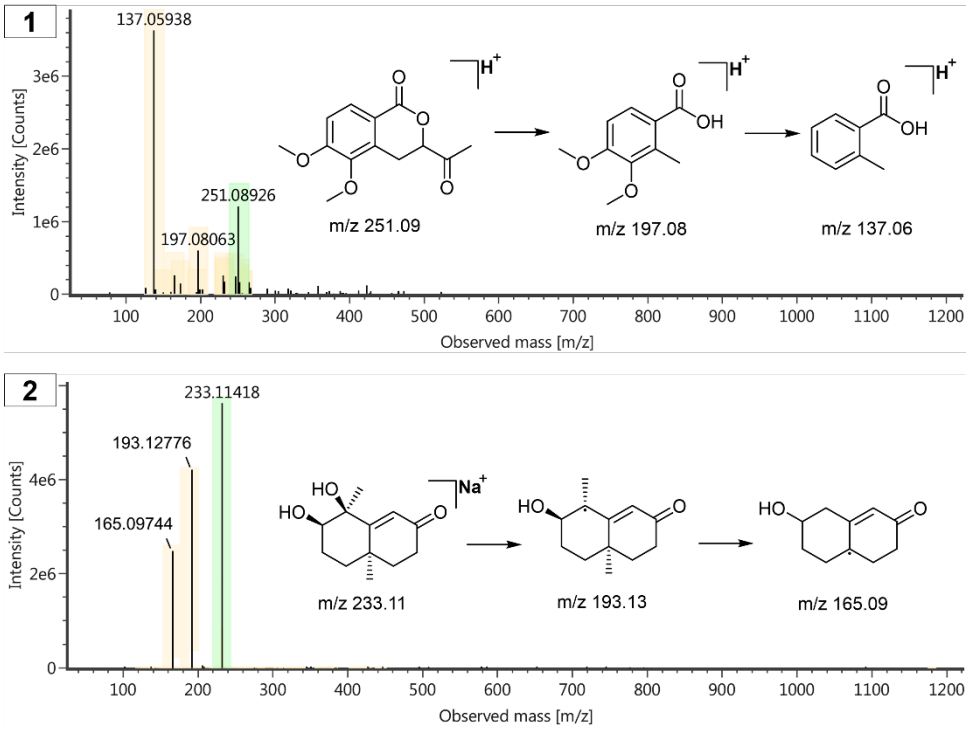
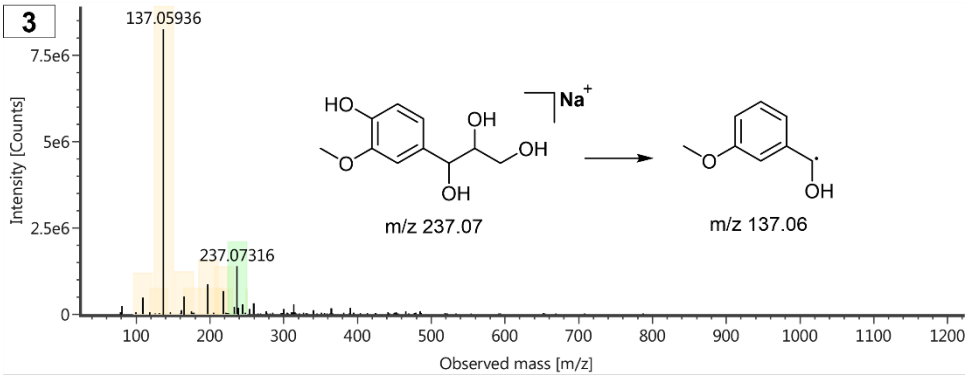


Fig.2. Fragmentation pattern of ESI-MS^n of identified compounds in Fraction 3 (**1**) 3-Acetyl-3,4-dihydro5,6-dimethoxy-2(1)H-benzopyrone, (**2**) Oxyphyllenone B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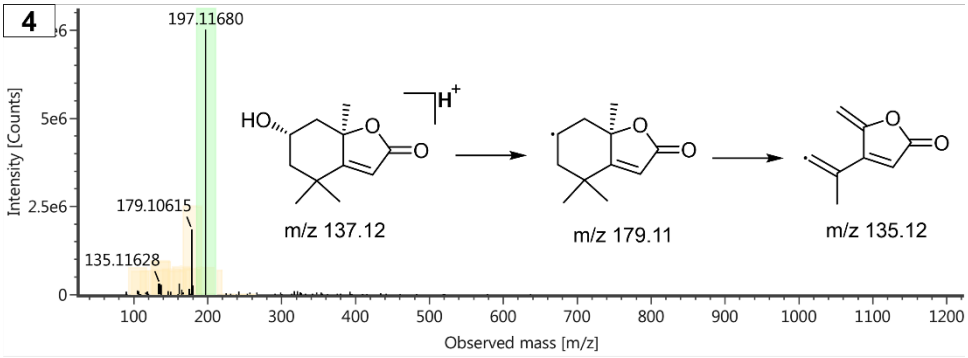
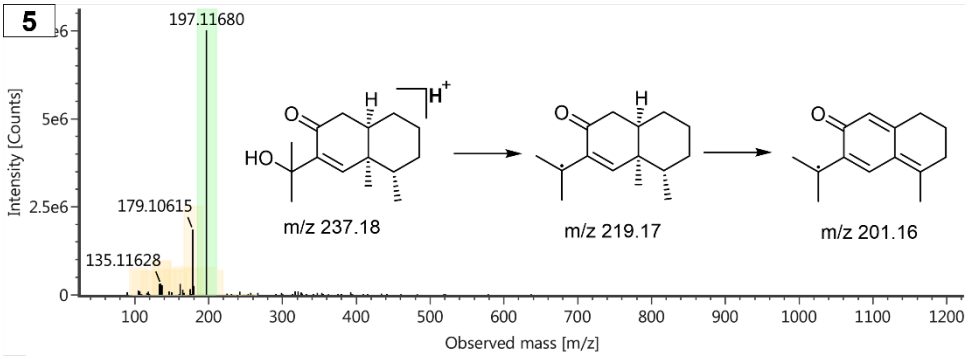
137.05936
237.07316
Intensity [Counts]
Observed mass [m/z]
m/z 237.07
m/z 137.06


4
197.11680
179.10615
135.11628
Intensity [Counts]
Observed mass [m/z]
m/z 137.12
m/z 179.11
m/z 135.12


5
197.11680
179.10615
135.11628
Intensity [Counts]
Observed mass [m/z]
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m/z 219.17
m/z 201.16

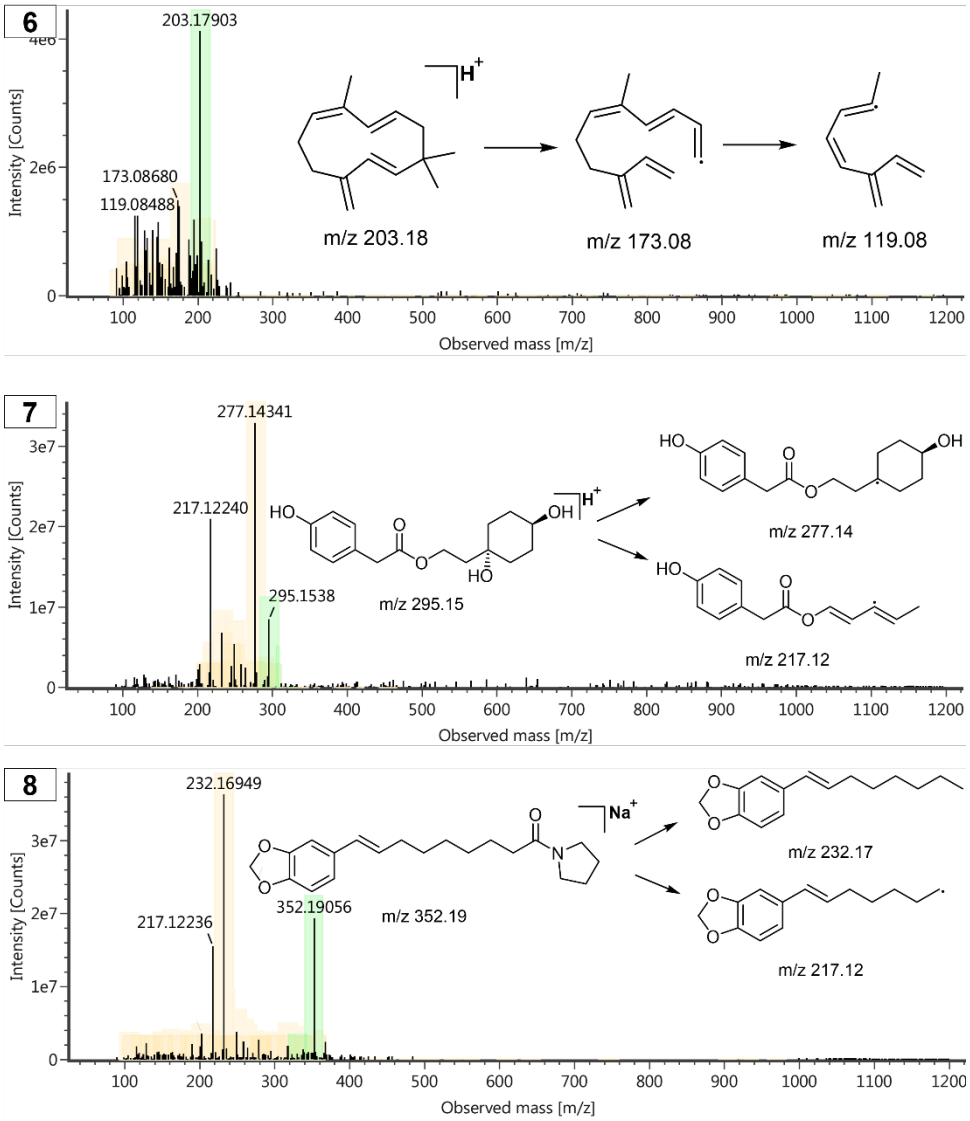

Fig.3. Fragmentation pattern of ESI-MS^n of identified compounds in Fraction 4 (**3**) 1-(4-Hydroxy-3-methoxy)-phenyl-1,2,3-propanetriol, (**4**) Digiprolactone, (**5**) Petasitolone, (**6**) δ-Humulene, (**7**) Rengyolester, (**8**) Piperolactam-C9:1 (8E)

Based on the interpretation of fragmentation data, the structure of the identified compounds from Fractions 3 and 4 of ethyl acetate extract *Nepthea* sp coral is shown in Figure 4.

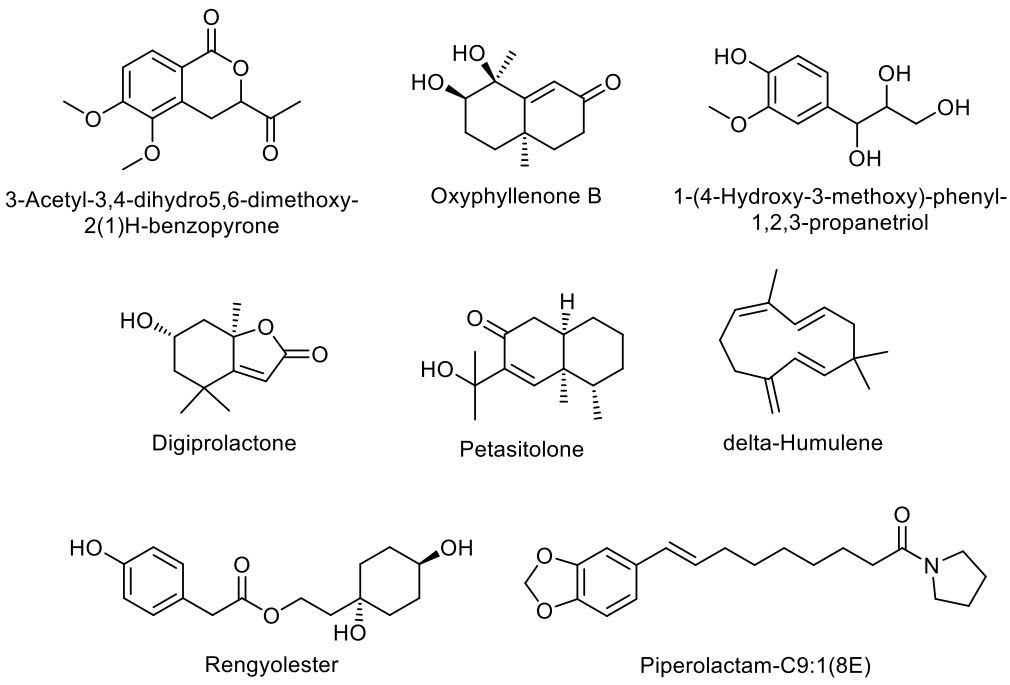

Fig.4. Identified compounds of Fraction 3 and 4

The absence of saponins, tannins, alkaloids and other phenolics compounds in Fraction 3 and 4 can be seen from the TPC value in Table 1, where is the TPC of Fractions 3<4. This causes the antioxidant potential of Fraction 4 to be better than Fraction 3 both DPPH and ABTS methods. The phenolics compounds have been identified in Fraction 4 are 1-(4-Hydroxy-3-methoxy)-phenyl-1,2,3-propanetriol and rengyolester. Some extracts that are reported to contain rengyoester and have antioxidant properties include non-polar fraction of ethyl acetate extract of *Nepthea* sp [43] and *Forsythiae fructus* [44]. Other compounds contained in Fraction 4 are not phenolic compounds but are antioxidants, namely digiprolactone [45] and δ-Humulene [46].

Fraction 3 has cytotoxic potential against breast cancer cells lines (MCF-7) better than Fraction 4 (Table 1 and Figure 1). This is caused by the alkaloids content of Fraction 3 which is more than Fraction 4 as shown by the results of phytochemical screening in Table 2, and is also supported by the results of the analysis of the chemical content of LC-MS/MS which shows a large amount of alkaloid compounds although they have not been identified or are still in the chemical formula that is $C_{15}H_{21}NO$, $C_{21}H_{33}NO_2$, $C_{45}H_{84}NO_{14}$, $C_{15}H_{23}NO_3$, $C_{15}H_{21}NO_3$ and $C_{15}H_{21}NO_2$. This consideration is based on the positive control used in the cytotoxic test is doxorubicine, and the active compound used in the handling of breast cancer is tamoxifen [47], both compounds are alkaloids. Doxorubicin and tamoxifen have structural formulas $C_{27}H_{29}NO_{11}$ and $C_{26}H_{29}NO$, respectively.

Molecular Docking Result

This soft coral fraction's cytotoxic and antioxidant activity was explored at the molecular level against ERα and XO receptors. The molecular docking process was confirmed to be valid with an RMSD value of 4-hydroxytamoxifen redocking of 1.158 to ERα (Figure 5A)

and hypoxanthine of 0.32 to XO. (Figure 5B). 4-hydroxytamoxifen and hypoxanthine have binding energies of -9.61 kcal/mol and -6.58 kcal/mol, respectively. 4-hydroxytamoxifene interacts with the ERα binding site, resulting in several interactions such as hydrogen bonds (Glu353 and Arg394) and hydrophobic interactions with Met388, Leu346, and Met421. Meanwhile, hypoxanthine exhibits three hydrogen bonds with Glu802, Arg880 and Thr1010 and hydrophobic interaction with Phe914, Ala1078, and Ala1079 on the XO active site. The formed interactions are identical to our prior studies, so using this simulation software supports validating the docking process [48].

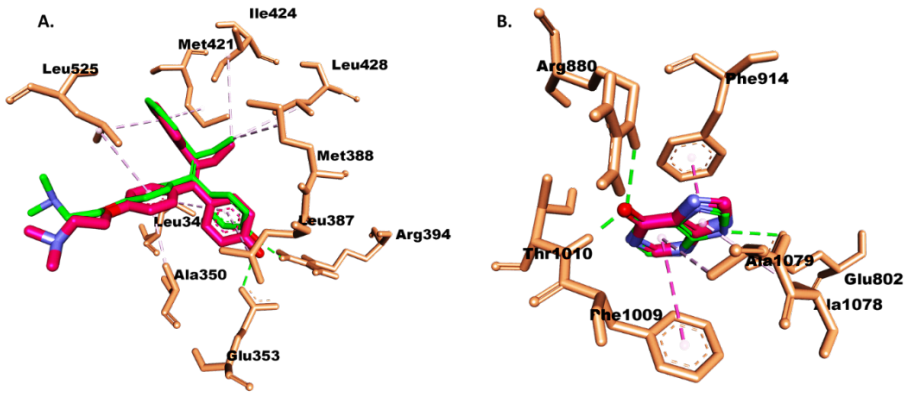

Fig.5. The 3D conformation and interaction of native ligand to its receptors. (A) co-crystallized (pink) and docked 4-hydroxytamoxifen (green) to ERα. (B) co-crystallized (pink) and docked hypoxanthine (green) to XO. Green and pink dashed lines illustrated hydrogen bonds and hydrophobic interactions

Overall, as many as eight compounds identified in the *Nepthea* sp soft coral fraction showed potential activity as ERα inhibitors associated with anticancer and XO inhibitors for antioxidant activity (Table 4). This potential was demonstrated by obtaining a negative binding energy value. The more negative binding energy values correlate with the compound's strong affinity to the receptor [49]. In ERα, compounds 2 and 8 have lower binding energy than the other compounds, with values of -7.04 kcal/mol and -7.63 kcal/mol, respectively. Interestingly, no hydrogen bonds were observed in compound 2. In compound 8, there was a hydrogen bond with Gly521. Meanwhile, the two compounds exhibit hydrophobic interactions with residues Leu346, Met388, Met421 and Leu525, which at the active site of ERα are similar to those of 4-hydroxytamoxifen interaction (**Figure 6**).

Table 4. Binding energy prediction from identified compounds in *Nepthea* sp fractions against ERα and XO

Compounds	ERα		XO	
	Binding energy (kcal/mol)	**Interactions***	**Binding energy (kcal/mol)**	**Interactions***
(1)	-5.75	**Val534,** Leu536	-7.46	**Gln767, Ser1080, Phe798**
(2)	-7.04	Leu346, Leu387, Met388, Leu391, Phe404, Met421	-7.71	**Gly799, Ser1082,** Met1038
(3)	-6.1	**Gly420, Glu423,** His524	-6.6	**Thr1077**, Met1038

Compounds	ERα		XO	
	Binding energy (kcal/mol)	**Interactions***	**Binding energy (kcal/mol)**	**Interactions***
(4)	-6.51	Leu346, Leu384, Leu525	-7.4	**Gln1040, Ser1082,** Arg912, Met1038
(5)	-6.79	Trp383, Leu525, Leu536	-8.16	**Ser1082, Thr1083,** Arg912, Met1038
(6)	-6.7	Leu346, Leu349, Ala350, Trp383, Leu387, Met388, Met421, Ile424, Leu428, Leu525	-7.17	Leu648, Phe649, Leu873, Val1011, Phe1013, Leu1014, Pro1076
(7)	-6.9	**Glu353,** Leu346, Leu391	-9.47	**Gln767, Arg880, Thr1077, Ala1079,** Phe914, Ala1078, Ala1079
(8)	-7.63	**Gly521,** Leu346, Ala350, Met421, Leu525	-9.94	**Val1011, Gln1194,** Phe914, Phe1009, Met1038, Ala1078, Ala1079

* Bold characters represent hydrogen bonds, while hydrophobic interactions are represented by regular letters.

We suspect that the difference in the hydrogen bond interactions causes the compounds from *Nepthea* sp to have a lower affinity than 4-hydroxytamoxifen. At the molecular level, there is a possibility that the *Nepthea* sp compounds work on other cancer targets that still need to be explored. In addition, several unidentified from this soft coral fraction may have a high affinity to the ERα receptor.

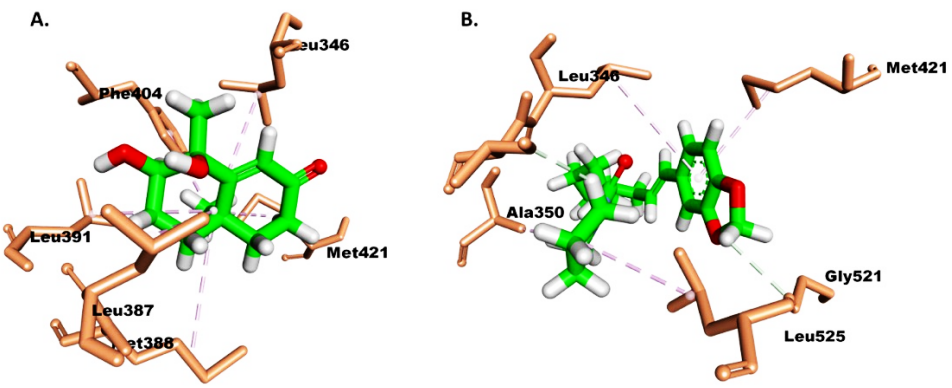

Fig.6. The interaction of identified compounds in *Nepthea* sp fractions with ERα. (A) compound 2 (Oxyphyllenone B), and (B) compound 8 (Piperolactam-C9:1 (8E))

Interestingly, the interaction of identified compounds in *Nepthea* sp fractions produced findings in line with the antiradical DPPH and ABTS tests, with compounds from fraction 4 having higher antioxidant activity than compounds from fraction 3. The best antioxidant activity in inhibiting XO was shown by compounds 7 and 8, with lower binding energy than hypoxanthine of -9.47 kcal/mol and -9.94 kcal/mol. In compound 7,

four hydrogen bonds were observed with Gln767, Arg880, Thr1077 and Ala1079 and compound 8 formed three hydrogen bonds with Val1011 and Gln1194. Furthermore, these two compounds include hydrophobic interactions with Phe914, Phe1009, Ala1078 and Ala1079 residues on the confirmed XO binding sites, comparable to those produced by hypoxanthine (**Figure 7**).

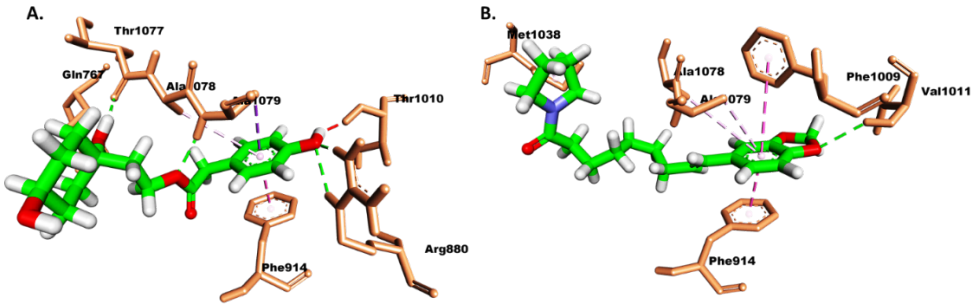

Fig.7. The interaction of identified compounds in *Nepthea* sp fractions with XO. (A) compound 7 (Rengyolester), and (B) compound 8 (Piperolactam-C9:1 (8E))

4. Conclusion

The semi-polar fraction of *Nepthea* sp. ethylacetate extract consist of Fractions 3 and 4 have the interesting potential both the diversity of compounds and their biological activities. The fraction 3 has better potential as anti-cancer especially breast cancer against MCF-7 cells lines which was supported by alkaloids contents. The Fraction 4 has Total Phenolic Contents better than Fraction 3, has good potential as antioxidant agents. At the molecular level, compounds 7 and 8 from *Nepthea* sp. revealed potential antioxidant activity by inhibiting XO, which was correlated with antiradical in vitro results. Meanwhile, the anticancer activity of compounds 2 and 8 demonstrated a higher affinity for inhibiting ERα than other compounds.

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