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EVALUATING THE EFFECT OF PYROLIGNEOUS EXTRACT AS NATURAL ANTIMICROBIAL AGENT UNDER DIFFERENT CONTACT TIMES

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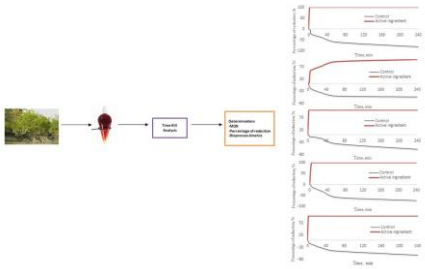
Graphical abstract



Abstract

Pyroligneous extract is a by-product of the charcoal making process. Pyroligneous extract application becomes a potential natural active ingredient to contribute in biocosmetics, bioprocess, and biopharmaceutical industry. In this study, five human harmful microorganisms which related urinary tract infection were selected: *Candida albicans* 10231, *Escherichia coli* 8739, *Enterococcus faecalis* 19433, *Proteus vulgaris* 33420 and *Klebsiella pneumoniae* 13883. Time-kill kinetics assay method was used to study natural pyroligneous acid as an antimicrobial agent to determine bacteriostatic and bactericidal activity of the minimum duration of killing (MDK) with a variety of contact time (0 – 240 minutes). From the result is showed that pyroligneous extract reached 100% of reduction effects with different MDK: *E.coli* (8739) at 2 minutes, *P.vulgaris* (33420) at 4 minutes, *K.pneumoniae* (13883) at 2 minutes, *C.albicans* (10231) at 2 minutes and *E. faecalis* (19433) at 240 minutes. For bioprocess kinetics analysis, the highest specific reduction rate and halve rate according to contact time: *E.coli* at 2 minutes (3.5450, 5.1144), *P.vulgaris* at 2 minutes (3.7192, 5.3657), *K.pneumoniae* at 2 minutes (3.5015, 5.0516), *C.albicans* at 2 minutes (3.4947. 5.0417) and *E.faecalis* at 4 minutes (3.8005, 5.4829). The results of this research provide convincing evidence to pyroligneous extract as an antimicrobial agent.

Keywords: Pyroligneous extract, Minimum duration of killing (MDK), Antimicrobial agent, Time-kill methods, *Rhizophora apiculata*

Abstrak

Ekstrak piroligneous ialah hasil sampingan dalam proses pembuatan arang. Aplikasi ekstrak piroligneous menjadi bahan aktif semulajadi yang berpotensi untuk menyumbang dalam industri biokosmetics, bioproses, dan biofarmaseutikal. Dalam kajian ini, lima mikroorganisma berbahaya terhadap manusia yang berkaitan dengan jangkitan saluran kencing dipilih: *Candida albicans* 10231, *Escherichia coli* 8739, *Enterococcus faecalis* 19433, *Proteus vulgaris* 33420 dan *Klebsiella pneumoniae* 13883. Kaedah ukuran kinetik tempoh membunuh digunakan untuk mengkaji asid piroligneous semulajadi sebagai ejen antimikrob untuk menentukan aktiviti bacteriostatic dan bactericidal yang tempoh minimum membunuh (MDK) dengan pelbagai masa reaksi (0 - 240 minit). Hasilnya ditunjukkan bahawa ekstrak piroligneous mencapai 100% kesan pengurangan dengan MDK yang berbeza: *E.coli* (8739) pada 2 minit, *P.vulgaris* (33420) pada 4 minit, *K.pneumoniae* (13883) pada 2 minit, *C.albicans* (10231) pada 2 minit dan *E. faecalis* (19433) pada 240 minit. Untuk analisis kinetik bioproses, kadar pengurangan khusus tertinggi dan kadar separuh mengikut masa reaksi:

E.coli pada 2 minit (3.5450, 5.1144), *P.vulgaris* pada 2 minit (3.7192, 5.3657), *K.pneumoniae* pada 2 minit (3.5015, 5.0516), *C.albicans* pada 2 minit (3.4947. 5.0417) dan *E.faecalis* pada 4 minit (3.8005, 5.4829). Hasil penyelidikan ini memberikan bukti yang meyakinkan terhadap ekstrak piroligneous sebagai agen antimikrob.

Kata kunci: Ekstrak piroligneous, Tempoh minimum membunuh (MDK), Ejen antimikrob, Kaedah masa membunuh, *Rhizophora apiculata*

1.0 INTRODUCTION

Urinary tract infections happen in patient population and increase complication associated risk [1]. This infection also facing the diagnostic challenge, and difficulties in obtaining urine sample to oligouric and anuric patients [2] which decrease the recovery rate. Most common bacterial infections in children are urinary tract infections, around 8% of children will facing urinary tract infection at least one time between the ages of 1 month and 11 years [3] and around 30% of infants and children facing recurrent infections during the first 6 months to 1 year after first-time urinary tract infection [4]. In the United States, around 1.5 million pediatric ambulatory visits every year due to urinary tract infections [5]. During 2013, the United States spent $630 million in health care management and treatment costs [6]. The type microbial strains caused urinary tract infection increasing, for example, *Acinetobacter junii* [7] and Gram-negative bacilli (GNB) such as Enterobacteriaceae normally will cause urinary tract infections and intra-abdominal infections [8-10]. This infection caused short term morbidity such as dysuria, fever and flank pain, and possible cause in long-term renal injury, for example permanent kidney scarring [11].

Unfortunately, some reports stated that targeted microorganism begins resistance to drugs [12, 13]. Those microbial strains have the genetic ability to evolve resistance to treat infections drugs. The consequence of complex genotypic mutational patterns on drug susceptibility has been investigated and phenotypic resistance was evaluated in some researches [14]. One of the reasons for this critical issue was augmented via the introduction of many antibiotics every year to the environment. The environment is heavily flooded with these toxic mixtures which significant cause microorganism resistance to antibiotics [15]. Currently, multidrug-resistant microbial strains are responsible for the rise in untreatable microorganism infections and increasing the death rates in the whole world, synthetic or artificial antimicrobial ingredient has been found with some side effects to the human being, and most of the harmful microorganisms are evolving resistance to antibiotics. Thus, the alternative green antimicrobial agents' production is important [16].

So, the increasing interest by researchers in the application of this matrix as a source of phenolic compounds, for use as active ingredients or food supplements for the pharmaceutical and cosmetic industries [17]. Also, phenolic compounds present biological activities, for example anti-inflammatory, antimicrobial, antioxidants, and others [18]. The polyphenols and derivative potential a variety of phytochemicals and pharmacological functions [19]. For instance, *quercus infectoria* extract proved that as an antimicrobial agent for decontaminating eggshells [20] and hydrogen peroxide in honey contribute strong antimicrobial activity, especially antifungal effect [21] and antimicrobial activity of pyroligneous extract due to the presence of mixtures such as phenolic compounds, organic acids and carbonyls [22-25].

In this study, pyroligneous extract will be studied as natural antimicrobial agents, especially against urinary tract infection-related strains. In Malaysia of the northern region, there are around 366 charcoal making production lines or kilns, with a total production of around three thousand and five hundred tonnes of charcoal with the sales around three million Ringgit Malaysia per month, also making condense chemical from gas released during charcoal production a flourishing industry [26]. This condense chemical called pyroligneous extract or wood vinegar or liquid smoke, is a crude condensate produced from the distillation of vapour produced in charcoal production. This plant extract is a complex mixture of compounds derived from the chemical breakdown of wood components via a condensation process of gases and vapours generated during the limited oxygen pyrolysis process [27, 28]. Pyroligneous extract is a highly oxygenated and complex aqueous liquid fraction, it's produced from the thermochemical breakdown or called pyrolysis of flora biomass components, for example lignin, hemicellulose and cellulose [29].

Chemically, pyroligneous extract is reddish brown liquid which contains about 10-20% aqua, 10% a mixture of carboxylic acids, especially acetic acid, and the rest are bioactive compounds such as alcohols, pyrolytic lignin, and aldehydes [22, 30, 31]. In the Lee *et al*. (2010), Sameshima, Sasaki, & Sameshima (2002) and Yatagai, Nishimoto, Hori, Ohira, & Shibata (2002) researches stated that acetic

and phenolic acid contents in the pyroligneous extract produced from charcoal manufacturing. Phenolic compounds such as 4-ethyl-2-methoxyphenol and 4-propyl-2-methoxyphenol from pyroligneous extract might have some effects of preservation and disinfectant which explain why lignin-rich fractions are more effective than bio-oil [22, 32,33].

Pyroligneous acid is applied in a variety of fields such as antimicrobial, antioxidant, and anti-inflammatory agents. This found in alternative cost-effective antimicrobial ingredients of green origin remains a challenge for the scientific application [34]. Some research also proved that the antimicrobial activity of pyroligneous extract against several plant pathogens and pathogenic bacteria [35]. Generally, pyroligneous extract is major used as deodorization agents, fertilizer additives, and a green aid for detoxification, sterilizer, and mild pain relief, and promote minor wound healing [26]. Pyroligneous extract also is used as a fungicide from wood decay Basidiomycetes by *Trichoderma* spp. [36]. In the research of Nakai, Kartal, Hata, & Imamura (2007) showed that pyroligneous extract from pyrolysis of acacia wood and sugi increased the wood resistant against brown-rot fungi [37].

However, in recent research studies regard antimicrobial agents against *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 8739), *Proteus vulgaris* (ATCC 33420), and *Klebsiellla pneunmoniae* (ATCC 13883) and *Candida albicans* (ATCC10231) with a variety of contact time have not been investigated further. Therefore, in this study the five strains that caused urinary tract infection and different treated contact time (0 – 240 minutes) with pyroligneous extract as a potential antimicrobial agent were investigated with time-kill method. Time-kill method is the most suitable testing for determining the fungicidal and bactericidal effect. It is a powerful tool for gaining results about the dynamic interaction between the target microbial strain and anti-microbial agent. The time-kill method shows a concentration-dependent or a time-dependent antimicrobial effect [38]. The antimicrobial effects against urinary tract infection-related strains' percentage of reduction and its bioprocess kinetics were also explored.

2.0 METHODOLOGY

2.1 Inoculum Preparation

Bacteria *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 8739), *Proteus vulgaris* (ATCC 33420), and *Klebsiellla pneunmoniae* (ATCC 13883) were cultured on suitable agar until it was ripe and fruiting at 35ºC for 18 to 24 hours. Yeast *Candida albicans* (ATCC10231) were cultured on appropriate agar (Nutrient agar CM0003B) till it was ripe and fruiting (44 – 52 hours) at 25ºC. By scraping the fruity culture, the spore collected were transferred to 10 ml sterilized tryptone sodium chloride solution in a universal bottle to obtain a microbial count of about $1.0x10^8$ CFU/ml. Each test microorganism was prepared in different universal bottles. The universal bottle was vortexed for 10 seconds to bring the spores into suspension. This suspension was then used as the inoculum for the test sample.

2.2 Time Kill Analysis Sample Preparation

The test (ASTM – E2783) was conducted in separate sterile universal bottles with 10ml of sample in it. The volume of the suspension inoculums used was 0.1% of the volume of the sample. The concentration of inoculum after inoculation is between $1.0x10^3$ – $1.0x10^4$ CFU/ml. The initial concentration of viable microorganisms in test preparation was determined by the plate count method at 0 minutes (as initial value). The inoculated sample with 16% pyroligneous extract was incubated in 22.5ºC until end of contact time [39]. The variety of contact time investigated were 0 minutes (as control), 2 minutes, 4 minutes, 6 minutes, 30 minutes, 60 minutes, and 240 minutes.

2.3 Microbial Activity Analysis

2.3.1 Mean Number of Cells, Mean Log, the Percentage of Reduction, Log Reduction and Log Growth

The microorganism activity was determined by the plate count method with colony counter (Funke Gerber, Colony Star 8502-3952). After inoculation, the sample was incubated at 22.5ºC until end of contact time with 16% of the pyroligneous extract with a variety of contact times (0, 2, 4, 6, 30, 60, and 240 minutes) respectively. Each experiment was performed in duplicates to ensure reproducibility of results. Then, microbial strains' bioactivity and its bioprocess kinetics were calculated with the Eq. 1 -7:

$$Mean\ nuumber\ of\ cell\ (m), CFU/g = \frac{Sample\ 1 + Sample\ 2}{2} \quad (1)$$

$$Mean\ log = log_{10}\, m \quad (2)$$

$$Percentage\ of\ reduction, \% = \frac{Initial\ value - Sample\ value}{Initial\ value}\ X\ 100\% \quad (3)$$

$$log\ growth = \log_{10} Sample\ value - \log_{10} Initial\ value \quad (4)$$

$$log\ reduction = \log_{10} Initial\ value - \log_{10} Sample\ value \quad (5)$$

$$Specific\ growth\ rate\ (\mu) = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (6)$$

$$Specific\ reduction\ rate\ (v) = \frac{\ln(N_1 - N_2)}{t_2 - t_1} \quad (7)$$

Where N_2 and N_1 represent cell number concentrations at time t_2 and t_1, respectively, whereas k_1 represents the time taken to duplicate the microbial division rate for control and k_2 represents the time take to halve the microbial halve rate for a sample, evaluated according to the Eq. 8 and 9.

$$k_1 = \frac{\mu}{0.693} \quad (8)$$

$$k_2 = \frac{v}{0.693} \quad (9)$$

3.0 RESULTS AND DISCUSSION

3.1 Effect of Antimicrobial Agent with Different Contact Time

Enterococci can act as opportunistic infectious agents, causing a variety of infections in the human body [40]. Anti-enterococcus activity of pyroligneous extract with a various contact time with 16% concentration presented in Figure 1. The anti-enterococcal activity of the pyroligneous extract increased as the contact time increased from 0 to 240 minutes. The control showed the negative percentage of reduction from -8% to -54% proved that the strain test in this testing is healthy to be valid used in the antimicrobial analysis. Pyroligneous extract showed a strong anti-enterococcus activity: 5% of reduction in the first 2 minutes, 48% of reduction in the next 4 minutes, 56% of reduction in 6 minutes, 73% of reduction in 30 minutes, 91% of reduction in 60 minutes and reached 100% of reduction with a minimum duration of killing in 240 minutes. The dead cell percentage is measured relative to the growth control by determining the active cell number (CFU/ml) of each test tube using the cell count method. Normally, the effect of bactericidal is obtained with 90% lethality for six hours, which is equal to 99.9% lethality percentage for twenty-four hours [41]. Pyroligneous extract majority contain phenolic compounds and Shahidi & Ho (2005) research found that phenolic compounds are defined as substances that possess one or more hydroxyl (-OH) substituents with an aromatic ring, including some functional derivatives such as esters, acids, glycosides, and methyl esters [42]. These compounds have anticarcinogenic, anti-inflammatory, and antioxidant effects [43]. Darah *et al*. (2013) also stated that *Rhizophora apiculata* pyroligneous extract which contain 5.5% acetic acid, 3.4% methanol and 6.5% wood tar. This considers as high content of volatile acids (8-10%) with the pH ranging from 2-3 which contribute to it's a mild corrosive feature [44] which might cause inhibition to microorganism growth. In addition, Davidson & Taylor (2007) stated that phenolic compounds showed antimicrobial effect against a lot of microorganisms which can inhibit the growth of foodborne bacteria and increase processed food shelf life [45].

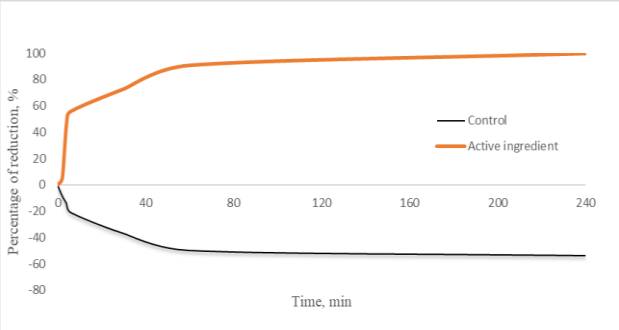


Figure 1 The percentage of reduction of *Enterococcus faecalis* 19433 with variety contact time

E. coli is responsible for the infection of meningitis, urinary tract, and gastroenteritis [46]. Antibacterial activity of pyroligneous extract with variety contact time with 16% concentration shown in Figure 2. The pyroligneous extract proved antibacterial activity with the contact time increasing from 0 to 240 minutes. The control is showed negative percentage of reduction from -7% to -58% proved that the strain used in this testing is active enough to be valid used in the time-kill analysis. Pyroligneous extract showed significant antimicrobial effect with 100% reduction with a minimum duration of killing at 2 minutes. Pyroligneous extract is heterogeneous chemical mixture which showing good antimicrobial activity [47, 48, 49]. Phenolic compounds are a majority group found in pyroligneous acid or liquid smoke [50]. Phenolic compounds have varying antimicrobial effects against foodborne harmful microorganisms such as *E. coli* and *Salmonella* following 24 hours and 60 hours incubation periods. Natural sources of phenolic compounds contain a lot of antibacterial components and have good potential to be used as a natural food preservative and antimicrobials for long term storage to partially or completely inhibit bacterial growth [51].

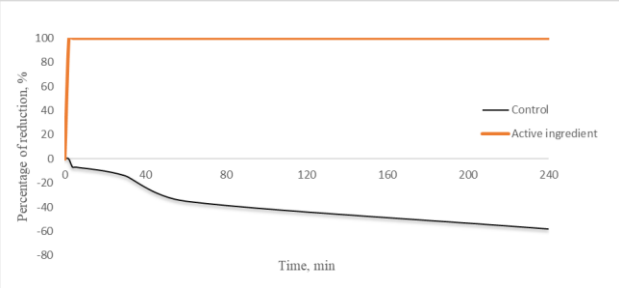


Figure 2 The percentage of reduction of *Escherichia coli* 8739 with variety contact time

The anti-proteus activity of pyroligneous extract with a various of contact time with 16% concentration presented in Figure 3. The pyroligneous extract indicated anti-proteus activity with the concentration increasing from 0 to 240 minutes. The control is showed a negative percentage of reduction from -3% to -74% proved that the strain chose in this testing is healthy enough to be used in the antimicrobial analysis. The minimum duration of killing which can be found from the time-kill curves are recommended as a quantitative measure of tolerance [52]. From the time-kill analysis, pyroligneous extract showed confirm strong anti-proteus effect: 68% of reduction in 2 minutes and reached 100% reduction with a minimum duration of killing in 4 minutes. Elo, Kuure, & Pelttari (2015) study indicated that proton exchange may be involved in the antimicrobial activity mechanisms of the mixtures [53] and it is believed that phenolic mixtures can deactivate microbial andesins, cell envelope transport proteins and enzymes [54]. Also, Phenolic acid diffuse into the cell may cause the efflux of cell components such as nucleic acid, proteins and inorganicions [55] and phenolic compounds can inhibit deoxyribonucleic acid gyrase which is involved in bacterial ribonucleic acid and deoxyribonucleic acid synthesis [56].

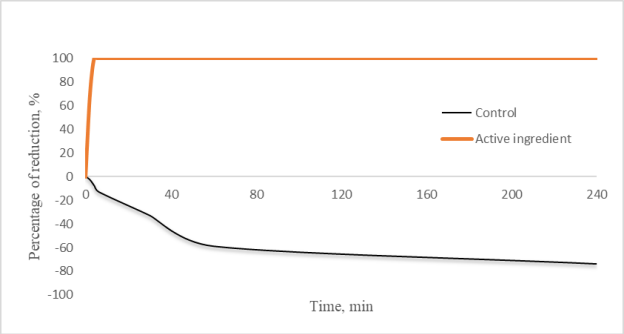


Figure 3 The percentage of reduction of *Proteus vulgaris* 33420 with variety contact time

Klebsiella pneumoniae is the contributing agent of urinary tract infections, respiratory tract, surgical wound sites, and lower biliary tracts [57]. Anti-klebsiella activity of pyroligneous extract with variety contact time with 16% concentration presented in Figure 4. The pyroligneous extract showed anti-klebsiella activity with the concentration increasing from 0 to 240 minutes which similar phenomenon with Figure 2. The control is showed a negative percentage of reduction from -5% to -63% proved that the strain selected in this testing is active enough to be used in the time-kill analysis. The minimum duration of killing is defined as, the time of an antibiotic treatment important to kill a known fraction of the microbial population at an antibiotic concentration that goes over the minimum inhibitory concentration. Likewise, to the minimum inhibitory concentration, that can be used to investigate the resistant level between microbial strains; the minimum duration of killing can be used to compare the tolerant level between target strains. An investigation framework that measures both the minimum duration of killing and the minimum inhibitory concentration would enable a clear distinction to be made between resistance (an increase in the minimum inhibitory concentration) and tolerance (an increase in the minimum duration of killing) [58]. Pyroligneous extract showed high potential strong anti-klebsiella with 100% of reduction with a minimum duration of killing at 2 minutes. The research of Kwon, Apostolidis, Kwon *et al*. (2008) discovered that phenolics can cause the redox process at the plasma membrane and sequester electrons from the respiration reaction [59]. These phenolics mixtures can also cause localized irregularity and disintegration in the outer membrane and lead to the cytoplasm leaking [60]. Any plant extract's antimicrobial activity depends on the antimicrobial agent's cell wall penetration ability. There are unique interactions between the cell wall compartments and antimicrobial agent [61]. The microorganism found more unusual morphology over time when treated by pyroligneous extract, it can be stated that the lethal action due to pyroligneous extracted treated with the shrinkage of the microorganism and followed by cavity formations [44]. It is because due to the induced membrane permeability changes that happened to the cell membrane caused the hydrogen bonds breakages that functions in maintaining the membrane shape [62, 63]. Normally, phenolic compounds are widely used in clinical dentistry as medication, disinfectants, and sedatives because despite being cytotoxic agents, they are non-mutagenic [64].

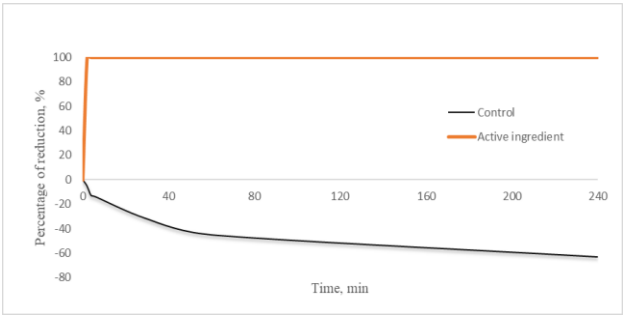


Figure 4 The percentage of reduction of *Klebsiellla pneunmoniae* 13883 with variety contact time

Candida albicans is a pathogenic yeast triggering mucocutaneous cavities of the skin, intestines, and vagina and infection of the human skin [65], caused by altering pathological and physiological conditions, for example, diabetes, infancy, pregnancy, steroidal chemotherapy, prolonged broad-spectrum antibiotic treatment and acquired immune deficiency syndrome [66]. Also, *Candida* sp. has been highly associated with a few opportunistic fungal infections [67]. Opportunistic microorganisms such as *Candida* sp. can cause from a simple

catheter-related peritonitis or fungemia to severe localized infections, or even extensive hematogenous dissemination [67, 68]. The anticandidal activity of pyroligneous extract with a variety of contact time with 16% concentration is shown in Figure 5. The pyroligneous extract indicated anticandidal activity with the concentration increasing from 0 to 240 minutes. The control is showed a negative percentage of reduction from -4% to -83% proved that the strain test in this testing is healthy enough to be valid used in the antimicrobial analysis. The antimicrobial agent effect in-depth, flow cytofluorometric methods, or time-kill test is recommended, which provide data on the nature of the bacteriostatic or bactericidal effect (concentration-dependent or time-dependent) and the microbe damage inflicted to the test microorganism [69]. Pyroligneous extract showed strong anticandidal with 100% of reduction and minimum duration of killing at 2 minutes only. Pyroligneous extract has shown potential as an insecticide and fungicide [70]. From the research of Bruce & Highley (1991) and Nakai *et al*. (2007) showed pyroligneous acid can be used as an efficient fungicide in the wood industry [36, 37]. Pyroligneous acid is useful for the control of termite and fungal infestations [37, 71]. Yang *et al*. (2016) found that antifungal and antibacterial effects of pyroligneous acid contributed to a combination of several ones instead of a single compound [72]. The phenolic extract contains a variety of functions depending on the source such as from *Elaeis guineensis* Jacq as antifungal potential [73], from *Pyrostegia venusta* Miers as antimicrobial and wound healing potential [74], from *Syngonanthus nitens* as vulvovaginal candidiasis [75] and from *Cassia fistula Linn*. as anticandidal potential [76].

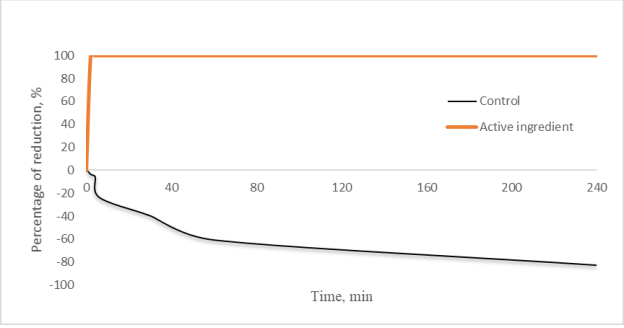


Figure 5 The percentage of reduction of *Candida albicans* 10231 with variety contact time

3.2 Antimicrobial Bioprocess Kinetics Analysis

Table 1 summarizes the bioprocess kinetics for the current study to potential antimicrobial agent - pyroligneous extract according to different strains (control: log growth, specific growth rate and division rate; sample: log reduction, specific reduction rate, and halve rate). The pyroligneous extract showed the promising reduction rate and halve rate to each microbe strain: *E. faecalis* 19433, *E. coli* 8739, *P. vulgaris* 33420, *K. pneumoniae* 13883 and *C. albicans* 10231 respectively. The highest log reduction (3.6675) for *E. faecalis* 19433 was achieved under 240 minutes of contact time condition with the highest specific reduction rate of 3.8005 min^{-1} and the highest halve rate of 5.4829 min^{-1} at 4 minutes respectively. The highest log reduction for *P. vulgaris* 33420 (3.3979) was achieved under 4 minutes contact time condition with the highest specific reduction and highest halve rate 3.7192 min^{-1} and 5.3657 min^{-1} at 2 minutes respectively. While the highest log reduction for *E. coli* 8739 (3.0792), *K. pneumoniae* 13883 (3.0414) and *C. albicans* 10231 (3.0354) were achieved under 2 minutes contact time condition with highest specific reduction rate of 3.5450 min^{-1}, 3.5015 min^{-1} and 3.4947 min^{-1} and highest halve rate 5.1144 min^{-1}, 5.0516 min^{-1} and 5.0417 min^{-1} respectively.

Table 1 Antimicrobial agent bioprocess kinetics according urinary tract infection related strains with different contact time

		Enterococcus faecalis 19433			*Escherichia coli* 8739			*Proteus vulgaris* 33420			*Klebsiellla pneunmoniae* 13883			*Candida albicans* 10231		
	Time, min	**Log growth**	**Specific growth rate [a]**	**Division rate [c]**	**Log growth**	**Specific growth rate [a]**	**Division rate [c]**	**Log growth**	**Specific growth rate [a]**	**Division rate [c]**	**Log growth**	**Specific growth rate [a]**	**Division rate [c]**	**Log growth**	**Specific growth rate [a]**	**Division rate [c]**
Control	**0**	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	2	0.0345	3.1073	4.4829	0.0000	0.0000	0.0000	0.0146	2.1587	3.1144	0.0223	2.3026	3.3219	0.0156	2.1587	3.1144
	4	0.0571	2.9290	4.2256	0.0300	2.5053	3.6144	0.0332	2.3026	3.3219	0.0537	2.5053	3.6144	0.0267	2.0037	2.8907
	6	0.0845	3.0546	4.4069	0.0300	0.0000	0.0000	0.0512	2.3026	3.3219	0.0587	1.6094	2.3219	0.0948	2.9568	4.2657
	30	0.1373	0.2857	0.4122	0.0580	0.2088	0.3012	0.1237	0.2546	0.3672	0.1192	0.2410	0.3477	0.1581	0.2496	0.3602
	60	0.1773	0.2228	0.3215	0.1288	0.2017	0.2910	0.2016	0.2118	0.3056	0.1606	0.1840	0.2655	0.2068	0.1953	0.2817
	240	0.1867	0.0294	0.0425	0.1996	0.0345	0.0498	0.2402	0.0321	0.0464	0.2126	0.0325	0.0470	0.2623	0.0339	0.0490
		Log reduction	**Specific reduction rate [b]**	**Halve rate [d]**	**Log reduction**	**Specific reduction rate [b]**	**Halve rate [d]**	**Log reduction**	**Specific reduction rate [b]**	**Halve rate [d]**	**Log reduction**	**Specific reduction rate [b]**	**Halve rate [d]**	**Log reduction**	**Specific reduction rate [b]**	**Halve rate [d]**
PE*	**0**	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	2	0.0240	2.7607	3.9829	3.0792	3.5450	5.1144	0.4949	3.7192	5.3657	3.0414	3.5015	5.0516	3.0354	3.4947	5.0417
	4	0.2872	3.8005	5.4829	3.0792	0.0000	0.0000	3.3979	3.3423	4.8219	3.0414	0.0000	0.0000	3.0354	0.0000	0.0000
	6	0.3557	2.9290	4.2256	3.0792	0.0000	0.0000	3.3979	0.0000	0.0000	3.0414	0.0000	0.0000	3.0354	0.0000	0.0000
	30	0.5705	0.2785	0.4018	3.0792	0.0000	0.0000	3.3979	0.0000	0.0000	3.0414	0.0000	0.0000	3.0354	0.0000	0.0000
	60	1.0654	0.2248	0.3244	3.0792	0.0000	0.0000	3.3979	0.0000	0.0000	3.0414	0.0000	0.0000	3.0354	0.0000	0.0000
	240	3.6675	0.0333	0.0480	3.0792	0.0000	0.0000	3.3979	0.0000	0.0000	3.0414	0.0000	0.0000	3.0354	0.0000	0.0000

*PE = Pyroligneous extract
[a] Specific growth rate, min^{-1}
[b] Specific reduction rate, min^{-1}
[c] Division rate, min^{-1}
[d] Halve rate, min^{-1}

4.0 CONCLUSION

The bioactivity reduction of pyroligneous extract as a potential natural-based antimicrobial agent from *Rhizophora apiculata* determined by the time-kill method. In this study, *E. coli* (8739), *K. pneumoniae* (13883), and *C. albicans* (10231) required a shorter minimum duration of killing compared to *P. vulgaris* (33420) and *E. faecalis* (19433). In addition, pyroligneous extract with 16% concentration able achieve to 100% microbial reduction under certain contact time against urinary tract infection relation strains. These in vitro testing proved that pyroligneous extract consider an antimicrobial agent with significant effect. In the future research more target microorganism even beneficial microorganism will be tested to enrich the potential of pyroligneous extract as natural antimicrobial agent.

References

[1] Gilbert, B., Robbins, P., Livornese, Jr. L. L. 2009. Use of Antibacterial Agents in Renal Failure. *Infect. Dis. Clin. North. Am.* 23(4): 899-924 viii.

[2] Wasim, S. E. N., Derrick, S., Mohamad, M., Islam, M. G. 2020. Treatment of Recurrent Urinary Tract Infections in Anuric Hemodialysis Patient, Do We Really Need Antimicrobial Urinary Concentration? ID Cases. 20: e00748.

[3] Hoberman, A., Chao, H. P., Keller, D. M., Hickey, R., Davis, H. W., Ellis, D. 1993. Prevalence of Urinary Tract Infection in Febrile Infants. *J. Pediatr.* 123: 17-23.

[4] Nuutinen, M., Uhari, M. 2001. Recurrence and Follow-up after Urinary Tract Infection Under the Age of 1 Year. *Pediatr. Nephrol.* 16: 69-72.

[5] Copp, H. L., Shapiro, D. J., Hersh, A. L. 2011. National Ambulatory Antibiotic Prescribing Patterns for Pediatric Urinary Tract Infection, 1998-2007. *Pediatrics*. 127: 1027-33.

[6] Millner, R., Becknell, B. 2019. Urinary Tract Infections. *Pediatr. Clin. North Am*. 66: 1-13.

[7] Abdelrhman, A. Z., Mohamed, Y., Tung, P. 2020. Acinetobacter Junii as a Rare Pathogen of Urinary Tract Infection. *Urology Case Reports*. 32: 101209.

[8] Lee, C. C., Lee, C. H., Hong, M. Y., Hsieh, C. C., Tang, H. J., Ko, W. C. 2018. Propensity-matched Analysis of the Impact of Extended-spectrum β-lactamase Production on Adults with Community-onset Escherichia coli, Klebsiella Species, and Proteus Mirabilis Bacteremia. *J. Microbiol. Immunol. Infect*. 51: 519-526.

[9] Jean, S. S., Lee, W. S., Lam, C., Hsu, C. W., Chen, R. J., Hsueh, P. R. 2015. Carbapenemase Producing Gram-Negative Bacteria: Current Epidemics, Antimicrobial Susceptibility and Treatment Options. *Future Microbiol*. 10: 407-25.

[10] Chen, G. J., Pan, S. C., Foo, J., Morel, C., Chen, W. T., Wang, J. T. 2019. Comparing Ceftolozane/ Tazobactam Versus Piperacillin/ Tazobactam as Empiric Therapy for Complicated Urinary Tract Infection in Taiwan: A Cost-utility Model Focusing on Gram-negative Bacteria. *J. Microbiol. Immunol. Infect*. 52: 807-15.

[11] Wennerstrom, M., Hansson, S., Jodal, U., Stokland, E. 2000. Primary and Acquired Renal Scarring in Boys and Girls with Urinary Tract Infection. *J. Pediatr.* 136: 30-4.

[12] Bandow, J. E., Brötz, H., Leichert, L. I. O., Labischinski, H., Hecker, M. 2003. Proteomic Approach to Understanding Antibiotic Action. *Antimicrob. Agents Chemothe*. 47(3): 948-955.

[13] Dadashi, M., Eslami, G., Goudarzi, H., Fallah, F., Dabiri, H., Hashemi, A., Ardeshiri, N., Nasiri, M. J. 2015. Evaluation of Antibacterial Effects of Cinnamon Extract and Essence on Bacteria Isolated from Patients with Urinary Tract Infection. *Int. J. Mole. Clinic. Microbio.* 5 (1): 523-527.

[14] Hertogs, K., de Béthune, M.cP., Miller, V., Ivens, T., Schel, P., Van Cauwenberge, A., Peeters, F. 1998. A Rapid Method for Simultaneous Detection of Phenotypic Resistance to Inhibitors of Protease and Reverse Transcriptase in Recombinant Human Immunodeficiency Virus Type 1 Isolates from Patients Treated with Antiretroviral Drugs. *Antimicrob. Agents Chemothe*. 42(2): 269-276.

[15] Davies, J., Davies, D. 2010. Origins and Evolution of Antibiotic Resistance. *Microbio. Mole. Bio. Rev*. 74(3): 417-433.

[16] Ghimire, B. K., Seong, E. S., Yub, C. Y., Kima, S. H., Chung, I. M. 2017. Evaluation of Phenolic Compounds and Antimicrobial Activities in Transgenic Codonopsis Lanceolata Plants via Overexpression of the γ-tocopherol Methyltransferase (γ-tmt) Gene. *South African Journal of Botany*. 109: 25-33.

[17] Anastasiadi, M., Pratsinis, H., Kletsas, D., Skaltsounis, A. L., Haroutounian, S. A. 2012. Grape Stem Extracts: Polyphenolic Content and Assessment of their In Vitro Antioxidant Properties. *LWT – Food Sci. Technol*. 48: 316-322. https://doi.org/ 10.1016/j.lwt.2012.04.006.

[18] Gouvinhas, I., Pinto, Rosa, Santos, Rafaela, Saavedra, Maria, J., Barros, Ana, I. 2020. Enhanced Phytochemical Composition and Biological Activities of Grape (Vitis vinifera L.) Stems Growing in Low Altitude Regions. *Sci. Hortic*. 265: 109248. https://doi.org/10.1016/j.scienta.2020.109248.

[19] Ramos, S. 2007. Effects of Dietary Flavonoids on Apoptotic Pathways Related to Cancer Chemoprevention. J. Nutr. Biochem. 18: 427-442.

[20] Ahmed, A. T., Mahmoud, A. E., Ahmed, I. I., Shaaban, H. M., 2018. Application of Quercus Infectoria Extract as a Natural Antimicrobial Agent for Chicken Egg Decontamination. *Revista Argentina De Microbiologia*. 50(4): 391-397.

[21] Bang, L. M., Buntting, C., Molan, P. 2003. The Effect of Dilution on the Rate of Hydrogen Peroxide Production in Honey and Its Implications for Wound Healing. *J. Alternative Compl. Med.* 9(2): 267-273.

[22] Lee, S. H., H'ng, P. S., Lee, A. N., Sajap, A. S., Tey, B. T., Salmiah, U. 2010. Production of Pyroligneous Acid from Lignocellulosic Biomass and Their Effectiveness against Biological Attacks. *J. Applied Sci.* 10: 2440-2446.

[23] Loo, A. Y., Jain, K., Darah, I. 2008. Antioxidant Activity of Compounds Isolated from the Pyroligneous Acid, Rhizophora apiculata. *Food Chem*. 107: 1151-1160.

[24] Vitt, S. M., Himelbloom, B. H., Crapo, C. A. 2001. Inhibition of Listeria Inocula and L. Monocytogenes in a Laboratory Medium and Cold-smoked Salmon Containing Liquid Smoke. *J. Food Safety*. 2: 111-125.

[25] Suzuki, T., Doi, S., Yamakawa, M., Yamamoto, K., Watanabe, T., Funaki, M. 1997. Recovery of Wood Preservatives from Wood Pyrolysis Tar by Solvent Extraction. *Holzforschung*. 51: 214-218.

[26] Lee, S. H., H`ng, P. S., Chow, M. J., Sajap, A. S., Tey, B. T., Salmiah, U., Sun, Y. L. 2011. Effectiveness of Pyroligneous Acids from Vapour Released in Charcoal Industry Against Biodegradable Agent under Laboratory Condition. *J. of Applied Sci.* 11 (24): 3848-3853.

[27] Sebestyen, T. T., Carlos A. 2022. Industrial Production of Activated Carbon using Circular Bioeconomy Principles: Case Study from a Romanian Company Grande. *Cleaner Engineering and Technology*. 7: 100443

[28] Ibrahim, D., Kassim, J., Lim, S. H., Rusli, W. 2014. Evaluation of Antibacterial Effects of Rhizophora Apiculata Pyroligneous Acidon Pathogenic Bacteria. *Malays J Microbiol*. 10(3): 197-204.

[29] Wu, Q., Zhang, S., Hou, B. 2015. Study on the Preparation of Wood Vinegar from Biomass Residues by Carbonization Process. *Bioresour Technol.* 179: 98-103.

[30] Grewal, A., Abbey, L., Gunupuru, L. R. 2018. Production, Prospects and Potential Application of Pyroligneous Acid in Agriculture. *J. Anal. Appl. Pyrolysis*. 135: 152-159.

[31] Crepier, J., Le Masle, A., Charon, N., Albrieux, F., Duchene, P., Heinisch, S. 2018. Ultra-high Performance Supercritical Fluid

Chromatography Hyphenated to Atmospheric Pressure Chemical Ionization High Resolution Mass Spectrometry for the Characterization of Fast Pyrolysis Bio-oils. *J. Chromatogr. B*. 1086: 38-46.
[32] Sameshima, K., Sasaki, M., Sameshima, I. 2002. Fundamental Evaluation on Termiticidal Activity of Various Vinegar Liquids from Charcoal Making. *Proceedings of the 4th International Wood Science Symposium, September 2-5, 2002, Serpong, Indonesia*. 134-138.
[33] Yatagai, M., Nishimoto, M., Hori, K., Ohira, T., Shibata, A. 2002. Termiticidal Activity of Wood Vinegar, Its Components and Their Homologues. *J. Wood Sci.* 48: 338-342.
[34] Juliana, L. S. d. S., Victoria, B. d. S. G., Angela, D. C., Rafael, G. L. 2018. Antimicrobial Potential of Pyroligneous Extracts – A Systematic Review and Technological Prospecting. *Brazilian J. of Microbiol.* 49s: 128-139.
[35] Hwang, Y. H., Matsushita, Y. I., Sugamoto, K., Matsui, T. 2005. Antimicrobial Effect of the Wood Vinegar from Cryptomer lajaponica Sapwood on Plant Pathogenic Microorganisms. *J. Microbiol. Biotechnol*. 15(5): 1106-1109.
[36] Bruce, A., Highley, T. L. 1991. Control of Growth of Wood Decay Basidiomycetes by Trichoderma spp. and other Potentially Antagonistic Fungi. *Forest Prod. J*. 41: 63-67.
[37] Nakai, T., Kartal, S.N., Hata, T., Imamura, Y. 2007. Chemical Characterization of Pyrolysis Liquids of Wood-based Composites and Evaluation of Their Bio-efficiency. *Build. Environ.* 42: 1236-1241.
[38] Pfaller, M. A., Sheehan, D. J., Rex, J. H. 2004. Determination of Fungicidal Activities against Yeasts and Molds: Lessons Learned from Bactericidal Testing and the Need for Standardization. *Clin. Microbiol. Rev*. 17: 268-280.
[39] Teo, C. L. 2022. Antimicrobial Study of Pyroligneous Extract from Rhizophora Apiculate against Urinary Tract Pathogens. *Jurnal Teknologi.* 84(1): 49-55
[40] Tendolkar, P. M., Baghdayan, A. S., Shankar, N. 2003. Pathogenic Enterococci New Developments in the 21st Century. *Cell. Mol. Life Sci.* 60: 2622-2636.
[41] Konaté, K., Mavoungou, J. F., Lepengué, A. N. 2012. Antibacterial activity against β-lactamase producing Methicillin and Ampicillin-resistants Staphylococcus aureus: Fractional Inhibitory Concentration Index (FICI) Determination, *Ann. Clin. Microbiol. Antimicrob*. 11: 18.
[42] Shahidi, F., Ho, C. T. 2005. Phenolics in Food and Natural Health Products: An Overview. Phenolic Compounds in Foods and Natural Health Products. *ACS Symposium Series 909, American Chemical Society, Washington, DC*. 1-8.
[43] Garrote, G., Cruz, J. M., Moure, A., Dominguez, H., Parajo, J. C. 2004. Antioxidant Activity of Byproducts from the Hydrolytic Processing of Selected Lignocellulosic Materials. *Trends Food Sci. Technol.* 15: 191-200.
[44] Darah, I., Jain, K., Lim, S. H. Wendy, R. 2013. Efficacy of Pyroligneous Acid from *Rhizophora apiculata* on Pathogenic *Candida albicans. Journal of Applied Pharmaceutical Science*. 3(07): 7-13.
[45] Davidson, P. M., Taylor. T. M. 2007. Chemical Preservatives and Natural Antimicrobial Compounds. Food Microbiology: Fundamentals and Frontiers. 3rd ed. P. M. Doyle and I. R. Beuchat, ed. ASM Press, Washington, DC. 713-745.
[46] Coutinho, H. D. M., Costa, J. G. M., Lima, E. O., Falcao, S. V. S., Siqueira, J. J. P. 2008. Enhancement of the Antibiotic Activity against a Multi-resistant Escherichia coli by Mentha arvensis L. and Chlorpromazine. *Chemotherapy*. 54: 328-330.
[47] Lu, X., Jiang, J., He, J., Sun, K., Sun, Y., 2019. Effect of Pyrolysis Temperature on the Characteristics of Wood Vinegar Derived from Chinese Fir Waste: A Comprehensive Study on Its Growth Regulation Performance and Mechanism. *ACS Omega 4*. 19054-19062.
[48] Hou, X., Qiu, L., Luo, S., Kang, K., Zhu, M., Yao, Y. 2018. Chemical Constituents and Antimicrobial Activity of Wood Vinegars at Different Pyrolysis Temperature Ranges Obtained from *Eucommia ulmoides* Olivers Branches. *RSC Adv*. 8: 40941-40949.
[49] Li, Z., Wu, L., Sun, S., Gao, J., Zhang, H., Zhang, Z., Wang, Z., 2019b. Disinfection and Removal Performance for Escherichia Coli, Toxic Heavy Metals and Arsenic by Wood Vinegar-Modified Zeolite. *Ecotoxicol. Environ. Saf*. 174: 129-136.
[50] Montazeri, N., Oliveira, A. C. M., Himelbloom, B. H., Leigh, M. B., Crapo, C. A. 2013. Chemical Characterization of Commercial Liquid Smoke Products. *Food Sci. Nutr.* 1: 102-115.
[51] Cetin, H., Newman, M. C. 2015. Antimicrobial Efficacy of Plant Phenolic Compounds against *Salmonella* and *Escherichia Coli*. *Food Bioscience*. 11: 8-16.
[52] Brauner, A., Fridman, O., Gefen, O., Balaban, N. Q. 2016. Distinguishing between Resistance, Tolerance and Persistence to Antibiotic Treatment. *Nat. Rev. Microbiol.* 14: 320-330. Doi: 10.1038/nrmicro.2016.34.
[53] Elo, H., Kuure, M., Pelttari, E. 2015. Correlation of the Antimicrobial Activity of Salicylaldehydes with Broadening of the NMR Signal of the Hydroxyl Proton. Possible Involvement of Proton Exchange Processes in the Antimicrobial Activity. *European J. of Medicinal Chemistry*. 92: 750-753.
[54] Stefanovic, O., Radojevic, I., Vasic, S., Comic, L. 2012. Antibacterial Activity of Naturally Occurring Compounds from Selected Plants. In: Bobbarala, V. (Ed.). Antimicrobial Agents. InTech. http://dx.doi.org/10.5772/33059.
[55] Campos, F. M., Couto, J. A., Figueiredo, A. R., Toth, IV, Rangel, A. O. S. S., Hogg, T. A. 2009. Cell Membrane Damage Induced by Phenolic Acids on Wine Lactic Acid Bacteria. *Int. J. Food Microbiol.* 135: 144-51.
[56] Mirzoeva, O. K., Grishanin, R. N., Calder, P. C. 1997. Antimicrobial Action of Propolis and Some of Its Components: the Effects on Growth, Membrane Potential and Motility of Bacteria. *Microb. Research*. 152: 239-246.
[57] Mishra, K., Basu, S., Roychoudhury, S., Kumar, P. 2010. Liver Abscess in Children: An Overview. *World Journal of Pediatrics*. 6: 210-216.
[58] Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., Balaban, N. Q. 2014. Optimization of Lag Time Underlies Antibiotic Tolerance in Evolved Bacterial Populations. *Nature*. 513: 418-421. Doi: 10.1038/nature13469.
[59] Kwon, Y. I., Apostolidis, E., Labbe, R. G., Shetty, K. 2008. Inhibition of Staphylococcus Aureus by Phenolic Phytochemicals of Selected Clonal Herbs Species of Lamiaceae Family and Likely Mode of Action through Proline Oxidation. *Food Biotechnology*. 21: 71-89.
[60] Lacombe, A., Wu, V. C. H., Tyler, S., Edwards, K. 2010. Antimicrobial Action of the American Cranberry Constituents; Phenolics, Anthocyanins, and Organic Acids, against Escherichia coli O157:H7. *International Journal of Food Microbiology*. 139: 102-107.
[61] Hyldgaard, M., Mygind, T., Meyer, R. L. 2012. Essential Oils in Food Preservation: Mode of Action, Synergies, and Interactions with Food Matrix Components. *Frontiers in Microbiol.* 3: 12.
[62] Neu, H. C., Gootz, T. D. 1996. Antimicrobial Chemotherapy. In: Baron S. (eds.). *Medical Microbiology*. Galveston (TX): University of Texas Medical Branch at Galveston.
[63] Gopala-Rao, T. V., Sen, S. K., Samal, A., Satpathy, S. 2010. Nystatin Induced Changes in Growth, Viability and Amino Acid Influx of Yeast *Saccharomyces cerevisiae*. *Int J Chem Res.* 2(1): 8-17.
[64] Chang, Y. C., Tai, K. W., Huang, F. M., Huang, M. F. 2000. Cytotoxic and Nongenotoxic Effects of Phenolic Compounds in Human Pulp Cell Cultures. *J. Endod.* 26: 440-443.
[65] Tsiotou, A.G., Sakorafas, G.H., Anagnostopoulos, G., Bramis, J., 2005. Septic shock; current pathogenetic concepts from a clinical perspective. Medical Science Monitor 11, RA76–RA85.
[66] Kennedy, W.A., Laurier, C., Gautrin, D., Ghezzo, H., Paré, M., Malo, J.L., Contandriopoulos, A.P., 2000. Occurrence and risk factors of oral candidiasis treated with oral antifungals in seniors using inhaled steroids. J. of Clinical Epidemiology., 53, 696–701.
[67] Martins, N., Ferreira, I.C.F.R., Barros, L., Silva, S., Henriques, M., 2014. Candidiasis: predisposing factors, prevention, diagnosis

and alternative treatment. Mycopathologia, 177(5-6), 223–240.
[68] Vazquez, G. D., Perusquia, O.A.M., Hundeiker, M., Bonifaz, A., 2013. Opportunistic yeast infections: candidiasis, cryptococcosis, trichosporonosis and geotrichosis. J. of the German Society of Dermatology., 11(5), 381–395.
[69] Mounyr, B., Moulay, S., Saad, K.I., 2016. Methods for in vitro evaluating antimicrobial activity: A review. J. of Pharmaceutical Analysis., 6, 71–79.
[70] Oramahi, H.A., Yoshimura, T., Diba, F., Setyawati, D., Nurhaida, 2018. Antifungal and antitermitic activities of wood vinegar from oil palm trunk, J. Wood Sci. 64, 311–317.
[71] Ratanapisit, J., Apiraksakul, S., Rerngnarong, A, Chungsiriporn, J., Bunyakarn, C., 2009. Preliminary evaluation of production and characterization of wood vinegar from rubber wood. Songklanakarin J. Sci. Technol., 31, 343–349.
[72] Yang, J.F., Yang, C.H., Liang, M.T., Gao, Z.J., Wu, Y.W., Chuang, L.Y., 2016. Chemical Composition, Antioxidant, and Antibacterial Activity of Wood Vinegar from Litchi chinensis. *Molecules*. 21: 1150.
[73] Vijayarathna, S., Zakaria, Z., Chen, Y., Latha, L.Y., Kanwar, J. R., Sasidharan, S. 2012. The Antimicrobial Efficacy of Elaeis Guineensis: Characterization, In Vitro and In Vivo Studies. Molecules. 17(5): 4860-4877.
[74] Roy, P., Amdekar, S., Kumar, A., Singh, R., Sharma, P., Singh, V. 2012. In Vivo Antioxidative Property, Antimicrobial and Wound Healing Activity of Flower Extracts of Pyrostegia venusta (Ker Gawl) Miers. *J. of Ethnopharmacology*. 140(1): 186-192.
[75] Araujo, M. G. F., Pacífico, M., Vilegas, W. 2013. Evaluation of Syngonanthus nitens (Bong.) Ruhl. Extract as Antifungal and in Treatment of Vulvovaginal Candidiasis. *Medical Mycology*. 51(7): 673-682.
[76] Jothy, S. L., Zakariah, Z., Chen, Y., Sasidharan, S. 2012. In vitro, In Situ and In Vivo Studies on the Anticandidal Activity of Cassia Fistula Seed Extract. *Molecules.* 17(6): 6997-7009.