Knowledge E
enriching | engaging | empowering

Research article

Antioxidant and Antibacterial Properties of Flavonoids in Gyrinops Versteegii Tea Leaves

Adi Parwata[1]*, Angga Kusuma[1], Bayu Pratama[1], and Putu Gede Adi Purwa Hita[2]

[1]Faculty of Mathematic and Natural Science, Udayana University, Indonesia
[2]Faculty of Health Science, Bali International University, Indonesia

ORCID
Adi Parwata:

Corresponding Author: Adi Parwata; email: okaadiparwata@unud.ac.id

Published 07 June 2022

Publishing services provided by Knowledge E

© Adi Parwata et al. This article is distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited.

Selection and Peer-review under the responsibility of the PGPR 2021 Conference Committee.

Abstract.
Gyrinops versteegii leaves are high in flavonoids, which have a variety of biological and pharmacological properties. The focus of this research was to determine the antioxidant and antibacterial activity of flavonoids in *Gyrinops versteegii* leaf water extracts. In this study, extraction was by maceration and partitioning, separation and purification was by chromatography, identification of compounds was carried out using UV-Vis and FTIR spectrophotometers, and antioxidant activity was measured with the DPPH method and antibacterial activity with the diffusion disc method. Flavonoids were thereby isolated. The ethyl acetate extract had the highest total flavonoid content, with 186.976 mg QE/100 g. With inhibition zone diameters of 19 and 13 mm, ethyl acetate extract was the strongest extract in inhibiting the growth of *S. aureus* and *E. coli* in the antibacterial activity test. Flavonoids were identified as flavonols after isolation, phytochemical screening, and UV-Visible and FTIR spectroscopic identification. The isolated flavonols had potent antioxidant activity (IC50 = 60.27 ppm) as well as potent antibacterial activity (inhibition zone diameters of 33.82 mm for *S. aureus* and 23.22 mm for *E. coli*). The findings therefore showed that tea made from *Gyrinops versteegi* leaves contains the flavonoid flavonol, which has antibacterial and antioxidant properties.

Keywords: *Gyrinops versteegii*, Flavonol, DPPH, antibacterial and antioxidant

1. Introduction

Maximum activity with irregular eating style and minimum exrcises can be an imbalance between the level of free radicals and antioxidant enzimatics in the body, known as oxidative stress. During maximum phycical activity, Number of oxygen molecules that enter increases 20 times while the oxygen consumption by muscle fibers increases by about 100 times. This situation can cause cells or organs to experience a lot of deficiency of oxygen which is called hypoxia. Increased oxygen intake can result in an increase in free radicals that can cause muscle cell damage due to the occurrence of fat peroxidation in muscle tissue (fat tissue peroxidation muscle) **[1].** One way to overcome the occurrence of oxidative stress is the oral consumption of natural exogenous antioxidants found in herbal medicinal plants as Gyrinops versteegii. Currently, the concern

about Gyrinops versteegii is only the sapwood, but it turns out that waiting for sapwood production takes a very long time (5-7 years) since planted **[2].** Therefore, our research team examined the content and bioactivity of the leaves.The research method in the process of finding lead compounds usually uses the High Throughput Screening (HTS) methods exactly study of bioactivity antioxidant and antibacterial of flavonoid in water extract of Gyrinops verstegii leaves [**2**] **[3].**

Flavonoid compounds can prevented the continuous reaction between free radicals and lipids, proteins and DNA in the body. Flavonoid can scavenging or give a proton to free radicals so that lipid peroxidation reactions and reactions of DNA damage can be prevented. Actually, the cells in the body produce endogenous antioxidants. However, it is unable to neutralize excess free radicals in the body's cells, resulting in an imbalance between the amount of endogenous antioxidants and free radicals. The most reactive free radicals in the body are oxygen and hydroxy radicals. Radicals will oxidize fats, especially in cell membranes or muscle tissue, which is known as fat tissue peroxidation which can cause damage to cell membranes and muscle tissue. The occurrence of muscle lipid peroxidation is characterized by increased malondialdehyde , a compound result of lipid peroxidation Damage to muscle tissue will be followed by damage cell proteins **[1]** **[2]** **[3].** This imbalance or oxidative stress situation can be overcome by oral consumption of exogenous antioxidants such as natural antioxidants. Currently, people prefer natural antioxidants compared to synthetic antioxidants because they have less side effects. This makes researchers conduct research on natural antioxidants, especially traditional medicinal plants. One of them is Gyrinop versteegii plants.

So far, the public have only focused on sapwood, whether they are used as export materials or as drugs. Rarely or no researchers are interested in researching the leaves. Accordance with the theory, secondary metabolites or active ingredients of a medicinal plant will be spread to all parts of the plant. One of them is Gyrinops versteegii leaves) [**2**}

Flavonols contained in medicinal plants can contribute significantly in increasing activity and stimulating the production of enzymatic antioxidants **[3].** Flavonols can also inhibit bacterial growth through inhibition of DNA gyrase, there by inhibiting the function of the cytoplasmic membrane and membrane breakdown by lipophilic components and can cause lysis of cellular components and damage the enzymatic mechanisms of bacterial cells. Flavonoids can also denature proteins and damage the permeability of bacterial cells, microsomes and lysosomes as a result of the interaction process between flavonoids and the bacterial wall. Flavonoids can be make complexes with extracellular proteins that disrupt the integrity of the bacterial cell membrane [**1**]**.**

Based on its used as traditional medicine and contains flavonoid compouds so Gyrinops versteegii leaves potentialy used as a source of natural antioxidants and antibacterial. Its needs to be analyzed both in vitro and in vivo of water extracts and isolate by measuring the antioxidant capacity with DPPH methods and antibacterial activity with diffusion disc methods.

2. Methodology

Material : Powder of Gyrinops versteegii leaves {sample), obtained from farmer's plant at the village of Marga, Subdistrict Marga, Tabanan, Bali, Indonesia, some chemical reagents produced by E Merck grade p.a. such as ethanol, ethyl acetate, HCl, NHCO3 pa, NH4Cl, methanol, and filter paper, Column chromatography techniques with various analytical grade reagents along silica gel (SiO2) were used as a stationary phase. TLC plates (Merck GF-254). nutrient agar (NA), Mueller Hinton agar (MHA), amoxicillin antibiotic disc (AML 25mcg CT 0061B OXOID), paper disc (blank disc), sterile physiological NaCl solution, 1% H_2SO_4 and 1% $BaCl_2$.

Instrument : UV-Vis (Jeol HX-110), FTIR (Jasco A-302), analytic Digital Balance Scale (Ohaus), rotary vacuum evaporator Brand Buchii, Vortex, water bath, pyrex measuring cup, pyrex test tubes, micro pipette, hot plate, autoclaf, incubator, laminar air flow, sacher

2.1. Analysis of the Nutrient Composition of Gyrinops Versteegii Leaves.

Analysis of nutrient composition in this study used the AOAC method or procedure. Parameters measured include crude fiber, protein, fat, vitamins and minerals and their chemical content

2.2. Isolation and Identification of Flavonoids Compound

Isolation and identification of sample followed prosedure [6]. Sample was shade dried for a period of 5 weeks and crushed in to coarse powder with the help of mechanical blender. Sample leaves powder is measured its water content. Its powder extracted with water at a temperature of 80 degrees for about 5 hours. This extract is evaporated with a rotary evapourator. This extract is then used to test the antioxidant activity and isolation of the flavonoids. The extract was again extracted through different solvent system starting from n-hexane followed by chloroform, ethyl acetate so get different

fractions i.e. n-hexane, chloroform and ethyl acetate. Each fraction was analyzed for its flavonoids and total flavonoid levels. The fraction that was positive for flavonoids and the highest levels of total flavonoids continued with separation and purification.

2.3. Analyzed of Flavonoid Contents

Analysis of Total Flavonoid Contents followed the procedure [7]. Extract of water dissolved in 5 mL ethanol in 10 mL volumetric flask, vortexed until homogeneous. Pipette 2,0 mL, put in a test tube, add 2,0 mL $AlCl_3$ 2%, vortexed until homogeneous then incubation in room temperature for 25 minutes. Furthermore absorbance at λ max = 415 nm.Total Flavonoid levels are integrated in the quersetin standard calibration curve (**mg QE/ 100 gram).**

2.4. Separation and Identification of Isolate

Separation begins with the determination of the best eluent with TLC GF_{254} by using several comparisons of eluents with different polarity. This eluent is best used as the mobile phase of column chromatography. The resulting eluent is collected according to the same separation pattern and then evaporated. The eluate analyzed its flavonoids. A positive eluate containing flavonoids was then tested for purity. Flavonoid isolates were identified qualitatively followed identified by UV-Vis and FTIR spectrophotometer. The isolated flavonoids were analyzed for their antioxidant activity

2.5. Antioxidant Capacity Analysis Isolated Flavonoid

The analysis begins with making of a solution gallic acid 0-100 mg/L solutions. Weighed 0.1 grams isolate, then diluted with methanol to a volume of 5 mL flask and then in the vortex so that a homogeneous solution. This homogeneous solution is centrifuged at 3000 rpm for 15 minutes . Isolate solution has been pipetted 0.5 mL of this homogeneous, then add 3.5 ml of 0.1 mM DPPH in methanol at a test tube and then in the vortex. This solution was incubated at 25^{0}C for 30 minutes so DPPH reacts with the sample. Isolate solution was measured absorbance at λ max = 517 nm. Antioxidant capacity was calculated using linear regression equation Y = ax + b [5]. Antioxidant activity can be shown from the value of its antioxidant capacity with its IC_{50} , which state that the concentration where 50% of the antioxidant resistance against free radical reactions (IC_{50}= x mg/mL or ppm)

2.6. Antibacterial Analysis of Isolated Flavonoid

Study of antibacterial activity testing was carried out using the agar diffusion method (disk diffusion). The test bacteria were inoculated on MHA media using the streak plate technique. A sterile cotton swab is dipped in the test bacterial suspension, then rotated several times and pressed against the inner tube wall to remove excess inoculum on the cotton. A cotton swab containing a suspension of the test bacteria was rubbed on the entire surface of the media until evenly distributed, then the petri dish was closed and allowed to stand for 3-5 minutes [9]

Disc paper (blank disc) was dipped into each test solution, then drained until the sample did not seep anymore. The disc paper that already contained the test solution was then affixed to the surface of the MHA media using tweezers and then allowed to stand for 15 minutes. The same treatment was also carried out for the negative control. As for the positive control, special discs were used in the form of antibiotic amoxicillin 25µg/disk which were also attached to the surface of the MHA media using tweezers. The petri dish was then incubated for 24 hours at 37°C with the petri dish inverted. The clear zone formed indicates the antibacterial power of the test solution against S. aureus and E. coli bacteria. The antibacterial activity test was carried out by measuring the diameter of the inhibition zone using a digital caliper [9].

2.7. Acute Toxicity Test of Water Extracts (tea) of Gyrinops Versteegii Leaves

Toxicity Test of a natural or synthetic product needs to be done before it is consumed by the public so that the public avoid poisoning the ingredients contained in the product. In this study toxicity tests were conducted to determine a safe dose of aqueous extract of agarwood leaves before it is used as a health supplement or herbal medicinal ingredients. Toxicity tests can be divided into acute toxicity test, subchronic and chronic Acute toxicity analysis with lethal dose value (LD_{50}) is a single dose of a substance or substance that is statistically estimated to kill 50% of experimental animals [17]. LD_{50} is calculated using Table Thomson-Weil formula : **Log LD_{50} = log D + d (f + 1)**

This study used a strain of mice white male Balb / C were normal, age 2.5 months, weight 22-30 grams. Mice were divided into 6 groups each consist of 5 tails. Water extract of **sample** given orally with increasing doses of 2 times the normal dose multiples. Calculation of adjusted oral dosing with calculations performed [18] which will be calculated human dose conversion to dose mice.

Extracts were administered orally by dissolving each treatment in 2 mL of distilled water previously in homogenkan the way in vortex. Observation results from the toxic extract made within 24 hours to there are no experimental animals that die for 24 hours, it means that there is no toxic effect.

3. Results and Discussion

In accordance with the Simplicia Standardization Rules, the water content of simplicia must first be determined, this is related to storage time and enzyme hydrolysis. The water content of dry powder simplicia of sample avarage 8,59%. This is in accordance with the quality standard of drugs simplicia. The results showed that the results were in accordance with the Standardization Rules of Materia Medika Indonesia, namely the name of the simplicia sample and the water content was less than 10%. Phytochemical screening water extract of Gyrinops versteegii leaves showed the content of phenolic compounds, flavonoids, steroids and tannins. The results of determining the water content of dry powder simplicia of sample are shown in the following table.

Table 1: Water Contents of dry powder simplicia

Code	Initial sample weight **(g)**	Final sample weight **(g)**	**Water Contents (%w/w)**
U1	1,1699	1,0694	8,59
U2	1,4630	1,3374	8,58
U3	1,1985	1,0958	8,59
	% Avarage of water contents		**8,59**

Analysis of the Nutrient Composition of Gyrinops Versteegii Leaves.

Analysis of the composition of nutrients in this study showed good nutritional value in improving community nutrition, as shown in the following table,

3.1. Isolation and Identification of Flavonoids Compound

Maceration of 100 g of dry powder of Gyrinops verstegii leaves obtained 400 mL of a blackish red concentrated extract. Next, 200 mL of this aqueous extract was partitioned with solvent extractins such as n-hexane, chloroform, and ethyl acetate . Each of the partitioned fractions was evaporated using a rotary evaporator to obtain 5.58 grams of n-hexane, 4.05 grams of chloroform, and 8.27 grams of ethyl acetate. Each of these three fractions was screened for their flavonoids and total flavonoids contents, Phytochemical screening with 10% NaOH showed that ethyl acetate of fraction contained flavonoid

TABLE 2: The composition of nutrients of sample.

Sample	Parameter	Value
Gyrinops versteegii Leaves	Water Contents (% w/w)	8,06
	Ash contents (% w/w)	8,98
	Protein contents (% w/w)	8,79
	Fat contents (% w/w)	4,55
	Crude fiber (% w/w)	33,43
	Vitamin C (mg/100 g)	13.797,99
	Provitamin A (β-karoten), (mg/100 gr)	77,34
	Ca (ppm)	2.489,85
	Fe (ppm)	6,06

compounds and the highest total flavonoid contents 840,12 mg QE /100 grams, as described the following table 2. Furthermore, isolation and identification of flavonoid compounds were carried out in the ethyl acetate fraction [10].

TABLE 3: Phytocemistry screening and Total Flavonoids Contens.

Extract	Total flavonoids (mg QE/ 100 g)	color test			**Flavonoids**
		NaOH 10%	Bate smith-Metcalfe	Willstater	
n-Hexane	136,27	brown	colorless	Greenish yellow	catecin
chloroform	30,04	yellow	colorless	yellow	Isoflavon
ethyl acetate	840,12	Brownish orange	colorless	red	Flavonol

3.2. Separation and identification isolate

Separation results with column chromatography obtained 4 fractions (A,B,C and D). From these four fractions, it turns out that fraction A positively contains flavonoids. This fraction was subsequently tested for purity by chromatography. The test results obtained that the pure isolates by thin layer chromatography. These isolates were further identified by UV-Vis and FTIR spectrophotometers.

Based on the results of measurements with a UV-Vis spectrophotometer showing two absorption bands at a maximum wavelength 373,20 nm (band I) and 257,20 nm (band II) as shown in the following Figure 1 :

According to the flavonoid theory, flavonols show an absorption band I at a wavelength of 378.90 nm and an absorption band II at a wavelength of 279.60 nm.

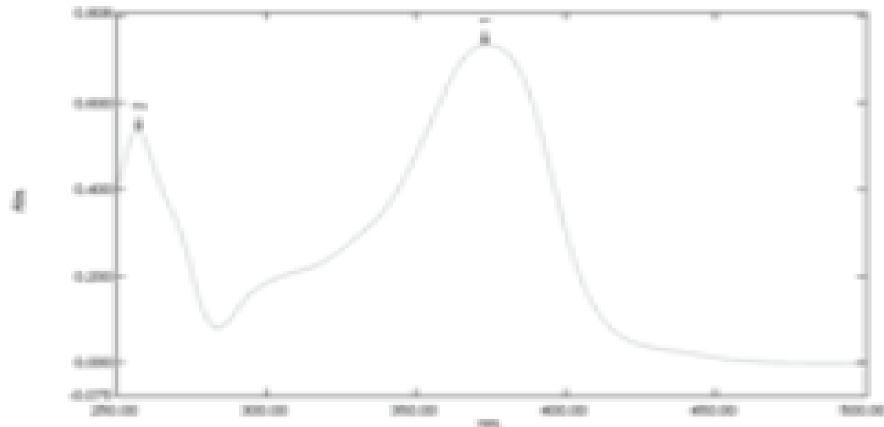

Figure 1: Spectra UV-Vis of isolate with Etanol.

3.3. Identification by FTIR spectrophotometers.

Identification by IR spectroscopy showed some peaks as shown the following Figure 2
.

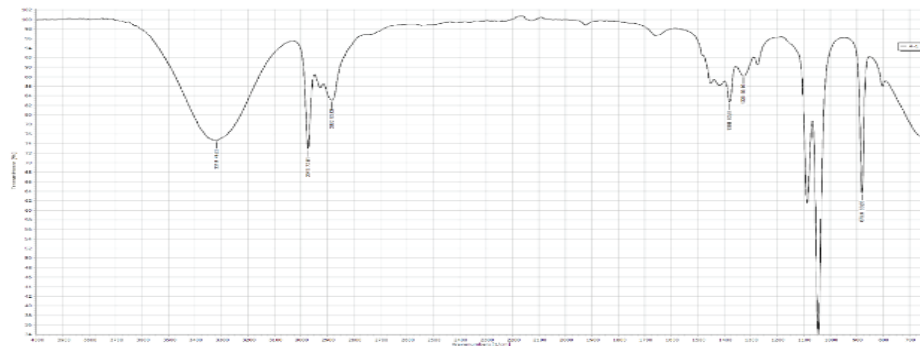

Figure 2: Spectra FTIR of Isolat.

In accordance with the theory, the results of the FTIR spectra used to prove the main functional groups in isolated flavonoids did indeed contain the main functional groups with their respective wave numbers: hydroxy (3318 cm^{-1}), carbonyl (1600-1700 cm^{-1}), >C-O alcohol (1000-1100 cm^{-1}) and >C=C< aromatic (1500 cm^{-1}). The results of the isolation and identification water extract of Gyrinops versteegii leaves are flavonoid aglycones namely flavonol type [2].

3.4. Antioxidant Capacity Analysis of Isolate

The results of the measurement of the antioxidant activity of the isolates showed that the isolated flavonoids showed strong antioxidant activity with an antioxidant capacity of IC_{50} = 60,27 ppm as shown in the following table,

Table 4: Antioxidant capacity of isolate.

Sample	Concentrations (ppm)	Absorbance		Linear equations
		blanco	% inhibit.	
Flavonol	20,4	0,529	0,413	y = 0,632x + 11,909 R^2= 0,9653
	40,8		0,306	
	61,2		0,263	
	81,6		0,2	
	IC_{50} (mg/L)		**60,27**	

IC_{50} is obtained from the graph between concentration (mg/L) versus % inhibition as shown in the following Figure 3:

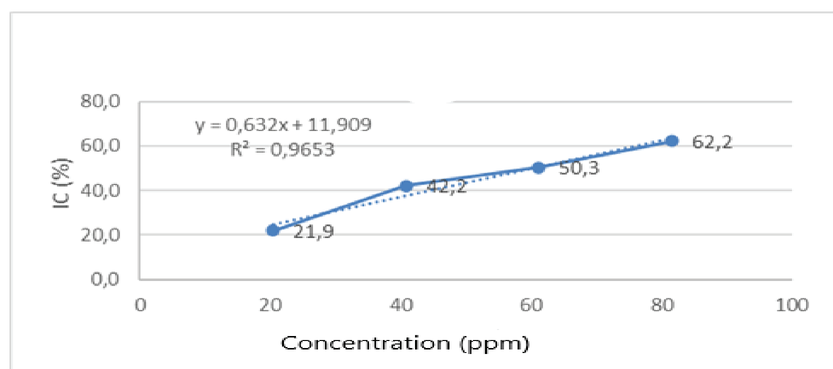

Figure 3: Antioxidant capacity of isolates.

Flavonols are the most abundant flavonoids in nature, especially medicinal plants. Flavonol aglycones are the basic structure of quercetin which is a pigment compound in plants and fruits and has very strong antioxidant activity so that quercetin is believed to provide protection against tissue damaged by exposure to drugs. Flavonols (quercetin) are abundant in tomatoes, onions, grapes, berries, broccoli, and oranges. As antioxidants, flavonol aglycones can prevent free radical oxidation reactions that come from within or like tar in cigarettes. Flavonol aglycones can prevent allergies through their mechanism of action by inhibiting the release of histamine from mast cells and other allergic compounds, thus acting as a natural antihistamine. By preventing these effects,

it turns out that flavonols (quercetin) are believed to be used in treating asthma and bronchitis. Flavonols can also reduce the risk of cancer cell growth, lower blood pressure and prevent brain damage [15],[16].

3.5. Acute Toxicity Test of Water Extract of Sample

Observations within 24 hours apparently no dead mice up to the highest dose of 16 384 mg / kgWW / day or 16,384 g / kg / day. These results when converted into the toxic threshold value table said no harm at all or safe for human comsumption (> 15 grams). as shown in the following table.

Table 5: Acut Toxicity Test of Water Extract of sample.

Group of mice	Dose (mg/kgWW/ day)	Dead of mice	Interpretation toxic
I	1024	No dead	No toxic/safe
II	2048	No dead	No toxic/safe
III	4096	No dead	No toxic/safe
IV	8196	No dead	No toxic/safe
V	16384	No dead	No toxic/safe

Acut Toxicity test showed that observations within 24 hours apparently no dead mice up to the highest dose of 16 384 mg / kgWW / day or 16,384 g / kg / day. These results when converted into the toxic threshold value of substance or chemical table (Table Weil) said no harm at all (> 15 grams). Furthermore, the highest dose can be used as an LD_{50} for research - further research [17]. This dose when converted to human weighing 70 kg will be 16384 x 387.9 = 635.5353,6 mg = 6355.3536 grams. The result of this calculation can be interpreted that the water extract of leaves Gyrinops versteegii leaves safe for human consumption [17]

3.6. Antibacterial Test of Isolate

The results of the antibacterial test showed that the isolates had very strong antibacterial activity with zones of inhibition against E. coli = 33.82 mm and S. aureus = 23.22 mm as shown in the following table,

As an antibacterial, flavonols denature proteins and damage the permeability of bacterial cells, microsomes and lysosomes. Flavonols can inhibit the function of the cytoplasmic membrane by damaging the fluidity of the membrane in the hydrophilic and hydrophobic regions so that the fluidity of the outer and inner layers of the membrane

Table 6: Antibacterial Test of Isolate.

No	Material	Bacteria	Zone of inhibit (mm)	Category
1	Isolate	*S. aureus*	33,82	very strong
		E. coli	23,22	very strong
2	Amoxycilline (Control)	*S. aureus*	23,01	very strong
		E. coli	23,46	very strong

will decrease (inhibition of DNA gyrase). Flavonols can form complex compounds with extracellular proteins that disrupt the integrity of bacterial cell membranes [9].

4. Conclusion

Isolated flavonols in water extract (tea) of *Gyrinops versteegi* leaves have activity as a strong antioxidants activity with IC_{50} = 60.27 ppm and strong antibacterial activity with inhibition zone diameters of 33,82 mm towards *S. aureus* and 23,22 mm toward *E. coli* as well as safe for human consumption. This proves that tea of *Gyrinops versteegi* leaves with flavonoid flavonol has the potential to antibacterial and natural antioxidants.

References

[1] Akhlaghi M, Brian B. Mechanisms of flavonoid protection against myocardial ischemia–reperfusion injury. Journal of Molecular and Cellular Cardiology. 2009;46:309–17.

[2] Parwata A, **Sukardiman S.,** Mulya HS, Widhiartini A. Inhibition of fibrosarcoma growth by 5-hydroxy-7-ethoxy-flavanons from kaempferia pandurata roxb. Biomedical & Pharmacology Journal, Oriental Scientific Publishing Company, Madhya Pradesh, India. 2016;9(3):941-48.

[3] Parwata A, Manuaba P, Yasa S. The potency of flavonoid compounds in water extract *Gyrinops versteegii* leaves as natural antioxidants sources. Biomedical and Pharmacology Journal. Oriental Scientific Publishing Company Madhya Pradesh, India. 2018;11(3):1501-1511.

[4] Parwata A, Laksmiwati, Sudiarta, Dina MN, Yasa S. The contents of phenol and flavonoid compounds in water extract of *Gyrinops versteegii* leaves have potentially as natural antioxidants and hypoglicemic in hyperglycemic Wistar rats. Oriental Scientific Publishing Company, **Madhya Pradesh, India**, 2018. Vol. 11(3), p. 1543-1552

[5] Almey A, Khan AJ, Zahir S, Suleiman M, Rahim AK. Total phenolic content and primary antioxidan activity of methanolic and ethanolic extract of aromatic plants' leaves. International Food Research Journal. 2010;17:1077-88.

[6] **Biswas R**., Dasgupta R, Mitra A, Roy A, Dutta SK, Achari PK, Dastidar B, Chatterjee TK. Isolation, purification and characterization of four pure compounds from the root extract of *Pluchea indica Less* and the potentiality of the root extract and the pure compounds for antimicrobial activity. European Bulletin of Drug Research. 2005;13:63-70.

[7] Chang S, Yang M, Wen HCJ. Estimation of total flavonoid content in propolis by two complementary colorimetric methods, **Journal of Food and Drug Analysis.** 2002;10:178-182.

[8] Grassi D, Desideri G, Ferri C. Flavonoids: Antioxidants against atherosclerosis. Nutrients. 2010;2:889-902.

[9] Maria Endah Hapsari . Uji aktivitas antibakteri ekstrak herba meniran (*Phyllanthus niruri*) terhadap pertumbuhan bakteri *Bacillus cereus* dan *Escherichia coli* [Skripsi]. Universitas Sanata Dharma; **Yogyakarta**, 2015.

[10] Harborne JB. Metode fitokimia penuntun cara modern menganalisa tumbuhan. (kosasih padmawinata). Bandung: **Institut Teknologi Bandung**; 1996.

[11] Harmita, Apt., Radji M. Buku Ajar Analisis Hayati. 3rd ed. Jakarta: Penerbit Buku Kedokteran EGC (**Asosiasi Ekstraksi et Gestion des Connaissances**); 2006.

[12] Jiang MZ, Yan H, Wen Y, Li XM. In vitro and in vivo studies of antioxidant activities of flavonoids from *Adiantum capillus-veneris* L. African Journal of Pharmacology. 2011;5(18):2079-85.

[13] Kunwar A, Priyadarsini KI. Free Radicals, oxidatives stress and importance of antioksidants in human health**. Journal of Medical and Allied Sciences.** 2011;1(2):53-60.

[14] Lecumberri E, Goya L, Mateos R, Alfa M, Ramos R, Izquierdo-Pulido M, Bravo L. A diet rich in dietary fiber from cocoa improves lipid profile and reduces malondialdehyde in hypercholesterolemic rats. Nutrition. 2007;23:332-41.

[15] Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoid. Spinger-verlag. 1970;3-56:165-171.

[16] Markham KR. Cara mengidentifikasi flavonoid, diterjemahkan oleh Kosasih Padmawinata, Penerbit. Bandung: **Institut Teknologi Bandung**; 1988.

[17] **Ngatidjan.** Metoda Laboratorium dalam Toksikologi, Bagian Farmakologi dan Toksikologi. Yogyakarta: **Fakultas Kedokteran Universitas Gajah Mada**; 2006.

[18] Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. The FASEB Journal Life Sciences Forum. 2007;22:659-661.