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NOOTKATONE AND ANTIOXIDANT COMPOUNDS IN *INOCARPUS FAGIFERUS* FOSB SEEDS

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ABSTRACT

Inocarpus fagiferus Fosb or Gayam is one of the species of the *Legumineceae* family whose seeds have been traditionally used for anti-inflammatory and coronary heart disease caused by plaque atherosclerosis. Gayam seeds in vivo potentially can be used as an antioxidant regarding atherosclerosis mechanism. This study aims to isolate and identify antioxidant activity toward DPPH of Gayam seeds. The result of antioxidant activity showed that IC_{50} values 156.00; 230.00; and 1303.76 mg/L of n-hexane, chloroform, and water extract respectively. Separation of n-hexane extract using column chromatography (stationer phase silica gel 60; mobile phase n-hexane-chloroform (2:1)) produced 4 fractions with the smallest IC_{50} value in fraction C being 330.24 mg/L. Analysis of mass spectra from the chromatography peaks of LC-MS/MS result showed that the isolate (fraction C) allegedly contains nootkatone as an antioxidant compound which is a mixture with other compounds (22E)-chola-5,22-dien-3-ol; 5,7-dimethoxyflavanone; (11β,16α)-11,17,21-trihydroxy-16-methylpregn-4-ene-3,20-dione; 11β-hydroxy-3,20-dioxopregn-4-en-21-oic-acid and unidentified compound with a mass of 321.0792 g/mol.

KEYWORDS: Antioxidants, atherosclerosis, *Inocarpus fagiferus* Fosb, LC-MS/MS, and Nootkatone.

1. INTRODUCTION

Reactive oxygen species (ROS) interact with various macromolecules caused to lipid peroxidation, damaging DNA strains (nucleic acid base), and protein changes.[1] Lipid membrane peroxidation causes damage or changes in the biological structure of the membrane, deactivates membrane binding with receptors or enzymes that can disrupt the normal functioning of cells, and contribute to cell damage caused by reaction peroxidation. Lipid peroxidation reactions in LDL cholesterol produce oxidized LDL species or ox-LDL[2] a species that endangers the health and survival of cells or can induce various cellular responses through the formation of secondary reactive species so that caused cell death (necrosis or apoptosis). Oxidative damage of various biomolecules contributes to the pathophysiology of both acute and chronic diseases.[3],[4],[5]

The cell damage caused to reactive oxygen species can be prevented by giving an antioxidant that can inhibit the oxidation process caused by free radicals by donating electrons to form a compound that is not reactive and relatively stable.[6],[7],[8] The human body produces endogenous antioxidants, but in certain conditions, the body required the antioxidant in huge quantities hence the need for antioxidants from outside or exogenous antioxidants as like synthesis or natural ingredients.[9],[10]

Gayam is one of the sources of natural antioxidants, which is seeds are traditionally used for anti-inflammatory and coronary heart disease caused by lipid plaque in arteries or atherosclerosis.[11],[12],[13] Antioxidant activity associated with atherosclerosis from ethanol extracts of Gayam seeds has been reported by Sukadana et al.[14] Atherosclerotic biomarkers such as blood MDA levels, SOD-2 expression of aortic endothelial cells, TNF-expression, and IL-6 Wistar rat liver cells prove that ethanol extract of Gayam seeds in vivo potential as an antioxidant is related to the mechanism of atherosclerosis.[15], [16] However, the process of separating the Gayam's ethanol extract has not been carried out to find out what compounds are contained in the Gayam seeds and which play a role in inhibiting the process of atherosclerotic plaque formation through antioxidant mechanisms. This study aims to trace the antioxidant potential of DPPH from various Gayam seed extracts and separate the antioxidant potential extracts to determine the content of their compounds.

2. METHODS

2.1 Materials

Gayam seeds (*Inocarpus fagiferus* Fosb) ethanol, n-hexane, chloroform, aqua dest, GF_{60} silica gel (Merck), 1,1-diphenylpicryl-2-picrylhydrazyl (DPPH), dichloromethane (DCM), $FeCl_3$1%, NaOH 10%, silica gel 60 (Merck), H_2SO_4 concentrated, anhydrous acetic

acid, Mg powder, Wagner, Mayer, and Dragendorff reagent and KBr.

2.2 Equipment

Analytical balance, stirring rods, Whatman paper, aluminum foil, vial bottles, set of glassware, rotary evaporators Buchi R 114 and vacuum Buchi B 169, desiccators, UV_{254} and UV_{366} nm lamps, a set of thin-layer chromatography (TLC), column chromatography, LCMS/MS (Waters ACQUITY UPLC®H-Class System).

2.3 Isolation and Identification Isolate of Gayam Seed

About 3000 g of dried powder of gayam seed was extracted using 800 mL of 96% ethanol solvent. The extract obtained was evaporated by a vacuum rotary evaporator to obtain a concentrated ethanol extract of about 34.04 g. Partition results of 10 g of concentrated extract ethanol with ethanol-water (7:3) with n-hexane and chloroform yielded 2.15 g of n-hexane, 1.28 g of chloroform, and 0.30 g of water extracts respectively. All extracts were tested for antioxidant activity toward DPPH and the data are plotted on graphs. The extracts with $IC_{50\ were}$ most active followed by separation, purification, and identification.

The n-hexane extract is most active (see Figures 1, 2, and 3) then it was separated using column chromatography (silica gel 60 stationary phase; n-hexane: chloroform (2:1) as a mobile phase). The results of the separation of 2 g of n-hexane extract using column chromatography resulted in 4 fractions and they were tested for antioxidant activity, phytochemical testing, and identification of compounds with Liquid Chromatography-Mass Spectrometry (LC-MS/MS).[17]

3. RESULTS AND DISCUSSION

IC_{50} values of n-hexane, chloroform, and water extracts obtained by making a graph by plotting concentrations in mg/L vs percentage of free radical reduction were 156.00 mg/L, 230.00 mg/L, and 1303 76 mg/L respectively as shown in Figures 1,2 and 3

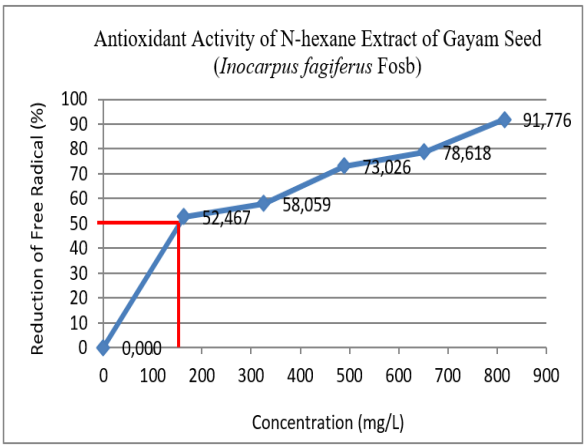


Figure 1: The IC_{50} Value of n-hexane extract of Gayam seed.

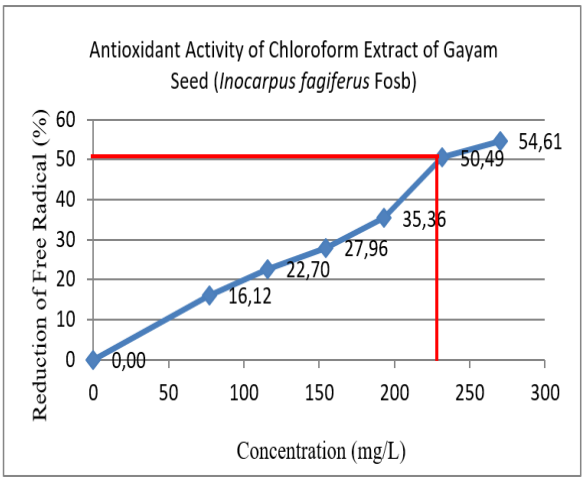


Figure 2: The IC_{50} Value of chloroform extract of Gayam seed.

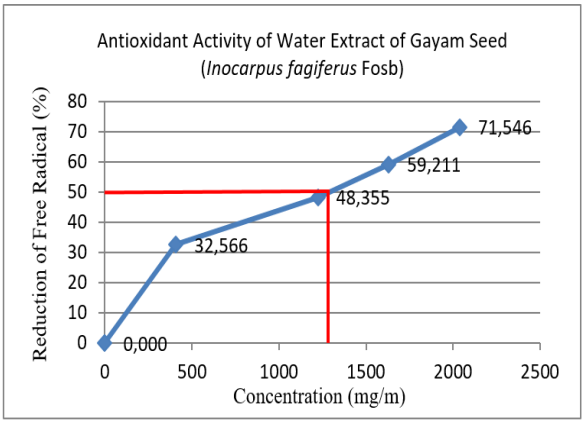


Figure 3: The IC_{50} Value of Water Extract of Gayam Seed.

The ability of n-hexane extract and chloroform to reduce DPPH free radicals are categorized as medium (100-500 mg/L), while water extracts were considered weak in reducing free radicals (≥500 mg/L).[18]

The separation of n-hexane extract using column chromatography resulted in 4 fractions as shown in Table 1.

Table 1: Fractions in the results of Column chromatography.

Fraction	Color	number of stains	Rf value	Mass (g)
A (13-25)	Clear yellow	1	0,56	0,031
B (31-35)	Clear yellow	2	0,68 ; 0,65	0,002
C (42-60)	Light yellow	1	0,56	0,064
D (61-67)	Light yellow	1	0,63	0,001
E (Ethanol)	yellow	2	0,38 ; 0,47	0,120

The fourth fraction was tested by antioxidant activity which is fractions C most active with IC_{50} values of 330.24 mg/L and it was categorized as having medium antioxidant activity of 100-500 mg/L.[18] The results test showed that the antioxidant activity of the separation results was weaker than n-hexane and chloroform extracts, presumably due to the synergistic effect of the compounds in reducing free radicals. The results of the antioxidant activity test fractions C are presented in Figure 4.

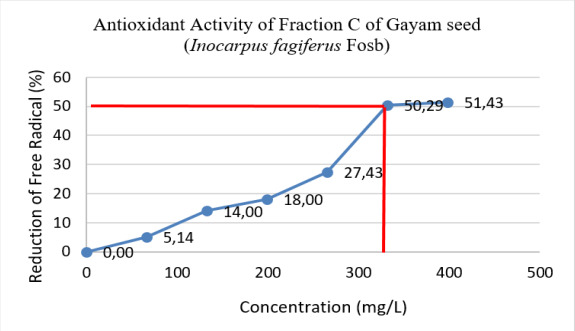


Figure 4: The IC_{50} value of the C fraction of n-hexane extract.

Identification Isolate (Fraction C) with Phytochemical test and LCMS/MS

The result of the phytochemical test was concluded that fraction C contained phenol, flavonoid, terpenoid, and steroid class compounds.

Before the C fraction was analyzed by LC-MS/MS was purified by SPE (Solid Phase Extraction) method using column chromatography and dichloromethane eluent (DCM)[17] yielded successively with a retention time of 9.14, 10.11, 12.26, 12.94, and 13.52 minutes. The molecular formula was obtained based on the fit n/a % approach from the masslynk software and then the molecular structure was compared using the Chemspider, HMDB, and Massbank database approach. Each peak was determined by matching its molecular ions to one of the spectra through the elemental composition sub-menu then a table appeared which showed the approximate molecular formula so that it could be predicted its compound content as shown in Table 2.

Table 2: Results of analysis of C fraction of n-hexane extract.

No.	Retention Time (tR)	$[M+H]^+$ (g/mol)	Predicted compound
1.	9.14	219.1754 $C_{15}H_{23}O$	*Nootkatone*
2.	10.11	343.2964 $C_{24}H_{39}O$	*(22E)-Chola-5,22-dien-3-ol*
3.	12.26	285.1134 $C_{17}H_{17}O_4$	*5,7-Dimethoxyflavanone*
4.	12.94	321.0792 $C_{25}H_{11}O$	Not identified
5.	13,52	377.2328 $C_{22}H_{33}O_5$	*(11β,16α)-11,17,21-Trihydroxy-16-methylpregn-4-ene-3,20-dione ; 11β-hydroxy-3,20-dioxopregn-4-en-21-oic-acid*

Active compounds identified using LC-MS/MS in n-hexane extracts in fraction C contained nootkatone an antioxidant compound; with the structural formula as in Figure 5.

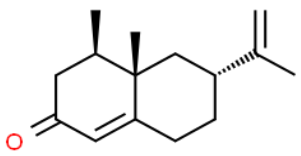


Figure 5: *Nootkatone* compound.

Nootkatone are sesquiterpenoid group, it has anti-inflammatory, reduces lung toxicity, and has antioxidants activities.[19] According to Amalia et al.[20], one of the sesquiterpenoid groups such as nootkatone has a conjugated double bond structure and has unpaired electrons so that it can donate electrons to the DPPH radical.

The antioxidant activity shown by nootkatone compounds may be caused by this compound which can reduce DPPH free radicals. The reaction begins with the process of radical formation or radicalization reaction on the nootkatone compound. In this process, two radical

compounds will be formed namely radical (H•) and carbocation radical then captured reaction of hydrogen radicals by DPPH compounds causes the DPPH radical to become neutral (DPPH-H) and the carbocation radical will react with the DPPH radical that is left in the solution to form the compound (o-diphenylpicril hydrazil-nootkatone) so this process is called the de-radicalization process.[21],[22] The mechanism is as follows:

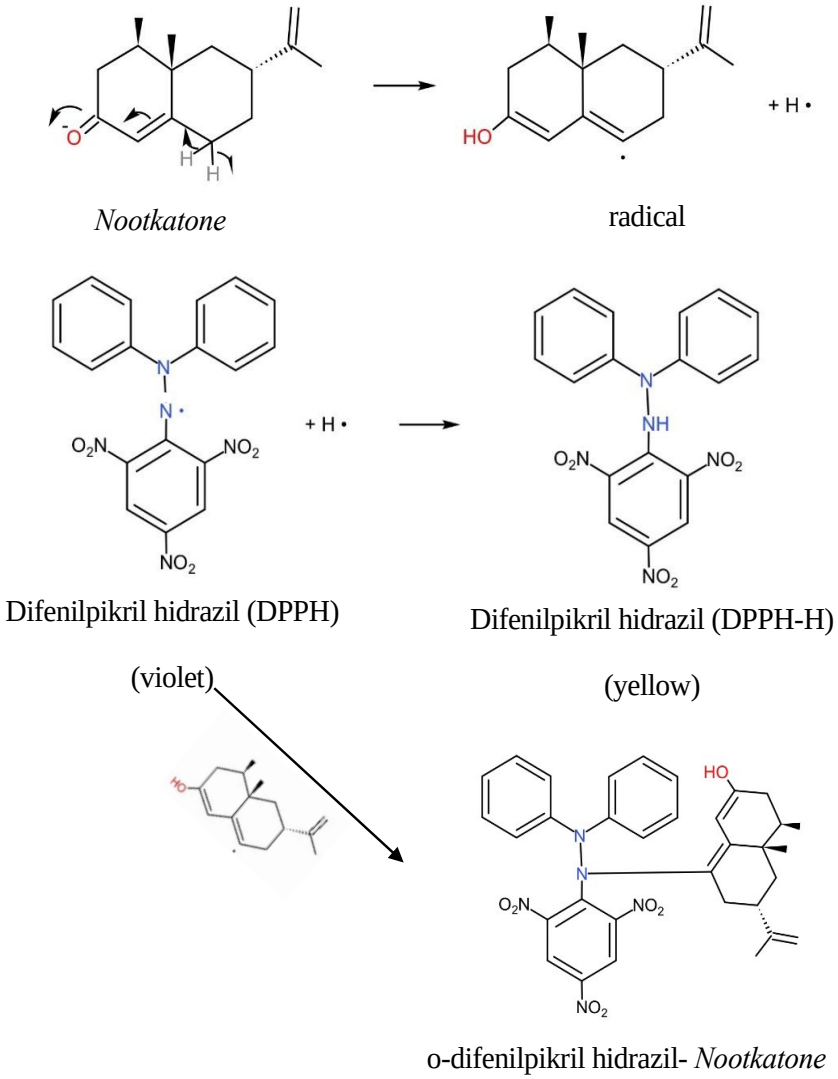


Figure 6: The mechanism of free radical scavenger DPPH in nootkatone compounds.

Other compounds that play an important synergistic role in antioxidant activity in isolates were (22E)-chola-5,22-dien-3-ol; 5,7-dimethoxyflavanone; (11β,16α)-11,17,21-trihydroxy-16-methylpregn-4-ene-3,20-dione; 11β-hydroxy-3,20-dioxopregn-4-en-21-oic-acid and an unidentified compound with a mass of 321.0792 g/mol. Three compounds belong to the steroid group, namely (22E)-chola-5,22-dien-3-ol; (11β,16α)-11,17,21-trihydroxy-16-methylpregn-4-ene-3,20-dione; and 11β-hydroxy-3,20-dioxopregn-4-en-21-oic-acid, while 5,7-dimethoxyflavanone belongs to the flavonoid group. The activity of these four compounds as antioxidants has never been reported. The structural formula of these compounds can be seen in figures 7, 8, and 9.

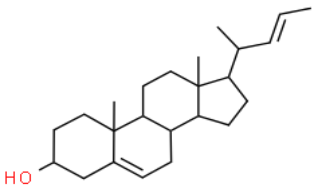


Figure 7: *(22E)-Chola-5,22-dien-3-ol.*

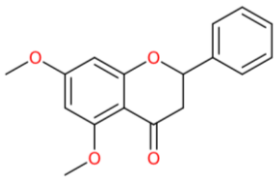
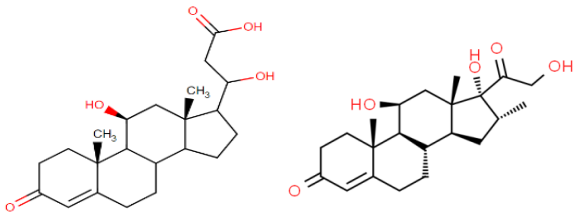


Figure 8: *5,7-Dimethoxyflavanone.*



(A) **(B)**

Figure 9: (A) *(11β,16α)-11,17,21-Trihydroxy-16-methylpregn-4-ene-3,20-dione;* (B) *11β-hydroxy-3,20-dioxopregn-4-en-21-oic-acid.*

4. CONCLUSIONS

Based on the experiment could to concluded that the antioxidant compound contained in the isolate (fraction C) of n-hexane extract of Gayam seed was nootkatone in synergist with other compounds like (22E)-chola-5,22-dien-3-ol; 5,7-dimethoxyflavanone; (11β, 16α) - 11,17,21-trihydroxy-16-methylpregn-4-ene-3,20-dione, 11β-hydroxy-3,20-dioxopregn-4-en-21-oic-acid and compounds which were not identified with a mass of 321.0792 g/mol.

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