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Effectiveness of Maceration Periods with Different Extraction Solvents on *in-vitro* Antimicrobial Activity from Fruit of *Momordica charantia* L.

Yang Lin Yeo, Yin Yin Chia, Chin Hong Lee, Heng Sheng Sow, Wai Sum Yap*
Department of Biotechnology, Faculty of Applied Sciences, UCSI University, Kuala Lumpur, Malaysia.

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ABSTRACT

Momordica charantia L. also known as bitter gourd, is one of the medicinal plants that have a long history usage as medicine in Asia for the treatment of various ailments. The present study was aimed to evaluate the effectiveness of maceration periods with various extraction solvents against four gram positive bacteria(*B. cereus*, *B. subtilis*, *E. faecalis* and *S. aureus*), four gram negative bacteria (*E. coli*, *K. pneumonia*, *P. aeruginosa* and *Serratia* spp.) and a fungus, *C. albicans*. Dried fruit powders were extracted at four maceration times (6 h, 12 h, 24 h and 48 h) using different solvents (hexane, petroleum ether, ethyl acetate, acetone, ethanol and distilled water). In the results, gram negative bacteria and fungus were found more susceptible as compared to gram positive bacteria. Solvents with low to intermediate polarity used such as hexane, petroleum ether and ethyl acetate demonstrated better antimicrobial activity as compared to other solvents used. On maceration times used, 6h was found to give the best inhibition zone of the antimicrobial activity with economic feasibility, while on the microorganisms tested, *E. coli* was found to be the most susceptible, followed by *C. albicans* and *K. pneumonia*. Statistical analysis demonstrated significant difference ($p < 0.05$), where maceration periods, extraction solvents used as well as the type of microorganisms have significant effect on the inhibition zone. Thus, this study revealed the importance of appropriate maceration periods in combination with different extraction solvents used, in giving a satisfactory and reliable result on the antimicrobial activity of the nine potent microorganisms.

INTRODUCTION

Over the years, the cases of infectious disease had been elevating in an alarming rate, despite the rapid advancement in the medical field. According to Ahmad and Beg (2001), infectious diseases remain as one of the leading cause of deaths worldwide, killing about 50,000 people daily. As defined by World Health Organization (WHO), infectious diseases is an evidence disease caused by pathogenic microorganisms, such as bacteria, viruses, multicellular parasites, fungi as well as prions. The common treatment for infectious disease is with the use of antimicrobial agents such as antibiotics or chemotherapeutic drugs. Allegedly, antimicrobial drugs should only be consumed according to the appropriate dosage, as over prescription of antimicrobial drugs is a vital contributor to drug resistance (Appiah and Vlas, 2002; Marchese and Schito, 2001). In 1980s, drug resistance towards pathogens was relatively low. However, the statistic is in an alarming stage now as numerous antibiotics and drug resistance to human pathogenic cases were reported globally. Despite the breakthrough of pharmacological industries in producing new antibiotics, nonetheless there is still an elevation of resistance towards these antibiotics by microorganisms (Nascimento *et al.,* 2000). To date, about 80% of all strains of *Staphylococcus aureus* were found to be resistant towards penicillin. Adding to the list, *Camplylobacter* spp, *E. coli*, *Proteus* spp., *P. aeruginosa*, *S. dysenteriae*, *S. enteritidis*, *S. paratyphi, S. typhi*, *S. aureus*, *S. faecalis*, and *C. albicans* are some of the example of drug resistance microorganisms (Appiah and Vlas, 2002; Barbour *et al.,* 2004; Benzic *et al.,* 2005). As the problem associated with microbial resistance continues to rise, yet there are still uncertainties in searching for the new antimicrobial drugs.

** Corresponding Author*
Wai Sum Yap, Department of Biotechnology,
Faculty of Applied Sciences, UCSI University,
Kuala Lumpur, Malaysia. Email: wsyap@ucsiuniversity.edu.my

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Therefore, controlling the use of antibiotics, creating research and experiment for better understanding of the genetic mechanisms of resistance, as well as developing new synthetic or natural drugs are some of the effective methods taken to cope with the problem of microbial resistance (Nascimento *et al.,* 2000). Whereby, researchers are screening and searching massively for any new antibiotics to combat the antibiotic resistance, where plant secondary metabolite still provides a mean to identify new antibiotics (Barnum, 1998). Thus, there are considerable interests in isolating, characterizing and utilizing natural antimicrobial compounds from various plant extracts in order to produce effective antimicrobial drugs (Mokbel and Hashinaga, 2005).

Nature has been a promising source of new therapeutic candidate compounds due to the tremendous chemical diversity found in various species of plants (Rocha *et al.,* 2001). Plants are considered as a potent and powerful source of drugs that have stood the test of time, where the modern medicine and chemistry development could not replace most of them (Ahmad *et al.,* 1998). Moreover, it has been revealed that more than 50% of all modern drugs contain natural products as the major component of modern pharmaceuticals used for the treatment of human diseases (Mayer *et al.,* 2010; Rosangkima and Prasad, 2004). Approximately, 400 herbs have been used widely as therapeutic medicinal substances as narcotics, purgatives and sudorifics (Borzelleca, 2001; Green-Hernandez *et al.,* 2001), where morphine was first obtained from opium by Serturner in year 1806. Other compounds such as cinchonine, quinine, caffeine and brucine were successfully isolated during the next five years (Ahmed, 1994).

Phytochemicals and biological constituents in plants have remarkable contribution towards the drug industry (Srinivasan *et al.,* 2007). The medicinal properties of plants are due to the presence of certain chemical substances which can elicit a definite physiological action in human body. The various plant parts such as roots, leaves and fruits contain bioactive compounds like alkaloidal constituents, essential oils, peptides and unsaturated long chain aldehydes, thus making them rich as a source of medicine. Moreover, Doughari *et al.,* (2008) have indicated that these bioactive compounds are effective against human pathogens such as bacteria, fungi and viruses, which could be of great significant in therapeutic treatments.

Momordica charantia L. or bitter gourd was chosen to evaluate its antimicrobial effects in the present study due to its high utilization in traditional medicine. This plant is widely distributed throughout the tropics and used as folk remedy for various ailments which include healing of wounds, infections, measles, hepatitis and fevers (Grover and Yadav, 2004; Gůrbůz *et al.,* 2000; Subratty *et al.,* 2005).

The entire plant of *Momordica charantia*, including the fruits, seeds, leaves and stem possess medicinal values and exert therapeutic effects such as antidiabetic, antiviral, antioxidant, anticancer and anti-HIV activities (Beloin *et al.,* 2005; Grover and Yadav, 2004; Kubola and Sirimornpun, 2008). Although numerous studies had been done on *Momordica charantia* L., most studies were done was for the treatment of diabetes (Han *et al.,* 2008; Leung *et al.,* 2009; Singh *et al.,* 2008), while only few studies were done on the antimicrobial activity (Braca *et al.,* 2008; Mahmood *et al.,* 2012), especially on fruit extract (Lu *et al.,* 2011; Mwambete, 2009). Nonetheless, to the best of my knowledge, none of these studies revealed the importance of proper maceration periods with various extraction solvents against the inhibition activity of the microorganisms tested, which was the main aim in this study.

Therefore, this study was to assess the effectiveness of maceration periods (6 h, 12 h, 24 h and 48 h) with various solvents (hexane, petroleum ether, ethyl-acetate, acetone, ethanol and distilled water) on *in-vitro* antimicrobial activity from fruit of *Momordica Charantia* L.

MATERIALS AND METHODS

Plant material

Throughout the study, the unripe fruit of *Momordica charantia* L. (Chinese phenotype) was supplied by a local farm located in Sitiawan, Perak, Malaysia.

Preparation of plant material

Fresh samples of *Momordica charantia* fruits collected were washed and the seeds were removed. The fruits were then sliced into small pieces and dried in drying oven at 50 ^{o}C until a constant weight was achieved. The dried fruits were then grounded to powder by miller with the mesh size of 813 micron (Quadro Comil, Canada) and vacuum packed until further use.

Preparation of crude extracts

2 g of dry powdered plant material was extracted with 20 ml of different solvents (hexane, petroleum ether, ethyl acetate, acetone, ethanol and distilled water) at solid to solvent ratio of 1:10 (w/v) for different maceration periods (6 h, 12 h, 24 h and 48 h). The extraction was carried out at room temperature with agitation at 150 rpm.

Filtration and solvent evaporation

After the respective maceration periods, the soaked powder-solvent mixtures were filtered through a Whatman No. 1 filter paper, concentrated to 1 ml with a rotary evaporator at 40-60 ºC and then diluted with 5% dimethyl sulfoxide (DMSO) at ratio 1 : 1 (v/v). The concentrated fruit extracts were then stored at -20 ºC until further use.

Test microorganisms

In-vitro antimicrobial studies were carried out on four Gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis* and *Staphylococcus aureus*), four Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Serratia* spp.) and a fungus (*Candida albicans*). All the microorganisms were obtained from the Laboratory of Microbiology, Faculty of Applied Sciences, UCSI University, Malaysia.

Culture media

Nutrient agar (NA) (Himedia), nutrient broth (NB) (Merck, Germany) and potato dextrose agar (PDA) (Merck) were used during the study. NA and NB were used for the cultivation of bacteria while PDA were used to culture yeast. All bacterial cultures were incubated at 37 ℃ for 24 hours whereas yeast cultures were incubated at 30 ℃ for 48 hours.

Antimicrobial susceptibility test

The Kirby Bauer disc diffusion method was used. The cell concentration of bacteria and yeast which were to be inoculated were standardized to 10^6 to10^7 cells/mL with haemacytometer (Chanwitheesuk *et al.,* 2005). The bacterial cultures were inoculated in NB and incubated at 37 °C for 24 h while yeast was incubated at room temperature for 48 h; in an orbital shaker (Infors AG, Switzerland) at 150rpm. Suspension for the respective microbes, measuring 100 µL each, were pipetted and spread evenly on NA (for bacteria) and PDA (for yeast) plates, respectively with a sterilized swab. Sterilized filter paper discs (diameter, 6 mm), was impregnated with 10 µL of fruit extracts and allowed to be air-dried for 10 to 15 minutes. The discs were subsequently placed on the surface of inoculated agar medium. Discs with 10 µg/disc of ampicillin served as positive control for bacteria while 30 µg/disc of tetracycline were used as positive control for yeast. Different extraction solvents (hexane, petroleum ether, ethyl acetate, acetone, ethanol and distilled water) and 5% DMSO were used as negative controls. All tests were carried out in triplicate. Zone of inhibition was then measured using a scale.

Statistical analysis

The data of inhibition zone (mm) obtained was submitted to analysis of variance using one-way Anova to detect if there is any significant difference when different variables (maceration periods, type of solvents and type of microorganisms) were used. The significances were then ranked using Tukey post-Anova analysis with 95 % confidence intervals. All the tests were carried out using SPSS version 17.1.

RESULTS AND DISCUSSION

In the results obtained, a broad-spectrum of antimicrobial activity was observed using different extraction solvents in combination with different maceration periods. In Figure 1(a), where hexane was used, strong inhibition zone was observed on *C. albicans* at 6 h maceration period with zone inhibition of 17.8 ± 0.5 mm. Interestingly, no inhibition was observed at 12 h and 24 h for *C. albicans*, whereas a moderate inhibition with 9.3 ± 0.3 mm was observed at 48 h maceration period. This suggests that when hexane was used, maceration period plays a very important role in determining the type and concentration of bioactive compounds extracted from the fruit of *Mormodica charantia* L. Hexane has a polarity index of near zero. Hence, the bioactive compounds extracted using this solvent should have similar polarity (Ncube, 2008), where the compounds responsible for the inhibition of *C. albicans* and *E. coli* in this study is said to be of low polarity. Similarly, in a study done by Adeloye *et al.,* (2007) on *Urena lobata* Linn. leaf extract using hexane, inhibition on *E. coli*, *C. albicans* and other pathogenic microorganisms was observed where tannins were found responsible to confer the antimicrobial activity. In another study done by Hassan *et al.,* (2006) using hexane, the leaf and root extracts of *Calotropis procera* were found to contain alkaloids, flavonoids, tannins, steroids, triterpenoids, saponins and saponin glycosides which were postulated to be responsible for the inhibition of *Trichophyton rubrum*, *Microsporum gypseum* and *Aspergillus niger*. On the other hand, when petroleum ether was used [as shown in Figure 1(b)], *E. coli* was shown to be the most susceptible towards the inhibition with 22.3 ± 0.4 mm at 48 h maceration, followed by *C. albicans* with 10.3 ± 0.7 mm at 6 h maceration, while low or no inhibition was observed for all other microorganisms. *E. coli* was also found to be susceptible towards hexane extract [Figure 1(a)] at 48h maceration with inhibition zone of 12.0 ± 0.5 mm. Thus, it can be postulated that the antimicrobial compounds which are responsible for *E. coli* inhibition, tend to be extracted at longer maceration time with lower polarity index solvent such as hexane. In this study, when hexane or petroleum ether was used, the antimicrobial compounds present possess better inhibition towards *C. albicans* with 6 h maceration while for *E. coli*, longer maceration period (48 h) was needed. Therefore, it is proposed that the antifungal agents responsible to inhibit the growth of *C. albicans* were unstable and might be decomposed, when longer maceration period was imposed (Chan *et al.,* 2009). Moreover, according to Trusheva *et al.,* (2007), longer maceration time would lead to chemical changes, particularly oxidation of phenolic compounds such as flavonoids. This might be the reason why no antifungal activity was observed for hexane macerated at 12 h and 24 h, respectively while mild antifungal activity (9.3 ± 0.3 mm) was observed at 48 h maceration might be due to the presence of other phytochemicals that conferred the activity. Contrarily, in *E. coli*, the antibacterial agents were relatively potent at 48 h maceration, indicating that maceration period of lesser than 48 h might not be sufficient to exhibit the antimicrobial activity and longer period was needed to increase the yield of the extracted material (da Silva Cunha *et al.,* 2006). As for ethyl-acetate [Figure 1(c)], similarly, *E. coli* was also found to be the most susceptible towards the inhibition at both 12 h and 24 h maceration periods with 12.7 ± 1.5mm and 12.0 ± 1.2mm, respectively. Only moderate inhibition could be observed on *P. aeruginosa* macerated at 24 h with 10.3 ± 0.5 mm while low inhibition was found in *C. albicans* with 8.0 ± 0.2 mm at 24 h maceration. Other microorganisms showed low or no inhibition. Ethyl-acetate has been widely used as an extraction solvent for numerous antimicrobial studies conducted. In a study conducted by Adeshina *et al.,* (2010) on leaf of *Alchornea cordifolia* using hexane, ethyl-acetate and methanol as extraction solvents, ethyl-acetate shown to be the best solvent used that conferred the antimicrobial activity against *P. aeruginosa*, *S. aureus*, *E. coli* and *C. albicans* with diameter of inhibition zone ranging from 10.0 – 35.0 mm.

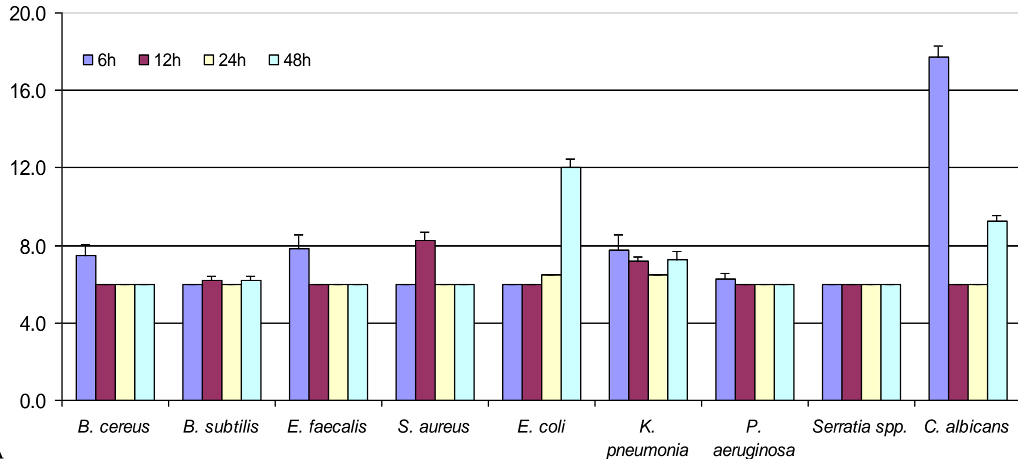

Fig. A

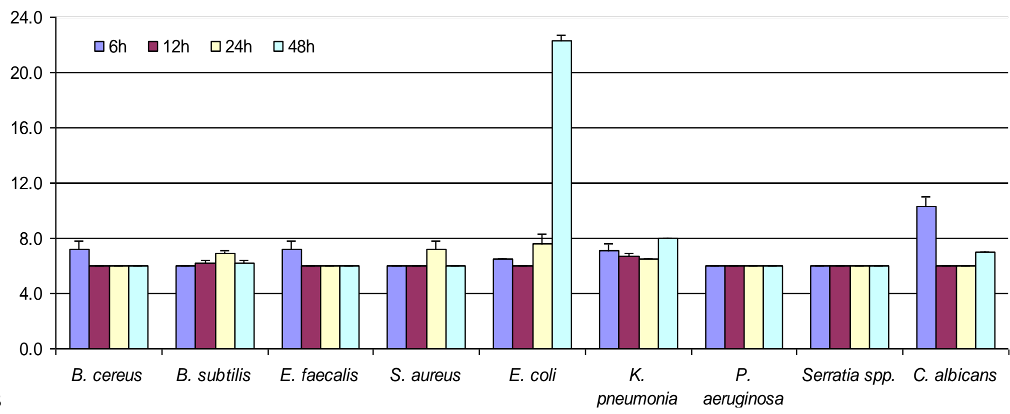

Fig. B

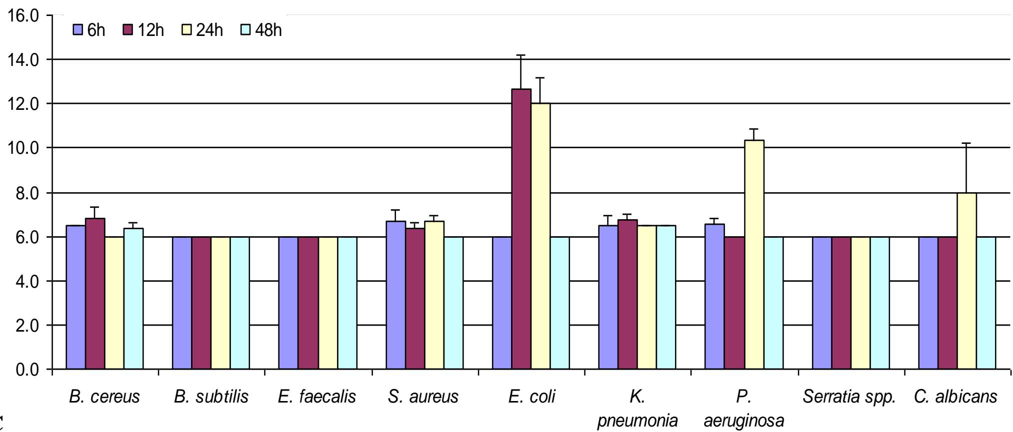

Fig. C

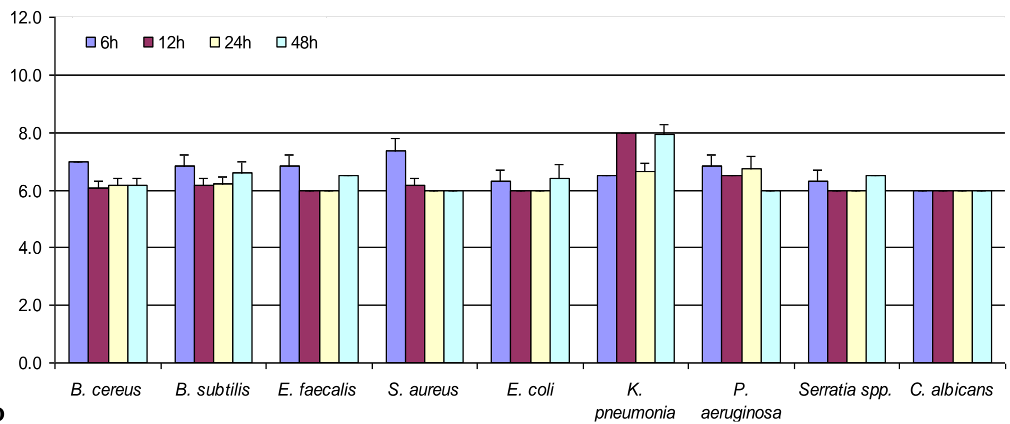

Fig. D

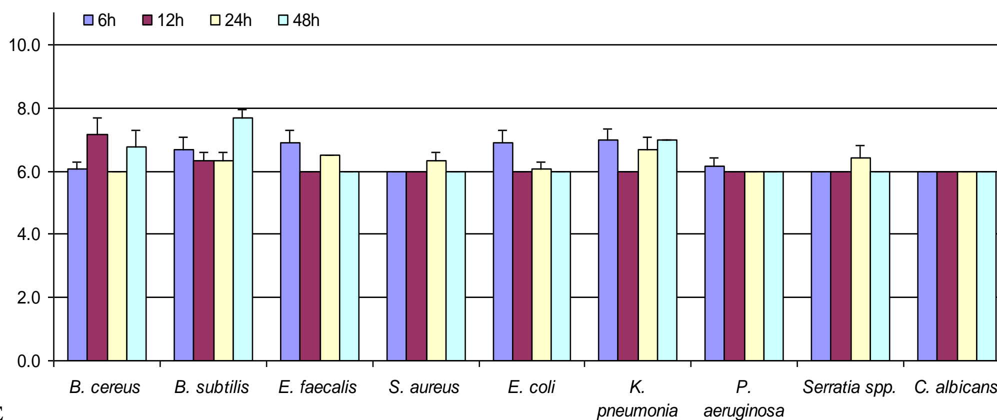

Fig. E

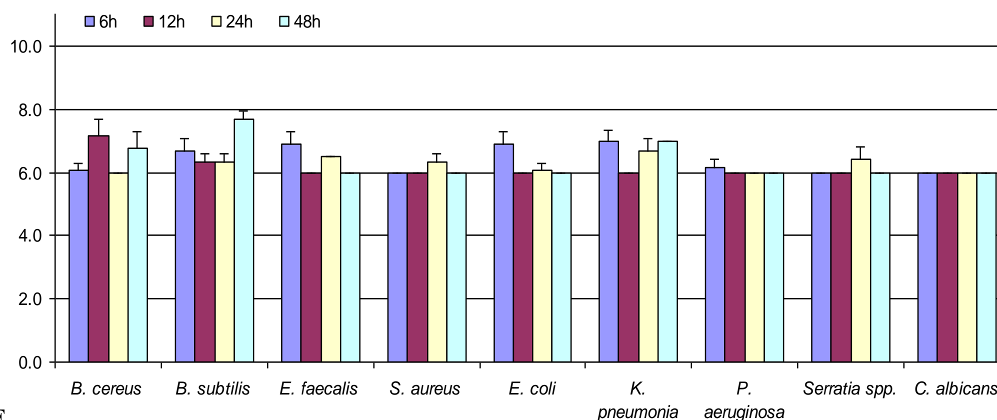

Fig. F

Fig. 1: Antimicrobial activity (disc diffusion method ~ zone of inhibition in mm) of *Momordica charantia* L. fruit using four maceration periods (6 h, 12 h, 24 h and 48 h) with different extraction solvents. **(A)** Hexane; **(B)** Petroleum ether; **(C)** Ethyl acetate; **(D)** Acetone; **(E)** Ethanol and **(F)** Distilled water.

The phytochemical screening of this plant revealed the presence of tannins, flavonoids, glycoside, resins and carbohydrates. In another study conducted by Gangadevi *et al.,* (2008) on the antimicrobial activity of *Acalypha indica* L. in leaf, stem and root using 3 different solvents (hexane, ethyl-acetate and methanol), greater zone of inhibition was found in ethyl-acetate extract against *B. subtilis*, *K. pneumonia* and *S.aureus*, thus indicating that the active ingredients in this plant are more readily dissolved or extracted in ethyl-acetate compared with other solvents used.

On the other hand, when more polar solvents such as acetone, ethanol and distilled water [Figure 1(d), 1(e) and 1(f)] were used as extraction solvents, only low inhibition (range from 8.0 ± 0.0 mm to 6.17 ± 0.3 mm) was observed in some of the microorganisms tested. Using acetone, *K. pneumonia* was found to be inhibited by all different maceration periods used while in *P. aeruginosa*, only extracts macerated at 6 h, 12 h and 24 h could inhibit the growth of this microbe, but not 48h. For ethanol extract, *B. subtilis* was inhibited by four different maceration periods used, but with only low inhibition, ranging from 7.7 ± 0.3mm to 6.3 ± 0.3mm. Distilled water extract on the other hand, showed the poorest of all solvents used where it could not inhibit most of the microorganisms tested. Only *E. faecalis*, *E. coli* and *C. albicans* showed low inhibition at 6 h maceration period. Thus, the finding from this study showed that non-polar and intermediate-polar solvents (hexane, petroleum ether and ethyl-acetate) were better solvent systems used in extracting antimicrobial compounds from fruit of *Momordica charantia* L. In a study done by Keskin and Toroglu (2001) on antimicrobial activities of different solvent extracts (methanol, acetone and ethyl-acetate) using different spices, their results revealed that none singular extraction solvent was effective against all microbes tested in any of the plant spices. Thus, the study concluded that there are differences in the antimicrobial effects of plant groups due to the phytochemical differences between species as well as the collection site (Keskin and Toroglu, 2001).

Nevertheless, the study conducted in this paper revealed that even in a singular plant (bitter gourd), when different extraction solvent was used, there were differences on the antimicrobial effects towards the microorganisms tested.

This is further confirmed when statistical analysis one-way ANOVA (Table 1) revealed that the inhibition zone (mm) depends on the type of extraction solvents, maceration periods as well as the strain of microorganisms used, was statistically significant where $p < 0.05$. In determining the best extraction solvents, further analysis with Tukey's multiple comparison tests revealed that hexane, petroleum ether and ethyl-acetate (Table 2) demonstrated better antimicrobial activity as compared to other solvents used. Thus, the results revealed that the active anti-

microbial compounds from fruit of bitter gourd are mostly in the categories of non-polar to intermediate polarity.

Table 1: Statistical analysis using One-way ANOVA on different parameters towards the inhibition zone (mm) of microorganisms tested.

	Variable	P-value
Inhibition zone (mm)	Type of solvents	< 0.05*
	Maceration periods	< 0.05*
	Type of microorganisms	< 0.05*

* P < 0.05 denoted statistically significant.

Table 2: Effect of extraction solvents on the inhibition zone (mm). Results were obtained from the nine microorganisms tested at four different maceration periods, with mean ± SD, where n = 108.

Extraction Solvent	Inhibition Zone (mm)
Hexane	6.90 ± 2.22^{a}
Petroleum ether	6.92 ± 2.22^{a}
Ethyl acetate	6.70 ± 1.60^{a,b}
Acetone	6.41 ± 0.52^{b,c}
Ethanol	6.31 ± 0.44^{b,c}
Distilled water	6.08 ± 0.25^{c}

* Different superscripts denoted statistical significance, with P < 0.05.

Table 3: Effect of different types of microorganisms on the inhibition zone (mm). Results were obtained from six extraction solvents used at four different maceration periods, with mean ± SD, where n = 72.

Type of Microorganism	Inhibition Zone (mm)
Gram postive bacteria:	
B. cereus	6.33 ± 0.48^{c,d}
B. subtilis	6.26 ± 0.41^{c,d}
E. faecalis	6.28 ± 0.51^{c,d}
S. aureus	6.29 ± 0.58^{c,d}
Gram negative bacteria:	
E. coli	7.68 ± 3.73^{a}
K. pneumonia	6.80 ± 0.63^{b}
P. aeruginosa	6.31 ± 0.89^{c,d}
Serratia spp.	6.05 ± 0.14^{d}
Fungus:	
C. albicans	6.96 ± 2.56^{b}

* Different superscripts denoted statistical significance, with P < 0.05.

On the other hand, on microbial susceptibility, analysis with Tukey's multiple comparison tests (Table 3) demonstrated that *E. coli* was the most susceptible microbe, followed by *C. albicans* and *K. pneumonia*.

In majority of the antimicrobial study conducted by other researchers, most of the antimicrobial agents obtained from plant extracts were more potent towards gram positive bacteria (Chia and Yap, 2011; Kaneria *et al.,* 2009; Rahman *et al.,* 2011; Somchit *et al.,* 2010; Srinivas *et al.,* 2010) while the results of this paper revealed the potency of *Momordica charantia* L. fruit extracts towards gram negative bacteria (*E. coli* and *K.pneumonia*) and fungi (*C. albicans*). The result from this finding is more prominent when the positive controls used in this study (ampicillin – 10ug/disc for bacteria and tetracycline – 30ug/disc for fungi in Table 5) were unable to inhibit *K. pneumonia*, while only low inhibition zone was demonstrated in both *E. coli* and *C. albicans*. Moreover, *E. coli* is well known for its multi-resistance towards drug (Sjölund *et al.,* 2008).

On the differences of maceration times toward the antimicrobial compounds extracted, it is notable that an increased in maceration period could improve the bioactive compounds extracted, where Zoecklein (2006) revealed that an increased contents of anthocyanin and tannin were found in wine,when longer maceration time was used. Subsequently, in a study done by Turkmen *et al.,* (2007) on antimicrobial activity of black tea using different extraction solvents, increasing of maceration time from 2h to 18h significantly increased the antibacterial activity of the extract, depending upon the microorganism tested as well as the solvents used.

In contrast to this study, the best maceration times found were 6h and 48h (no significance different) when Tukey's multiple comparisons test was imposed (Table 4). On maceration time, in a research done by da Silva Cunha *et al.,* (2006), the prolonged extraction periods did not enhance richer propolis extracts was found, nevertheless a decreased in the activity was observed.

Table 4: Effect of different maceration periods on the inhibition zone (mm). Results were obtained from nine microorganisms tested using six different extraction solvents, with mean ± SD, where n = 162.

Maceration Time	Inhibition Zone (mm)
6 h	6.74 ± 1.70^{a}
12 h	6.33 ± 1.00^{b}
24 h	6.42 ± 1.04^{a,b}
48 h	6.71 ± 2.39^{a}

* Different superscripts denoted statistical significance, with P < 0.05.

Table 5: Positive controls. Ampicillin (10ug/disc) was used for both gram positive and gram negative bacteria, while tetracycline (30ug/disc) was used for fungi. Results were mean ± SD, where n = 3.

Type of Microorganism	Inhibition Zone (mm)
Ampicillin (10ug/disc)	
Gram postive bacteria:	
B. cereus	8.11 ± 0.23
B. subtilis	8.30 ± 0.07
E. faecalis	14.54 ± 0.79
S. aureus	23.30 ± 1.11
Gram negative bacteria:	
E. coli	6.27 ± 0.01
K. pneumonia	6.00 ± 0.00
P. aeruginosa	18.17 ± 0.24
Serratia spp.	6.28 ± 0.10
Tetracycline (30ug / disc)	
Fungus:	
C. albicans	9.53 ± 0.09

CONCLUSIONS

This study revealed that maceration periods and type of extraction solvents are significantly influenced the inhibition zone (mm) of the nine potent microorganisms tested. Therefore, it is of great important to use an optimum maceration period in combination with suitable extraction solvents in order to obtain the desirable antimicrobial compounds from fruit of *Momordica chrantia* L. against the microorganisms tested.

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