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Research Article

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Antioxidant and acetylcholine esterase inhibition activity of the extract from *Centella asiatica* obtained by Ultrasound pre-treatment followed by Microwave-assisted extraction method

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ABSTRACT

The extraction of compounds from *Centella asiatica* a member of the family Apiaceae by adopting Ultrasound assisted Extraction (UAE), Microwave assisted extraction (MAE) and Ultrasound pre-treatment followed by Microwave-assisted extraction were reported. The yield of the extract, phenol content, antioxidant activity and Acetylcholine esterase activity were found to be more in the extract obtained by Ultrasound pre-treatment followed by Microwave-assisted extraction method. The yield of the extract is more by 30.8% to the UAE and MAE methods. The phenol content is 1289 ± 0.57 µg GAE/mL which is 27% higher than the MAE method and 46% higher than the UAE method. HPLC analysis showed that the *C. asiatica* extract comprised of Madecassoside in major quantity followed by madecassic acid, asiatic acid and asiaticoside. In the antioxidant activity by DPPH assay and AChE inhibitory effect the IC_{50}, value for *C. asiatica* extract obtained by UAE pre-treated and followed by MAE method is 38.24 µg mL^{-1} and 26.7±0.49 mg/mL respectively which are substantially higher than the other two methods. So, Ultrasound pre-treatment followed by Microwave-assisted extraction method is found to be a preferable method to get the desired compounds from *C. asiatica*.

KEYWORDS: *Centella asiatica*, Apiaceae, Ultrasound, Microwave, Extraction

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INTRODUCTION

It is obvious from different studies reported worldwide that from *C. asiatica* a member of the family Apiaceae (Sabaragamuwa *et al.*, 2018) more than seventy constituents have been identified and reported. From *C. asiatica* to extract these compounds several methods have been adopted. The solvent, extraction method and time decides the efficiency of extraction. Apart from availability and cost the preferred compounds to be extracted also decides the choice of the method of extraction (Idris & Nadzir, 2021). Triterpenes like asiatic acid, madecassic acid, asiaticoside and madecassoside, are the most common bioactive constituents isolated from *C. asiatica*. Also saponins like brahmoside and centelloside, glycosides, alkaloids (Monton *et al.*, 2019). In addition to this phenolic compound like kaempferol, rutin, apigenin, quercetin, naringin, and catechin, were also found *C. asiatica*. Among these compounds triterpenes finds their use in cosmeceuticals particularly for their anti-wrinkle, wound healing and anti-cellulite effects because these compounds activate the formation of collagen and thereby increase the fibronectin synthesis in the fibroblasts on the human skin. Madecassoside and asiaticoside stimulate the collagen Type III production, induces glycosaminoglycan synthesis, improve the hydroxyproline content and tensile strength in tissues where wound is present and in turn expedites the wound-healing process (Maquart *et al.*, 1990; Hou *et al.*, 2016; Azerad, 2016; Sabaragamuwa *et al.*, 2018).

Since the quantity of bioactive compounds is relatively less, one has to carefully choose the extraction method to get the preferred compounds from *C. asiatica*. A diverse number of methods were used till now to get high-quality extract of *C. asiatica* with a shorter extraction time and at moderate cost. Maceration and Soxhlet extraction are traditional extraction methods which have broad applicability. However, they consume longer extraction hours, high temperature, higher resistance to transfer of mass, less efficiency of extraction, and also consumes large quantity of solvent (Zhuoyan, 2011; Zhao *et al.*, 2016; Idris & Nadzir, 2021).

Recently emerging technologies like supercritical fluid extraction, Ultrasound-assisted extraction (UAE) and Microwave assisted extraction (MAE) were used to extract the bioactives from *C. asiatica*. In UAE using ultrasonic wave's acoustic cavitation are produced in the solvent and causes the cells to disrupt. However, the disadvantages of this technique under high frequencies bioactive compounds are affected by free radical formation and also exposing the plant material for a longer time, the ultrasound waves may degrade the yield of the compounds. In MAE it operates in an oxygen-rich environment at higher temperature. This will have a destructive nature on the preferred bioactive constituents, especially when the compounds are oxygen- and heat-sensitive (Shen *et al.*, 2009; Borhan *et al.*, 2013; Sellathoroe *et al.*, 2019; Idris *et al.*, 2020).

Hybrid technologies like a combination of natural deep eutectic solvents (NADESs) such as acetylcholine chloride: water: malic acid (1:2:2): water (40:60) and MAE were reported to provide a high yield of bioactive compounds and also results in high antioxidant activity. Similarly, combination of natural deep eutectic solvents with UAE was reported to extract asiaticoside from *C. asiatica* (Suppalak *et al.*, 2021; Thong-on *et al.*, 2021). In the present work the compound extraction from0Z *C. asiatica* by adopting Ultrasound pre-treatment followed by Microwave-assisted extraction was studied for the first time. The antioxidant activity of the extract obtained by this method was high when compared with the extract obtained by UAE and MAE. Further the compounds extracted were identified by HPLC of the extract and the standard compounds.

MATERIALS AND METHODS

General

Microwave oven model: GMX 20 SA 2 BLM Microwave (700 W), Digital ultrasonic cleaner LMUC-4, Ultrasonic frequency 40 KHz were used for the experiments. The above extracts were analysed by TLC using the solvent system petroleum ether: Ethyl acetate (8:2) and iodine vapour is used as the detection agent.

Plant Material

Fresh leaves of *Centella asiatica* was collected from Coimbatore, Tamil Nadu, India, and dried in air under shade for 10 days, crushed into small pieces (1 kg). Three types of extraction method were carried for the extraction.

Ultrasonic Assisted Extraction

50 gm of *C. asiatica* soaked in 500 mL of water and introduced into Ultrasonicator bath for 1hr at 45 °C at 40 KHz power and cooled for 1hr at room temperature. The above process was repeated for 5 times till a clear filtrate was obtained. Experiments were performed keeping the mass of material as 25, 50 and 75 g extracted for at different time periods 10, 15, 25 and 30 min. The yield of the extracts was monitored.

Microwave Assisted Extraction

The ultrasonicated pretreated extract obtained above was introduced into Microwaves (700 W) for 2 min and cooled for 15 min at room temperature. The above process was repeated for 5 times till a clear filtrate was obtained. The extract was concentrated using rotary evaporator at 45 °C till dryness and the dried sample were dissolved using methanol and then analysed by TLC.

Experiments under various temperatures at 25, 45 and 60 °C were carried out keeping different time of extractions like 30, 45 and 60 min. The yield obtained in all the experiments was noted.

Microwave Assisted Extraction

50 gm of *C. asiatica* soaked in 500 mL of distilled water and introduced into Microwaves in an oven for 2 min and cooled for 15 min at room temperature. The above process was repeated for 5 times till a clear filtrate was obtained. The resulted extract was concentrated using rotary evaporator at 45 °C till dryness and the dried sample was dissolved using Methanol.

Ultrasonic Assisted Extraction

50 gm of *C. asiatica* soaked in 500 mL of distilled water was introduced into Ultrasonicator bath for 1 hr at 45 °C at 40 KHz power and cooled for 1 hr at room temperature. The above process was repeated for 5 times till clear filtrate was obtained. The extract was concentrated using rotary evaporator at 45 °C till dryness and the dried sample was dissolved using Methanol and then analysed by TLC.

HPLC Analysis

Waters Allaince e2695 HPLC system consisting of a photodiode array detector, Waters 600 E System Controller, Rheodyne injector and Empower software was used for this analysis. A C18 column (Excil ODS 5 µm of the size 150 mm length and 4.6 mm diameter was used for the separation. A solvent system comprising 0.05% phosphoric acid and 100% acetonitrile were used at a flow rate of 1 mL/min. A nylon membrane syringe filter (0.45 µm) was used to filter the aqueous solutions of the standard and sample extract before injection. 20 µL of the solutions were injected into the system and the analysis was made in triplicate.

In-vitro Bioactive Properties of the Extracts

Antioxidant activity

C. asiatica extracts obtained by pre-treatment with ultrasonicated waves followed by microwaves method, microwave assisted method and ultrasonicated method were determined by DPPH assay (Brand-Williams *et al.*, 1995; Valko *et al.*, 2007; Beatrice *et al.*, 2020) to assess their antioxidant property. Methanol solution of the extracts with different concentration like 20, 40, 60, 80 and 100 µg/mL were made and used for the

assay. 3 mL DPPH workable solution is taken in a test tube, and mixed with 100 µL of leaf extract. The test tubes were placed in darkness for about 30 min. The absorbance was of this solution was recorded at 517 nm. The same quantity of DPPH solution along with 100 µL of methanol is used as a control. Ascorbic acid was used as a standard material. The percentage of antioxidants was determined using the formulae

$$\% \text{ of antioxidant activity} = [(Ac - As) \div Ac] \times 100$$

where: Ac - Absorbance of the control solution; As - Absorbance of test solution.

Slightly modified Folin-Ciocalteu method was used to quantify the total phenolic content of the extracts (Park *et al.*, 2008).

The acetylcholinestrase inhibitory activity (AChE) of the test extracts were estimated using Ellman method (Blois, 1958; Ellman *et al.*, 1961; Hurmat *et al.*, 2020). Donepezil was used as the standard material.

RESULT AND DISCUSSION

Fresh leaves of *C. asiatica* collected from Coimbatore, Tamil Nadu, India was air dried and crushed into small pieces. This was subjected to two emerging techniques UAE and MAE individually and also in combination. Even though Maceration and Soxhlet extraction have broad applicability, the efficiency of extraction is low in spite of long time of extraction at high temperature. The solvent consumption is also high. Hence, in the present work initially the plant material was subjected to UAE followed by MAE. Though many reports established that MAE is a feasible alternative to the conventional techniques, it gets operated at high temperature and in an environment richer in oxygen. These two conditions could raise destructive effects on the preferred bioactive compounds. When UAE is carried out alone, the sample gets exposed to the ultrasound activity for a longer time which may reduce the yield of the bioactive constituents. To overcome this difficulty, we in the present work the extraction of compounds was carried out from *C. asiatica* by Ultrasound pre-treatment followed by microwave -assisted extraction (MAE) and were studied for their biological activity for the first time.

In Ultrasound-assisted extraction (UAE), ultrasonic waves disrupt the cells and this disruption facilitates the release of the phytoconstituents and increases the surface contact area between the compounds and the solvents. Ultrasound causes opening of the pores on the surface, scrap and disintegrate the particles, which enhance the transfer of mass from the cytoplasm of the cell to the solvent surrounding it. It is responsible for the breaking of the cell wall and accelerates the solutes to go into the solvent medium. The enhanced rate compound transfer helps to shorten the time of extraction. Furthermore, less solvent is required for this process and the degradation of compounds which are heat-sensitive is also minimised. Microwave extraction technique utilizes the quick heating of samples in aqueous medium. In this method the key concept is that initially the solvent absorbs the microwave energy and then it transfers the same to the sample in the form of heat, by two different mechanisms, by ionic conduction and dipole rotation. The process of getting the active compounds extracted from the sample to solvent is highly influenced by the temperature and the solvent nature.

Performing the experiments under various conditions like mass of material (25, 50, 75 g), and time of extraction (10, 15, 25 and 30 min) the conditions to be used for MAE was optimised. The highest yield under the optimised condition is (1.64 g) 3.28% at 700 watt microwave power, 50 g mass, and 15 min extraction time. Similarly experiments under various conditions like temperature (25, 45, 60 °C), and time of extraction (30, 45 and 60 min), the conditions were optimised for the UAE. The highest yield obtained the optimised condition is (1.83 g) 3.36 % at 45 °C, 40 KHz power, 50 g mass, and 60 min extraction time.

The total phenolic content of the extract was determined by Folin-Ciocalteu method. The phenol content is found to be 697.2±0.34 and 942.2±0.59 µg GAE/mL by UAE and MAE respectively. The above optimised conditions were adopted for the combination of extraction by Ultrasound pre-treatment and followed by microwave -assisted extraction (MAE). 50 gm of *C. asiatica* in water was introduced into Ultrasonicator bath for 1hr at 45 °C at 40 KHz power and cooled for 1 hr at room temperature. Five times it was repeated in order to get a clear solution. The ultrasonicated pretreated extract was introduced into Microwave (700 W) for 2 min and cooled for 15 min at room temperature. The same was followed for 5 times to get a clear solution. The resulted extract was concentrated at 45 °C in a rotary evaporator till it gets dried, and the dried sample was dissolved using Chloroform and then analysed by TLC. The yield of the extract is (2.43 g) 4.86%. The phenol content is 1289 ± 0.57 µg GAE/mL which is higher than the other two methods. It is almost 27% higher than the MAE method and 46% higher than the UAE method.

Determination of the Antioxidant Activity

This property of the extracts obtained by UAE, MAE and UAE pre-treatment followed by MAE methods were estimated by DPPH assay (Brand-Williams *et al.*, 1995; Valko *et al.*, 2007; Beatrice *et al.*, 2020). As a reference antioxidant compound L-ascorbic acid was used. Similarly extracts of 20, 40, 60, 80 and 100 µg mL^{-1} concentrations were used. The relationship between the UAE, MAE and UAE pre-treatment followed by MAE sample concentration and the percentage inhibition effects was plotted against each other and analyzed by linear regression. The determined activity is presented in terms of IC_{50} values, which is the concentration of sample scavenging 50% of the DPPH radicals (Figure 1). The IC_{50}, value for *C. asiatica* obtained by MAE and UAE are very less whereas the IC_{50}, value for *C. asiatica* obtained by UAE pre-treated and followed by MAE is substantial and the value is 38.24 µg mL^{-1} and that of the standard L-ascorbic acid is 18.76 µg mL^{-1}.

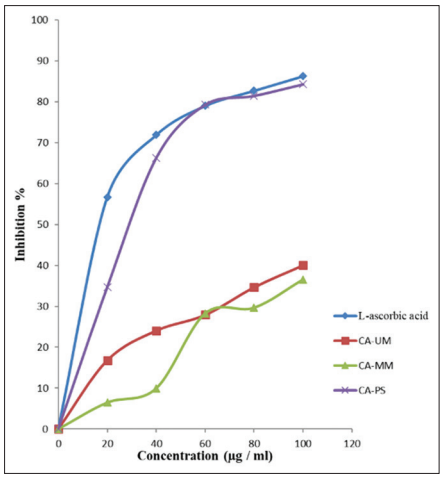

Figure 1: Antioxidant activity of *C. asiatica* extract (CA-UM: Ultrasound assisted extraction method, CA-MM: Microwave assisted extraction method, CA-PS: Ultrasound pre-treatment followed by Microwave-assisted extraction method)

High Performance Liquid Chromatography

The extract obtained by Ultrasound pretreatment and followed by microwave -assisted extraction (MAE) method was analysed by HPLC. An isocratic method using the solvents 0.05% phosphoric acid (solvent A) and 100% acetonitrile (solvent B) in a ratio of 1:1 at a flow rate of 1 mL/min and a PDA detector at 205 nm is used detect the four triterpenes present in the C. *asiatica* extract (Figure 2). The peaks obtained in the chromatogram with retention times 1.854, 3.12, are identified as glycosides madecassoside, asiaticoside, and at retention times 4.18 and 5.21 min are identified as their respective acids madecassic acid and asiatic acid respectively when compared with the retention times of the standard compounds. The extract of C. *asiatica* leaves obtained by this method comprised of Madecassoside in the highest concentration, followed by madecassic acid, Asiatic acid and asiaticoside (Figure 3). However, when (Zainol *et al.*, 2008) investigated the leaves from different acessions of C. *asiatica* available in the southern part of Malaysia using an isocratic system of water and methanol in a ratio of 2:8 he could able to separate only three compounds asiaticoside, madecassoside and asiatic acid of which asiatic acid is of high concentration. The triterpenes present in C. *asiatica* are not always the same because of different locations and diverse environmental conditions.

In-vitro Acetylcholinesterase (AChE) Inhibitory Activity

Ellman (Blois, 1958; Ellman *et al.*, 1961) method was followed to determine the AChE inhibitory effect of the test extract obtained as above. When acetylcholinestrase enzyme hydrolyzes acetylcholine it forms thiocholine which in turn reacts with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), to form 2-nitrobenzoate-5-mercaptothiocholine and thio-2-nitrobenzoate, and could be detected and estimated by an absorption band at 412 nm in the UV spectrum. The readings were recorded in triplicate. Donepezil was used as a standard substance. The IC_{50} values (mg/mL) are reported in Table 1.

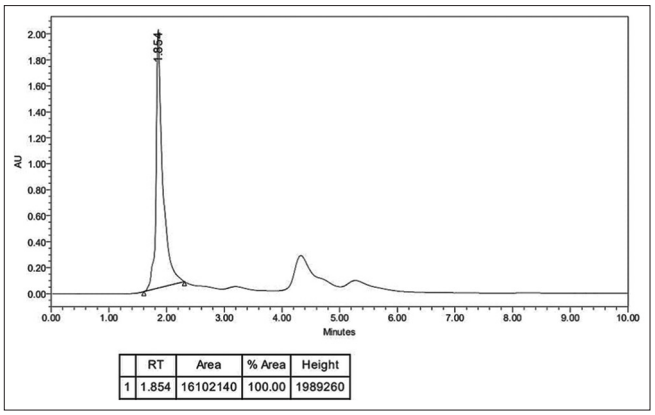

Figure 2: Representative chromatogram of compound from *C. Asiatica* by Ultrasound pre-treatment followed by Microwave-assisted extraction method

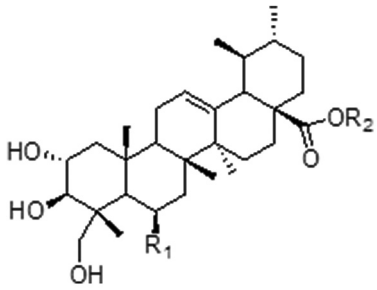

Figure 3: Structure of Madecassoside, Asisticoside, Madecassic acid and Asiatic acid from *C. Asiatica*
Madecassoside - R_1 = OH; R_2 = Glu-Glu-Rha
Asiaticoside - R_1 = H; R_2 = Glu-Glu-Rha
Madecassic acid - R_1 = OH; R_2 =H
Asiatic acid - R_1 = H; R_2 =H

Table 1: The IC_{50} values Of Acetylcholinestrase Inhibitory Assay of *C. asiatica* obtained by UAE, MAE and UAE pre-treatment followed by MAE methods

S. No.	Extracting method	IC_{50} values (mg/mL)
1	UAE	52.3±0.34
2	MAE	48.2±0.56
3	UAE pre-treated followed by MAE	26.7±0.49
4	Standard compound Donepezil	0.045±0.29

The AChE inhibitory effect was more for the C. *asiatica* extract obtained from UAE pre-treated followed by MAE method and the value is 26.7±0.49 mg/mL. This was followed by the extracts obtained by MAE method and UAE method respectively.

CONCLUSION

The extraction of compounds from C. *asiatica* by adopting Ulttrasound assisted Extraction (UAE), Microwave assisted extraction (MAE) and Ultrasound pre-treatment followed by Microwave-assisted extraction were reported. The compounds present in the extract were identified as madecassoside, asiaticoside, madecassic acid and asiatic acid. The yield of the extract, phenol content, antioxidant activity and Acetylcholine

esterase activity were found to be more in the extract. This method is found to be a preferable method to get the desired compounds from *C. asiatica*.

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