Phytochemicals and Antioxidant Activity of Aqueous and Ethanolic Extracts of Mentha aquatica L.

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Abstract: In Vietnam, Mentha aquatica L. is often used as a spice in dishes, fragrance and pharmaceuticals. The tea made from the plants’ leaves has been used as a traditional medicine for fevers, headaches, digestive disorders and mouthwash. The present study aimed to explore the phytochemical profile of aqueous and ethanolic extracts of M. aquatica L. leaves as well as their total phenolics content, total flavonoid content and antioxidant activity. Various bioactive constituents were detected in M. aquatica L. extracts, including alkaloid, flavonoid, terpenoid, tannin, coumarin, anthraquinone and saponin. Furthermore, the total content of phenolics of aqueous extract was higher than ethanolic extract. We then evaluated the antioxidant capacity of these two extracts by using DPPH and ABTS scavenging assays. Results have shown that the aqueous extract exhibited scavenging activity more actively against both free radicals, as compared to the aqueous extract. Overall, the study proposed that M. aquatica L. leaves can be an enriched source of phytochemicals that can be used as natural antioxidants in multiple industries.

1. Introduction

Mentha L. belongs to Lamiaceae family, which comprises of more than 7000 species found all over the world from temperate to tropical regions [1]. Most Mentha species favor wet environments and moist soils. Mentha species are widely used in cooking as spices, seasonings and teas. Besides, their isolated essential oils and bioactive compounds are used in perfumery, cosmetics and flavour industries, as toothpaste, breath fresheners, drinks, chewing gum, desserts, candies and aseptic mouthwash [2, 3]. Mentha species is one of the world’s most ancient herbs that are commonly used in traditional medicines as a main alternative therapy for flatulence, indigestion and nausea [4]. The tea traditionally made from the leaves has been used to treat fevers, headaches and digestive disorders.

A wide range of flavonoids have been isolated from Mentha plant. For instance, (S)-naringenin isolated from the ethanolic extract of M. aquatica by bioassay-guided fractionation could bind to the GABA benzodiazepine site, which then lead to either a sedative effect or anti-convulsive activity, depending on its binding receptor subtype [5]. A majority of phenolic compounds such as eriodictyol-O-rutinoside and rosmaricin acid were also found in hydroethanolic extracts of M. aquatica [6]. This
extract displayed high antioxidant activity and effectively exhibited cytoprotective effect on HEPG2 cells line [7-9].

Due to limited knowledge about *M. aquatica* leaves extract, the present study performed a preliminary screening of the phytochemicals and total contents of phenolics and flavonoids of ethanol and aqueous extracts from *M. aquatica* leaves. Taking into account the presence of several antioxidants, we then decided to evaluate their antioxidant activity using ABTS and DPPH scavenging assays.

2. **Materials and methods**

2.1. **Chemicals and Equipment**
Ethanol, methanol, 2,2‘-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reagents such as Folin-Ciocalteu’s reagent and other general purpose laboratory chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the reagents and chemicals were of analytical grade.

2.2. **Plant collection**
*M. aquatica* was collected at a local market located in Ho Chi Minh City (Vietnam), washed with distilled water and let dry at 40°C in an oven. The dried plant was grounded into fine powder in order to pass the 100 mm sieve (Fig.1).

![Figure 1. Visual images of *M. aquatica* L.(A) leaves and (B) leaves powder extract.](image)

2.3. **Plant extraction**
Plant powder (10 g) was extracted with 300 mL ethanol and distilled water at 60°C for 1 h. The resulted extracts were filtered by vacuum-filtered through a sintered glass funnel with Whatman No.1 filter paper and allowed to dry at 40°C at reduced pressure using a rotatory vacuum evaporator. Crude extracts were obtained and used for various analyses.

2.4. **Phytochemical test**
Plant phytochemicals are primary and secondary metabolites and these substances have a wide range of biological activities. Therefore, several phytochemical tests were performed to detect the presence of these natural compounds in various ethanolic and aqueous extracts [10].

2.5. **Estimation of total alkaloids content**
The crude extracts were dissolved into 4 mL of 1% HCl and filtered. Several tests such as Bouchardat’s test, Meyer’s test, Dragendorff’s test were then employed as previously described [1] [11].

2.6. **Estimation of tannins content**
Crude extracts were dissolved with distilled water and placed in a bain-marie, filtered and added with 5% FeCl₃. The content of tannins was estimated by the appearance of dark blue or greenish black color [10].
2.7. **Estimation of anthraquinones content**
The ethanolic crude extracts were dissolved into 5 ml of chloroform, filtered and added with 1 ml of 10% NaOH. The presence of anthraquinones was indicated by appearance of pink precipitates [10].

2.8. **Content of flavonoid**
Wiltstatter’s test was employed to estimate the flavonoid content [10]. Crude extracts were dissolved into ethanol and filtered. The filtrate was divided equally into 2 test tubes, added 0.05g magnesium powder and 1 ml HCl, and then heated for 5 min. The presence of flavones, flavanones, flavanol and xanthone was indicated by the formation of orange, red or purple colored precipitates; whereas the presence of isoflavone, isoflavanone and aurone was indicated by discoloration.

2.9. **Terpenoids content**
Liebermann – Burchard’s test was employed to estimate the terpenoids content [10]. Crude extracts were dissolved in 1 ml of anhydride acetic and mixed with chloroform. After filtering, 1 ml of H$_2$SO$_4$ was added. A reddish-brown ring separator was taken as positive result.

2.10. **Coumarins content**
Crude extracts were dissolved into ethanol and filtered. The filtrate was divided equally into 2 test tubes, added 0.5 ml 10% KOH to the first tube, and 0.5 ml distilled water into the second tube. Both tubes were heated for 2 min, then allowed to cool down and observed under 365 nm UV lamp. The first tube fluorescing more strongly than the second tube indicated the presence of coumarins [10].

2.11. **Saponins content estimation**
Crude extracts were dissolved in distilled water, heated and filtered. After being cooled down, the filtrate was filled with distilled water to 10 ml and vigorously shaken. Establishment of a stable persistent froth was taken as positive result [12].

2.12. **Estimation of reducing sugar**
Crude extracts were dissolved in distilled water heated and filtered. The filtrate was heated and added with Fehling’s solution A and B. The presence of reducing sugar was indicated by the appearance of red precipitate [10].

2.13. **Determination of Total Phenolic Content (TPC)**
The TPC of two different extracts of *M. aquatica* L. were measured using Folin-Ciocalteu’s reagent as previously described by Pham et al [13]. The crude extracts were diluted to the appropriate concentration in methanol. Then, the diluted sample solution and 10% Folin-Ciocalteu solution were added. The mixture was homogenized using a Vortex machine, and placed at room temperature. After 5 min, Na$_2$CO$_3$ solution 7.5% was added to the solution shaken well and further incubated in darkness for 1 h at room temperature. Absorbance measurement was at 765 nm against a control sample, which contained the reaction mixture with methanol instead of plant extract using UV-visible spectrophotometer (Cary 60, Agilent Technologies, Palo Alto, CA, USA). Gallic acid solution prepared in methanol was employed to construct a calibration curve. TPC was expressed in milligrams of gallic acid equivalent per gram of extracted compounds (mgGAE / g DW).

2.14. **Determination of Total Flavonoid Content (TFC)**
The TFC of *M. aquatica* L. extracts were estimated according to Mahboubi et al [14]. The crude extracts were diluted in methanol and added with 10% AlCl$_3$, 1M CH$_3$COOK and distilled water. The mixture was subjected to 30-min incubation at room temperature. Absorbance measurement was at 415 nm against a control sample, which contained the reaction mixture with methanol instead of plant extract using UV-visible spectrometer. Quercetin solution prepared in methanol was used to construct the calibration curve. TFC was expressed in milligrams of quercetin equivalent per gram of extracted compounds.
compounds (mgQE/ g DW).

2.15. Antioxidant Activity Assay

2.15.1. DPPH radicals scavenging activity. DPPH scavenging activity of *M. aquatica* L. aqueous and ethanol extracts determination involved the use of the modified method described by Pham et al [13]. Methanol was then added to the stock solution (containing DPPH and methanol), giving working solution with 1.1 ± 0.02 of absorbance value at 517 nm. The prepared working solution was added to the ethanol extract at different concentrations (0-310 µg/ml) and subjected to incubation at room temperature without light for 30 min. Similar procedure was repeated for aqueous extract (0-212.63 µg/ml). Absorbance measurement was at 517 nm using UV-visible spectrometer and methanol was used as a blank. The control used was Vitamin C. IC₅₀ values represent the sample concentration required to scavenge half of free DPPH radicals. The percentage of inhibited DPPH radicals was calculated using the following formula:

\[
\% \text{ inhibition} = \frac{A_C - A_A}{A_C} \times 100
\]

Where \( A_A \) and \( A_C \) are the absorbance of the antioxidant and control, respectively.

2.15.2. ABTS radicals scavenging activity. Evaluation of ABTS scavenging activity followed the method described by Pham et al. with a few modifications [13]. To prepare the stock solution, 7.4 mM ABTS solution was mixed with 2.6 mM K₃S₂O₈ and placed in the darkness at room temperature for 15 h. Methanol was added to the stock solution to obtain working solution with the absorbance value of 1.1 ± 0.02 at 734 nm. 0.5 mL of ethanol extract at different concentrations (0-217 µg/ml) was added to working solution. Similar procedure was repeated for aqueous extract (0-212.63 µg/ml). The mixture was subjected to incubation at room temperature for 30 min. The mixture absorbance was measured at 734 nm using UV-visible spectrometer and methanol was used instead of plant extracts as a blank. The control used was Vitamin C. IC₅₀ values represent the sample concentration required to scavenge half of ABTS free radicals. The percentage of inhibited ABTS radicals was calculated using the following formula:

\[
\% \text{ inhibition} = \frac{A_C - A_A}{A_C} \times 100
\]

Where \( A_C \) and \( A_A \) are the absorbance of the antioxidant and control, respectively.

2.16. Statistical Analysis

All experiments were repeated three times. The graphs were constructed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). Date were analyzed by two-way ANOVA and expressed as mean ± standard error of mean (SEM).

3. Results and Discussion

3.1. Phytochemical test

The use of different solvents have been evidenced to affect the phytochemical content of the obtained extract. For example, in the present study, results from phytochemical tests indicated that the two extracts from *M. aquatica* L. leaves were enriched with flavonoids, phenolics, tannins, coumarin and saponin which are soluble in water and ethanol [15]. In addition, as compared to chloroform, acetone and ethyl acetate, extracts of water and ethanol are safe to use in foods. Considering the suitable solubility and safety, water and ethanol were used as the solvents for *M. aquatica* L. leaves extraction. Although chloroform, acetone and methanol were previously used to extract *M. aquatica* L. roots and aerial parts, resulting in a different profile of phytochemical constituents (e.g. steroids, flavonoids and triterpenoids) and higher phenolics content [16], whether these solvents are suitable for extraction of *M. aquatica* L. leaves requires further study. Overall, it can be concluded that different parts of the plant
require careful selection of an appropriate solvent to obtain the extract with desirable content of bioactive compounds.

Phytochemical screening of the two different *M. aquatica* extracts showed positive results for tannins, flavonoids and terpenoid (Table 1). In contrast, reducing sugars were absent in both extracts. The alkaloids were found present in ethanolic extract, yet absent in aqueous extract. On the contrary, coumarin and saponin were only found present in aqueous extract, while not being detected in ethanolic extract.

**Table 1.** Phytochemical analysis of *M. aquatica* L. extracts. ‘±’ indicates the presence and ‘=’ indicates the absence of the compounds.

| Name of compound | Ethanol extract | Aqueous extract |
|------------------|----------------|----------------|
| Alkaloid         | ±              | =             |
| Tannin           | ±              | ±             |
| Anthraquinone    | ±              | =             |
| Flavonoids       | ±              | ±             |
| Terpenoid        | ±              | ±             |
| Coumarin         | =              | ±             |
| Saponin          | =              | ±             |
| Reducing sugar   | =              | =             |

![Figure 2. Images of phytochemical of (A-F) ethanolic extract and (a-d) aqueous extract of *M. aquatica*. A,a: Terpenoid, B,b: Tannin, C,c: Flavonoids, D: Anthraquinone, d: Saponin, E: Alkaloids in Mayer’s test and F: Alkaloids in Bouchardat’s test.](image)

**3.2. Determination of TPC and TFC**

Phenolics are widely recognized as the largest phytochemical molecules from plants that exhibit antioxidant, antibacterial, anti-cancer, anti-inflammatory and cardio protective activities [17, 18]. Determination of TPC involved the use of Folin-Ciocalteu’s reagent and expressed in the form of GAE (the standard curve equation: \( y = 0.09206x + 0.03255 \), \( R^2 = 0.9981 \)), while TFC was expressed as QE (the standard curve equation: \( y = 0.00735x - 0.01790 \), \( R^2 = 0.9998 \)). The TPC and TFC of *M. aquatica* extracts were measured (Table 2). Results have shown that the amount of flavonoids present in ethanolic extracts of *M. aquatic* was four-time higher than aqueous extract; whereas the amount of phenolic
compound present in both extracts showed no significant difference. Such high TPC in *M. aquatic* leaves aqueous extract can be comparable to other *Mentha* plants such as *M. piperita* (14.00 ± 0.12 mgGAE/g), *M. pulegium* L. (6.1 ± 0.5 mgGAE/g), *M. spicata* L. (12.0 ± 0.3 mgGAE/g) and *M. rotundifolia* L. (4.6 ± 0.1 mgGAE/g) [19]. Under the situation where phenolic compounds have been well-known to mainly associate with antioxidant activity of plant extracts, these findings TPC present in a wide range of *Mentha* species is required to exploit more plant sources with potential and high-value bioactivity [12, 20-22].

Table 2. TPC and TFC of *M. aquatica* L. extracts. Data was represent as mean ± SE. Mean values superscripted by a and b are considered as significant different at *p*<0.05.

| Plant extract    | TPC (mgGAE/g) | TFC (mgQE/g) |
|------------------|---------------|--------------|
| Ethanolic extract| 7.51 ± 0.91a  | 93.88 ± 3.30a|
| Aqueous extract  | 9.35 ± 1.04a  | 23.24 ± 0.05b|

3.3. **Antioxidant activity**

Previous studies have shown that phenolic compounds exhibit peroxide decomposition and free radical scavenging in biological systems [23]. Overall, the antioxidant activity of both *M. aquatica* extracts was significantly higher than that of ascorbic acid (Table 3). In particular, the aqueous extract of this plant have scavenged both free radicals two-time more actively than the ethanolic extract. This results can be explained by the presence of higher concentration of phenolic compounds, as compared to the ethanolic extract. Similar observation was also reported by Tongco et al. (2014) [15]. However, in general, the antiradical effect of tested *M. aquatica* extracts was more significant than *Garcinia mangostana* L. and *Marrubium vulgare* L. extracts [24]. In short, with the association between phenolics and antioxidant activity found in *M. aquatica* leaves extracts, the present study have preliminarily proposed that ethanol and water could be considered as suitable solvents for *M. aquatica* leaves extraction.

Table 3. IC$_{50}$ values of DPPH and ABTS scavenging activities of *M. aquatica* extracts and control. The superscripted values (i.e. a, b and c) indicated significant difference at 95% confidence interval.

| Plant extract      | DPPH (µg/ml) | ABTS (µg/ml) |
|--------------------|--------------|--------------|
| Ethanolic extract  | 306.97 ± 13.78a | 209.29 ± 18.5a |
| Aqueous extract    | 142.98 ± 19.93b | 157.93 ± 16.42a |
| Control            | 5.33 ± 0.28c  | 2.33 ± 0.10b  |
Figure 3. DPPH scavenging activities of (A) ethanolic and (B) aqueous extracts of *M. aquatica* as compared to the (C) control.

Figure 4. ABTS scavenging activities of (A) ethanolic and (B) aqueous extracts of *M. aquatica* as compared to the (C) control.
4. Conclusion

In conclusion, the study has pursued the potential pharmacological effects of *M. aquatica* L. Significant total polyphenols and flavonoid contents, along with antioxidant activity suggest the possibility of *M. aquatica* L. as a potentially attractive material for food, cosmetic or pharmaceutical industries [25, 26]. Further investigations are expected to support these considerations and help to set up suitable standards for effective applications of this herbal plant in the future.

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References

[1] Brahmi F, Khodir M, Mohamed C and Pierre D 2017 *Chemical Composition and Biological Activities of Mentha Species Aromatic and Medicinal Plants - Back to Nature*, ed H A El-Shemy (IntechOpen) chapter 3 pp 47–78
[2] de Sousa D P, Lima T C and Steverding D 2016 *Planta Med.* 82 1346–50
[3] Tran Q T, Le T T T, Pham M Q, Do T L, Vu M H, Nguyen D C, Bach L G, Bui L M and Pham Q L 2019 *Molecules* 24 895
[4] Bach L T, Hue B T B, Tram N T T, Thu D N A and Dung L T 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* 736 022083
[5] Jäger A K, Almqvist J P, Vangsøe S A K, Stafford G I, Adsersen A and Van Staden J 2007 *S. Afr. J. Bot.* 73 518–521
[6] Tran N Y T, Nhan N P T, Thanh V T, Nguyen D V, Thin P V, Vy T A, Lam T D and Truc T T 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* 736 022064
[7] Pereira O R, Macias R I R, Domingues M R M, Marin J J G and Cardoso S M 2019 *Antioxidants (Basel)* 8 267
[8] Pham T N, Tran B P, Tran T H, Nguyen D C, Nguyen T N P, Nguyen T Q, Vo D V N, Le N T H, Le X T, Nguyen T D and Bach L G 2019 *IOP Conf. Ser.: Mater. Sci. Eng.* 479 012012
[9] N.P. Minh, T.H.P. Trang, N.T.T. Trang, L.G. Bach 2019 *Res. on Crops* 20 180–186.
[10] M.T. Nguyen, V.T. Nguyen, L.V. Minh, L.H. Trieu, M.H. Cang, L.B. Bui, X.T. Le, V.T. Danh 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* 736 062011.
[11] Ciulei I 1982 *Methodology for Analysis of Vegetable Drugs Practical Manual on the Industrial Utilisation of Medicinal and Aromatic Plants*, (Bucharest: Scientific Research) pp 1–62
[12] Nhan N P T, Hien T T, Nhan L T H, Anh P N Q, Huy L T, Nguyen T C T, et al. 2018 *Solid State Phenom.* 279 235–239.
[13] Pham H N T, Nguyen V T, Vuong Q V, Bowyer M C and Scarlett C J 2017 *J. Food Process. Preserv.* 41 e12879
[14] Mahboubi M, Kazempour N and Boland Nazar A R 2013 *Jundishapur J. Nat. Pharm. Prod.* 8 15–19
[15] Oloruntola A and Omotosho O 2019 *Curr. Dev. Nutr.* 3 nzz040.P20-019-19
[16] Ferhat M, Erol E, Beladjila K A, Çetintaş Y, Duru M E, Öztürk M, Kabouche A and Kabouche Z 2017 *Pharm Biol* 55 324–329
[17] Tungmunnithum D, Thongboonyou A, Pholboon A and Yangsabai A 2018 *Medicines (Basel)* 5 93
[18] Thuy N V, Tien N M, Quy N N, Cang M H, Quan P M, Bui L M, Minh L V and Muoi N V 2020 *Asian J. Chem.* 32 1230–34
[19] Siddeeg A, Salih Z A, Mukhtar R M, Ali A O 2018 *Gezira Journal of Engineering and Applied Sciences* 13
[20] Bhuyan D J and Basu A 2017 *Utilisation of Bioactive Compounds from Agricultural and Food Production Waste* ed Q V Vuong (Boca Raton: CRC Press) chapter 2 pp 27–59.
[21] Mai H C, Le T T T, Diep T T, Le T H N, Nguyen D T and Bach L G 2018 *Asian J. Chem.* **30** 293–297

[22] Minh N P, Bach L G, Chau M H, Loan L Y, Tram V T B and Van Truyen T 2019 *Journal of Pharmaceutical Sciences and Research* **11** 279–83

[23] Nguyen V T, Nguyen M T, Tran Q T, Thinh P V, Bui L M, Le T H N, Le V M and Linh H T K 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **736** 022063

[24] Karakaş N, Karadağ A E, Yılmaz R, DemiRci F and Okur M E 2019 *J. Res. Pharm.* **23** 711–8

[25] Nguyen M T, Nguyen V T, Le V M, Trieu L H, Lam T D, Bui L M, Nhan L T H and Danh V T 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **736** 062012

[26] Nguyen N Q, Minh L V, Trieu L H, Bui L M, Lam T D, Hieu V Q, Khang T V and Trung L N Y 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **736** 062017