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ANTIMICROBIAL STUDY OF PYROLIGNEOUS EXTRACT FROM *RHIZOPHORA APICULATE* AGAINST URINARY TRACT PATHOGENS

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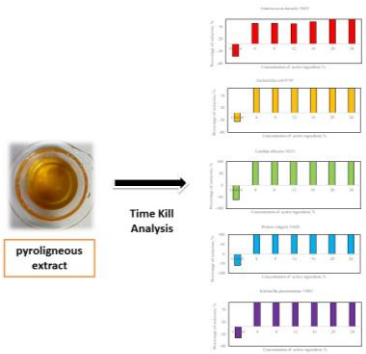
Graphical abstract



Abstract

Various parts of the human body are host to certain microorganism species, such as fungi and bacteria which may cause urinary tract infections (UTIs). In this research, the pyroligneous extract as antimicrobial agent on urinary tract infection-related pathogenic microorganism were studied. The Time-kill kinetics assay was used to study an antimicrobial agent over time. A series of various concentration (0 – 24%) of the pyroligneous extract was tested. Each strain's bioactivity was quantified after 60 minutes of incubation at 22.5 °C. From the result showed that pyroligneous extract reached 100% of reduction effects with different concentration (*E.faecalis* (19433) at 20%, *E.coli* (8739) at 4%, *P.vulgaris* (33420) at 4%), *K.pneumoniae* (13883) at 4%, *C.albicans* (10231) at 4%). For bioprocess kinetics analysis, the highest specific reduction rate and halve rate according concentration: *E.faecalis* at 24% (0.1426, 0.2058), *E.coli* at 4% (0.1219, 0.1759), *P.vulgaris* at 4% (0.1311, 0.1891), *K.pneumoniae* at 4% (0.1175, 0.1695), *C.albicans* at 4% (0.1175, 0.1695).These findings shows the importance of pyroligneous extract as antimicrobial agent, especially in urinary tract infection, as a natural antimicrobial agent in public health industries.

Keywords: Pyroligneous extract, Concentration, Optimization, Time-kill methods, Antibacterial agent

Abstrak

Pelbagai bahagian tubuh manusia adalah tuan rumah kepada spesis mikroorganisma tertentu, seperti kulat dan bakteria yang boleh mengakibatkan jangkitan saluran kencing (UTIs). Dalam penyelidikan ini, ekstrak piroligneous sebagai ejen antimikrob terhadap mikroorganisma yang berkaitan jangkitan saluran kencing telah dikaji. Ujian kinetik Membunuh-masa digunakan untuk mengkaji ejen antimikroba dari masa ke masa. Satu siri kepekatan berlainan (0 - 24%) ekstrak piroligneous telah diuji. Setiap bioaktiviti mikroorganisma dihitung setelah 60 minit inkubasi pada suhu 22.5 °C. Daripada hasil kajian menunjukkan bahawa ekstrak piroligneous mencapai 100% kesan pengurangan dengan kepekatan yang berbeza (*E.faecalis* (19433) pada 20%, *E.coli* (8739) pada 4%, *P.vulgaris* (33420) pada 4%), *K. pneumoniae* (13883) pada 4%, *C.albicans* (10231) pada 4%). Dalam analisis kinetik bioproses, kadar pengurangan spesifik tertinggi dan kadar separuh mengikut kepekatan: *E.faecalis* pada 24% (0.1426, 0.2058), *E.coli* pada 4% (0.1219, 0.1759), *P.vulgaris* pada 4% (0.1311, 0.1891), *K.pneumoniae* pada 4% (0.1175, 0.1695), *C.albicans* pada 4% (0.1175, 0.1695). Penemuan ini menunjukkan pentingnya ekstrak piroligneous sebagai ejen antimikroba, terutamanya dalam jangkitan saluran kencing, ini sebagai agen antimikrobial semula jadi terhadap industri kesihatan awam.

Kata kunci: Ekstrak piroligneous, Kepekatan, Pengoptimuman, Kaedah membunuh-masa, Ejen antibakteria

1.0 INTRODUCTION

Among the widespread infection diseases that infect adults are urinary tract infections (UTIs), around 150 million patients in the world each year facing this problem, and 10.5 million outpatient visits in the United States [1]. According to a study carried out in the United States, 12% of young women face a minimum urinary tract infection problem by the age of 32, and around 50 – 70% of women have a history of urinary tract infection [2]. Another survey of female college student stated that a rate of around 0.7 episodes of cystitis per person per 12 months and twenty-five percent of respondents having recurrent urinary tract infections [3]. In Russia, around twenty percent of women have at least 1 episode of cystitis by the age of eighteen to twenty years old, and the number of urinary tract infections that happen with age increased [4]. In Rafalskiy and Moiseeva (2018) survey, one thousand one hundred and eighty-five Russian college students in 2005 and 2017 discovered that nineteen to twenty-one percent of the students reported an episode of dysuria in their lifetime, and 22.9 – 28.5% of student faced recurrent infections [5].

In some studies 70 – 95% of urinary tract infections are caused by *Escherichia coli*, and 5 -20% are infected by *Klebsiella pneumoniae*. Patient are infected by *Proteus mirabilis*, other bacteria of the Enterobacteriaceae family [6] and *Pseudomonas aeruginosa* [7], which all microorganism are gram-negative. Urinary tract infections infected by gram-positive bacteria are considered less compared to gram-negative strains, mainly caused by *S. saprophyticus*, *Enterococcus spp.* and group B *Streptococcus* (GBS) [6]. The genitourinary tract's functional and structural abnormalities are relevant for infection, including urinary catheterization, caused by urinary tract infections (UTIs) [8].

Unfortunately, many of these microbes are antibiotics resistant due to prolonged stays in hospital or critical care units, and form a serious threat to public health, caused highlighting the immediate requirement for novel or alternative treatments [9, 10]. Urinary tract infections (UTIs) also consider serious infections which require antibiotics treatment and cost more than 6 billion dollars [11].

Even though several antibiotics such as amino-penicillins are used clinically to treat urinary tract infections (UTIs) [12] especially to *Escherichia coli*, high resistance issues become a challenging and multi-drug-resistant microbe caused urinary tract infections (UTIs)'s prevalence is globally increasing [13]. Thus, discovering of new antimicrobial agents for urinary tract infections with cost-effective and green chemical is a critical challenge for public health and the scientific community.

The pyroligneous extract is synthesized from gas condensation during charcoal production [14]. The pyroligneous extract is famous for its properties of organoleptic. It is content (10 – 20%) of a complex mixture of water and many organic compounds. For example formic acid, catechols, guaiacols, syringols, methanol, acetone, vanillin, isoeugenol, furan carboxaldehydes, ketones, pyrone, esters, and more than two hundred organic compounds with phenolic compounds, which are pyrolytic produced of hemicelluloses and lignin [15].

In several scientific studies stated that pyroligneous acid tested as antibacterial agent [16], antioxidant agent [17, 18], efficient antifungal agent [19, 20], and anti-pathogenic bacteria agent [21].

However, in recent studies, the antimicrobial activities of pyroligneous extract against urinary tract infections related strains have not been investigated further. Therefore in this research, the variety of urinary tract infection-related strains (*Candida albicans* (10231), *Enterococcus faecalis* (19433), *Escherichia coli* (8739), *Klebsiella pneumoniae* (13883) and *Proteus vulgaris* (33420)) and different concentration of pyroligneous extract (0 – 24%) as antimicrobial agent were investigated. The antimicrobial effects of the pyroligneous extract on the urinary tract infection-related strains' percentage of reduction and its bioprocess kinetics were investigated.

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 – 24 hours. Yeast *Candida albicans* (ATCC10231) had been cultured on appropriate agar (Nutrient agar CM0003B) till it was ripe and fruiting (44 – 52 hours) at 25ºC. The spore collected had been 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 by scraping the fruity culture. Each test microorganisms were prepared in a different universal bottle. 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 the sample in it. The volume of the suspension inoculums used was 0.1% from the volume of the sample used. The concentration of inoculum after inoculation is between $1.0x10^3$ – $1.0x10^4$ CFU/mL. The initial concentration of viable microorganisms in the test preparation was determined by plate count method at 0 minutes (as initial value). The inoculated sample with pyroligneous extract was incubated at 22.5ºC until the end of contact time (60 minutes). The

concentration of the pyroligneous extract from *Rhizophora apiculate* used were 0% (as control), 4%, 8%, 12%, 16%, 20%, and 24%.

2.3 Microbial Activity Analysis

2.3.1 Mean Number of Cells, Mean Log, 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 the end of contact time (0 and 60 minutes) with a variety of extract concentrations (0, 4, 8, 12, 16, 20, 24%). Each experiment was performed in duplicates to ensure reproducibility of results. Then, microbial strains' bioactivity and its bioprocess kinetics were calculated with the Equation 1-7:

$Mean\ number\ of\ cell\ (m), CFU/g = \frac{Sample\ 1 + Sample\ 2}{2}$	(1)
$Mean\ \log = log_{10} m$	(2)
$Percentage\ of\ reduction, \% = \frac{Intial\ value - Sample\ value}{Initial\ value}\ x\ 100\%$	(3)
$\log growth = log_{10} sample\ value - log_{10} initial\ value$	(4)
$\log reduction = log_{10} initial\ value - log_{10} sample\ value$	(5)
$Specific\ growth\ rate\ (u) = \frac{\ln (N_2 - N_1)}{(t_2 - t_1)}$	(6)
$Specific\ reduction\ rate\ (v) = \frac{\ln (N_1 - N_2)}{(t_2 - t_1)}$	(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 sample, evaluated according to the Equation 8 either 9.

$K_1 = \frac{u}{0.693}$	(8)
$K_2 = \frac{v}{0.693}$	(9)

3.0 RESULTS AND DISCUSSION

3.1 Effect of Antimicrobial Agent with Different Pyroligneous Extract Concentration

Anti-enterococcus activity of pyroligneous extract with various concentration with 60 minutes contact time presented in Figure 1. The pyroligneous extract showed anti-enterococcus activity increasing with the increasing concentration from 4 to 24%. The control showed the negative percentage of reduction (-50%) and it proved that this testing's strain test is healthy to be valid used in the antimicrobial analysis. Pyroligneous extract showed strong anti-enterococcus 82-83% of reduction at 4-12% concentration, 91% of reduction at 16% concentration and reached 100% of reduction at 20-24% concentration. These results are similar to the findings by previous researchers; Harada et. al. (2013) stated that pyroligneous extract used as antimicrobial testing from bamboo (*Phyllostachys pubescens*) to *Enterococcus faecalis* ATCC29212 [22]. Nevertheless, the detailed study on anti-enterococcus activity of the *Rhizophora apiculata* of pyroligneous extract is none. This is, to the best our knowledge, the first report of anti-enterococcus activity (on urinary tract infection related strain – *Enterococcus faecalis* 19433) of *Rhizophora apiculate* pyroligneous extract.

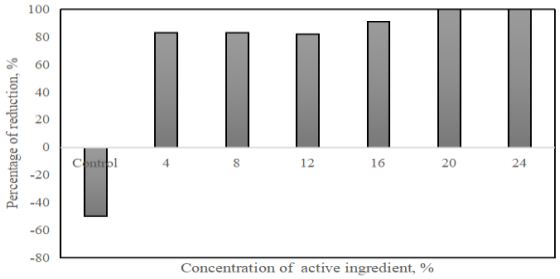


Figure 1 Percentage of reduction of *Enterococcus faecalis* 19433 with variety pyroligneous extract concentration

Antibacterial activity of pyroligneous extract with various concentration with 60 minutes contact time shown in Figure 2. The pyroligneous extract proved antibacterial activity, with the increasing concentration from 4 - 24%. The control is showed the negative percentage of reduction (-35%) confirmed that the strain used in this testing is active enough to be used in the time-kill analysis. The pyroligneous extract showed super-strong antibacterial with 100% of reduction at 4 - 24% concentration. Thus, pyroligneous extract may serve as potential antibacterial and antiseptic agents and many reports revealed similar findings [23, 24]. Current research proves that charcoal extract has strong antibacterial activity, which is one of the reasons charcoal extract is widely used in traditional oriental medicine [25, 26].

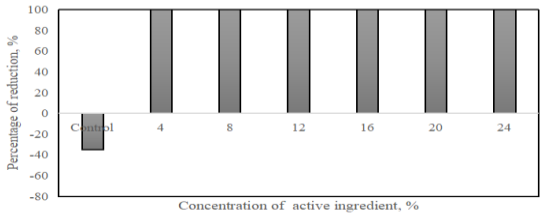


Figure 2 Percentage of reduction of *Escherichia coli* 8739 with variety pyroligneous extract concentration

The anti-proteus activity of pyroligneous extract with various concentration with 60 minutes contact time presented in Figure 3. The pyroligneous extract indicated anti-proteus activity, with the concentration increasing from 4 to 24%. The control is

showed negative percentage of reduction (-59%) and proved that the strain chose in this testing is healthy enough to be used in the antimicrobial analysis. The pyroligneous extract showed anti-proteus with 100% of reduction at 4 - 24% concentration. Pyroligneous extract contains phytochemical constituents such as flavonoids [27] and is known to prevent gastric ulcer due to the astringent and antimicrobial effect, which appear to be responsible for gastro-protective bioactivity by Okolo *et al*. (2012) [28].

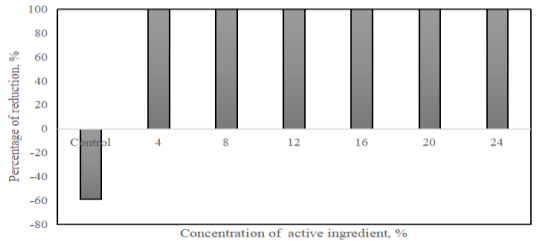


Figure 3 Percentage of reduction of *Proteus vulgaris* 33420 with variety pyroligneous extract concentration

The anti-klebsiella activity of pyroligneous extract with various concentration with 60 minutes contact time is presented in Figure 4. The pyroligneous extract showed anti-klebsiella activity with the increasing concentration from 4 to 24%, similar to Figures 2 and 3. The control is showed the negative percentage of reduction (-45%) proved that the strain selected in this testing is active enough to be used in the time-kill analysis. The pyroligneous extract showed high potential strong anti-klebsiella with 100% reduction at 4 - 24% concentration.

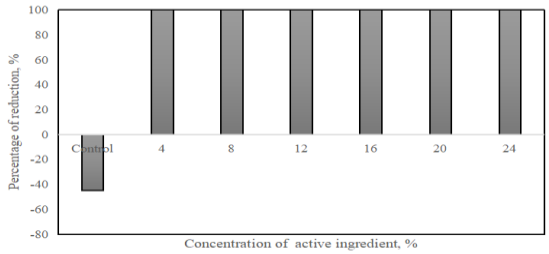


Figure 4 Percentage of reduction of *Klebsiellla pneunmoniae* 13883 with variety pyroligneous extract concentration

The anticandidal activity of pyroligneous extract with various concentration with 60 minutes contact time is shown in Figure 5. The pyroligneous extract indicated anticandidal activity, with the increasing concentration from 4 to 24%. The control showed negative percentage of reduction (-61%) proved that this testing's strain test is healthy enough to be used in the antimicrobial analysis. The pyroligneous extract showed efficient strong anticandidal activity with 100% of reduction at 4 - 24% concentration. A similar discovery also proved that pyroligneous extract becomes a useful anticandidal agent [27]. Many antifungal agents, including anticandidal can treat systemic and superficial candidiasis [29]. The development of drug-resistant microorganism and dose-limiting toxic effects has hindered antifungal treatment. Many researchers have to look for alternative compounds with anticandidal activity [30, 31, 32].

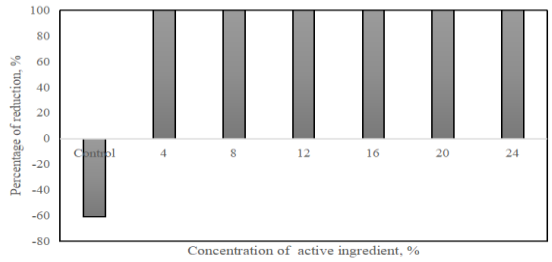


Figure 5 Percentage of reduction of *Candida albicans* 10231 with variety pyroligneous extract concentration

3.2 Antimicrobial Bioprocess Kinetics Analysis

Table 1 summarizes the bioprocess kinetics of the 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 a promising reduction and halve rate to each microbe strains: *E. faecalis* 19433, *E. coli* 8739, *P. vulgaris* 33420, *K. pneumoniae* 13883 and *C. albicans* 10231 respectively. The highest log reduction (3.7160) for *E. faecalis* 19433 was achieved under 24% pyroligneous extract concentration condition with the highest specific reduction rate of 0.1426 min^{-1} and highest halve rate 0.2058 min^{-1}. While the highest log reduction for *E. coli* 8739 (3.176), *P. vulgaris* 33420 (3.415), *K. pneumoniae* 13883 (3.061) and *C. albicans* 10231 (3.061) were achieved under 4% pyroligneous extract concentration condition with highest specific reduction rate of 0.1219 min^{-1}, 0.1311 min^{-1}, 0.1175 min^{-1} and 0.1175 min^{-1} and highest halve rate 0.1759 min^{-1}, 0.1891 min^{-1}, 0.1695 min^{-1} and 0.1695 min^{-1} respectively. These showed as *E. faecalis* 19433 required higher concentration than other strains. The succession of any plant extract's antimicrobial activity depends on the antimicrobial agent's ability to penetrate the cell wall [27]. There are unique interactions between the bioactive molecule and the strains of cell wall compartments [33]. This interaction with the constituents' target microbial site will either aid or hinder the bioactive compounds' penetration into the strains. Some research stated that the harmful effect of the extract on the fungus's microbial wall could be the key reason for the reduction in yeast budding rate, because the microbial wall is necessary for cell division [34].

Table 1 Antimicrobial agent bioprocess kinetics according urinary tract infection related strains with different extract concentration

	***Enterococcus faecalis* 19433**			***Escherichia coli* 8739**			***Proteus vulgaris* 33420**			***Klebsiellla pneunmoniae* 13883**			***Candida albicans* 10231**		
Concentration, (%)	**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	0.1773	0.1337	0.1930	0.1288	0.1098	0.1584	0.2016	0.1195	0.1724	0.1606	0.1124	0.1622	0.2068	0.1188	0.1715
	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]**
4	0.7819	0.1413	0.2039	3.176	0.1219	0.1759	3.415	0.1311	0.1891	3.061	0.1175	0.1695	3.061	0.1175	0.1695
8	0.7597	0.1411	0.2036	3.161	0.1213	0.1751	3.407	0.1307	0.1886	3.000	0.1151	0.1661	3.061	0.1175	0.1695
12	0.7473	0.1378	0.1989	3.041	0.1167	0.1684	3.380	0.1297	0.1872	3.041	0.1167	0.1684	2.998	0.1150	0.1660
16	1.0654	0.1392	0.2009	3.079	0.1182	0.1705	3.398	0.1304	0.1882	3.041	0.1167	0.1684	3.035	0.1165	0.1681
20	3.6532	0.1402	0.2023	3.021	0.1159	0.1673	3.371	0.1294	0.1867	3.041	0.1167	0.1684	2.991	0.1148	0.1656
24	3.7160	0.1426	0.2058	3.000	0.1151	0.1661	3.342	0.1283	0.1851	3.000	0.1151	0.1661	2.957	0.1135	0.1637

[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 current study conclusively demonstrates the potential of pyroligneous acid from *Rhizophora apiculate* as antimicrobial agent, it is a great alternative natural source of green compounds with promising antimicrobial activity to urinary tract infection-related microorganisms (*E. faecalis* 19433, *E.coli* 8739, *P. vulgaris* 33420, *K. pneumoniae* 13883 and *C. albicans* 10231) that could be a novel resource in the development of public health and wellness products sectors.

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