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Combining Essential Oils of *Piper betle* and *Myristica fragrans* for Enhanced Antimicrobial Properties

SIONG FONG SIM[1,*], FAZIA MOHD SINANG[1], DIANA KERTINI[1], FELECIA COLLICK[1], MELISSA EDWARD DANKAN[2], VANESSA RENTING NIXION LUNCHA[2] & NOOR SYAZANA ABDUL MAJID[2]

[1] Department of Chemistry, Universiti Malaysia Sarawak, Faculty of Resource Science and Technology, 94300 Kota Samarahan, Sarawak, Malaysia; [2] MARA Junior Science College, Jalan Sultan Tengah, Petra Jaya, 93050 Kuching, Sarawak, Malaysia

ABSTRACT

This paper reports the chemical compositions and antimicrobial activity of essential oils extracted from *Myristica fragrans* and *Piper betle* in individual and combined fractions. Enhanced antimicrobial activity is anticipated when the volatile oils are combined as compounds present in different extracts could complement each other resulting in synergistic effect offering a broader spectrum of microbial resistance. The GC-MS analysis indicates that chemical compositions of *M. fragrans* and *P. betle* vary with *M. fragrans* containing more early-eluting compounds. The combined extract is characterised by compounds present in both extracts, some appear to co-elute in the mixture. The antimicrobial activity of the single and combined extracts against *Staphylococcus aureus*, *Escherichia coli* and *Aspergillus flavus* were evaluated. *P. betle* demonstrates stronger antimicrobial activity than *M. fragrans*; the combined extract exhibit improved performance especially on *A. flavus*.

Keywords**:** Essential oils, antibacterial, antifungal, *M. fragrans*, *P. betle*

INTRODUCTION

Essential oil has long been recognised for its antimicrobial activity primarily as antibacterial and antifungal agent. A study by Pawar & Nabar (2010) evidenced that mixing the essential oil could increase the coverage of microbial resistance. The synergistic and additive antimicrobial effect was similarly demonstrated elsewhere (Lachowicz *et al*. 1998; Das *et al*. 2012; Nguefack *et al.* 2012). Hypothetically, if the volatile oil extracted from two plants exerts antibacterial and antifungal activity, respectively, the combined extract would be more potent than the individual fraction. Likewise, if the extracts from both plants exhibit positive response against two different bacterial strains, the blended fraction is anticipated with a greater spectrum of microbial resistance. In this paper, we attempt to evaluate the chemical composition and antimicrobial activity of the single and blended essential oils of *P. betle* and *M. fragrans*. Essential oils from both plants have been known with promising antimicrobial properties nevertheless there is no information on the blended extract as yet (Dorman & Deans, 2000; Datta *et al*. 2012; Suprapta & Khalimi, 2012; Arambewela *et al.* 2005; Muchtaridi *et al*. 2011).

MATERIALS & METHODS

1.1 Extraction of essential oil

The fresh *P. betle* leaves and *M. fragrans* seeds were cut into smaller pieces and subjected to hydrodistillation using a Clevenger apparatus according to Meshkatalsadat *et al.* (2009). Approximately 100 g of samples were placed into a 2 L flat bottom flask with 1.5 L distilled water and several granules of anti-bumping agents. The samples were hydrodistilled for 6 hrs and the volatile oil extracted was dried with a small amount of anhydrous sodium sulphate.

*Corresponding author: *sfsim@frst.unimas.my*

1.2 Chemical analysis

Two milligrams of essential oil was dissolved in 2 mL of dichloromethane (DCM) for gas chromatography mass spectrometry (GC-MS) analysis. For the combined extract, 1 mL of the dissolved volatile oil of *P. betle* and *M. fragrans* was drawn and mixed. The essential oil was analysed on a Shidmazu GC-MS system model QP500. A medium polarity capillary column; BPX-5 column (29.5 m × 0.25 mm), with film thickness of 0.25 µm was used with helium as the carrier gas. One microlitre of sample was injected using splitless injection according to the following scheme: 50 °C for 5 mins with 6.5 °C/min up to 280 °C. The final temperature was held for 10 mins. The total runtime for each sample was 50 mins. For MS detection, the mass fragments were detected between 40 and 1000 amu. The ion source temperature was 200 °C. Note that the detector was activated after 10 mins. The compounds detected were identified based on 90% correlation of the mass spectrum with the pattern of the NIST library (Adams, 1995).

1.3 Antibacterial activity

S. aureus and *E. coli*, representative of gram-positive and gram negative culture, respectively, were tested on the single and blended essential oil. The bacterial cultures were wild type bacteria confirmed by sequencing. The active cultures were prepared by inoculating fresh Mueller Hinton Broth (MHB) with single colony bacteria streaked from stock cultures and incubated at 37 °C overnight. A standardised optical density (OD) at 600 nm of 0.60 was attained. The antibacterial activity was determined using the disc diffusion method. The agar plates were prepared and swabbed with the bacterial culture using sterile cotton uniformly. A 10 µL of essential oil (single and blended) at concentrations 10 mg/mL, 20 mg/mL and 50 mg/mL, prepared in 30% dimethyl sulfoxide (DMSO), were loaded on sterile filter paper discs of 6 mm in duplicates. The disc was placed on the surface medium and the plates were incubated at 37 °C for 24 hrs. The inhibition zones were measured in milimeter. Commercial antibiotic, streptomycin and penicillin, were used as positive control and DMSO was used as negative control. The method is described in Emami *et al.* (2006).

1.4 Antifungal activity

The antifungal activity was tested on *A. flavus*. A 100 µL essential oil (10 mg/mL, 20 mg/mL and 50 mg/mL, respectively) was spread onto petri dishes of potato dextrose agar (PDA). A circular block of mycelia from the freshly prepared stock culture was punched and placed onto the plates. The diameter of the fungus was obtained on the fifth day after incubation at 30^{o}C as a measure of growth (Serrano *et al.* 2004). Note that in this study only the negative control of DMSO was used.

RESULTS & DISCUSSION

Figure 1 illustrates the chromatograms of the single and combined essential oils of *P. betle* and *M. fragrans*. Visually, the compounds eluted at the earlier retention time in *M. fragrans*, between 10 and 15 mins, are not found in *P. betle*; the peaks detected after 23 mins in *M. fragrans* on the contrary are less prominent suggesting that the chemical constituents of both plants vary nonetheless some compounds are found in common.

Table 1 summarises the compositions of some chemical compounds identified in *P. betle* and *M. fragrans*. The combined extracts attained the features of both with five compounds identified in common; these include β-phellandrene, linalool, α-terpineol, eugenol and α-cadinol. There are also unique compounds to each extract; combining the essential oil as a result produces a mixture with richer chemical compositions. For example, the well reported antifungal compounds, α-humulene and β-elemene, are distinctively found in *P. betle* whilst 1,8-cineole and *p*-cymene are unique to *M. fragrans*. In the mixture, they present concurrently implying possible synergistic effect that could lead to improved antimicrobial selectivity and efficacy (Hossain *et al.* 2008); this is also demonstrated in the findings of Henry *et al.* (2009). Despite the additive effects, some compounds identified in the single isolate are interestingly absent in the

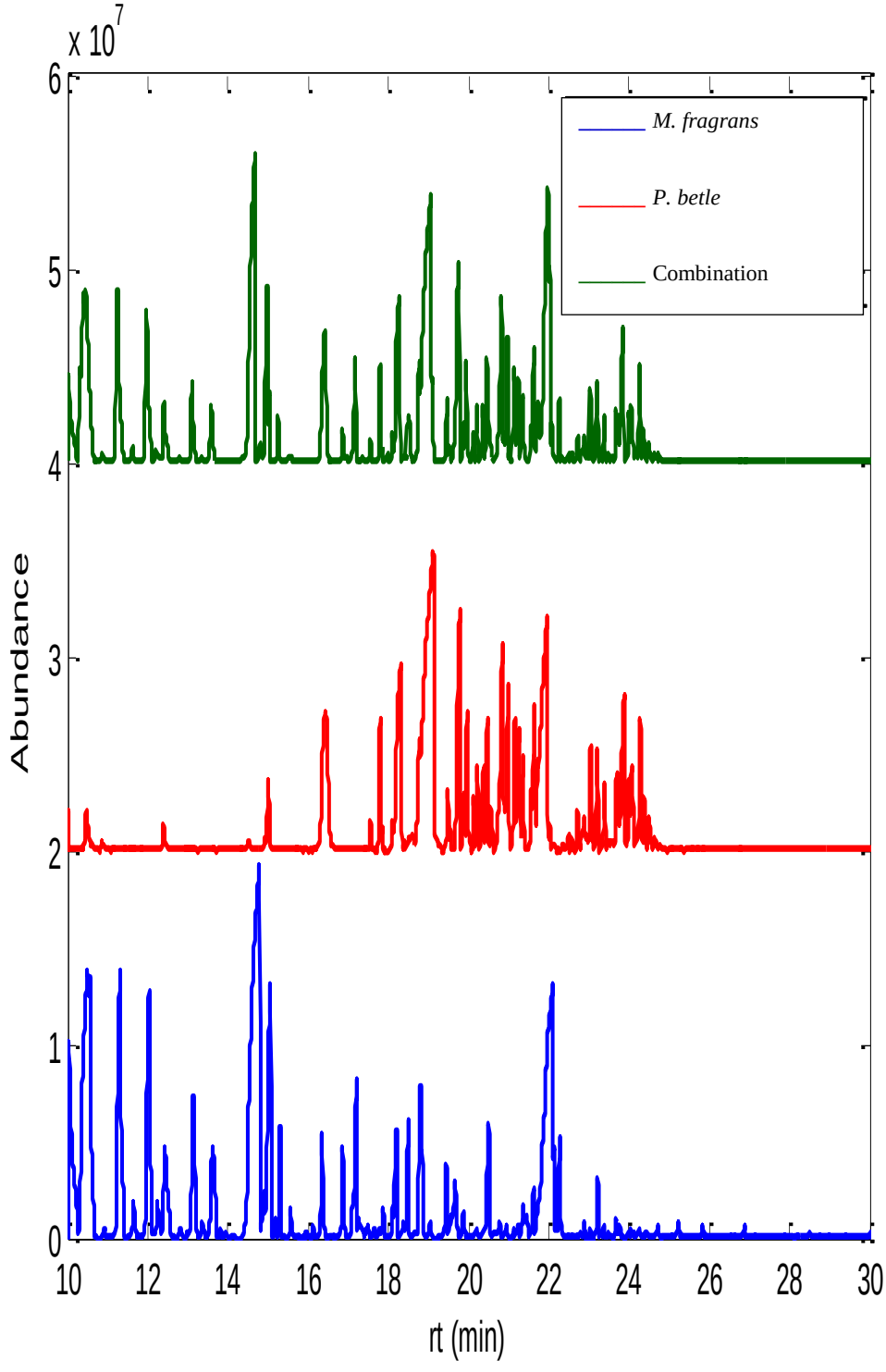


Figure 1. GC chromatograms of single and combined essential oil of *P. betle* and *M. fragrans*

Table 1. Some compounds identified in single and blended essential oils of *M. fragrans* and *P. betle*

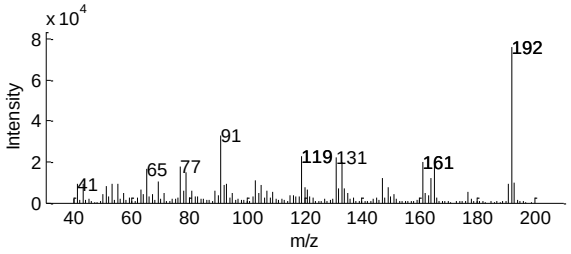
Compounds	Retention time, min	*M. fragrans* (%)	*P. betle* (%)	Combined (%)
Camphene	10.04	4.39	nd	2.53
p-cymene	10.50	7.40	nd	2.27
β-phellandrene	11.32	5.51	1.01	3.69
1,8-cineole	11.64	1.54	nd	0.86
Linalool	12.42	3.11	1.75	1.98
α-terpineol	15.06	4.67	2.62	2.90
trans-piperitol	15.30	2.14	nd	1.18
β-citronellol	15.56	1.15	nd	-
Chavicol	16.43	5.39	nd	3.47
Borneol	16.86	2.06	nd	1.08
Safrole	17.21	3.18	nd	2.09
γ-elemene	17.80	nd	3.13	1.78
α-terpinolene	17.85	1.21	nd	0.60
4-allylphenyl acetate	18.30	nd	5.90	3.61
Eugenol	18.50	2.62	1.63	1.79
Caryophyllene	19.79	nd	5.23	4.04
Humulene	20.47	nd	3.01	2.37
Ylangene	21.15	nd	3.32	1.89
α-muurolene	21.25	nd	3.43	2.06
α-amorphene	21.35	nd	2.70	1.76
Myristicin	21.75	7.67	nd	nd
Eugenol acetate	22.00	nd	9.69	nd
Elemicin	22.28	2.33	nd	1.48
β-elemene	22.73	nd	1.71	0.99
Elixene	23.05	nd	2.72	1.57
2-acetyloxy-4-allyphenyl acetate	23.89	nd	4.13	2.74
α-cadinol	24.25	0.71	3.12	1.93
Myristic acid	25.84	0.82	nd	nd

Key: "nd" indicates not detected.

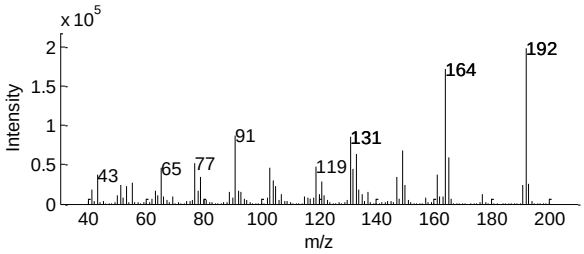
blended extract. For example, myristicin and eugenol acetate are eluted in *M. fragrans* and *P. betle*, respectively, at similar retention time with prominent abundance. These compounds are found to co-elute in the mixture where the peak detected fails to be matched with the typical mass spectrum of myristicin or eugenol acetate according to the similarity percentage of 90%.

Figure 2 shows the mass spectra of myristicin and eugenol acetate detected in *M. fragrans* and *P. betle*. The mass spectrum of the compound eluted in the mixture is 87% and 84% similar to myristicin and eugenol acetate, respectively. This confusion resulting from co-elution suggests that the operating conditions would require further optimization.

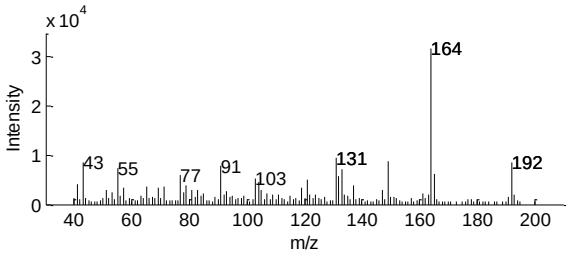
Table 2 summarises the antibacterial activity of single and blended essential oils against *S. aureus* and *E. coli* at 10, 20 and 50 mg/mL. No statistical significance is deduced between the inhibitory diameters of single and blended essential oils ($p > 0.05$). Comparing the performance with the positive control, streptomycin, the inhibitory activity of *P. betle* is considerably encouraging especially on



Myristicin in *M. fragrans* (21.75 min)



The peak eluted in the mixture (21.98 min)



Eugenol acetate in *P. betle* (22.00 min)

Figure 2. Mass spectra of myristicin, eugenol acetate and the peak detected in the mixture at similar retention time.

S. aureus. Penicillin however is relatively less effective. For *M. fragrans*, weak antibacterial activity is seen on *E. coli* with no inhibition identified on *S. aureus*. This observation concurs the finding of Ameen (2012) where gram positive bacteria are evidenced to be more resistant due to the presence of extra layer of protection restricting the diffusion of hydrophobic compounds (Prabuseenivasan *et al.* 2006). The lower inhibitory effect of *M. fragrans* does not deny its antibacterial activity but may suggest that the effective concentration have not been attained. The combined essential oil demonstrates enhanced antimicrobial activity although statistically insignificant; this may be associated to the complementary effects of the chemical compounds present in both plants. According to literature, eugenol and linalool are compounds with moderate antibacterial activity; other compounds such as camphene, *p*-cymene, caryophellene, terpinene, borneol, chavicol are reported with weak inhibition property (Damián-Badillo *et al.* 2008; Fabri *et al.* 2012). Safrole and α-terpineol on the other hand possess relatively greater activity than the aforementioned weak antibacterial compounds (Jantan *et al.* 2008). The presence of these compounds corroborates the antibacterial property of the extracts. Both *M. fragrans* and *P. betle* exhibit affirmative antifungal activity where treatments at varying concentrations demonstrate statistical significance ($p < 0.05$). The results imply that *P. betle* is stronger than *M. fragrans;* when the extracts are combined, the antifungal performance is improved nonetheless no significant different is corroborated statistically. It is speculated that no antagonistic effects are present as the antimicrobial activity of the combined extract is unhampered on the contrary improved compared to the single extracts.

Table 2. The antibacterial activity of essential oil of *M. fragrans* and *P. betle* against *E. coli* and *S. aureus*

Sample	mg/mL	Inhibition zone (mm) *E. coli*	Inhibition zone (mm) *S. aureus*
P. betle	10	8	-
	20	10	9
	50	10	10
M. fragrans	10	-	-
	20	8	-
	50	7	-
Combined	10	-	-
	20	10	10
	50	11	10
Positive control (Streptomycin)		18	12
(Penicillin)		-	8
Negative control (DMSO)		-	-

Key: "-" indicates no observable inhibition zone.

Table 3. The antifungal activity of essential oil of *M. fragrans* and *P. betle* against *A. flavus*

Sample	mg/mL	Diameter (mm)	% Inhibition
P. betle	10	54	13
	20	50	19
	50	38	39
M. fragrans	10	58	6
	20	50	19
	50	46	26
Combined	10	50	19
	20	45	27
	50	21	66
Control (DMSO)	-	61	-

Key: "-" indicates no observable inhibition zone.

CONCLUSIONS

Variation is identified between the chemical compositions of volatile extracts from *P. betle* and *M. fragrans*, the latter contains more eluting compounds at the earlier retention time for example, camphene, *p*-cymene and 1,8-cineole. When the extracts are combined, the mixture is characterised by a wider range of chemical constituents suggesting possible additive effects and a broader spectrum of antimicrobial resistance. Generally, *P. betle* demonstrates stronger antimicrobial activity than *M. fragrans*. The antifungal performance is enhanced when the extracts are combined. Although the differences are not statistically supported, the elevated inhibitory activity is observed when the concentration is increased and the extracts are blended.

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