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Synergy effect of legal highs with antibiotics

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Legal High Plants와 항생제의 항균활성 비교

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Abstract : In this study would like to find extending or increasing the efficacy of the antibiotic substance for the strains with resistance to antibiotics or persister cells by inhibition of the resistance. This study was used different species of 'legal high' plants leaves from *Leonotis leonurus, Mitragyna speciosa*, and seeds from *Ipomoea murucoides* with antibiotics which are Amoxicillin, Chloramphenicol, Ciprofloxacin, Kanamycin, Oxacillin, and Vancomycin. Legal highs were extracted with methanol. Minimum inhibitory concentration(MIC) testing for a range of antibiotics with extracts of plant was fulfilled by broth dilution methods. In this essay, it was determined in a microdilution assay utilizing suspended in ISB up to a final concentration of 512μg/ml in 96 wells microtitre plates, threefold and serial dilutions. After that, the microplates were kept in incubator between 35° C and 37° C for overnight. *Leonotis leonurus, Mitragyna speciosa*, and *Ipomoea murucoides* of Legal highs (512μg/ml) investigated small activity to inhibit against pathogens which are susceptible *Staphylococcus aureus*, resistant *Staphylococcus aureus*, susceptible *Enterococcus faecalis*, resistant *Escherichia coli*.

Keywords : Active Research, Legal Highs, antibacterial, multidrug resistant, antibiotic

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1. Introduction

Hundreds of years ago, average life expectancy was only 20 to 30 years old because of diseases such as smallpox, pneumonia, cholera, and so forth which were related to bacteria in children and adults (Kaplan et al., 2000). Scientists have been persevering efforts to save humanity from diseases caused by microorganisms for a long time and antibiotics have played an important role for humanity among a diverse group of medicines since their discovery (Montañez-Izquierdo, 2012).

Salvarsan was developed by Paul Ehrlich in Germany in 1910. It was the first chemotherapeutic agent in the world and was synthesized by the 606th experiment. The drug was used in syphilis treatment because of the major side effect (Lloyd et al., 2005). The first natural antibiotic compound was penicillin which was discovered accidently by Scottish scientist and Nobel laureate Alexander Fleming in 1928 (Allen et al., 2007). Penicillin is a substance to inhibit bacterial growth by fungus which belonging to Penicillium notatum. According to Quirke (2001), penicillin was difficult to purify and produce so that it could not be used in the early 1950's commercially. In the years following their initial discoveries and availability for clinical utilization, antibiotic use rose exponentially and several new classes of both man-made and naturally occurring antibiotics were discovered (Lesche, 2007). The quest of finding new drugs introduced new antibiotics into the pipeline and was followed by the introduction of streptomycin, tetracycline, chloramphenicol and macrolides in the 1950's and later by trimethoprim and quinolones (Islam, 2008). For some decades after their introduction, antibiotics seemed to have solved the problem of bacterial infectious diseases (Davies, 2010). Scientists have been persevering efforts to save humanity from diseases caused by microorganisms even now. There are major examples of developed antibiotics with group of antibiotics from first discovery to recent year that standard years were onto the pharmaceutical market year(Table 1).

Table 1. Timeline of antimicrobial classes

Year	*Antibiotics*	*Group*	*Year*	*Antibiotics*	*Group*
1910	Salvarsan	Chemotherapy (From arsenical)	1961	Trimethoprim	Chemotherapy (Dihydrofolate reductase inhibitor)
1935	Prontosil	Sulfonamide	1964	Cefalotin	Cephalosporin
1942	Benzylpenicillin	Penicillin	1967	Nalidixic acid	Quinolone
1942	Gramicidin S	Peptide antibiotic	1968	Clindamycin	Lincosamide
1944	Streptomycin	Aminoglycoside	1972	Amoxicillin	Aminophenicillin
1948	Chlortetracycline	Tetracycline	1985	Imipenem	Carbapenem
1949	Chloramphenicol	Amphenicol	1987	Ciprofloxacin	2^{nd}– gen Fluoroquinolone
1952	Erythromycin	Macrolide	1987	Rifaximin	Ansamycin
1955	Vancomycin	Glycopeptide	2000	Linesolid	Oxazolidinone
1958	Colistin	Polymyxin	2001	Telithromycin	Ketolide
1960	Metronidazole	Nitroimidazole	2009	Telavancin	Lipoglycopeptide

* This timeline is not development year for them. It indicates release year when a given drug was onto the pharmaceutical market (http://en.wikipedia.org/wiki/Timeline_of_antibiotics).

This paper was performed to find extending or increasing the efficacy of the antibiotic substance for the strains with resistance to antibiotics or persister cells by inhibition of the resistance. Therefore, firstly, to examine the extracts of Leonotis leonurus, which has reported to suppress effect to bacteria. Secondly, to examine synergistic effect on combining Leonotis leonurus, Mitragyna Speciosa, and Ipomoea murucoides of extracts with antibiotics.

2. Background

2.1. Definition of Antibiotics

Originally, an antibiotic was a substance produced by a microorganism that selectively inhibits the growth or life of another microorganism. However, some of the antibiotics synthesized without being derived from microorganisms, are semi-synthetic drugs which make some part of existing structure of the antibiotic change, and are an artificially synthesized microbial drug but it is detected by microorganism at first. These kinds of medicines can be called 'antimicrobial agent' rather than 'antibiotic' which is a more accurate representation. In addition, this word is not appropriate to antibiotics which act on the microorganisms such as fungi, mold, or viral agents except bacteria. If it includes these kinds of drugs, the medicines can be called antimicrobial agents for a more exact expression(NHS, 2020).

2.2. Antibiotic Resistance

Over the last few years, antibiotics seemed to be overcoming the battle in comparison with infectious disease. Wish for this reason, antibiotics can save the lives of many patients and wounded in the battlefield. Antibiotics in feed could promote not only development but also growth of livestock and fish. However, in spite of the successful development of various classes of antibiotic, there have been followed almost always many kinds and strains of bacteria which resist to antibiotics (Sjölund, 2004). The problems have been increased in health crisis widely nowadays. Although antibiotic resistant bacteria started to appear soon after the clinical introduction of antibiotics, the problem was limited and was at first dismissed as of little concern. The leading causes to the emergence and spread of antibiotic resistance include absence of regulation in the proper use of antibiotics, transmission of antibiotic resistance genes in the community through normal microflora, improper disposal of antibiotics used in animals and agriculture. Obtaining a resistant antibiotic to bacteria, it will be a desperate effort to survive from exposed to antibiotics (WHO, 2020). One of the salient features of the organism, it can be variation to survive and adapt to the environment. Bacteria can adapt to the environment especially very fast compared to other organisms because they have ability of gene mutation which makes useless antibiotics. Mankind has been against the resistance of bacteria to continue to develop new antibiotics such as methicillin, vancomycin, and so on. If new antibiotic take to develop at least 10 years, it will be obtained soon by fast antibiotic resistance traits. Therefore, it is the largest expectation to overcome the resistance in the next generation. There are two ways to get antibiotic resistance from bacteria. One of the theories is selective pressure which is obtained by method of adaptable mutants resistance or spontaneous mutation(France el. al, 2019). The other theory is horizontal gene transfer method that resistance is caused by cross-resistance or gene transfer. At this time, the genetic material in the transfer of antibiotic resistance is primarily a plasmid and resistant bacteria of various kinds of antibiotics are emergence at the same time.

2.3. Legal Highs

Legal highs are well known as herbal highs,

herbal dietary supplements, or part pills from natural sources and over 450 substances in Europe(Bježančević et al., 2019). These can include fungal materials or synthetic chemicals compounds of recreational drugs such as the cocaine, opium, or amphetamine classes which are made in the form of powders or tablets. 'Legal highs' also can purchase easily from retail stores or online shopping mall legally (Arunotayanun & Gibbons. 2012). Many types of antibiotics are developing and using to inhibit or to kill bacteria in these days. Among them, excellent physiological activity of medicinal plants has increased in interest and studies because these can be easily recognized by consumer as natural products (Chen et al., 2003). Medicinal plants mean as urpflanze of herbal plants used for disease. These may be included wood plants, fungi such as mushrooms, and bacteria which produce antibiotics and other drugs as well. Medicinal plants can be classified even edible plants and poisonous plants which are used as drugs (Choi, 1998). They have been used for prevention and treatment of disease in the West and East for a long time already (Yang et al., 2004). The precise mechanism of action is not known until now because existing bioactive substances in plants are diverse and complicated. However, they are widely well known various bioactivity effects as antimicrobial, antioxidant, anticancer, and so on (Windisch et al., 2008). Moreover, they can be affected on the physiology and metabolism of multiple factors in plant such as inhibition of pathogens growth, improvement of immunity function, or secretion acceleration of digestive enzymes (Guo et al., 2004). It provides unlimited opportunities for the development of new drugs such as plant extracts, either pure compounds or standard extract because of excellent chemical diversity supplements.

3. Materials and Methods

3.1. Extract of Plants

Leonotis leonurus is well known as lion's tail or wild dagga and the plant can find Eastern, Wester Cape, and South Africa commonly (Oyedemi et al., 2010). It is also as known for psychoactive, anti-inflammatory, and dysentery medicines (Wu et al., 2012). The family of L. leonurus is Lamiaceae which consist of flavonoid, tannins, alkaloids, saponins, and lactones (Jimoh et al., 2010). These penolic compounds can link with biological activity (Steenkamp et al., 2004). As known as, Mitragyna speciosa is called "Biak", Ketum", and "Kratom" as tropical plant indigenous. This plant has been prescribed as a useful ingredient to treat of opiate addiction as an alternative medicine (Parthasarathy et al., 2009). Ipomoea murucoides is the morning glory family and distributed worldwide in tropical area (Pereda-Miranda et al., 2010). According to Hooton et al (1984), combining antibiotics with plants will be much more successful synergy effect of therapeutic result than antibiotics only to treat serious bacterial infection.

The materials were powdered and weighed following that an amount of solvent to submerge the plant material by some hours in ultrasonication (Table 2). Solvents were η

Table 2. Dry weight and extract solvents

Family	Species	Common name	Part
Lamiaceae	Leonotis leonurus	Lion's tail & Wild dagga	Leaf
Rubiaceae	Mitragyna speciosa	Kratom, Biak, Ketum	Leaf
Convolvulaceae	Ipomoea murucoides	Morning glory	Seed

-hexane(HEX). The eluent was filtered using filter paper. After this time, the legal highs extract concentrated in a rotary vacuum evaporator at 40℃ and the solvent was completely removed by enriching decompression.

3.2. Antibiotic Agents

The antimicrobial agents used in this study were obtained from suppliers as listed in Table 3.

3.3. Bacterial and Isolates

1 bacteria (Table 4) were isolated and collected for Gram-negative and Gram-positive to grow on the agar plate which was autoclaved nutrient agar (3.5g), 200ml distilled water, and 4 capsules of LB BROTH, LENNOX at 37℃ for overnight in incubator at the laboratory in school of pharmacy (SOP). Bacteria were inoculated using a loop added to a 2ml phosphate buffered saline (PBS) solution and to standardise the inoculum it. The calculated quantity bacteria were diluted in Iso-SensitestTM Broth (ISB) was read the absorbance.

3.4. Minimum Inhibitory Concentration (MIC) testing

The minimum inhibitory concentration (MIC) inspects visible growth of microorganism for production possibilities in microplate wells including dilutions of the antimicrobial agents after overnight at 37℃ of incubation (Andrews, 2006). MIC testing for a range of antibiotics was performed by the broth dilution method which is modern high throughput screening methods. It was performed between 7.2 and 7.4pH because aminoglycosides activity can be affected by pH.

MIC testing was determined in a microdilution assay utilizing an inoculum of 50μl of prepared each strain, suspended in ISB up to a final concentration of 128μg/ml in 96 wells microliter plates, twofold and serial dilutions using multichannel pipettes. After that, the microplates were kept in incubator at 37℃ for overnight.

The extracts were assessed by using the microdilution method with a 96 wells microlitre plate (Eloff, 1988). For this test, the solutions were prepared from extracts in a

Table 3. Antibiotic agents, their abbreviations and sources

Antibiotic	Abbreviation	Source	Solution
Amoxicillin	AMX	Sigma-Aldrich, Poole, UK	Sodium hydrogen -carbonate
Chloramphenicol	CHP	Sigma-Aldrich, Poole, UK	Ethanol
Ciprofloxacin	CIF	Bayer AG, Leverkusen, Germany	Sterile distilled water
Kanamycin	KAN	Sigma-Aldrich, Poole, UK	Sterile distilled water
Oxacillin	OXA	Sigma-Aldrich, Poole, UK	Sterile distilled water
Vancomycin	VAN	Sigma-Aldrich, Poole, UK	Sterile distilled water

Table 4. Bacteria species

Bacteria	Number	Type	Gram
Escherichia coli	NCTC 10418	Susceptibility	-
Escherichia coli	Clinical isolated	Resistance	-
Staphylococcus aureus	12961	Susceptibility	+
Staphylococcus aureus	13373	Resistance	+

concentration dissolved in DMSO (dimethyl-sulfoxide) with an equal volume of bacterial suspensions (50μl) approximately. These were diluted with Iso-SensitestTM Broth (ISB) with a concentration range 512μg/ml-0μg/ml. ISB was autoclaved adding 4.7g of ISB in 200ml of distilled water. After that, the microplates were kept in incubator at 37℃ for overnight. Every experiment was repeated at least three times.

4. Results and Discussion

4.1. Antibiotic Activities of Antibiotics

The antibiotic activity of antibiotics (128μg/ml) which are amoxicillin (AMX), chloramphenicol (CHP), ciprofloxacin (CIF), kanamycin (KNA), oxcacillin (OXA), and vancomycin (VAN) of MICs showed no antimicrobial activity against susceptible S. aureus, and resistant S. aureus, susceptible E. coli, and resistant E. coli. The MICs are summarized in the Table 5.

4.2. Antibiotic Activities of Legal Highs

The antibiotic activity methanol extracts of 512μg/ml from L. leonurus, I. murucoides, M. speciosa showed antimicrobial activity against susceptible S. aureus, resistant S. aureus, susceptible E. coli. but there was no activity to inhibit resistant E. coli.

Some studies show that L. leonurus methanol extract (4000μg/ml) of MIC was inhibited the growth all bacteria, Gram negative and Gram positive; E. coli, S. aureus (Steenkampa et al., 2004). Jimoh et al. (2010) showed that the antibacterial activity of Acetone extract were affected to inhibit E. coli at 1000μg/ml of MIC and S. aureus at 2500 μg/ml. Thus, Methanol extract of L. leonurus were active against E. coli (1000 μg/ml) and S.aureus (5000 μg/ml). Ethanol extract from L. leonurus (1560μg/ml) with 100μg/ml of neomycin were inhibited against S. aureus, B. subtilis, E. coli (Stafford et al. 2005). Moreover, one of the studies showed that MeOH extract (6250μg/ml) and Alkaloid extract (3120μg/ml) from M. speciosa with 30μg/ml of CHP showed antimicrobial activity against Bacillus subtilis and Salmonella typhi (Parthasarathy et al., 2009). MeOH and CHL extract (25μg/ml) from I. murucoides with norfloxacin 128μg/ml showed antibacterial activity against resistant S. aureus. This study was mixed norfloxacin 128μg/ml, MeOH extract, and CHL extract with Kanamycin,

Table 5. Inhibition of antibiotics

antibiotics (128μg/ml)	Susceptible S. aureus	Resistant S. aureus	Susceptible E. coli	Resistant E. coli
Amoxicillin	0.25	8	4	〉128
Chloramphenicol	4	4	4	〉128
Ciprofloxacin	2	1	0.125	〉128
Kanamycin	2	32	1	〉128
Oxacillin	0.125	0.5	〉128	〉128
Vancomycin	1	0.5	〉128	〉128

Table 6. Inhibition of L. leonurus extracts

Extract from Legal Highs(512μg/ml)	Susceptible S. aureus	Resistant S. aureus	Susceptible E. coli	Resistant E. coli
L. leonurus HEX	64	128	256	〉512
I. murucoides HEX	64	256	128	〉512
M. speciosa HEX	16	32	128	〉512

Chloramphenicol against E. coli so that these are antimicrobial active against E.coli (Corona-Castañeda at al., 2013).

4.3. Mitragyna speciosa, Ipomoea murucoides with antibiotics

The antibiotic activity of η-Hexane extracts of 128μg/ml from L. leonurus, I. murucoides and M. speciosa with antibiotic agents which are AMX, CHP, CIF, KNA, OXA, and VAN showed antimicrobial activity against susceptible S. aureus, and resistant S. aureus, susceptible E. coli, and resistant E. coli. The MIC result of L. leonurus extracts with antibiotic summarized in Table 7.

5. Conclusions

In terms of the MIC results of combining six antibiotics with CHL extract from I. murucoides, the best inhibitor against susceptible S. aureus was 1μg/ml of AMX and OXA, resistant S. aureus was 1μg/ml of OXA, and susceptible E. coli was 1 μg/ml of CIF. In terms of MeOH extract from I. murucoides with antibiotics, there are indicated that the best growth inhibition was demonstrated susceptible S. aureus was 1μg/ml of AMX and OXA, resistant S. aureus was 2μg/ml of VAN, and susceptible E. coli was 0.25μg/ml of CIF. In part of MeOH extract from M. speciosa with six antibiotics, the result of minimum

Table 7. MIC result of Mitragyna speciosa, Ipomoea murucoides with antibiotics

Antibiotics (128μg/ml) with Extract from legal highs (512μg/ml)	Susceptible S. aureus	Resistant S. aureus	Susceptible E. coli	Resistant E. coli
AMX	0.25	8	4	〉128
L. leonurus HEX	2	16	8	〉128
I. murucoides HEX	1	32	16	〉128
M. speciosa HEX	2	16	1	〉128
CHP	4	4	4	〉128
L. leonurus HEX	64	8	8	〉128
I. murucoides HEX	8	8	8	〉128
M. speciosa HEX	4	4	4	≥128
CIF	2	1	0.125	〉128
L. leonurus HEX	2	8	1	〉128
I. murucoides HEX	4	4	0.25	〉128
M. speciosa HEX	1	1	0.25	〉128
KAN	2	32	1	〉128
L. leonurus HEX	8	64	16	〉128
I. murucoides HEX	16	64	16	〉128
M. speciosa HEX	4	64	32	〉128
OXA	0.125	0.5	〉128	〉128
L. leonurus HEX	1	1	〉128	〉128
I. murucoides HEX	1	16	〉128	〉128
M. speciosa HEX	0.25	0.5	〉128	〉128
VAN	1	0.5	〉128	〉128
L. leonurus HEX	4	4	〉128	〉128
I. murucoides HEX	4	2	〉128	〉128
M. speciosa HEX	4	4	〉128	〉128

* AMX: amoxicillin, CHP: chloramphenicol, CIF: ciprofloxacin, KNA: kanamycin, OXA: oxcacillin, VAN: vancomycin

inhibitory concentration indicated susceptible S. aureus was 0.25μg/ml of OXA, resistant S. aureus was 0.5μg/ml of OXA, and susceptible E. coli was 0.25 μg/ml of CIF. However, resistant E.coli was not inhibition activity of any agents which means 〉128μg/ml against bacteria.

As known as, Mitragyna speciosa is called "Biak", Ketum", and "Kratom" as tropical plant indigenous. This plant has been prescribed as a useful ingredient to treat of opiate addiction as an alternative medicine (Parthasarathy et al., 2009). Ipomoea murucoides is the morning glory family and distributed worldwide in tropical area (Pereda-Miranda et al., 2010). According to Hooton et al (1984), combining antibiotics with plants will be much more successful synergy effect of therapeutic result than antibiotics only to treat serious bacterial infection. Comparing with the existing antibiotics, overall weaker antibacterial activity of legal highs has been a number of difficulties to the therapeutic development potentially for resistant bacteria and persisters. However, the extract of plant and the conventional antibiotic can combine to use a due synergistic combination as one of the solution (Adwan & Mhanna, 2008).

One of the studies showed that MeOH extract (6250μg/ml) and Alkaloid extract (3120μg/ml) from M. speciosa with 30μg/ml of CHP showed antimicrobial activity against Bacillus subtilis and Salmonella typhi (Parthasarathy et al., 2009). MeOH and CHL extract (25μg/ml) from I. murucoides with norfloxacin 128μg/ml showed antibacterial activity against resistant S. aureus. This study was mixed norfloxacin 128μg/ml, MeOH extract, and CHL extract with Kanamycin, Chloramphenicol against E. coli so that these are antimicrobial active against E.coli (Corona-Castañeda at al., 2013).

There have increased so many kinds and strains of bacteria which resist to antimicrobial because of the frequent use of antibiotic. Comparing with the existing antibiotic, overall weaker antibacterial activity of legal highs has been a number of difficulties to the therapeutic development potentially for resistant bacteria and persisters.

In this study, there were measured antibacterial of Leonotis leonurus, Mitragyna speciosa, Ipomoea murucoides of Legal highs (512μg/ml) with antibiotics (128μg/ml) which are amoxicillin, chloramphenicol, ciprofloxacin, kanamycin, oxacillin, and vancomycin investigated no activity to inhibit against pathogens which are susceptible S. aureus, resistant S. aureus, susceptible Escherichia coli, resistant Escherichia coli. However, there were no activities to inhibit against any bacteria. The reason may be tested small amount of Legal highs. Most of antibacterial activity assays were at least 1000μg/ml of extract but this assay was 512μg/ml. One of the assays was showed no antibacterial activity of K. pneumoniae even 5000 μg/ml (Jimoh et al., 2010).

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