

ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS IN MEDICINAL PLANT *Centella asiatica* AND STUDY THE EFFECTS ON FUNGAL ACTIVITIES

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doi: 10.15414/jmbfs.2021.10.4.631-635

ARTICLE INFO

Received 21. 3. 2020
Revised 17. 10. 2020
Accepted 27. 10. 2020
Published 1. 2. 2021

Regular article



ABSTRACT

Medicinal plants are sources of important therapeutic aids for alleviating human ailments. The present research investigation was carried out to study anti-fungal activity of *C. asiatica* were tested against *C. albicans*, *Aspergillus niger*, and *Penicillium* sp. using two methods, disc diffusion method and broth dilution method. *C. asiatica* crude methanol extract was found to be the most effective against fungal activity. Compared to disc diffusion, broth dilution was a more appropriate method to quantitatively determine the anti-Candida activity of plant extract, whereby the MIC values of the crude extracts was determined. The result from disc diffusion assay demonstrates that plant extracts have an inhibitory effect. However, the broth dilution method result reveal that *C. asiatica* crude methanol extract has lower MIC values, meaning it has more prove that *C. asiatica* has an anti-fungal effect. Bioactive analysis results reveled that bioactive compounds present in the leaf, stems, roots, and the whole plant extract from *Centella asiatica* are the major chemical constituents are n-Hexadecanoic acid (99%), cis-Vaccenic acid (91%), 5-Hydroxymethylfurfural (88%), Tetradecanoic acid (86%). Further study is required to find out the specific phytochemical which is responsible for its medicinal value.

Keywords: *Centella asiatica*, GC-MS, bioactive compounds and phytochemical

INTRODUCTION

Despite tremendous progress in human medicines, infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health (Bhuyar, 2017; Bhuyar *et al.*, 2020a; Bhuyar *et al.*, 2018). Their impact is particularly large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance. Clinical microbiologists have great interest in screening of medicinal plants for new therapeutics (Saengsawang *et al.*, 2020). The active principles of many drugs found in plants are secondary metabolites. The antimicrobial activities of plant extracts may reside in a variety of different components, including aldehyde and phenolic compounds (Bhuyar *et al.*, 2019a). The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants (Bhuyar *et al.*, 2020b). Hence the sensitivity study of bacterial strains to the plant *Centella asiatica* was evaluated.

Centella asiatica belongs to Apiaceae family and *Centella* genus. It is a tasteless, odorless plant that thrives in and around water. It has small fan-shaped green leaves with white or light purple-to-pink or white flowers and it bears small oval fruit. The whole plant is used for medicinal purposes (Loiseau *et al.*, 2012). It is widely used as a blood purifier as well as for treating high blood pressure, for memory enhancement and promoting longevity (Jagtap *et al.*, 2009). In Ayurveda, *Centella asiatica* is one of the main herbs for revitalizing the nerves and brain cells (Vogel *et al.*, 1990). Eastern healers relied on *Centella asiatica* to treat emotional disorders, such as depression, that were thought to be rooted in physical problems. In the Western medicine, during the middle of the twentieth century, *Centella asiatica* and its alcohol extracts reported to have shown positive results in the treatment of leprosy (Zainol *et al.*, 2003).

Variou well-known fungus causing fungal infections in humans. *Candida albicans* is an opportunistic pathogen typically colonizes the skin and mucosal epithelium of healthy individuals (Zainol *et al.*, 2008). In response to changes in the host environment, this yeast may become pathogenic causing various non-life-threatening infections, such as oral thrush and vaginitis (Bhuyar *et al.*, 2020). However, in cancer patients and immunosuppressed individuals, the yeast colonization may progress to invasion, resulting in severe systemic candidiasis. Invasive fungal infections are associated with high mortality rates and over 40% in bloodstream infections caused by *Candida albicans* (Cushnie *et al.*, 2005).

Despite appropriate anti-fungal therapy, mortality from candidemia is over 30% (Bhuyar *et al.*, 2020c). With the increasing number of immunocompromised patient population, these fungal infections are becoming more prevalent. In addition, *C. albicans* can also colonize various biomaterials, such as urinary and vascular catheters, and ventricular assist devices causing increasing incidence of candidiasis in hospital patients (Wang *et al.*, 2006). Another fact is up to 75% of sexually active women have at least, at time experienced vaginal candidiasis, an inflammatory condition caused by yeast predominately *C. albicans*. This condition results in severe genital itching, vagina odor and abnormal discharge (Inamdar *et al.*, 1996; Khammee *et al.*, 2020).

Responding to this worldwide concern, the proposed research is carried out to analyze and characterized the specific bioactive component in *Centella asiatica*. For instance, a bioactive component produce can be used to analyze mechanism activity of fungal and bacteria by adding the extraction of *C. asiatica*. The proposed research is done to compare the effect of plant extraction and specific drug to the growth production of bacterial and fungal by monitoring mechanism activity of pathogen in agar plate and broth dilution. Having said this, obviously, the objective of this proposed research is to reduce costs in terms of buying external expensive drug and to efficiently safe use for human purpose as it come from natural sources chemical.

MATERIAL AND METHODS

Fungal and bacterial strain

The type strain of *Candida albicans* ATCC 10231, *Aspergillus niger* and *Penicillium* sp. tested is subculture on Sabouraud dextrose agar (SDA) and Potato dextrose agar (PDA). Then, incubated for 24 h at 37°C. Inoculum obtained from the overnight agar culture.

Preparation of plant crude methanol extract

Centella asiatica were purchased from the local retail market. All plant materials washed thoroughly under running tap water and air dried at room temperature for two days and ground into a fine powder. The air-dried and powdered plant material are extracted with 400 ml of 95.5% methanol, kept in the room temperature for 72 h with periodic stirring. Thereafter the extract was filtered by

Whatman no 4. filter paper to remove the marc. The supernatant was collected and evaporated in a rotary evaporator under reduced pressure at a temperature of 64 °C (methanol bp) until an oily dark paste was formed then transferred to water bath to allow further evaporation of methanol. The plant extracts were stored in refrigerator at -80°C for 2 days before transferred to freeze dry for further usage.

Fungal inoculum preparation

The inoculum is prepared using 24-hour plate cultures of *Candida albicans*, *Aspergillus niger*, and *Penicillium* sp. The inoculums were prepared by taking at least three to five well-isolated colonies of the same morphology with a sterile loop and transferred into a tube containing 4 to 5 ml of Potato dextrose broth. The turbidity of the suspension is adjusted until it achieved the turbidity of the 0.1 McFarland standard (Bhuyar et al., 2019b). This resulted in a suspension containing approximately of 1 x 10⁸ CFU/ml. To aid comparison the test and standard are compared against a white background with a contrasting black line (Sampson et al., 2001).

Antifungal Assay

Antifungal assay used to study the plant extracts for antifungal activity. Firstly, the plant extracts are dissolved in methanol. Under aseptic conditions, 0.2 mL of standardized inoculum are spreaded on to sterile Sabouraud dextrose agar (SDA) plates and Potato dextrose agar (PDA) to achieve a confluent growth. The plates are then allowed to dry. Sterile filter paper discs of 5 mm diameter are loaded with 50 µL of the methanolic extracts (100 mg/mL). The paper discs were allowed to evaporate and then placed aseptically on the surface of the inoculated agar plates. Standard Fluconazole and Nystatin (30 µg) disc for antifungal activity were used as positive control while methanol (30 µL/disc) are used as negative control. Plates were then incubated at 37°C for 24 h. The experiment was performed in triplicates. At the end of the incubation period, the antifungal activity was measured by evaluating by zone of inhibition around each paper disc. The mean value of the diameter of the inhibition zone of the triplicates were selected as the final value.

Determination growth rate

The growth rate process is determined by broth dilution method as described by (Booncong, 2000). The fungal of *Aspergillus niger*, *Penicillium* sp. and *Candida albicans* were grown in Potato Dextrose Broth for overnight at 27°C temperature. The fungal sample was diluted until absorbance to get below than 0.1Abs. Extracts of stems, roots, leaves, and whole plants reconstituted in 95.5% methanol was pipetted into cuvettes in each of sample is 30µl in concentration. Same procedure was followed with 1000µl of fungal sample in Potato dextrose broth (PDB). Extract-free solution was used as a negative control. The growth rate measurement process (absorbance) was started with 0 second followed with 30 minutes interval until the growth rate in steady state.

The growth rate process will show the growing process of fungal in presence of inhibitor which is parts of *Centella asiatica* and other controls. The measurement of growth whether increase or decrease is observed in every 30 minutes. Minimum Fungicidal Concentration (MFC) used to determine if the plant extracts were fungistatic or fungicidal using (Hamid et al., 2002) method. To determine the growth rate, 1000 µL of fungal broth collected (*Aspergillus niger*, *Penicillium* sp. and *Candida albicans*) and pipette into sterile cuvettes. After that, 30ul of extract sample (each fungal with four different sample of stems, roots, leaves, and whole plants) are pipette into the cuvettes. The absorbances are measure in every 30 minutes which they were observed for growth curve of fungus.

Chemical Composition Analysis by GCMS

To test the bioactive compounds, present, preliminary phytochemical analysis was carried out by using the standard method to identify the components in the various plants extracts of *Centella asiatica*. The methanol extracts of *C. asiatica* was subjected to the analysis of chemical components by gas chromatography-mass spectrometer (GC-MS) (Ramli et al., 2020; Bhuyar et al., 2019b). A high-temperature column (Inert cap 1MS; 30 m x 0.25 mm id x 0.25µm film thickness) was used whereby a high- temperature column was applied. The injector and detector temperature were set at 280°C while the initial column temperature was set at 100°C. A 5µl sample volume was injected into the column and ran using split (1:10) mode. After 1 min, the oven temperature was raised to 225°C at a ramp rate of 12.5°C/min (hold time 4 min). The oven temperature was then raised to 300°C at a ramp rate of 7.5°C/min (hold time 5 min). The helium carrier gas was programmed to maintain a constant flow rate of 17.5 ml/min and the mass spectra were acquired and processed. Finally, the mass was compared with NIST library search and authentic standards to identify the compound (Borhan, et al., 2013).

RESULTS AND DISCUSSION

Antifungal activity of *Centella asiatica*

Antifungal activity determined by the disc diffusion assay as described by (Devi and Prabakaran, 2014) Antifungal activity of each plant extract was evaluated based on the diameter of the clear inhibition zone surrounding the paper discs. If there is no inhibition zone, it is assumed that there is no antifungal activity. Table 1, 2 and 3 shows the antifungal activity of stems, roots, leaves, and whole plant methanol extract against *Candida albicans*, *Aspergillus niger*, *Penicillium* sp. At the end of the analyses, all the plant extracts were found to have an inhibitory effect against the tested fungi strain. The potency and activity of antifungal is usually determined by zone of inhibition on agar plates. Inhibitory activity of the paper disc diffusion test revealed that the crude methanolic extracts of *C. asiatica* showed that there has inhibition process occur and visible of difference degrees of growth inhibition in difference fungal.

Based on the Table 1 and Figure 1a of antifungal activity for *Aspergillus niger*, it shows that there is not any inhibition zone visible in stems, roots, leaves, whole plants, water crude extracts and control. From the previous study, the Fluconazole and nystin is an antibiotic that can kill the fungal infection and grow (Brinkhaus et al., 2000). So, positive control should show an inhibition that kill the fungal instead of none. The negative results on the control show an error occur as the control must give positive result of zone inhibition. Table 2 and Figure 1b shows *Penicillium* sp. anti-fungal activity, the results above show that only control (Nystatin antibiotic) give a positive result which is visible of inhibition zone. No zone inhibition results visible on all parts of *C. asiatica* (stems, roots, leaves, whole plants, crude water extracts). Its shows that the part of *C. asiatica* do not have any antifungal properties to kill *Penicillium* sp. For the *C. albicans*, the figure 1c and table 3 above shows, there is a positive result as zone of inhibition is visible. From the observation, stems, roots, leaves, whole plant shows that they have an inhibitory property to against *C. albicans*. Compared to the other of water plant extract, both of control, which is nystatin in, and fluconazole antibiotic give a negative result as no zone inhibition visible. Based on the previous study, both of this antibiotic are strong enough to kill the *C. albicans* but here there shows no inhibition.

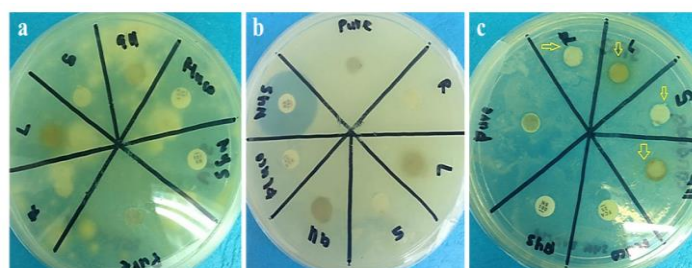


Figure 1 (a) Zone inhibition of *Aspergillus niger* (b) Zone of inhibition of *Penicillium* sp. (c) Zone of inhibition of *Candida albicans*

Table 1 Result of zone of inhibition of *Aspergillus niger* of methanolic extraction of *Centella asiatica* in difference parts.

Parts of Plants	Positive	Negative
Roots	-	-
Stems	-	-
Leaves	-	-
Whole plants	-	-
Crude water extract	-	-
Nystin	-	-ve
Fluconazole	-	-ve

Table 2 Result for zone of inhibition of *Penicillium* sp. of methanolic extraction of *Centella asiatica* in difference parts.

Parts of Plants	Positive	Negative
Roots	-	-
Stems	-	-
Leaves	-	-
Whole plants	-	-
Crude water-extract	-	-
Nystin	+ve	-
Fluconazole	-	-ve

Table 3 Result for zone of inhibition of *Candida albicans* of methanolic extract of *Centella asiatica* in difference parts.

Parts of Plants	Positive	Negative
Roots	+	
Stems	+	
Leaves	+	
Whole plants	+	
Crude water extract	-ve	
Nystin		-ve
Fluconazole		-ve

The broth dilution method is used to determine the growth rate of plant extracts. By using this method, the antifungal activity can be noticed even at low concentration. The result of this test revealed that the *C. asiatica* crude methanol extract have an antifungal property that can inhibit antifungal against *C. albicans*, *Penicillium* sp., and *Aspergillus niger niger*. All the part of *Centella asiatica* of the plant extract contain active compound that cause mitochondrial damage to the yeast (Gray et al., 2018; Ling et al., 2000).

Growth Rate

The broth dilution method is used to determine the inhibition process that presence during the time interval of plant extracts against fungal. By using this method, the antifungal activity can be noticed even at low concentration by observation the absorbance using spectrophotometer. The result of this test revealed that the *C. asiatica* methanol extract shows there have antifungal activity against *C. albicans*, *Aspergillus niger*, and *Penicillium* sp. All of the plant extract contains active compounds that cause mitochondrial damage to the yeast (Diallo, 1991; Guzewska et al., 2006). The difference between the antifungal activities towards difference part of *Centella asiatica* may be due the type of difference bioactive compounds in difference part of plant extracts such as stems, roots, leaves, whole plants and crude water extracts. This finding is supported by previous research (Rakotoniriana et al., 2008; Gupta, et al., 1999) who had found that the biological effects of the essential oils depend on the type and quantity of active compounds. The effectiveness against fungus of *Centella asiatica* oil is affected by their chemical compositions and compounds.

Table 4 Growth rate of *Aspergillus niger* in different parts of *Centella asiatica* plant samples

Time interval (minutes)	Roots	Stems	Leaves	Whole Plants
0	0.071	0.134	1.135	0.565
30	0.067	0.112	0.957	0.411
60	0.056	0.097	0.851	0.364
90	0.054	0.081	0.727	0.235
120	0.052	0.077	0.665	0.209
150	0.049	0.072	0.592	0.180
180	0.144	0.167	0.639	0.245

From the table 4, it shows that the growth rate of *Aspergillus niger* in different parts of *C. asiatica* plants sample. From the graph, the growth curves (GC) of the fungi *Aspergillus niger* showed that the PDA medium at room temperature of 27°C well suited the in vitro assays in the cuvette. The each of the sample of stems, leaves, roots, and whole plants is observed from every 30 minutes interval from the first 0 minutes to the minutes of 180. From the reading of table 4 above, the absorbance of all the part of *C. asiatica* did significantly affect fungal Growth Rate (GR) and markedly inhibited or completely prevented the growth of fungi tested.

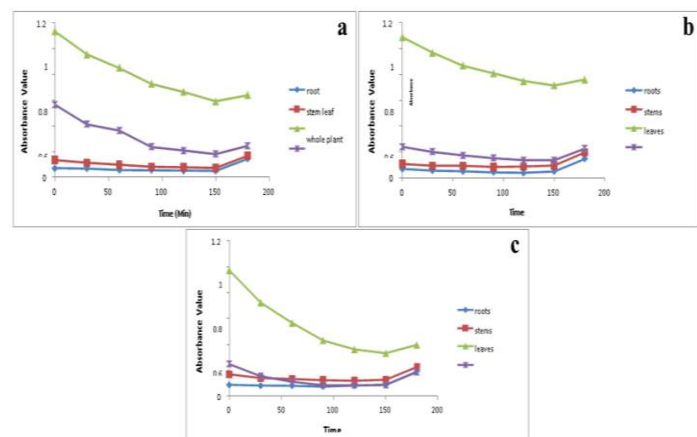


Figure 2 (a) Effect of different parts of *Centella asiatica* on *Aspergillus niger* (b) Effect of Different parts of *Centella asiatica* on *Penicillium* sp. (c) Effect of different parts of *Centella asiatica* on *Candida albicans*

From the table and figure 2a above the inhibition process continuing decreasing from 0 min until minutes of 150. After minutes of 150 to minutes of 180, the reading of absorbance is increased in all the parts of plants. A time-response relationship was generally observed for each fungus, with average fungal GR decreasing when the time is increased.

Figure 2 b above clearly shows the reduction of growth rate (GR) of *Penicillium* sp., from minutes of 0 to minutes of 150. It means that the bioactive compound in the stems, leaves, roots, and whole plants inhibit the fungi growth. It shows the plant of *C. asiatica* have an antifungal property in each of parts. When the time is increased from zero minutes to 150 minutes, the growth rate of the fungi is decrease, and is well depicted in figure 2b above. There is growth actively occur in the minutes of 150 to minutes of 180 where the inhibition process of fungi is stop and start to increase. It is clearly stated at the table 5 above where it shows the reading absorbance of each sample of stem, leaves, roots and whole plants.

Table 5 Growth rate of *Penicillium* sp. in different parts of *Centella asiatica* plants sample

Time interval (minutes)	roots	stems	leaves	Whole plants
0	0.071	0.109	1.089	0.242
30	0.057	0.096	0.969	0.202
60	0.052	0.095	0.868	0.175
90	0.043	0.086	0.81	0.154
120	0.04	0.089	0.75	0.138
150	0.05	0.097	0.715	0.138
180	0.147	0.198	0.761	0.227

Table 6 Growth rate of *Candida albicans* in different parts of *Centella asiatica* plants sample

Time interval (minutes)	roots	stems	leaves	whole plants
0	0.089	0.17	0.971	0.248
30	0.083	0.141	0.723	0.155
60	0.081	0.133	0.567	0.112
90	0.075	0.124	0.43	0.085
120	0.083	0.12	0.362	0.085
150	0.088	0.128	0.333	0.089
180	0.191	0.226	0.398	0.187

From the results below, it shows that growth rate of *C. albicans* towards *C. asiatica* extracts of stems, leaves, roots, and whole plants were tested against fungi. Therefore, the antifungal activities from all part of *C. asiatica* were tested using the same methods of broth dilution and was observed by measuring the growth rate process through absorbance measurement. It shows that all parts of stems, leaves, roots and whole plants can inhibit the process of fungi growth (Bhuyar et al., 2020d). It means that the *C. asiatica* have an antifungal property that can prevent the process of fungi from growth. From the table 6, it is clearly stated the reading of absorbance is decrease when the time is increased. But in the minutes of 150, the process of inhibition of fungal is depleted and start to growth back.

According to results obtained it shows that the growth rate of fungal *Penicillium* sp., *Aspergillus niger*, and *C. albicans*. The inhibitory effects of different part of *C. asiatica* such as stems, roots, leaves and whole plants on the growth rates (GR) of three pathogenic fungi (*Aspergillus niger*, *C. albicans*, *Penicillium* sp.) were examined using a cuvette and a spectrophotometer reader. The antifungal activity of the fungus is observed within 30 minutes interval. From that, we can see that all the part of *C. asiatica* showing a positive result as they can inhibit the fungal growth. So, it clearly shows that the bioactive component in the *C. asiatica* have an antifungal property in the stems, roots, leaves, and whole plant can inhibit the process of fungal growth as any compound with fungicidal properties could be potentially has inhibitory activity on fungal growth or sporulation. In the minutes of 150, the growth of fungal seems continuing active and start to growth.

Bioactive Compounds

In the last few years gas-chromatography mass-spectrometry has become firmly established as a key technological platform for metabolite profiling in both plant and non-plant species (Bhuyar et al., 2019c). Until relatively recently only a limited number of plant research laboratories had access to gas-chromatography mass-spectrometry instrumentation, however, such machines are increasingly becoming more common place. The table 7 shows that the bioactive compound that presence in the *Centella asiatica* plant extract of methanol. The major compound is n-Hexadecanoic acid which percentage of 99. The second major compound presence is cis-Vaccenic acid at peak 8 with percentage 91. Followed by 5-Hydroxymethylfurfural 88 percent, Tetradecanoic acid and so on. The identified compounds possess many biological properties. For instance, n-Hexadecanoic acid, cis-Vaccenic acid, 5-Hydroxymethylfurfural and Tetradecanoic acid Previously, 5-HMF known to exhibited novel antioxidant

activity, also act as inhibitor that effect of 5-HMF on human cancer cell proliferation was investigated by MTT assay, flow cytometric analysis, and the TUNEL and DAPI costaining assay. It is displayed higher antiproliferative activity on human melanoma A375 cells than other cell lines potent antiangiogenic agent in colorectal cancer and in HUVEC cells. The other compounds such as n-Hexadecanoic acid, are reported to be antimicrobial.

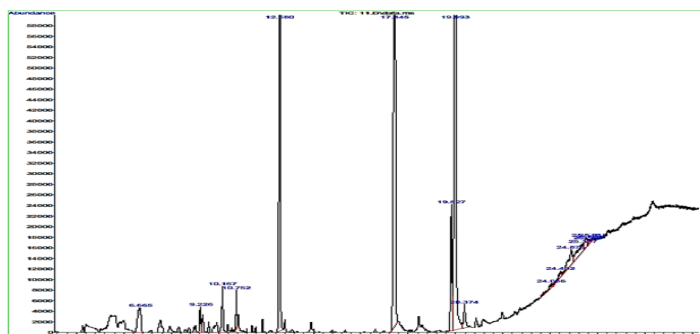


Figure 3 Total ionic chromatogram biological active compounds of methanolic extract of whole part of *Centella asiatica*

Table 7 Total ionic chromatogram biological active compound of methanol extract of whole plants of *Centella asiatica*

Peak	Retention time	Compounds	Percentage
5	12.580	5- Hydroxymethylfurfural	88
6	17.443	n-Hexadecanoic acid	99
7	19.829	Tetradecanoic acid	86
8	19.995	cis-Vaccenic acid	91
10	24.035	4,7,10,13,16-Pentaoxonadeca-1,18122476058185-54-9 78-diene	78
11	24.430	2,5,8,11,14,17-Hexaoxaoctadecane	64
12	24.876	Propane, 1,2,3- trimethoxy-	53
13	25.374	Silane, methylenebis	64
14	25.517	15-Crown-5	64
17	25.809	3,6,9,12- Tetraoxatetradecan-1- ol	56

By referring and comparing with the previous research, we can determine the substances presence in the *C. asiatica* that act as therapeutic interest are the saponin- containing triterpene acids and their sugar esters, the most important being: asiatic acid, madecassic acid and the three asiaticosides, asiaticoside, asiaticoside A and asiaticoside B (Brinkhaus et al. 2000; Devkota et al., 2010). The structures of the three triterpenoid trisaccharides asiaticoside, asiaticoside-A and asiaticoside-B, have been elucidated by spectroscopic analysis as the [O-β-L-rhamnopyranosyl-(1→4)-O-β-D-glucopyranosyl (1→6)]-O-β- glucopyranose esters of 2β,3β,23β-trihydroxy-urs-12-ene-28-oic acid, of 2β,3β,6β,23v-tetrahydro-urs-12-ene-28-oic acid and of 2β,3β,6β,23β-tetrahydroylean- 12-ene- 28-oic acid (Devkota et al., 2010). The main active principles of *C. asiatica*, are the triterpenoids glycosides asiaticoside and madecassoside (asiaticoside A), used for the quantification of this species as described in the European Pharmacopoeia, and their respective aglycons (asiatic acid and madecassic acid). Significant differences in active constituent contents have been observed between samples of *Centella asiatica* originating from different countries (Chen et al., 2007). Depending on the source of the material, the amount of the triterpenoids may vary widely from 1% to 8%. Fig. 1 (Brinkhaus et al., 2000) As a conclusion, by comparing the result of previous research and this research, less similarity of the compound presence in this experiment maybe due to improper procedure.

CONCLUSION

Thousands of years, human utilize plant extracts and essential oil in daily life as natural remedy against various infections. Until today, it is widely used in food preservation and pharmaceuticals alternative medicine. The anti-fungal activity of *C. asiatica* were tested against *C. albicans*, *Aspergillus niger*, and *Penicillium* sp. using two methods, disc diffusion method and broth dilution method. *C. asiatica* crude methanol extract was found to be the most effective against fungal activity. Compared to disc diffusion, broth dilution was a more appropriate method to quantitatively determine the anti-Candida activity of plant extract, whereby the MIC values of the crude extracts can be determined. The result from disc diffusion assay demonstrates that plant extracts have an inhibitory effect. However, the broth dilution method result reveal that *C. asiatica* crude methanol extract has lower MIC values, meaning it has more prove that *C. asiatica* has an anti-fungal effect. In conclusion, *C. asiatica* crude methanol extract was found to be a as antifungal agent against fungal infection especially *C. albicans*, *Aspergillus niger*, and *Penicillium* spp. Hence, it represents an alternative source of natural antifungal substances.

Acknowledgments: The authors gratefully acknowledged Universiti Malaysia Pahang for the financial assistance through the Internal Research Grant No. RDU190337/ RDU1903125 and Flagship Grant No. RDU182205. Author [Prakash Bhuyar] is thankful to UMP for providing Doctoral Research Scholarship DRS as a financial support.

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