Total Phenolic and Flavonoid Contents and Antimicrobial activity of *Acorus calamus* L. Rhizome Ethanol Extract

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Abstract

The rhizomes of Acorus calamus L. are widely used as medicinal plant for diseases caused by microbes. The antimicrobial activity of rhizomes is suspected to have a positive correlation to the content of phenolic and flavonoid compounds. This study aims to determine the total phenolic and flavonoid contents and to evaluate the antimicrobial activity of ethanol extract of Acorus calamus L. rhizome. An extraction process was carried out at room temperature by maceration methods.

The determination of total phenolic and flavonoid contents was conducted by a UV-Vis Spectrofotometer using standard gallic acid and quercetin respectively. Additionally, the antimicrobial activity was evaluated by the agar disc diffusion method. The total phenolic and flavonoid contents were successively 2398.40 mg GAE/100g and 190.46 mg QE/100g dry rhizome extract. The extracts demonstrated antibacterial activity against Escherichia coli and Staphylococcus aureus and antifungal activity against Candida albicans. Minimum inhibitory concentration of the extracts against Escherichia coli, Staphylococcus aureus and Candida albicans was 2, 3 and 3% respectively with the inhibition zone of 9.80, 9.50 and 8.67 mm.

Keywords: *Acorus calamus* L., Antimicobial Activity, Flavonoid contents, Phenolic contents.

Introduction

The environment plays a major role in improving public health. However, poor public health conditions such as the incidence of various diseases are also influenced by the surrounding environment. An inadequate environment can potentially have a direct impact on human health, it can spread dangerous agents, or indirectly by disrupting the ecosystems that sustain life[1]. A dirty environment is good for breeding various disease vectors such as microbes, including both bacteria and fungi. Microbial pathogens can cause various diseases in living organisms by infection. The microbes that often cause the disease are *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*.

Medicines such as tetracycline, amoxicillin, miconazole are often applied to treat infectious diseases. However, these medicines can lose effectiveness due to microbial resistance[2]. Hence, new medicines from natural sources such as antibiotics from plants are required. A variety of plant species are traditionally used in some countries for treatment of infectious diseases such as *Allium sativum*, *Bunium persicum*, *Oryza sativa*[3], *Vitex negundo*, *Piper nigrum*, *Duranta repens* and *Acorus calamus*[4].

Among the plant widely used as traditional medicine to treat diseases caused by microbials is *A. calamus* L. This plant has many uses. Rahamoz-Haghighi et al[5] reported that *A. calamus* rizhome ethanol and methanol extracts could inhibit the growth of *S. aureus*, *Staphylococcus epidermidis*, *E. coli* with similar effects. Rawal et al[6] revealed that acetone, aqueus, ethanol and petroleum ether extracts of *A. calamus* rizhome could potentially inhibit the growth of *Fusarium oxysporum f.sp. lycopersici* with Minimum Inhibitory Concentration (MIC) of 500, 750, 250, 250 mg/mL respectively. Funde[7] investigated anticancer, antioxidants and antimicrobial activity of *A. calamus* and stated that *A. calamus* was useful for multi-diseases therapeutic research.

Anisah et al[8] reported that ethanol extract of *A. calamus* rhizome contains alkaloids, flavonoids and polyphenols. Rahamoz-Haghighi et al[9] reported that *A. calamus* ethanol extract contains phenyl propanoids, monoterpenes, sesquiterpenes and α-asarone. The compounds demonstrated antibacterial activity. Based on preliminary tests, the methanol extract of rhizome collected in Denpasar contains triterpenoids, steroids, flavonoids, polyphenols and alkaloids. The antimicrobial activity of *A. calamus* rhizomes is probably caused by the content of the compounds in the rhizomes such as essential oils, flavonoids and polyphenols.

Rita et al[10] reported that the essential oils of *A. calamus* could potentially inhibit the growth of *C. albicans* with the minimum inhibitory concentration (MIC) of 1%. The essential oils also inhibit the growth of *Fusarium solani*, a pathogenic fungus causing stem rot diseases on dragon fruit stems[11,12]. The results demonstrate that the oils strongly inhibited *F. solani* at a concentration of 10% with an inhibition zone of 10 mm, MIC of 2 %. The results also indicate that the growth of colony, spores and fungal biomass increased with the increase of essential oil concentration.

Besides essential oils, the antimicrobial activity of *A. calamus* rizhome was associated with flavonoid and phenolic content. Mahboubi et al[13] revealed that the

antimicrobial efficacy of the plant extracts is correlated with their phenolic and flavonoid contents. Hence, it is necessary to investigate antimicrobial activity to ethanol extract of the rhizomes collected in Bali and to determine the total content of phenolic and flavonoid compounds.

Material and Methods

Plant Material: *A. calamus* L. was identified at LIPI-UPT Center for Plant Conservation Botanical Garden "Eka Karya" Bali. Rhizomes of *A. calamus* were collected around Denpasar Bali. The rhizomes were respectively cleaned, cut and dried at room temperature for 15 days. Next, they were powdered and stored for later analysis.

Microbial Agents: Two strains bacteria *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) and a fungal pathogen, *C. albicans*, were applied to the antimicrobial assay. These microorganisms were obtained from culture collection of Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Udayana University. The isolates were purified and maintained at 4°C until later use.

Extraction: Around 750 g of *A. calamus* rizhome powder was extracted with 10 L of ethanol 96% for 24 h at room temperature (25°C). The extract was filtered through a Whatmann filter paper and then evaporated under vacuum and stored at 4°C until further analysis.

Determination of Total Phenolic and Flavonoid Contents
***Total Phenolic contents*:** Folin-Ciocalteu reagent was applied for the determination of total phenolic contents[14]. A total of 0.1158 gram extract was dissolved in 96% ethanol to obtain a volume of 5 mL. Dilution was performed 100 times; 1 µL of filtrate was dissolved in ethanol to obtain 100 µL of solution. 100 µL of Folin-Ciocalteu reagent and 800 µL of 5% sodium carbonate were added so the total solution volume became 1000 µL. The mixture was then allowed to stand for 90 minutes, before the absorbance was measured at a wavelength of 760 nm. A series of gallic acid solutions with various concentrations were also prepared.

The absorbance of each concentration was measured at a wavelength of 760 nm. From the standard gallic acids standards, a calibration curve was made to get the equation line of y = ax + b. The total phenolic contents were expressed as mg gallic acid equivalents /100 g of extract. The total phenols can be determined by using the following formula:

$$F_1 = \frac{C.V.F.\ 10^{-6}}{m}\ 100\% \quad \text{..... (1)}$$

where F_1 = total phenol, C = equality of gallic acid (g/mL), V = total volume of extract (mL), F = the dilution factor and m = weight of sample (g).

Total Flavonoid contents: Total flavonoids were determined by the aluminum chloride method[14]. A total of 0.1035 grams of samples were dissolved in 96% ethanol to obtain a volume of 5 mL. Dilution was performed 5 times. 100 µL of filtrate was dissolved in ethanol to obtain 500 µL of solution. 500 µL of 2% aluminum chloride was added, so the total volume of the solution became 1000 µL. The mixture was then allowed to stand for 90 minutes before the absorbance was measured at a wavelength of 415 nm.

A series of quercetin solutions with various concentrations were also prepared. The absorbance of each concentration was measured at a wavelength of 415 nm. From the quercetin standards, a calibration curve was made to obtain equation line of Y = ax + b. The total flavonoid contents were expressed as mg quercetin equivalents/100 mg extract. The total flavonoids can be determined by using the following formula:

$$F_2 = \frac{C.V.F.\ 10^{-6}}{m}\ 100\% \quad \text{..... (2)}$$

where F1 = total flavonoids, C = equality of quercetin (g/mL), V = total volume of extract (mL), F = the dilution factor and m = weight of sample (g).

Antimicrobial Activity Assay: Antimicrobial activity assay of *A. calamus* rhizome ethanol extract was conducted by the well diffusion method at various concentrations of 0 (negative control), namely, 0.5, 1, 2, 3, 4, 6, 8 and 10% with three repetitions[10]. In order to determine the optimum concentration, the assay was performed on the extracts with various concentrations greater than 10. The concentrations applied were 12, 14 and 16%.

Petri dish containing 10 mL of PDA (*Potato Dextro Agar*) media and 200 µL of suspension of the microbes (*E. coli, S. aureus* and *C. albicans*) were allowed to solidify. After the suspension was solid, the diffusion wells were made using a cork borer and each well was filled with 20 µL of the extract and incubated at a temperature of 37°C for 48 hours. The antimicrobial activity was determined by the diameter of the inhibition zone.

Results and Discussion

Determination of Total Phenolic and Flavonoid Contents
The curves of the calibration to determine total phenolic and flavonoid contents are presented in figures 1 and 2. Based on the calibration curves, the calibration equation of gallic acid (figure 1) obtained was y=0.047x + 0.0635 (R^2=0.9899), while that of quercetin (figure 2) was y=0.0313x+0.0272 (R^2= 0.9961). The calculation to determine the total phenolic and flavonoid contents is summarized at table 1. According to table 2, the total phenolic and flavonoid contents were 2398.40 mg GAE/100g and 190.46 mg QE/100g dry rhizome extract successively.

The data showed that the total phenol was higher than total flavonoids, likely due to only a few phenol compounds being flavonoids especially quercetin. The most likely compound is tannin, especially hydrolysable tannis. According to Khanbabaee and Ree[15], tannins consist of hydrolysable and condensed tannins. The hydrolysable tannins include both the gallotannins and the ellagitannins. The condensed tannins consist of cathecin (flavan-3-ol) units.

Antimicrobial Activity Assay: Antimicrobial activity assay against *E. coli*, *S. aureus* and *C. albicans* of *A. calamus* rhizome ethanol extract was performed at concentrations of 0 (negative control), namely, 0.5, 1, 2, 3, 4, 6, 8 10, 12, 14 and 16 %. The antimicrobial assay results are shown in table 3.

The data showed that the inhibition towards the microbes increased with the increase of the concentration applied. Rhoades and Roller[16] revealed that in general, the inhibition tends to increase with the increase of the extracts concentration. Minimum inhibitory concentration (MIC) of the extract against *E. coli*, *S. aureus* and *C. albicans* was 2, 3 and 3% respectively with the inhibition zone of 9.80, 9.50 and 8.67 mm.

Overall, *E.coli* was more sensitive to the extract compared to *S. aureus* and *C. albicans*, except at a concentration of 6%. This indicates that gram-negatives bacteria are more sensitive compared to gram positive. These findings are in contrast to the findings of Okigbo and Mmeka[17] who reported that *S. aureus* is the most susceptible to the plant extracts followed by *E. coli* and then *C. albicans*. The reason why *E.coli* is more susceptible to the extracts than other microbes is unclear.

The data in table 2 shows that the optimum concentration of the extract to inhibit the growth of *E. coli* was 8.0 % because the inhibition zone was not significantly different from the concentration of 8.0 to 16%. Meanwhile, the optimum concentration of that to inhibit *S. aureus* and *C. albicans* was 12.0 and 10.0% respectively. Figure 3 shows the inhibitory zone of the extract with various concentrations towards *E. coli*, *S. aureus* and *C. albicans*. The extract at a concentration of 10% could strongly inhibit the growth of all microbes used (figure 4). The inhibition zone less than 5 mm was categorized as weak inhibition, between 5 and10 mm was moderate, larger than 10 to 20 mm was strong and higher than 20 mm was very strong inhibition[14].

Camargo et al[18] reported the antimicrobial effects of phenolic acids and flavonoids of peanut by-products. Their results showed that phenolic acid-rich extracts showed the lowest minimum inhibitory capacity (MIC) which means that the antibacterial effect is highest. Meanwhile, Xuan et al[19] studied the total phenolic and flavonoid contents of commercial vegetable edible oils marketed in Japan. All the oils studied possess antimicrobial activity on both *S. aureus* and *E. coli*.

The total phenolic and flavonoid contents contribute to the antimicrobial activity of this *A. calamus* rhizome. The antimicrobial activities of the phenolic compounds are associated to the ability to bind extracellular and soluble proteins, thus enabling complexation with bacterial cell walls[20].

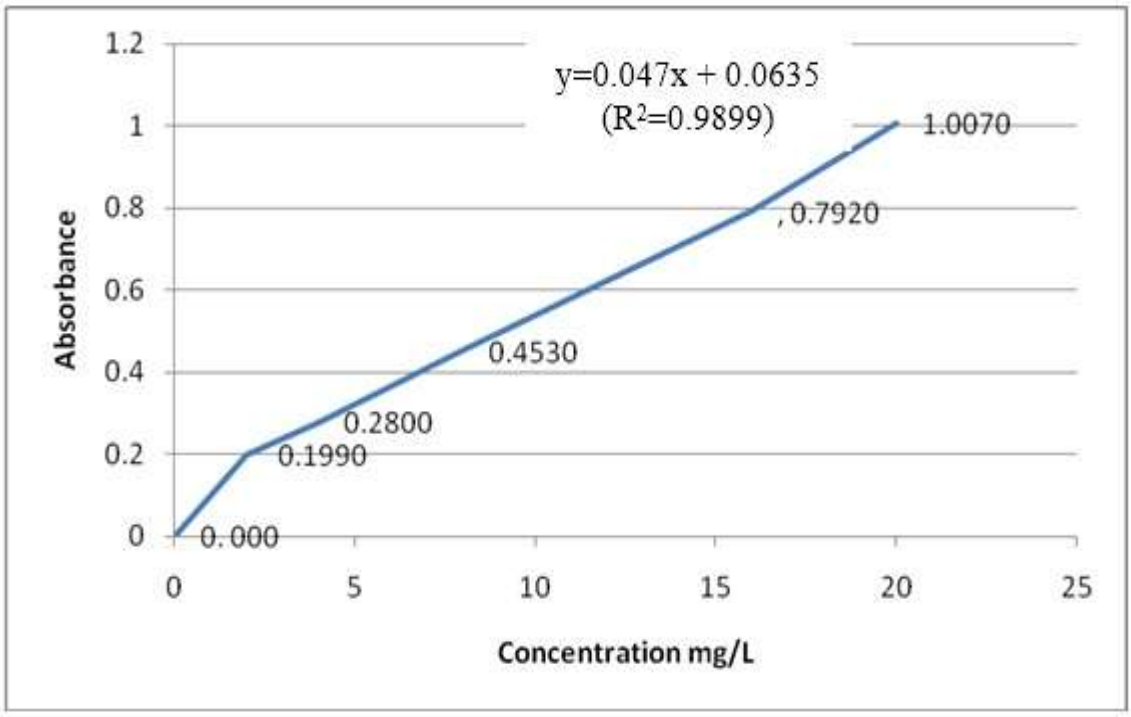

Figure 1: Calibration Curve of Standard Gallic Acid

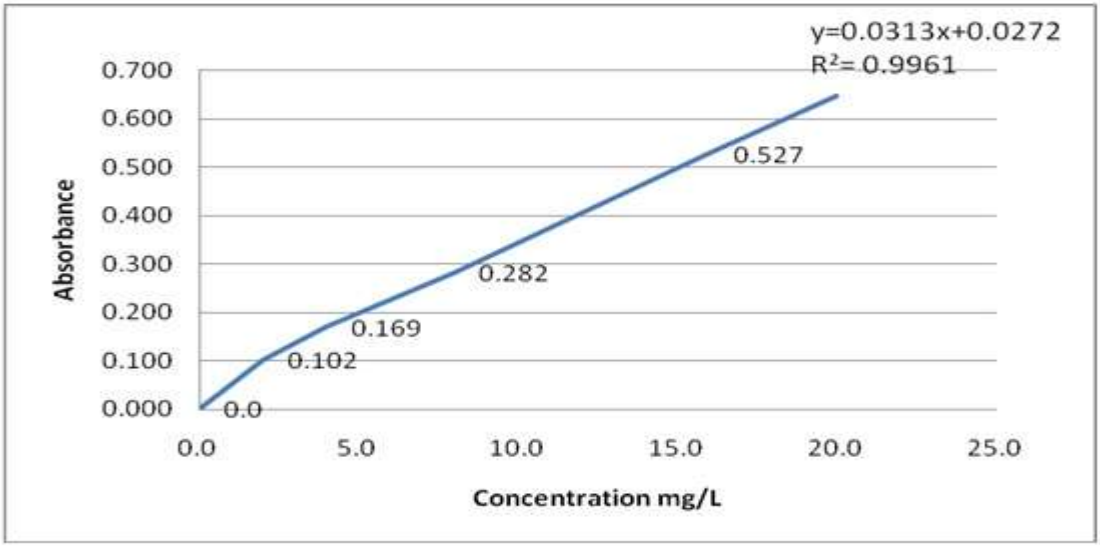

Figure 2: Calibration Curve of Standard Quercetin

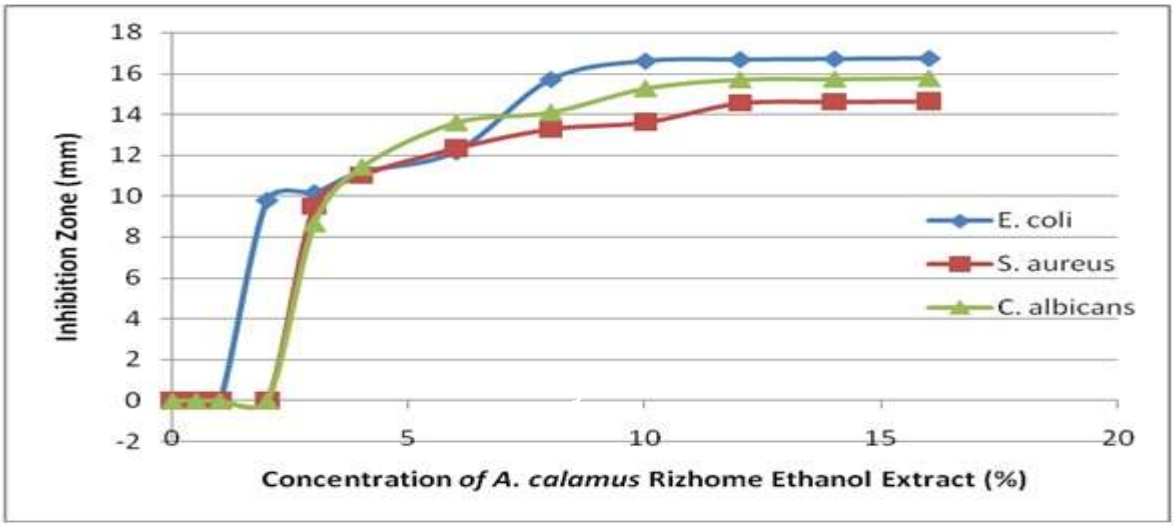

Figure 3: Graph of Inhibitory Activity of *A. calamus* Rizhome Ethanol Extract against *E. coli, S. aureus* and *C. albicans*

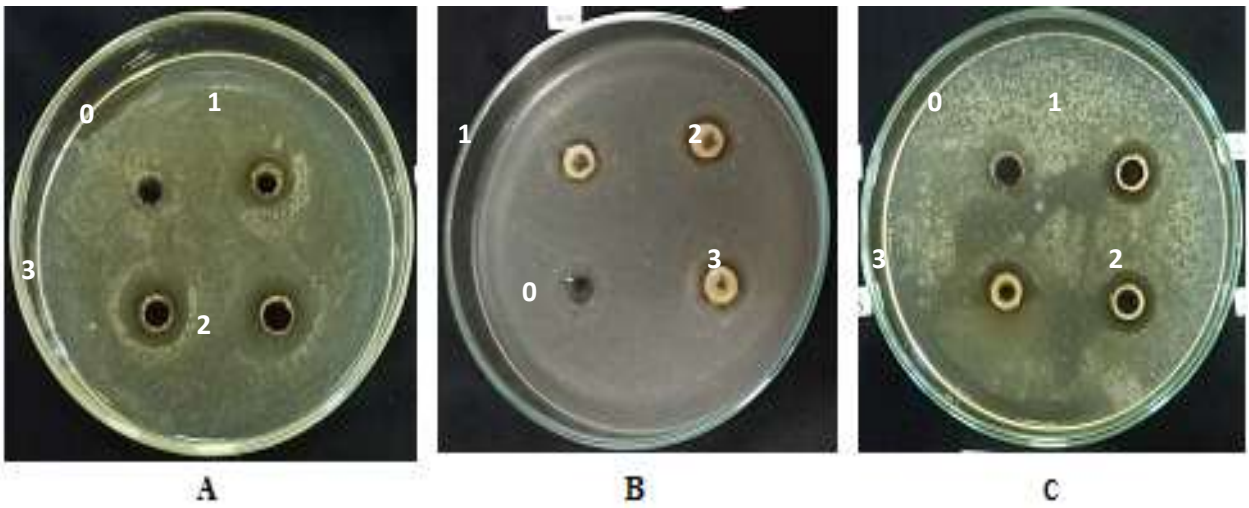

Figure 4: Inhibitory Activity of *A. calamus* Rizhome Ethanol Extract against A) *E. coli*, B) *S. aureus* and C) *C. albicans* at concentration of 10% with three repetitions (0: negative control; 1: the 1st repeat; 2: the 2nd repeat; 3: the 3rd repeat)

Table 1
Total Flavonoid and Phenolic Contents of *A. calamus* Rhizome Ethanol Extract

Comp.	Sample Weight (g)	Abs (Y)	Cons(x) mg/L	Volume (mL)	Dilution	Contents	
						%	mg/100g
Phenols	0.1158	0.325	5.5651	5.00	10	2.3984	2398.40
Flavonoids	0.1035	0.274	7.8850	5.00	5	0.1905	190.46

Table 2
Inhibition Zone of The Growth of *E. coli, S. aureus* and *C. albicans* of *A. calamus* Rhizome Ethanol Extract at Various Concentrations

Treatment (%)	Avarage of Inhibition Zone (mm)		
	E. coli	*S. aureus*	*C. albicans*
0 (negative control)	0^{a}	0^{a}	0^{a}
0.5	0^{a}	0^{a}	0^{a}
1.0	0^{a}	0^{a}	0^{a}
2.0	9.80^{b}	0^{a}	0^{a}
3.0	10.17^{b}	9.50^{b}	8.67^{b}
4.0	11.17^{bc}	11.00^{c}	11.43^{c}
6.0	12.17^{c}	12.33^{d}	13.60^{d}
8.0	15.70^{d}	13.27^{e}	14.10^{d}
10.0	16.60^{d}	13.60^{e}	15.27^{e}
12.0	16.67^{d}	14.53^{f}	15.70^{e}
14.0	16.70^{d}	14.60^{f}	15.73^{e}
16.0	16.73^{d}	14.63^{f}	15.77^{e}

* Values followed by the same letters in the same column are not significantly different according to the Duncan's Multiple Range Test at *P<5%.*

Conclusion

The total phenolic and flavonoid contents of *A. calamus* rizhome ethanol extract were successively 2398.40 mg GAE/100g and 190.46 mg QE/100g dry rhizome extract. The extract possessed the ability to inhibit the three pathogens. Minimum inhibitory concentration of the extracts against *E. coli, S. aureus* and *C. albicans* was 2, 3 and 3% respectively with the inhibition zone of 9.80, 9.50 and 8.67 mm. The optimum concentration to inhibit *E. coli, S. aureus* and *C. albicans* was 8, 12 and 10% respectively.

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References

1. Remoundou K. and Koundour P., Environmental Effects on Public Health: An Economic Perspective, *Int. J. Environ. Res. Public Health*, **6**, 2160-2178 **(2009)**

2. Balouiri M., Sadiki M. and Ibnsouda S.A., Methods for in vitro evaluating antimicrobial activity: A review, *J. Pharma. Anal*., **6**, 71–79 **(2016)**

3. Amber R., Adnan M., Tariq A., Khan S.N., Mussarat S., Hashem A., Al-Huail A.A., Al-Arjani A.F. and Abd Allah E.F., Antibacterial activity of selected medicinal plants of northwest Pakistan traditionally used against mastitis in livestock, *Saudi J Biol Sci*., **2(8)**, 1-25 **(2017)**

4. Padder B.M., Yasmeen S. and Ganaie M., Antibiotic/Antibacterial activity of *Vitex negundo, Duranta repens*, *Acorus calamus* and *Piper nigrum*, *Brit. Biotechnol. J*., **6(1)**, 16-22 **(2015)**

5. Rahamoz-Haghighi S., Asadi M.H., Riahi-Madvar A. and Baghizadeh A., Antibacterial effect of *Acorus calamus* extractions against gram positive and negative bacteria, *Ethno-Pharmaceutical Products*, **1(1)**, 1-7 **(2014)**

6. Rawal P., Adhikari R.S., Danu K. and Tiwari A., Antifungal activity of *Acorus calamus* against *Fusarium oxysporum f.* sp. lycopersici, *Int. J. Curr. Microbiol. App. Sci*., **4(1)**, 710-715 **(2015)**

7. Funde S.G., Phytochemicals evaluation, anticancer, antioxidant and antimicrobial activity of *Acorus calamus* different solvent extracts, *J. Chem. Pharm. Res*., **7(6)**, 495-504 **(2015)**

8. Anisah, Khotimah S. and Yanti A.H., The antibacterial activity of rhizome Jeringau (*Acorus calamus* L.) extract on the growth of *Staphylococcus aureus* dan *Escherichia coli*, *Protobiont*, **3**, 1-51 **(2014)**

9. Rahamoz-Haghighi S., Asadi M.H., Riahi-Madvar A. and Baghizadeh A., Antibacterial effect of *Acorus calamus* extractions against gram positive and negative bacteria, *Ethno-Pharmaceutical Products*, **1(1)**, 1-7 **(2014)**

10. Rita W.S., Kawuri R. and Swantara I.M.D., The essential oil contents of Jeringau (*Acorus calamus* L.) Rizhomes and Their Antifungal Activity against *Candida albicans*, *J. Health Sci. and Med.*, **1(1)**, 33-38 **(2017)**

11. Rita W.S., Asih I.A.R.A. and Yuliari N.M., Potency of essential oils of *Acorus calamus* Linn Rhizome to inhibit the growth of *Fusarium solani*, pathogenic fungi causing stem rot diseases on dragon fruit stem, *Cakra Kimia*, **4(2)**, 113-119 **(2016)**

12. Rita W.S., Suprapta D.N., Sudana I.M. and Swantara I.M.M., First Report on *Fusarium solani*, a Pathogenic Fungus Causing Stem Rot Disease on Dragon Fruits (*Hylocereus* sp.) in Bali, *J Biol Agric Health*, **3(17)**, 93-99 **(2013)**

13. Mahboubi A., Asgarpanah J., Sadaghiyani P.S. and Faizi M., Total phenolic and flavonoid content and antibacterial activity of *Punica granatum* L. var. *pleniflora* flowers (Golnar) against bacterial strains causing foodborne diseases, *BMC Complement Altern Med*., **15**, 366 **(2015)**

14. Rita W.S., Swantara I.M.D., Asih I.A.R.A., Sinarsih N.K. and Suteja I.K.P., Total flavonoid and phenolic contents of n-butanol extract of *Samanea saman* leaf and the antibacterial activity towards *Escherichia coli* and *Staphylococcus aureus*, AIP Conference Proceedings, **1718**, 060005 **(2016)**

15. Hanbabaee K. and Ree T.V., Tannins: Classification and Definition, *Nat. Prod. Rep*., **18**, 641–649 **(2001)**

16. Rhoades J. and Roller S., Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods, *Appl. Environ. Microbiol*., **66(1)**, 80-86 **(2000)**

17. Okigbo R.N. and Mmeka M.C., Antimicrobial Effects of Three Tropical Plant Extracts on *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*, *Afr. J. Trad.*, **5(3)**, 226 – 229 **(2008)**

18. Camargo A.C., Regitano-d'Arce M.A.B., Rasera G.B., Canniatti-Brazaca S.G., Prado-Silva L., Alvarenga V.O., Sant'Ana V.S. and Shahidi F., Phenolic acids and flavonoids of peanut by-products: Antioxidant capacity and antimicrobial effects, *Food Chemistry*, **237**, 538-544 **(2017)**

19. Xuan T.D., Gangqiang G., Minh T.N., Quy T.N. and Khanh T.D., An Overview of Chemical Profiles, Antioxidant and Antimicrobial Activities of Commercial Vegetable Edible Oils Marketed in Japan, *Foods*, **7(2)**, 1-14 **(2018)**

20. Puupponen-Pimiä R., Nohynek L., Meier C., Kähkönen M., Heinonen M., Hopia A. and Oksman-Caldentey K.M., Antimicrobial properties of phenolic compounds from berries, *J Appl Microbiol*, **90(4)**, 494-507 **(2001)**.