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ORIGINAL ARTICLE

Comparative Antioxidant and Anti-inflammatory Activity of Different Extracts of *Centella asiatica* (L.) Urban and Its Active Compounds, Asiaticoside and Madecassoside

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ABSTRAK

Keupayaan Centella asiatica (CA) untuk bertindak sebagai antioksidan dan agen anti-radang telah banyak dilaporkan. Namun begitu, kaedah pengekstrakan CA untuk memperoleh hasil yang terbaik masih dipersoalkan. Dalam kajian ini, kami menilai tiga kaedah pengekstrakan CA dan membuat perbandingan ekstrak dari segi aktiviti antioksidan dan anti-radang, dan juga kandungan sebatian bioaktif, asiaticoside dan madecassoside. Centella asiatica diekstrak menggunakan pelarut etanol, metanol dan juga air. Kandungan sebatian fenolik ekstrak diukur menggunakan kaedah reagen Folin-Ciocalteu. Kandungan asiaticoside dan madecassoside ditentukan dengan kaedah HPLC. Aktiviti antioksidan diukur dengan asai 2,2-diphenyl-1-picrylhydrazyl (DPPH) dan asai penurunan kuasa Ferric Reducing Antioxidant Power (FRAP). Aktiviti anti-radang ditentukan dengan kebolehan ekstrak untuk merencatkan enzim tapakjalan keradangan, COX-1 dan COX-2, serta kebolehan ekstrak melindungi sel fibroblas aruhan 12-O-tetradecanoylphorbol-13-acetate (TPA) daripada menghasilkan prostaglandin E_2 (PGE_2). Hasil kajian menunjukkan aras sebatian fenolik, asiaticoside dan madecassoside tertinggi dalam ekstrak etanol, diikuti metanol dan esktrak akues (masing-masing 17.76 g/100g, 15.52 g/100g, 13.16 g/100g untuk sebatian fenolik, 42.86 mg/g, 36.37 mg/g, 2.82 mg/g untuk asiaticoside and 18.66 mg/g, 15.87 mg/g, 3.75 mg/g untuk madecassoside). Ketiga-tiga ekstrak menunjukkan aktiviti antioksidan sederhana berbanding kawalan positif. Kesemua ekstrak, asiaticoside dan madecassoside merencat COX-1 dan COX-2 dan menyekat penghasilan PGE_2 -aruhan TPA. Ekstrak etanol dan metanol merupakan perencat COX yang lebih kuat dan lebih poten daripada ekstrak akues. Oleh itu, walaupun ekstrak akues menunjukkan kebolehan antioksidan yang lebih tinggi, dari segi aktiviti anti-radang, pelarut hidrofobik iaitu etanol dan metanol ternyata lebih baik untuk mengekstrak Centella asiatica.

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Kata kunci: Antioksidan, anti-radang, ekstrak Centella asiatica, asiaticoside, madecassoside

ABSTRACT

The potential of *Centella asiatica* (CA) as an antioxidant and anti-inflammatory agent has been well described. However the extraction method which gives the best yield is debatable. In this study, we evaluated three different methods of extractions and compared the extracts in terms of antioxidant, anti-inflammatory activities as well as the contents of its bioactive compounds, asiaticoside and madecassoside. *Centella asiatica* was extracted using ethanol, methanol and aqueous extraction methods. The extracts were then measured for their phenolic contents using Folin-Ciocalteu reagent. Asiaticoside and madecassoside were determined using HPLC. Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DHPP) and ferric reducing antioxidant power (FRAP) assays. Anti-inflammatory activities were determined by the ability of the extracts to inhibit the inflammatory pathway enzyme, COX-1 and COX-2 as well as their ability to protect fibroblasts against 12-*O*-tetradecanoylphorbol-13-acetate (TPA) -induced production of prostaglandin E_2 (PGE_2). Results showed that the level of phenolic constituents, asiaticoside and madecassoside were highest in the ethanol, followed by methanol and then aqueous extracts (17.76 g/100g, 15.52 g/100g, 13.16 g/100g for phenolics, 42.86 mg/g, 36.37 mg/g, 2.82 mg/g for asiaticoside and 18.66 mg/g, 15.87 mg/g, 3.75 mg/g for madecassoside respectively. All extracts showed considerable antioxidant activity compared to the positive controls. The extracts, asiaticosside and madecassoside inhibited both COX-1 and COX-2 and suppressed the TPA-induced production of PGE_2. The ethanol and methanol extracts were stronger COX inhibitors and more potent suppressor of PGE_2 formation than aqueous extract. Thus although the aqueous extract showed higher antioxidant potential, in terms of anti-inflammatory activities, the hydrophobic solvents, ethanol and methanol, proved to be the better extraction method for *Centella asiatica*.

Key words: Antioxidant, anti-inflammatory, *Centella asiatica* extracts, asiaticoside, madecassoside

INTRODUCTION

Inflammation is part of normal host response to infection and injury. Although inflammation helps clear infection, and along with repair, makes wound healing possible, both inflammation and repair have considerable potential to cause harm. Prolonged presence of inflammatory agents may lead to chronic inflammation.

Chronic inflammation can result from various inflammatory agents such as virus, bacteria or antigens that stimulate the activation of inflammatory cytokines (Hussain et al. 2003; Bartsch & Nair 2006). Chronic inflammation processes

increase oxidative and nitrasative stress and lipid peroxidation by inducing oxidant-generating enzymes such as NADPH oxidase, inducible nitric oxide synthase (iNOS) thereby producing excess of diverse reactive oxygen and nitrogen species (ROS and RNS). ROS, RNS and lipid peroxidation products (LPO) are able to cause damage to cell, protein, mRNA, DNA and lipid in both nucleus and mitochondria (Ohshima et al. 2003; Sawa & Ohshima 2006). Lipid peroxides (LPO) such as malondialdehyde (MDA), for example, form adducts with DNA bases. This then leads to alterations in the expressions of signaling molecules (Chung et al. 1996; Bartsch 1999), (West & Marnett 2006), and the functions of enzymes and proteins involved in inflammation such as cyclooxygenase-2 (COX-2) and iNOS (Ohshima 2003).

Chronic inflammation is abnormal and the excessive ROS, RNS and LPO produced, facilitate carcinogenesis and other chronic diseases (Hussain et al. 2003; Bartsch & Nair 2005).

Several medicinal herbs have been shown to inhibit inflammation as well as antioxidant activity. The antioxidant activity can further protect from oxidative and nitrosative stress damages induced by inflammation process. Compounds from fenugreek spice (Liu et al. 2012), and polyphenols from green tea (Tipoe et al. 2007) have been found to exhibit anti-inflammatory and antioxidant activities. Locally known as *pegaga, Centella asiatica* is a perennial creeper commonly found in moist areas in tropical and subtropical countries (Brinkhaus et al. 2000). *C. asiatica* has been used extensively in Ayurvedic and Chinese traditional medicines to treat various ailments such as wounds, ulcer and leprosy (Cheng & Koo 2000; Shetty et al. 2006). The bioactive pentacyclic triterpenoid compounds of *C. asiatica*, asiaticoside and madecassoside, have been widely studied, and reported to have various biological properties such as wound healing (Shukla et al. 1999), ulcer healing (Guo et al. 2004), anti-apoptosis (Bian et al. 2008), and also immunomodulatory property (Jayathirtha & Mishra 2004).

Various methods have been described to prepare *C. asiatica* extract. However, there is a lack of comparative data on the best method for extracting these bioactive compounds in terms of antioxidant and anti-inflammatory properties. In this study, we compared three different extraction methods for *C. asiatica* using ethanol, methanol and water and then evaluated the extracts in terms of the phenolic, asiaticosside and madecassoside contents, antioxidant and anti-inflammatory activities.

MATERIALS AND METHODS

Plant Material

Centella asiatica was bought from a local market in Kuala Lumpur and the sample was deposited at UKM herbarium with voucher number UKMB29984. The whole plant was washed thoroughly with tap water, and dried at 40 ^{0}C until constant weight and coarsely grounded. Pure standards of asiaticoside and madecassoside were obtained from Biopurify (Chengdu, China).

Extracts Preparation

Sohxlet extraction was used for the preparation of methanol and ethanol extracts. 50g of *Centella asiatica* coarse plant powder was extracted with 1 litre of either methanol or ethanol for 24 hours and then filtered to collect the extracts. The extracts were then concentrated under vacuum and excess solvent were removed using rotary evaporator.

For the preparation of aqueous extract, 50 g of coarse plant powder in 1 litre of distilled water was boiled for 1 hour and the extract filtered. Extract were then freeze-dried.

Determination of Asiaticoside and Madecassoside Content

The level of asiaticoside and madecassoside in all CA extracts were determined using HPLC. 1mg of ethanol or methanol CA extracts were dissolved in 1ml of methanol. As for the aqueous extract, 1mg of extract was dissolved in ultra pure water. Sample extracts were filtered through 0.2 m filter. A volume of 20 l was injected using an autosampler into the HPLC (Shimadzu, Japan) equipped with a diode array detector. The solvent used for gradient elution were 1% ortophosphoric acid and acetonitrile. The concentration of 1% ortophosphoric acid was set at 5% for the first 5 minutes and then decreased to 80%, 50% and 20% for the next 10, 5 and 7 minutes respectively. The concentration of the 1% ortophosphoric acid was then increased to 50%, 80% and 95% for 5 minutes for each concentration. The analytical column used was ACE C18 with packing material of 5 m particle size at a flowrate of 1.0ml/min at room temperature. Samples were read at 210nm wavelength. Pure standards of asiaticoside and madecassoside concentration range (15 g/ml to 500 g/ml) were used to calibrate standard curve and retention times.

Total Phenolic Content Assay

Folin-Ciocalteu reagent (diluted 10-fold) was used to determine the total phenolic content of all CA extracts (Velioglu et al. 1998). 100 l of each sample was mixed with 0.75 ml of Folin-Ciocalteu reagent and allowed to stand at 22°C for 5 minutes before adding 0.75 ml sodium bicarbonate (60 g/L). The mixture was incubated at 22°C for 90 minutes and then read at 725 nm wavelength. Rutin was used as standard for the construction of calibration curve. The assay was carried out in triplicates.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The radical scavenging activity of each extract was determined using *2,2-diphenyl-1-picrylhydrazyl* (DPPH) assay as described byUllah et al, 2009 (Ullah et al. 2009). 0.25ml aliquots of each of the CA extracts or bioactive compounds (10-1000 g/ml) were added to 0.75ml of freshly prepared ethanol DPPH solution (0.02mM). The optical density changes were measured at 517nm after 30 minutes. Blanks were used to remove the influence of colour of the samples. Ethanolic solution of DPPH was used as negative control. Butylated hydroxytoluene (BHT) and

ascorbic acid were used as positive controls in the same concentration range (50-1000 g/ml) as were used for the samples. Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula :

% free radical scavenging activity =

$$\left[1 - \left(\frac{Sample\ OD}{Control\ OD}\right)\right] \times 100$$

Ferric Reducing Antioxidant Power (FRAP) Assay

Working FRAP reagent was prepared by mixing 25 ml of acetate buffer pH 3.6, 25 ml of TPTZ (2,4,6-tripyridyl-s-triazine) solution, 2.5 ml of $FeCl_3.6H_20$ solution and 3 ml of distilled water (Benzie & Strain 1996). The reaction of mixture of aliqout of CA extracts and bioactive compounds (25µl) with working FRAP reagent (175µl) was monitored for up to 4 minutes and the optical density changes were read at 593 nm. FRAP values were obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ion in known concentration using $FeSO_4.7H_2O$ solution.

COX Enzymes Assay

Anti-inflammatory activity of CA extracts and bioactive compounds was evaluated by its ability to inhibit the inflammatory pathway enzyme, cyclo-oxygenase (COX) which catalyses the production of PGE_2. This was determined using COX Inhibitor Screening Assay Kit (Cayman Chemical ACE, USA). 20µl of extract solutions (30 g/ml) or bioactive compounds (5 g/ml) were added to the COX-1 and COX-2 tubes respectively. The mixtures were incubated for 10 minutes at 37°C. The reaction was initiated by the addition of 10µl of arachidonic acid to all the test tubes. The tubes were shaken vigorously and allowed to stand for another 2 minutes at 37°C. After that, 50µl of 1 M HCl was added to each test tube to stop the reaction. Test tubes were removed from the water bath and 100µl of the saturated stannous chloride solution was added to each test tube. The tubes were then shaken vigorously before leaving them to stand for 5 minutes at room temperature. The prostaglandin E_2 (*PGE_2*) produced were then quantified using a commercial Prostaglandin E_2 Monoclonal-EIA Kit (Cayman Chemical ACE, USA).

Suppression of TPA-Induced Prostaglandin E2 Production

The ability of the extracts, asiaticoside and madecassoside to suppress TPA-induced inflammation was evaluated by measuring the production of PGE2 which is produced during the inflammatory process. Fibroblast cells were incubated with TPA and either of the extracts, asiaticosside or madecassoside for 24 hours. The amount of PGE2 produced was measured using the Prostaglandin E_2 Monoclonal-EIA Kit (Cayman Chemical ACE, USA).

Cells (5 x 10^3) were seeded in *Dulbecco modified Eagle's medium* (DMEM) supplemented with 10% fetal calf serum (FCS) in a 96-well plate and were incubated in a humidified atmosphere with 5% CO_2 at 37°C, for

24 hours. Cells were then incubated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and either of the extracts, asiaticosside and madecassoside for a further 24 hours. The supernatant from the tissue culture was then collected and quantified by EIA procedure.

RESULTS AND DISCUSSION

Total Phenolic and HPLC Analysis

The level of total phenolic constituents, asiaticoside and madecassoside in the three different extracts are shown in Table 1.

The results showed that ethanol is the most effective solvent in extracting phenolic compounds as well as the bioactive compounds, asiaticoside and madecassoside. The total concentration of phenolic compounds extracted in this study for the three different extraction solvents was higher than that previously reported by Zainol et al. (2003). The levels of asiaticoside and madecassoside in both ethanol and methanol extracts of CA were in agreement with previous study which reported that values of triterpenic acid sugar esters including asiaticoside and madecassoside of 1-8% of plant material (Kartnig & Hoffmann-Bohm 1992) and higher than that reported by Jayathirta & Mishra (2004), who reported a yield of only 0.18 % of asiaticoside.

However the yield of asiaticoside (0.28%) using aqueous extraction produced lower yield (4%) than that reported by Guo et al. (2004). The low yield of asiaticoside and madecassoside in our aqueous extract could be due to the use of high temperature during extraction of plant materials. It has been reported that, high temperatures would decrease the concentration of asiaticoside, and madecassoside (Kormin 2005). Distribution of asiaticoside and madecassoside in C. *asiatica* plant were also organ specific, with the highest concentration found in leaves (Aziz et al. 2007). Plant origin could also be a factor for variations in asiaticoside content, as Das & Mallick (1991) has reported that *C. asiatica* growing at higher altitude contained more asiaticoside than plants from lower altitude.

Antioxidant Activity by DPPH and FRAP Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the antioxidant activity of CA extracts based on their ability to trap unpaired electrons of DPPH. Extracts and compounds that could decolorize DPPH solution (purple) to yellow suggest their antioxidant potential. The DPPH scavenging activity of CA extracts in the concentration range of 50 - 1000 g/ml are shown in Figure 1. Among CA extracts, the aqueous extract showed the strongest antioxidant activity at 1000 g/ml reaching a 79.4 % of DPPH radicals scavenging activity compared to methanol extract and ethanol extract with 77.3 % and 65.7 % respectively, at the same concentration level. However, both asiaticoside and madecassoside did not show any detectable DPPH scavenging activity, hence data is not shown.

The ferric-reduction ability of plasma (FRAP) assay also enables the

Table 1: Total phenolic content (g Rutin equivalent/100g) of CA extracts and bioactive compounds content (mg/g of extract), asiaticoside and madecassoside from HPLC analysis. Results were expressed as means ± SD, n=3.

Compounds	Total Phenolic Content (g/100g)	Asiaticoside (mg/g)	Madecassoside (mg/g)
Ethanol Extract	177.6	42.86	18.66
Methanol Extract	155.2	36.37	15.87
Aqueoous Extract	131.6	2.82	3.75

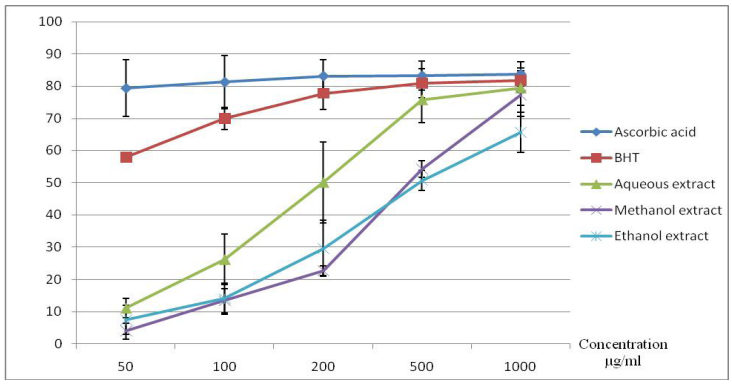

Figure 1: The DPPH scavenging activity of CA extracts. Ascorbic acid and BHT were used as positive controls. Results were expressed as means ±SD (n=6).

evaluation of antioxidant potential based on the ability to reduce ferric ion (Fe3+) to ferrous ion (Fe2+). The antioxidant potential is shown as FRAP reagent decolorizes from purple to blue, as a result of the formation of ferrous-tripyridyltriazine (TPTZ) complex. The FRAP values of CA extracts in the concentration range of 500 g/ml - 2000 g/ml are shown in Table 2. At all concentrations, all CA extracts reduced the ferric-diTPTZ less than ascorbic acid and tocotrienol-rich fraction (TRF) that were used as positive controls. Among the extracts, aqueous extract showed slightly better ferric reducing ability than ethanol and methanol extracts at all concentrations tested. Consistent with DPPH assay, both asiaticoside and madecassoside did not show appreciable ferric reducing activity, hence data not shown.

Despite having the lowest total phenolic content among the CA extracts, aqueous extract possessed the strongest free radical scavenging activity as determined by the DPPH assay. Based on a previous study, the antioxidant activity of polar extracts could be due to the presence of compounds with free hydroxyls, such as flavonoids (Mensor et al. 2001). Levels of flavonoids in the aqueous extract of CA could be higher compared to ethanol and methanol extract due to it being extracted in most polar water, compared to less polar ethanol and methanol solvent. Flavonoids make

Table 2: Ferric Reducing Antioxidant Power (FRAP) value of CA extracts and positive controls ascorbic acid and TRF. Results were expressed as means ± SD (n=3). EE; Ethanol extract, ME; Methanol extract, AE; Aqueous extract, AA; Ascorbic Acid, TRF; Tocotrienol-rich fraction. [a,b,c,d] $p<0.05$ vs. EE,ME,AE and AA, respectively at similar concentration.

Concentration (g/ml)	Compounds				
	EE	ME	AE	AA	TRF
500	122±36.8	141±20.5	189±8.7	3681±18.5[a,b,c]	1733±27.6[a,b,c,d]
1000	223±9	314±11.6	417±7.6[a]	3584±100.9[a,b,c]	3211±159.9[a,b,c]
1500	558±23.7	461±61.8	604±12.5	3954±59.6[a,b,c]	3866±155.5[a,b,c]
2000	689±29.4	685±73.5	863±216.5	4109±35.5[a,b,c]	4901 + 20[a,b,c,d]

good antioxidant agents since they possess a number of free hydroxyls which can scavenge free radicals (Cao et al. 1997; Basile et al. 2005). In contrast to our findings, a previous study on CA extraction has shown that ethanol extract had the highest antioxidant activity when compared with aqueous extract (Hamid et al. 2002). However, the differences might be due to different methods of extraction and assays used to measure the antioxidant activity. The results also suggest that the antioxidant activity of CA resides in water soluble compounds. This is also in agreement with several reports that high polarity solvents are effective in extracting natural antioxidants (Duh et al. 1992; Tian & White 1994).

Anti-Inflammatory Activity

The concentration of CA extracts (30 g/ml) and the two bioactives (5 g/ml) used for the anti-inflammatory studies were predetermined using MTS assay to ensure cell viability (data not shown).

The CA extracts and bioactive compounds were tested for COX-1 and COX-2 enzyme inhibitory activity. At 30 g/ml, the ethanol, methanol and aqueous extracts inhibited COX-1 and COX-2 enzymes by 97.84% and 97.91%, 98.18% and 96.16%, 83.06% and 72.10%, respectively. Asiaticoside and madecassoside at 5 g/ml concentration inhibited COX-1 and COX-2 enzymes by 98.08% and 83.67%, and 91.57% and 89.79%, respectively. Aspirin was used as positive control at 5 mg/ml concentration and inhibited COX-1 and COX-2 by 80.98% and 88.86%, respectively (Fig.2). Among CA extracts, ethanol and methanol extracts showed stronger inhibition of COX-1 and -2 than the aqueous extract. Asiaticoside and madecassoside inhibitory activity of COX-1 and COX-2 enzymes were similar to commercial NSAIDs, aspirin.

The anti-inflammatory activity of CA extracts and compounds were further studied using TPA-induced inflammation in human fibroblasts. Treatments with 30 g/ml concentration of ethanol, methanol and aqueous extracts of CA on the TPA-induced fibroblast cells produced significantly less PGE_2 compared to control (TPA alone). Treatment of the cells with asiaticoside and madecassoside at 5 g/ml also significantly reduced the TPA-induced formation of PGE_2 (Figure 3).

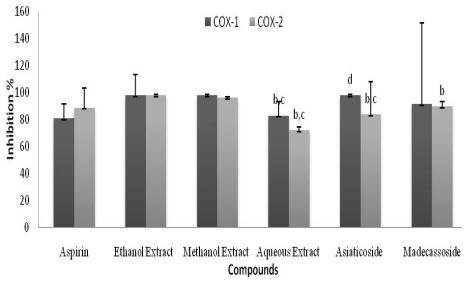

Figure 2: COX-1 and COX-2 enzyme inhibitory activities of CA extracts at 30 g/ml, and bioactive compounds at 5 g/ml. Commercial NSAIDs, aspirin at 5 mg/ml concentration was tested as positive control. Results were expressed as means ±SD (n=4). a, b, c, d p<0.05 vs. aspirin, ethanol, methanol and aqueous extract, respectively.

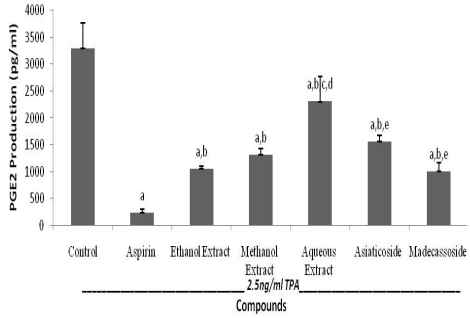

Figure 3: PGE2 production of TPA-induced fibroblast cell treated with CA extracts (30 g/ml) and bioactive compounds (5 g/ml). Aspirin was used as positive control at 5 g/ml. Results were expressed as means ±SEM (n=6). a, b, c, d, e p<0.05 vs. control, aspirin, ethanol, methanol, and aqueous extract, respectively.

Figure 3 also showed that ethanol and methanol extracts of CA were more potent than aqueous extract in suppressing the TPA-induced formation of PGE2 while Figure 2 showed that these extracts were better inhibitors of the COX activity.

Bioactive compounds of CA, asiaticoside and madecassoside suppressed PGE_2 formation at similar level. Both compounds were also found to inhibit TPA-induced inflammation in fibroblasts by modulating COX activity as shown by their inhibitory effects in COX-1 and -2 enzymes assay. Our findings also agree with previous work on collagen-induced arthritis mice in which treatment with asiaticoside were reported to inhibit inflammation by reducing the expression of COX-2 and inflammatory cytokines (Li et al. 2007). Previous work on similar model of arthritis, have found that oral administration of madecassoside to reduce the expression of COX-2 and PGE_2 production, but did not change the expression of COX-1 (Li et al. 2009).

The more potent anti-inflammatory activity of ethanol and methanol extracts of CA in regressing inflammation in TPA-induced fibroblasts than the aqueous extract could be due to higher content of phenolics, and or bioactive compounds asiaticoside and madecassoside in both ethanol and methanol CA extracts compared to the aqueous extract. Asiaticoside and madecassoside, both are shown to have anti-inflammatory activity, and these compounds may react synergistically with each other or with other phenolic constituents to give CA its anti-inflammatory activity.

CONCLUSION

The present study demonstrated that hydrophobic solvent extraction of *Centella asiatica* yielded better yields in terms of phenolic, asiaticosside and madecassoside contents and

that this is associated with better anti-inflammatory potential.

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