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Antimicrobial Activities of *Phyllanthus urinaria* Extracts Before and After Combined with *Pandanus tectorious* and *Lactobacillus rhamnosus* PN04

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ABSTRACT

*Phyllanthusurinaria*plant was used in liver relating diseases in Vietnam. The study was to know more about the bioactivities of *Phyllanthusurinaria*leaf extracts before and after combined with *Pandanustectorious*fruit extracts and *Lactobacillus rhamnosus*extract. This was accomplished by examining the influence of the total aqueous, chloroform and ethylacetateextracts. The biological tests were done on*Candida albicans,Salmonella typhi, Pseudomonas aeruginosa*and *Staphylococcus aureus.*Agar diffusion test was applied to determine the antimicrobial activities.Based on the inhibition zone diameter, the result showed that *Phyllanthusurinaria*aqueousextraction had the inhibition on the *Pseudomonas aeruginosa*(21.75 ± 0.96 mm)and *Staphylococcus aureus*(24.75 ± 0.96 mm). The activities decreased after combined with *Pandanustectorious*fruit extractson *Pseudomonas aeruginosa*(14.75 ± 0.50 mm) and *Staphylococcus aureus*(14.75 ± 0.50 mm)and *Lactobacillus rhamnosus*(14.75 ± 0.50 mm).The total aqueous extracts fractionated with chloroform and ethylacetate still showed activities that suggested the polar and nonpolar antimicrobial compounds existed in *Phyllanthusurinaria.* However, the activities of these fractionated extracts in the combination showed the weaker activities than in the using only *Phyllanthusurinaria*extracts. Especially, there was no activity of the ethylacetate extracts in the combination with *Lactobacillus rhamnosus.* The study is the first report of the combination of *Phyllanthusurinaria* with *Pandanustectorious* and *Lactobacillus rhamnosus* PN04.

Key word: *Phyllanthusurinaria, Pandanustectorius, Lactobacillus rhamnosus*, Antimicrobial activity.

INTRODUCTION

Traditional medicine has been used in some communities for thousands of years without understanding on safety, effectiveness and quality. Many kinds of traditional medicines caused the side effects (Niggeman and Gruber, 2003). Some of them are safety, effectiveness and quality. Therefore, finding the bioactivities of traditional medicine was necessary.*Phyllanthusurinaria* (*P. urinaria*), one of the herbal plants belonging to the genus *Phyllanthus* (Euphorbiaceae), is widely distributed in China,South India and Southern America. The ethylacetate extract of *P. urinaria* was shown to exhibit anticancer activity by inducing apoptosis through the inhibition of telomerase activity and Bcl-2 expression (Huang et al., 2003; Huang et al., 2004a and 2004b).The water extract prepared from *P. urinaria* has an anticancer effect on Lewis lung carcinoma cells through a similar pathway (Huang et al., 2006). One of the most commonly used traditional herbs in Vietnam is *Phyllanthusurinaria*. People usually used this herb to treat many diseases as hepatitis, diuretic, jaundice, edema, pimples. Sometimes, people combined *Phyllanthusurinaria*with some other medical herbs to treat various kinds of diseases as *Pandanustectorious*(also called pineapple wood, family of Pandanaceae) which was used to treat cough, hemorrhoids, dysentery. *Pandanustectorius* (*P. tectorius*) is a large shrub or small tree of immense cultural, health, and economic importance in the Pacific where it can withstand drought, strong winds, and salt spray. The nutritious fruits are edible varieties, those with low amounts

of calcium oxalate crystals. One 100 g portion of edible pericarp is mainly comprised of water (80 g) and carbohydrates (17 g). There are also significant levels of betacarotene (19 µg to 19 mg) and vitamin C (5 mg), and small amounts of protein (1.3 mg), fat (0.7 mg), and fiber (3.5 g) (Englberger et al. 2003, Englberger et al. 2006a and 2006b).Besides that, in order to improvement the dysentery, constipation and hepatitis, people used probiotic popularly in their life. Probiotics are living microorganisms, which, when ingested or locally applied in sufficient numbers, provide the consumer with one or more proven health benefits(De Keersmaecker et al., 2006; Chateris, 1998).One of probiotic used in colon treatment was *Lactobacillus rhamnosus*(Avlami, 2001). *Lactobacillus rhamnosus is usually isolated from milk, human vagina. Therefore, in order to study the combination of Phyllanthusurinaria*with *Lactobacillus rhamnosus, Lactobacillus rhamnosus PN04 isolated in plant named HottuyniacordataThunb was chosen for the study (Nguyen et al., 2013).*

Additionally, to confirm that if people combined *Phyllanthus urinaria* with *Pandanus tectorious* (fruit extracts), the antimicrobial activities of *Phyllanthusurinaria* extracts with or without combination with *Pandanustectorious*(fruit extracts) was done.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Five pathogen strains were*Salmonella typhi, Candida albicans, Pseudomonas aeruginosa*and *Staphylococcus aureus*. These bacteria have been cultured and maintained in LB (Luria-Bertani) broth and agar.

Lactobacillus rhamnosus PN04isolated from *Houttuynia cordata. Thunb*(Nguyen et al., 2013) was used in this study. This strain was maintained in MRS agar and MRS broth. The MRS medium contains peptone, glucose, yeast extract, tween 80, dipotassium phosphate, sodium acetate, ammonium citrate, magnesium sulphate and manganese sulphate(De Man et al., 1960).

Herbs

Dry *Phyllanthus urinaria and Pandanus tectorius* were bought commercially from medical store located in Hai Thuong Lan Ong street, district 5, Ho Chi Minh City, Vietnam.

Total water extraction

Fifty grams of dry *Phyllanthusurinaria*and fifty grams of *Pandanustectorius*were boiled with water enough (50 mL) for 1 hour to obtain a volume of 20 mL.The extracts were collected, thenanother water portion (50 mL) was added into this mixture and boiled continuously for 1 hour to obtain 20 mL. The second extract was collected and combined with the former for antimicrobial tests.

Fractionated extraction with chloroform

The water extraction was fractionated with chloroform. 10 mL of total aqueous extract was mixed with 3 mL of chloroform. The mixture of total was shaken well for 30 minutes and the lower layer was collected.This procedure was performed in triplicate. The chloroform extracts were combined for antimicrobial tests.

Fractionated extraction with ethylacetate

After completely extracted with chloroform, this extract was shakenwith 3 mL of ethylacetate for 30 minutes. The mixture was separated in three layers. The lowest layer was collected and the extraction with ethylacetatewas done for more twice. All the lowest extracts were combined and ready for antimicrobial activity test.

Antimicrobial activity tests

Antimicrobial effects were tested on the pathogens by the agar diffusion method. The tested microorganismswere propagated twice and then grown for 18-24 h in 10 ml of appropriate growth media. Turbidity of the culture broth was compared with McFarland tubes to give an estimate of bacterial population (10^6 CFU/mL). Supernatant of the cell after expression were collected after centrifugation at 12,000 rpm for 15 min) and the clear supernatantwas sterilized by filtration (0.45 ìm), thus yielding cell-free filtrates. The wells (ø 6 mm) were then prepared and filled using 100 ìL of cell-free filtrate. The inoculated plates were incubated for 18-24 h at appropriate temperatures, and the diameter of the inhibition zone was measured in millimeters with calipers. The measurements recorded werefrom the edge of the zone to the edge of the wall.

RESULTS AND DISCUSTION

Antimicrobial activities of the water extracts

The objective of this project was to find out the antimicrobial activities of *P.urinaria* and *P. urinaria* combined with *P.tectorius*when extract with water. The result showed that the different amount applied into wells gave significant difference ($p<0.05$). From Table 1, the inhibition of the total extract of *P.urinaria*on *S.aureus*(24.75 ± 0.96 mm) was significantly stronger than *P.aeruginosa*(21.75 ± 0.96 mm)based on the inhibition zone diameter.The total extract of the combination between *P.urinaria* and *P.tectorius*showed the weaker activities than the total extract of the *P.urinaria*on*S. aureus*(14.75 ± 0.50 mm)was bigger than *P. aeruginosa*(14.75 ± 0.50 mm).There was no activity on *Candida albicans* and *Salmonella typhi*.The antimicrobial activities of the aqueous extract were not broad. Regarding to the inhibition on *S. aureus*, *P.urinaria*might be used

Table 1: Antimicrobial activity test of the water extract of *Phyllanthusurinaria*according to the inhibition zone diameter (mm)

Pathogens	Inhibition zone diameter (mm)	
	Phyllanthus	***Phyllanthus and Pandanus***
Pseudomonas aeruginosa	21.75 ± 0.96	14.75 ± 0.50
Staphylococus aureus	24.75 ± 0.96	14.75 ± 0.50
Candida albicans	0.00 ± 0.00	0.00 ± 0.00
Salmonella typhi	0.00 ± 0.00	0.00 ± 0.00

Mean±SD

Table 2: Antimicrobial activity test of the chloroform extract of Phyllanthusurinaria according to the inhibition zone diameter (mm)

Pathogens	Inhibition zone diameter (mm)	
	Phyllanthus	***Phyllanthus and Pandanus***
Pseudomonas aeruginosa	*21.75 ± 0.96*	*10.25 ± 0.50*
Staphylococus aureus	*18.25 ± 0.50*	*14.25 ± 0.50*

Mean±SD

Table 3: Antimicrobial activity test of the ethylacetate extract of Phylanthus urinaria according to the inhibition zone diameter (mm)

Pathogens	Inhibition zone diameter (mm)	
	Phyllanthus	***Phyllanthus and Pandanus***
Pseudomonas aeruginosa	*16.25 ± 0.50*	*17.75 ± 0.50*
Staphylococus aureus	*9.5 ± 0.58*	*11.5 ± 0.58*

Mean±SD

Table 4: Antimicrobial activity test of *Lactobacillus rhamnosus* according to the inhibition zone diameter (mm)

Pathogens	Inhibition zone diameter (mm)			
	Early exponential phase	**Late exponential phase**	**Stationary phase**	**Death phase**
Salmonella typhi	0.00 ± 0.00	0.00 ± 0.00	11.00±1.00	10.00± 1.00
Pseudomonas aeruginosa	0.00 ± 0.00	7.67± 2.03	14.25 ± 0.50	11.67 2.31
Staphylococus aureus	0.00 ± 0.00	5.33 ± 0.58	18.5 ± 0.58	8.33 ± 2.08[a]
Candida albicans	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 5: Antimicrobial activity test of the aqueous, chloroform, ethylacetate extract of *Phyllanthus*in the combination of*Pandanus* and *Lactobacillus*according to the inhibition zone diameter (mm)

Pathogens	Inhibition zone diameter (mm)					
	L. rhamnosus	***Phylanthus* and L. *rhamnosus***	***Phylanthus* and *Pandanus* and *L. rhamnosus***	**Choroform extract of Phylanthus and *L. rhamnosus***	**Choroform extract of *Phylanthus* and *Pandanus* and *L. rhamnosus***	**Ethyl acetate extract of *Phylanthus* and *L. rhamnosus***	**Ethylacetate extract of *Phylanthus* and *Pandanus* and *L. rhamnosus***
Pseudomonas aeruginosa	14.25 ± 0.50	4.75 ± 5.50	4.75 ± 5.50	16.25 ± 0.50	13 ± 0.82	12.25 ± 0.96	00 ± 0.00
Staphylococcus aureus	18.5 ± 0.58	14.25 ± 0.96	14.25 ± 0.96	15 ± 0.82	13.25 ± 1.50	15.25 ± 0.50	00 ± 0.00

MEAN±SD

in skin infection and sepsis. According to Richard RM (1973), psoriatic patients were studied with regard to the quantities of *S.aureus* on involved and uninvolved skin. About 50% of the patients carried *S.aureus*, usually in low numbers. All patients with erythroderma harbored *S.aureus*, mostly on their skin. In atopic dermatitis, sepsis and skin infections, toxin C and in psoriasis, toxin B was most often detected. *S.aureus* was present in more than 50% of patients with atopic dermatitis and psoriasis. The severity of AD and PS significantly correlated to enterotoxin production of the isolated *S aureus* strains(Nordwi, 2005). As a result, *Phyllanthusurinaria* can aid for the skin diseases as psoriasis and atopic dermatistis.

P.aeruginosa is an important bacterial pathogen, particularly as a cause of infections in hospitalised patients, immunocompromised hosts and patients with cystic fibrosis. Surveillance of nosocomial *P. aeruginosa* infections has revealed trends of increasing antimicrobial resistance, including carbapenem resistance and multidrug resistance. Mechanisms of antimicrobial resistance include multidrug efflux pumps, ß-lactamases and downregulation of outer membrane porins. Mechanisms of virulence include secreted toxins and the ability to form biofilms. The effective treatment of infections caused by *P. aeruginosa* includes prevention when possible, source control measures as necessary and prompt administration of appropriate antibacterial agents. Antibacterial de-escalation should be pursued in patients with an appropriate clinical response, especially when antibacterial susceptibilities are known (Driscoll, 2007). Multidrug-resistant *P. aeruginosa* may require treatment with less commonly used antibacterials (e.g. colistin), but newer anti-pseudomonalantibacterials are expected to be available in the near future.*P. urinaria* may be used in bacteremia, diarrhea, ecthymagangrenosum in treating multidrug-resistant *P. aeruginosa*.

Although*Phyllanthus* had an effect on DNA polymerase of virus (Joseph, 2011), the mechanism of this plant on the examined bacteria should be studied more. However, there were the significant differences ($p<0.05$) between total extract solutions of *P.urinaria* before and after combined with *P.tectorius* on*P.aeruginosa*, *S.aureus*. The total extract of *P.urinaria*showed the stronger than the combination with *P.tectorius.*The study informed

that the combination of these two herbs in treating diseases related to these kinds of bacteria should be considered.

Antimicrobial activity of the extractsfractionated with chloroform and ethylacetate

The Table 2 and Table 3 showed the activities of the extracts fractionated with chloroform or in the extract after ethylacetate. In *P.urinaria*, there are some antimicrobial compounds in both polar and nonpolar. Interestingly, the activities decreased after these chloroform and ethylacetateextracts of *P.urinaria*in the combination with *P.tectorius*.From Table 2, the inhibition of the chloroform extract of *P.urinaria*on *S.aureus*(18.25 ± 0.50mm) was significantly stronger than *P.aeruginosa* (17.25 ± 0.50mm) based on the inhibition zone diameter.The combination of the chloroform extract of *P.urinaria* and *P.tectorius* showed the weaker activities on *S.aureus* (14.25 ± 0.50mm) and *P.aeruginosa* (10.25 ± 0.50 mm).However, The combination of the chloroform extract of *P.urinaria* and *P.tectorius*

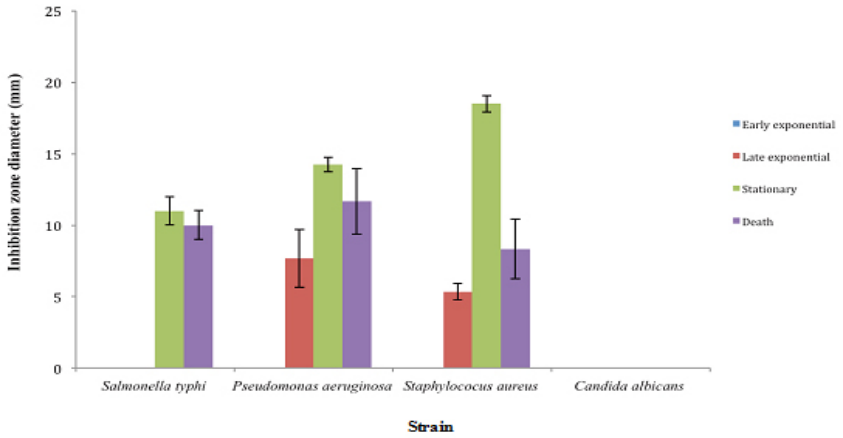

Fig 1: Antimicrobial activities of *Lactobacillus rhamnosus* PN04in different phases

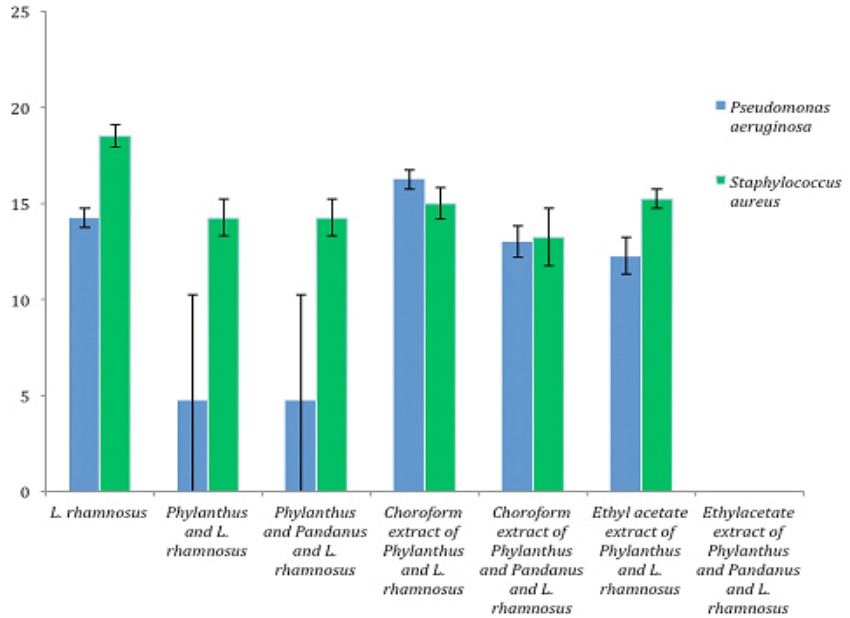

Fig 2: Comparison of antimicrobial activities of *Lactobacillus rhamnosus* PN04 with the extracts of *Phyllanthusurinaria*and *Pandanustectorius*

showed the similar activity on*S.aureus*(14.75 ±0.50 mm). The antimicrobial characteristics of *P.urinaria* and *P.tectorius* might have interestingmehanisms. The compounds in these plants might be antagonists. The identification of *P.urinaria* compounds will be done so far.

From Table 3, the inhibition of the ether extract of *P. urinaria* on *S.aureus* (17.75 ± 0.50mm) was significantly stronger than *P.aeruginosa* (16.25 ± 0.50mm) based on the inhibition zone diameter. The combination of the ether extract of *P.urinaria* and *P.tectorius* showed the weaker activities on *S.aureus* (11.50 ± 0.58 mm)and *P.aeruginosa* (9.5 ± 0.58 mm). The inhibition of the ether extract of *P.urinaria* on *S.aureus*and *P. aeruginosa* was insignificantly different from the inhibition of the chloroform extract of *P. urinaria* stronger than in*P.aeruginosa* ($p<0.5$).

Antimicrobial activity of *Lactobacillus rhamnosus*PN04

In order to study the effects of *Lactobacillus rhamnosus* PN04 on the extracts of *P.urinaria*, the antimicrobial activity of *Lactobacillus rhamnosus* was tested. As showing in Table 4 and Figure 1,*L. rhamnosus*PN04 isolated in *Hottuyniacordata*Thunb had the highest inhibition on *Salmonella typhi*(11.00±1.00), *Pseudomonas aeruginosa* (14.25 ± 0.50), *Staphylococcus aureus* (18.5 ± 0.58) in exponential phase. Regarding to the inhibition of *Phyllanthusurinaria* on *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the supernatant of *Lactobacillus rhamnosus* PN04 was used in the combination of *Phyllanthusurinaria* on *S. aureus* and *P. aeruginosa*.

Antimicrobial activity *of Lactobacillus rhamnosus*and*Phyllanthusurinaria* and *Pandanustectorus*

As shown in Table 5 and Figure 2, *L. rhamnosus* reduces the activity of *P. urinaria* on *Pseudomonas aeruginosa*. Especially, there was resistance on *P. aeruginosa* and *S. aureus* when the ethyacetate fraction combined with *Pandanustectorius* and *L. rhamnosus*. However, the ethyl acetate fraction and *L.rhamnosus* showed the insignificant difference of the activities on two pathogens. In this case, the activities were not significant different from the extracts without combination. Similarly, the chloroform fractions did not show the significance on the activities of the combined extracts. To understand the combination, more studies are carried out. The combination in using these plants and *L. rhamnosus* was complicated. People should not used*L. rhamnosus* and *P.urinaria* together in treating some diseases that related to intestinal like dysentery, constipation.

In Viet Nam, the traditional method that people used to extract this kind of herbal is water extraction, so that some characteristics might loss or might not be secreted during the extraction. This lead to the result that samples only inhibited the activities of two indicators bacteria as *P. aeruginosa* and *S. aureus*. According to the previous study, *P.amarus*, a plant related with *Phyllanthusurinaria* when extract with ethanol may inhibit the activity of *Salmonella typhi*(Oluwafemi and Debiri, 2008). Moreover, the *Phyllanthusurinaria* can also inhibit *Pseudomonas aeruginosa, Staphylococcusaureus* and another microorganism*as Escherichia coli, Bacillus cereus,Klebsiellaaerogenes, Proteus vulgaris, Shigellaboydis when extract* of *Phylanthusurinaria with acetone or methanol* (Daburet. al., 2007). Our study has found the antimicrobial activities on *S. aureus* and *P. aeruginosa* of the water extract of *P. urinaria*. Also, the study warned the people who used these plants and *L. rhamnosus* for any treatment should be carefully.

CONCLUSION

The study showed the antagonistic characteristics of *Phyllanthusurinaria* and *Pandanustectorius* and *Lactobacillus rhamnosus* on *Staphylococcus aureus* and *Pseudomonas aeruginosa*.With the action on *Staphylococcus aureus* and *Pseudomonas aeruginosa*, *Phyllanthusurinaria* could be used in treating psoriasis, atopic dermatitis, bacteremia, diarrhea, ecthymagangrenosum. However, the mechanism of action should be done.

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