ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Synergistic Antimicrobial Effects of Different Ratio Combination of *Smilax myosotiflora*, *Persicaria odorata* and *Syzygium aromaticum* with antibiotics

Pang Jyh Chyang*, Mahfuzha Mustapa, Kaswandi M. Ambia

Universiti Kuala Lumpur, Institute of Medical Science Technology, 43000 Kajang, Selangor, Malaysia

Article History:

Received on: 02.10.2018
Revised on: 15.12.2018
Accepted on: 17.12.2018

Keywords:

Antibacterial, Antifungal, Persicaria odorata, Smilax myosotiflora, Syzygium aromaticum

ABSTRACT

Antibiotic resistance to infectious microbe has become a major threat to clinical treatment. In order to control the misuse of antibiotics, an alternative antimicrobial agent is crucially needed to cater to this issue. This study was carried out to assess the efficacy of antibacterial and antifungal activities of all single or combination extracts of S*milax myosotiflora (SM), Persicaria odorata (PO)* and *Syzygium aromaticum (SA)* with standard antibiotics (gentamicin or ketoconazole) as the positive controls. All plant extracts were prepared using methanol extraction procedure. Antibacterial activities of the crude extracts were determined by agar well diffusion method. The mixed extracts were prepared by adding 25µg/µl concentration of each crude extract based on the volume ratio of 1:1, 1:2, 2:1, 1:1:1, 1:1:2, 1:2:1 and 2:1:1. Single extracts of *SM, PO* and *SA* demonstrated positive effects on the growth of *Bacillus subtilis, Salmonella typhi,* and *Candida albicans.* All crude extracts displayed increased diameter of inhibition zones when combined with positive controls. Findings of this study suggested that the synergism activity is present, and extracts do not react antagonistically with antibiotics.

* Corresponding Author

Name: Dr. Pang Jyh Chyang
Phone: +6012-3580973
Email: jcpang@unikl.edu.my

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9iSPL2.1749

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INTRODUCTION

In developing countries where modern medicines are expensive, natural resources present as an alternative medication for the human to supersede synthetic drugs and antibiotics. According to the World Health Organization (WHO), the usage of traditional medicine is practised daily by 80% of the population especially in Africa and Asia. In addition, herbal extracts have the potency to act as an antioxidant agent to scavenge free radicals (Rasha *et al.,* 2014). Some of the natural plant resources that have been widely used nowadays are *Smilax myosotiflora (SM), Persicaria odorata (PO)* and *Syzygium aromaticum (SA).*

Smilax myosotiflora, locally known in Malaysia as 'ubi jaga', is a low, herbaceous climbing plant. It is traditionally used by indigenous folk for its aphrodisiac effects. It is believed to improve blood circulation, strengthen male energy as a general tonic, increase body metabolism, possess antioxidant activity as well as anticancer activity (Dasuki *et al.,* 2012).

Persicaria odorata or known as 'kesum' among Malaysian is a herb where its leaves are commonly used in Southeast Asian cooking as flavouring materials in food preparation. *P. odorata* is a tender perennial herb measuring 30 to 35 cm in height with pointed leaves about 6 to 15 cm in length with distinctive dark purple marking in the centre of the leaves. The plant is believed to have a range of medicinal properties such as anti-inflammatory and antidiuretic (Sasongko *et al.,* 2011).

Meanwhile, *Syzygium aromaticum* (cloves) are the aromatic dried flower buds of a tree in the family Myrtaceae (Srivastava and Malhotra, 1991). *S. aromaticum* contains a significant amount of potent anti-inflammatory component called Eugenol. It

has been used in dentistry in the application of root canal therapy, temporary fillings, and general gum pain. Abd Azim (2015) reported that the plant extract showed strong antioxidant activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as compared to vitamin C. Besides that, it has also been used as an antimicrobial agent on the tooth and effectively sooth toothache temporarily (Ghelardini et al. 2011).

Due to its antioxidant, anti-inflammatory and anticancer effects of *SM, SA* and *PO*, it is hypothesized that the combination of these three crude extracts could lead to some synergistic effects in enhancing anti-microbial activity. Hence, this study was conducted to determine the synergistic effects of different ratio combinations of *SM, SA* and *PO* with a standard antibiotic to inhibit bacteria and fungus growth.

MATERIALS AND METHODS

Preparation of Extract

The plants were purchased from a local wet market and dried in the oven at 40°C for five days. Approximately 100g of dried *SM, SA* and *PO* were grounded. Then, it was soaked separately in 500mL of methanol for three days and filtered through a 0.45µm syringe filter. Using a Rota evaporator, the pure extracts were evaporated and subsequently transferred into culture plates, weighted and stored in the refrigerator before use.

Culture Media Inoculum Preparation

Sabouraud Dextrose Agar, Mueller-Hinton agar and Nutrient agar were prepared according to standard protocols and kept at 4°C before use.

Disc Diffusion Method

Well diffusion method was used for antimicrobial survival test. The test was performed according to the protocol described by Perez (1990). A sterilized cotton swab was dipped into microbe suspension and the microbe inoculum was swept onto agar plate surface three times by spinning the agar plate 60º clockwise. The plate was left for several minutes to allow inoculums to be absorbed into the agar before forming 6mm agar well by using a sterile Durham tube. A total of 50µl (25µg/µl) extract solution, 50µl (10µg/µl) positive control, 50µl negative control (distilled water) and different ratios of extract mixture was suspended with distilled water and pipetted into each respective well. Following that, the plates were incubated at 30ºC for fungus and 37ºC for bacteria. Finally, the inhibition zone was measured using Vernier calliper or ruler. The experiment was performed in triplicate.

Data Analysis

All grouped data were statistically evaluated using a parametric test of one-way ANOVA computed using the SPSS software. The analysis was conducted to test whether there are significant differences in the inhibition effects of the extracts that gave positive results. The differences were considered significant if the p-value is less than 0.05.

RESULTS AND DISCUSSION

Approximately 25µg/ml of S*milax myosotiflora (SM), Persicaria odorata (PO)* and *Syzygium aromaticum (SA)* crude extract were used to determine their antimicrobial effects on *Bacillus subtilis*, *Salmonella typhi* and *Candida albicans*. *SA* gave the most antimicrobial effect among the three-crude extract (28±0.2mm *Salmonella typhi*; 17±0.1mm *Bacillus subtilis*; 21±0.2mm *Candida albicans*), followed by *SM* (13±0.5mm *Salmonella typhi*; 10±0.1mm *Bacillus subtilis*; 16±0.2mm *Candida albicans*) and *PO* (9 ±0.3mm *Salmonella typhi*; 5±0.1mm *Bacillus subtilis*; 21±0.2mm *Candida albicans*).

Studies on *SM* are still lacking compared to other types of smilax genus plant. Hossain (2013) in his study revealed that *Smilax zeylanica Linn* possesses antibacterial and antioxidant properties. In this study, *SM* showed the significant mean of inhibition zone 47±0.1mm when combined with gentamicin at a ratio of 1:2 towards *Bacillus.* This inhibition towards *Bacillus subtilis* is even greater when compared to the inhibition zone by gentamicin alone (35± 0.5mm). According to Mishra and Kalyani (2014), *SA* extract was reported to have high activity of antimicrobial properties against a pathogen which are due to the presence of a phenolic compound, eugenol. Furthermore, Wendakoon and Sakaguchi (1995) also reported that eugenol has many types of action mode against a pathogen by altering the membrane, affects the transport of ions and ATP, and changes the lipid profile of different bacteria.

Meanwhile, *PO* showed markedly increased inhibition, and double the single extract mean inhibition zone 40±0.3mm towards *Candida albicans* in triple mixture extracts when combined with *SM* and ketoconazole (1:1:1 and 1:1:2 ratio). A study by Ridzuan (2013) reported that *PO* contains aldehyde and terpene, which could contribute to positive antimicrobial activity. Thus, *PO* significantly inhibits the growth of *Candida albicans* in both single and combinations extract.

Majority of the plant extracts combination showed enhancements in the inhibition zone when combined with the positive control, with the exception of several combinations against *Candida albicans.* In contrast, a combination of extracts resulted in

Table 1: Zone of inhibition in different ratio combination of two different extracts and antibiotics against Bacillus subtilis, Salmonella typhi and Candida albicans

Extracts	Zone of inhibition (mm)								
	Bacillus subtilis			*Salmonella typhi*			*Candida albicans*		
Ratio	1:1	1:2	2:1	1:1	1:2	2:1	1:1	1:2	2:1
SM: PO	0	0	0	0	0	0	8±0.1	9±0.1	8±0.1
SM: SO	0	0	0	0	0	0	9±0.1	16±0.4	7±0.2
PO: SO	8±0.1	12±0.2	12±0.1	11±0.1	10±0.2	11±0.2	20±0.3	19±0.2	23±0.1
SM: G	45±0.2	47±0.2	45±0.1	35±0.3	36±0.3	34±0.3	-	-	-
PO: G	40±0.1	38±0.1	40±0.4	34±0.2	35±0.1	35±0.1	-	-	-
SO: G	45±0.3	40±0.1	43±0.3	35±0.1	38±0.3	35±0.2	-	-	-
SM: KETO	-	-	-	-	-	-	17±0.3	20±0.1	13±0.2
PO: KETO	-	-	-	-	-	-	33±0.4	38±0.2	30±0.3
SO: KETO	-	-	-	-	-	-	47±0.4	45±0.3	42±0.1

G: Gentamicin; KETO: ketoconazole

Table 2: Zone of inhibition in different ratio combination of three different extracts and extracts with antibiotics against Bacillus subtilis, Salmonella typhi and Candida albicans

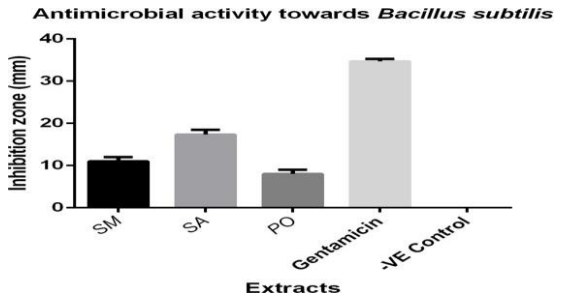
Extracts	Ratio	SM: PO: SO	SM: PO: G	SM: SO: G	SO: PO: G	SM: SO: KETO	SM: PO: KETO	SO: PO: KETO
	1:01:01	17±0.1	46±0.3	46±0.2	45±0.3	-	-	-
Bacillus	1:02:01	20±0.2	45±0.3	47±0.3	45±0.4	-	-	-
subtilis	1:01:02	21±0.4	45±0.2	46±0.2	45±0.3	-	-	-
	2:01:01	15±0.1	46±0.4	45±0.3	45±0.3	-	-	-
	1:01:01	13±0.3	38±0.1	36±0.2	37±0.2	-	-	-
Salmonella	1:02:01	14±0.3	37±0.1	36±0.2	35±0.3	-	-	-
typhi	1:01:02	14±0.2	43±0.4	35±0.3	38±0.1	-	-	-
	2:01:01	11±0.3	45±0.2	35±0.2	35±0.3	-	-	-
	1:01:01	0	-	-	-	20±0.2	35±0.4	30±0.3
Candida	1:02:01	0	-	-	-	25±0.2	40±0.3	20±0.4
albicans	1:01:02	0	-	-	-	18±0.2	40±0.3	18±0.4
	2:01:01	0	-	-	-	27±0.3	30±0.1	17±0.3

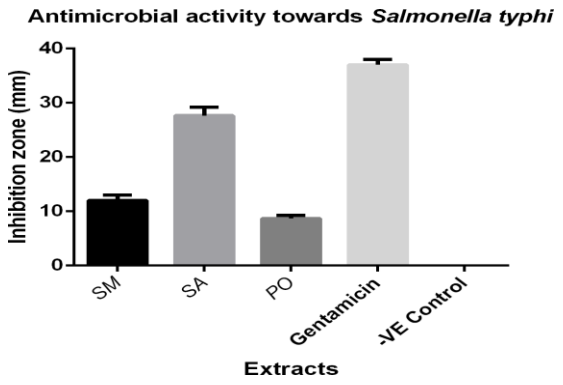
G: Gentamicin; KETO: ketoconazole

antagonistic effects as demonstrated by the suppression

of antimicrobial activities compared to single extracts. This is shown in double mixture extracts of *SM, PO* and *SA* which minimally inhibit the growth of both *Bacillus subtilis* and *Salmonella typhi*.

The differences in the degree of inhibition zone for *Bacillus subtilis* and *Salmonella typhi* were due to the differences in antimicrobial activity against gram-positive and gram-negative bacteria. According to Arullapan (2014), the antimicrobial activity of extract is more effective against gram-positive than gram-negative bacteria. This is due to the presence of thin lipopolysaccharide outer membrane layer on gram-negative bacteria which could resist the penetration of plant extracts. In contrast, gram-positive bacteria only have a mesh-like peptidoglycan layer which was more accessible to permeation by the extracts. A study by Nagi (2008) demonstrated that gram-negative bacteria have an effective permeability barrier, comprised of the outer membrane, which restricts the penetration of compounds and multidrug resistance pumps that extrude toxins cross this barrier. It is plausible that apparent lower antimicrobial activity of single plant extract is largely due to this permeability barrier.





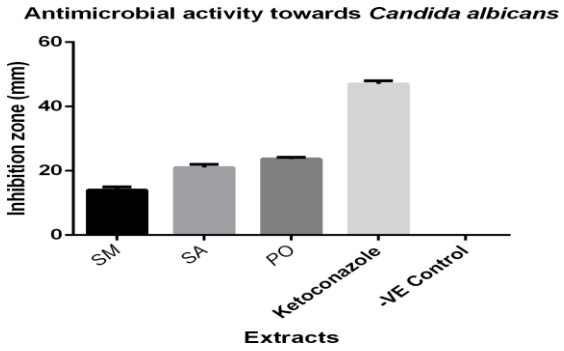


Figure 1: Mean difference in diameter of the inhibition zone (mm) between extracts and different microbes; (A) Bacillus subtilis, (B) Salmonella typhi and (C) Candida albicans

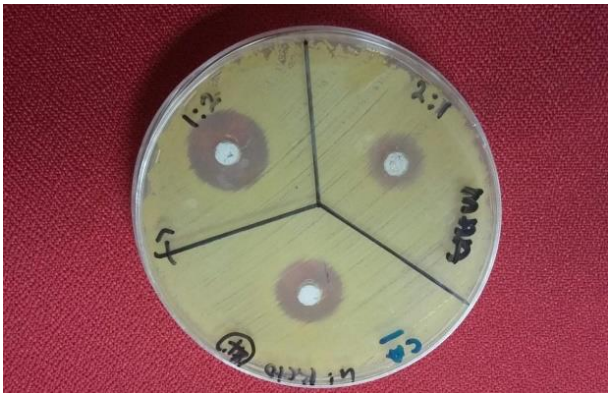


Figure 2: Effects of SO and ketoconazole mixture towards the growth of Candida albicans

CONCLUSION

In conclusion, all extracts positively inhibited the growth of *Bacillus subtilis*, *Salmonella typhi* and *Candida albicans.* combination of the extracts with standard antibiotics appeared to have synergistic effects which can be used to expand the antimicrobial spectrum. However, further studies are required to explore their potential antimicrobial effects on other species of bacteria and fungus.

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