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# *In Vitro* Xanthine Oxidase Inhibitory Activity of *Morinda citrifolia* L. (Noni) Leaf and Identification of Its Xanthine Oxidase Inhibitors

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**Abstract**

Hyperuricemia causes an excessive level of uric acid in the body, which leads to the formation of gouty arthritis and high uric acid-related diseases. To cure this condition, xanthine oxidase (XO), a key enzyme in uric acid production, was targeted. A routinely used XO inhibitor drug, allopurinol, has side effects. Therefore, an alternative XO inhibitor with a high activity and fewer side effects is strongly desired. In this research, we evaluated the XO inhibitory activity of *Morinda citrifolia* L. (noni) leaves extracted using 2 solvents, 95 % ethanol and water. The total phenolic and flavonoid content was analyzed. Moreover, the phenolic profile was investigated using liquid column chromatography-mass spectrometry (LC-MS). The results showed that at a concentration of 100 µg $mL^{-1}$, the ethanolic (McE) and water (McW) extracts could exhibit XO inhibitory activity above 90 %, and the $IC_{50}$ values predicted using dose-response logarithmic function curves were 2.24 ± 0.2 and 7.2 ± 0.1 µg $mL^{-1}$, respectively, while the $IC_{50}$ of allopurinol was 1.02 ± 0.1 µg $mL^{-1}$. The total phenolic and flavonoid content in McE was higher than that in McW. LC-MS analysis revealed that all extracts contained several phenolic compounds. Interestingly, known potent XO inhibitors were found in both extracts. Kaempferol, esculetin, saponarin, physcion and γ-aminobutyric acid were found in McE, while McW contained esculetin, saponarin and rutin. Taken together, *M. citrifolia* L. leaves could be used as an effective alternative XO inhibitor for curing hyperuricemia and high uric acid-related diseases caused by XO activity.

**Keywords:** *Morinda citrifolia* leave, Xanthine oxidase, Phenolic compounds, LC-MS, XO inhibitors

## Introduction

Hyperuricemia is characterized by excess uric acid levels in the body. In many cases, the excess uric acid level leads to the deposition of urate monohydrate crystals in joints and other tissues [1]. In addition, hyperuricemia can increase the risk of cardiovascular diseases [2]. The prevalence of hyperuricemia and gout is increasing due to lifestyle changes [3], increasing obesity, and aging of the population [4]. These results have a significant impact on the global health system [5].

The key enzyme involved in uric acid formation is xanthine oxidase (XO). This enzyme catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid in the catabolism of purine substrates. Substrate reduction is an oxidation process resulting in oxygen-derived free radicals being concomitantly formed. Free radicals contribute to the oxidative damage of living tissues [6] and are implicated in several diseases, including neurodegenerative diseases, cardiovascular diseases, immunological disorders, renal diseases, and cancer [7].

Currently, medicine employed to cure hyperuricemia and gout include uricosuric drugs, which activate the excretion of uric acid, and XO inhibitors. However, the reduction of uric acid formation by inhibiting XO is the most desirable and efficient treatment to relieve hyperuricemia and gout. The effective XO inhibitor widely used is allopurinol, a hypoxanthine analog. However, this drug has unexpected side effects, such as allergic reactions, hepatotoxicity, and potentially acute renal failure [8]. Thus, alternative XO inhibitors with high inhibition activity and fewer side effects are strongly desired.

Medicinal plants are a source of pharmaceutical substances and have been used as a primary healthcare modality worldwide, especially in developing countries. Natural XO inhibitors have been reported from several plant species. Among the phytoconstituents in plants, phenolic compounds especially flavonoids [9-14], and coumarins [15-18] have been proven to be potent XO inhibitors.

*Morinda citrifolia* L. (noni) belongs to the Rubiaceae family. The noni parts, especially fruits and leaves, have been used as food ingredients and medicine for more than 2,000 years. In Thailand, dried noni

fruit or powdered leaves have been consumed as tea to moderate blood pressure, muscle pains, and vomiting [19]. Noni fruit and leaves contain numerous pharmaceutical compounds that exhibit biological activities, such as anticancer [20], anti-infection [21], antioxidant [22,23], analgesic, and anti-inflammatory properties [24]. Moreover, noni fruit was shown to possess an active component against XO activity [25]. In Thai folk wisdom, noni leaves have been used for curing gout. However, the XO inhibitory activity of noni leaves and their bioactive compounds have remained unexplored. Thus, this study aimed to investigate the potential XO inhibitory activity of noni leaves *in vitro* along with assessing their bioactive compound content using LC-MS. Ultimately, the information obtained may support the development of noni leaves and their constituents as a pharmaceutical remedy and a new alternative XO inhibitor for the relief of hyperuricemia and high uric acid-related diseases caused by XO activity.

## Materials and methods

### Plant materials and extraction

Fresh *M. citrifolia* L. (noni) leaves were collected in February, 2019 at Khun Talay, Mueang, Surat Thani. The leaves were washed and oven-dried at 60 °C for 24 h. The dried plant materials were ground and subsequently extracted using 2 different solvents, 95 % ethanol and water (ratio of powdered leaves 1 g: solvent 10 mL). The mixtures were then shaken at 250 rpm at 30 °C for 3 days in the dark. Subsequently, the suspensions were filtered using Whatman No. 1 filter paper and the obtained filtrate fractions were concentrated using a vacuum rotary evaporator. Eventually, the noni leaf crude extracts were prepared as ethanolic (McE) and water (McW) extracts used for further experiments.

### Total phenolic content analysis

The total phenolic content was determined according to Nguyen *et al.*' protocol [26] with some modifications. Briefly, gallic acid at concentrations of 20, 40, 80, 160 and 320 µg $mL^{-1}$ were prepared and used to generate a standard curve. The extracts (1 mg $mL^{-1}$) were mixed with 2.5 mL of 10 % (v/v) Folin-Ciocalteu reagent and the reactions were incubated at room temperature for 5 min. Next, the reactions were added to 2.0 mL of 20 % (w/v) $Na_2CO_3$ and further incubated at 50 °C for 10 min. Finally, the absorbance was measured at 765 nm using a spectrophotometer. The experiments were performed in triplicate. The total phenolic content was expressed as milligrams of gallic acid equivalents per gram extract (mg GAE $g^{-1}$ extract).

### Total flavonoid content analysis

The total flavonoid content was determined according to the Maulana *et al.*' protocol [27] with some modifications. Briefly, concentrations of 20, 40, 80, 160 and 320 µg $mL^{-1}$ quercetin were prepared and used to generate a standard curve. Approximately 0.5 mL of the extracts (1 mg $mL^{-1}$) was mixed with 0.5 mL of 2 % (w/v) aluminum chloride ($AlCl_2$), and the mixture was incubated for 15 min at room temperature. Next, the absorbance was measured at 415 nm using a spectrophotometer. The experiments were performed in triplicate. The total flavonoid content was expressed as milligrams of quercetin equivalents per gram extract (mg GAE $g^{-1}$ extract).

### *In vitro* xanthine oxidase inhibitory activity assay

To evaluate the inhibitory activity of noni leaf crude extracts against XO activity, the *in vitro* xanthine oxidase inhibition was assayed in accordance with previous methods [28] with some modifications. The assay contained the following components: 100 µL of the plant crude extracts (at a final concentration of 100 µg $mL^{-1}$), 300 µL of 50 mM phosphate buffer (pH 7.5), and 100 µL of XO enzyme solution (0.2 units $mL^{-1}$ in phosphate buffer (pH 7.5)). Allopurinol was used as a positive control. Subsequently, the mixtures were incubated at 37 °C for 15 min, and 200 µL of xanthine solution (150 mM xanthine in phosphate buffer (pH 7.5)) was then included and further incubated at 37 °C for 30 min. The reaction was then terminated by adding 200 µL of 0.5 M hydrochloric acid (HCl) and spectrophotometrically measured at an absorbance of 295 nm using a UV spectrophotometer. Each experiment was performed in triplicate.

The XO inhibitory activity was calculated using the following equation:

$$\% \text{ XO inhibition} = (1 - A/B) \times 100$$

A is the activity of the XO without extracts
B is the activity of the XO with extracts/allopurinol

**The $IC_{50}$ concentration evaluation**

The noni leaf crude extracts and allopurinol were diluted to various concentrations of 0.5, 1, 5, 10, 20, 40, 60, 80, and 100 µg $mL^{-1}$. The diluted samples were assayed to determine the XO inhibitory activity following the above section. Dose-response logarithmic function curves were generated for calculating the $IC_{50}$.

**Identification of phenolic compound profiles using LC-MS**

Analyses were performed on a liquid chromatograph-quadrupole time-of-flight mass spectrometer (LC-QTOF MS), 1290 Infinity II LC-6545 Quadrupole-TOF, Agilent Technologies, USA. The column was a Zorbax Eclipse Plus C18 Rapid Resolution HD column (150 mm length×2.1 mm inner diameter, particle size 1.8 µm). The mass spectrometer was operated in negative ion mode with the following parameters: Capillary voltage, 4.0 kV; cone voltage, 2,000 V; and extractor, 2 V. The source temperature was 100 °C, the desolvation temperature was 325 °C, the cone gas flow was 13 L $min^{-1}$, and the desolvation gas flow was 350 L $h^{-1}$. A mobile phase consisted of 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 0.2 mL $min^{-1}$ and the following gradient: 0 - 2 min, 10 % B; 2 - 20 min, 10 - 60 % B; 20 - 21 min, 60 - 80 % B; 21 - 25 min, 80 % B; 25 - 26 min, 80 % - 10 % B; 26 - 30 min, 10 % B. The injection volume was 2 µL, and the column temperature was 25 °C [29]. The bioactive compounds were identified on the basis of their retention times, MS spectra and molecular-ion identification using MassHunter Work Station Software compared with the Library METLIN database.

**Statistical analysis**

The XO inhibition was expressed as the mean ± standard deviation (mean ± SD) from triplicate measurements by using Microsoft Office Excel 2010. The logarithmic function curves of the dose-response data were also obtained using Microsoft Office Excel 2010 to calculate $IC_{50}$. The difference in mean values was analyzed through one-way ANOVA and considered significant when $p < 0.05$.

## Results and discussion

***Morinda citrifolia* L. (noni) leaf extracts inhibited xanthine oxidase (XO) activity *in vitro***

After performing an *in vitro* xanthine oxidase inhibitory activity assay, the results showed that the extracts and allopurinol could inhibit XO activity in a dose-dependent manner (**Figure 1**). At a concentration of 100 µg $mL^{-1}$, all samples exhibited XO inhibitory activity above 90 %.

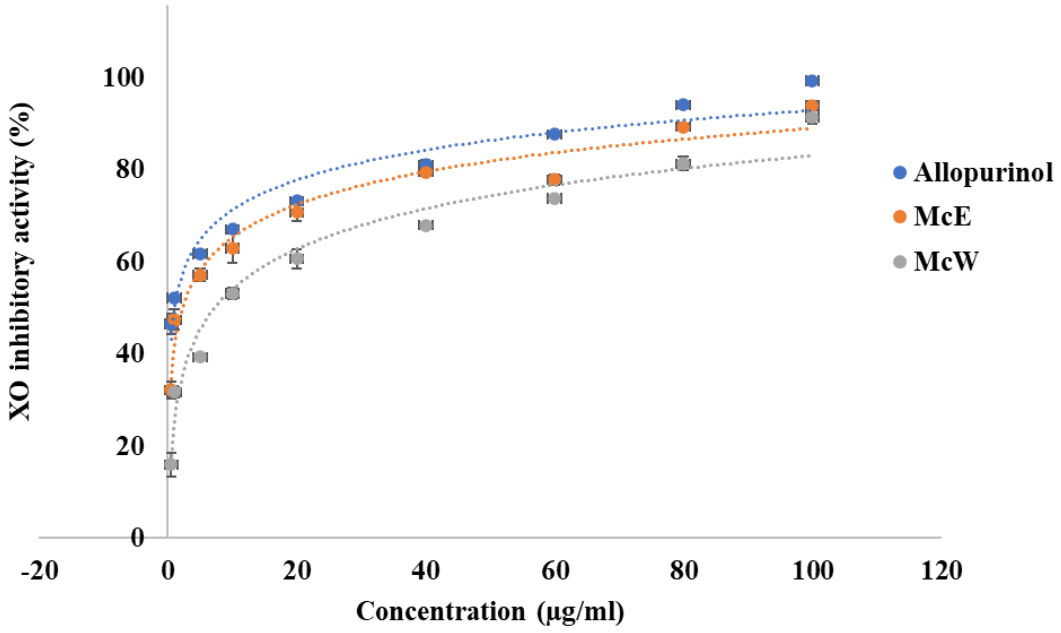


**Figure 1** XO inhibitory activity of ethanolic (McE) and water (McW) extracts of noni leaves at various concentrations (0.5 - 100 µg $mL^{-1}$).

To compare the XO inhibitory activity between samples, the $IC_{50}$ value was considered. The $IC_{50}$ value of all samples was calculated using the equations of the dose-response logarithmic function curve. The results showed that allopurinol still possessed the highest XO inhibitory activity, with an $IC_{50}$ of 1.02 ± 0.1 μg $mL^{-1}$ followed by McE and McW, with $IC_{50}$ values of 2.24 ± 0.2 and 7.2 ± 0.1 μg $mL^{-1}$, respectively (**Table 1**). Overall, it could be concluded that noni leaves contained XO inhibitory activity and might be potential sources of XO inhibitors.

**Total phenolic and flavonoid contents in noni extracts**

The results (**Table 1**) showed that the total phenolic and flavonoid contents in the McE extract were significantly higher than those in the McW extract, with values of 35.04 ± 1.2 mg GAE $g^{-1}$ extract, and 11.8 ± 0.7 mg QU $g^{-1}$ extract and 29.50 ± 0.9 mg GAE $g^{-1}$ extract, and 8.3 ± 1.3 mg QU $g^{-1}$ extract, respectively. Therefore, it was concluded that noni extracts contained both phenolic and flavonoid substances. Thus, it might be implied that one of them might be an XO inhibitor resulting in noni extracts possessing XO inhibitory activity.

**Table 1** The total phenolic and flavonoid contents and $IC_{50}$ values of *M. citrifolia* L. leaf crude extracts.

| Samples | Total phenolic content (mg GAE $g^{-1}$ extract) | Total flavonoid content (mg QU $g^{-1}$ extract) | XO inhibitory activity $IC_{50}$ (μg $mL^{-1}$) |
|---|---|---|---|
| Allopurinol | - | - | 1.02 ± 0.1 [a] |
| McE | 35.04 ± 1.2 [a] | 11.8 ± 0.7 [a] | 2.24 ± 0.2 [b] |
| McW | 29.50 ± 0.9 [b] | 8.3 ± 1.3 [b] | 7.2 ± 0.1 [c] |

**Note**: Different letters indicate significant differences at $p < 0.05$ according to Duncan's Multiple Range Test.

**Identification of the bioactive compound profile in noni leaf extracts**

Most bioactive compounds in plants that possess XO inhibitory activity are phenolic compounds especially flavonoids and their derivatives [9-14], and coumarins [15-18]. Thus, both noni leaf extracts possessing the XO inhibitory activity were thought to contain bioactive compounds acting as XO inhibitors in particular phenolic compounds as well as other plants. To answer this question, we used LC-MS.

After LC-MS analysis, the chromatogram of bioactive compounds of McE and McW extracts was shown in **Figure 2**. The identified phenolic compounds present in McE and McW extracts are shown in **Tables 2** and **3**, respectively. The results showed that both extracts contained various phenolic compounds. Some phenolic compounds were found in both extracts, including esculetin, verbasoside, quercetin 3-rutinoside-3'-apioside, kaempferol 3-(2''-rhamnosylgalactoside) 7-rhamnoside, schaftoside 4'-glucoside, saponarin, isorhamnetin 3-O-[b-L-rhamnofuranosyl-(1->6)-D-glucopyranoside], vestitone 7-glucoside, and 3-[3-carboxy-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-5-benzofuranyl]-2-propenoic acid. While 4-aminocatechol, luteolin 6-C-glucoside 8-C-arabinoside, niazicinin A, 3',7-dimethoxy-4',5,8-trihydroxyflavone 8-glucoside, hieracin, oxynarcotine, kaempferol, 1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, myrsinone, ventinone A and physcion were only found in McE in contrast to dihydroferulic acid 4-O-glucuronide, 4-glucogallic acid, 3,3',5-trihydroxy-4'-methoxy-6,7-methylenedioxyflavone 3-glucuronide, flaviolin, 4,7-dihydroxy-2H-1-benzopyran-2-one, caffeoyl C1-glucuronide, isofraxidin, gentisic acid, trans-caffeic acid [apiosyl-(1->6)-glucosyl] ester, 1-O-2'-hydroxy-4'-methoxycinnamoyl-b-D-glucose, rutin, quercetin 3-xylosyl-(1->3)-rhanosyl-(1->6)-[apiosyl-(1->2)-galactoside], scutellarein 7,4'-dirhamnoside and kaempferol 3-xylosylglucoside were only found in McW extract.

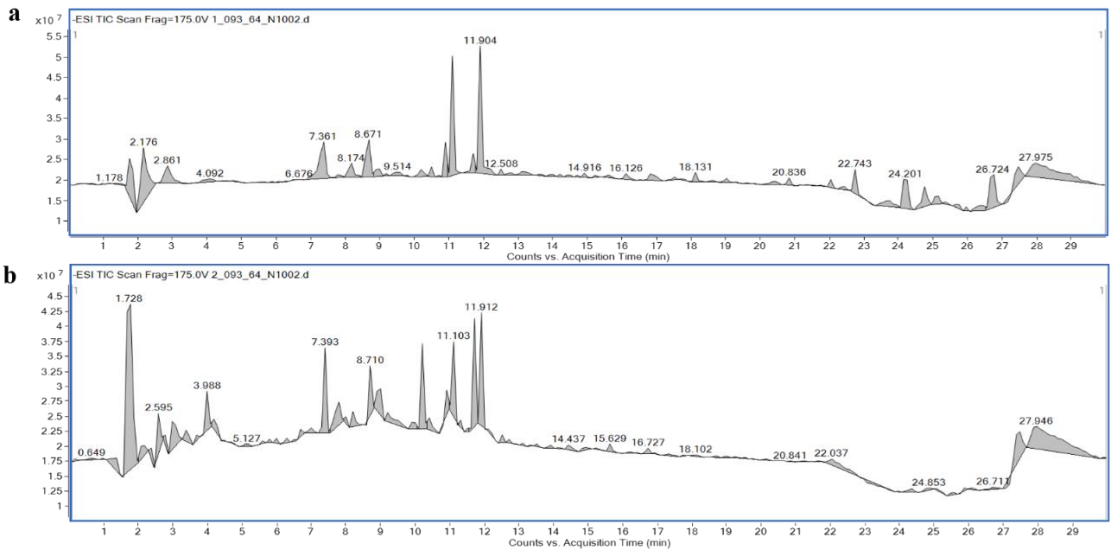


**Figure 2** LC-MS chromatogram of bioactive compounds from McE (a) and McW (b) extracts.

Interestingly, phenolic compounds known as potent XO inhibitors were present in both extracts including esculetin [14], kaempferol [11], rutin [30], physcion [31] saponarin [32] (**Figure 3**). In detail, McE contained esculetin, kaempferol, physcion, and saponarin while McW contained esculetin, rutin and saponarin. In addition, McE also presented nonphenolic compounds acting as an XO inhibitor, which was γ-aminobutyric acid [33].

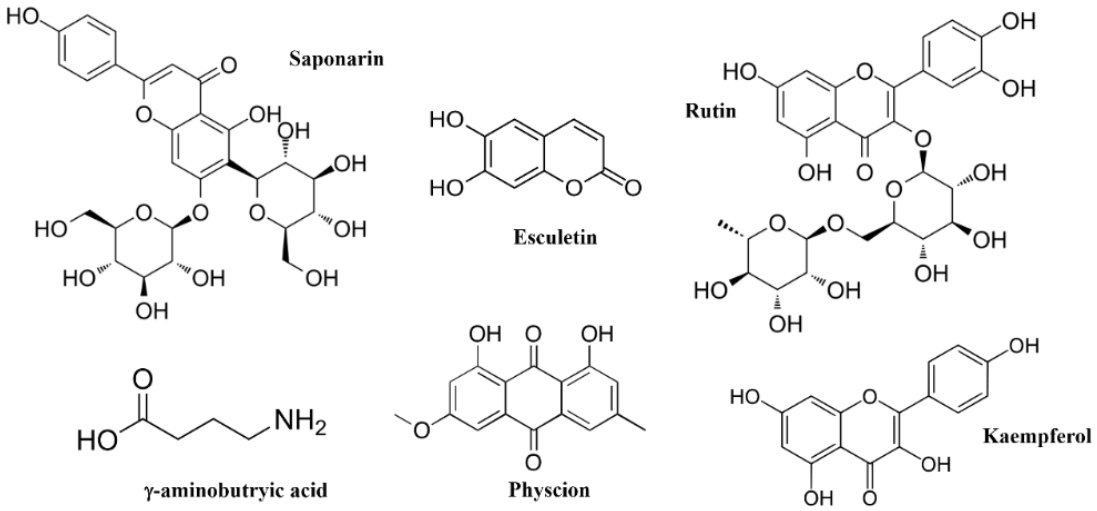


**Figure 3** Structures of the reported known XO inhibitors identified in McE and McW extracts.

**Note:** The retention time of γ-aminobutyric acid after LC-MS analysis was 1.936 min, and $[m/z]^-$ was 102.0555.

**Table 2** Phenolic compounds in McE leaf extracts.

| Compounds | Bioactive constituents | Formula | Tr (min) | [m/z]⁻ |
|---|---|---|---|---|
| 1 | Esculetin | $C_9H_6O_4$ | 9.48 | 177.0192 |
| 2 | 4-aminocatechol | $C_6H_7NO_2$ | 9.668 | 124.0401 |
| 3 | Verbasoside | $C_{20}H_{30}O_{12}$ | 10.145 | 461.1655 |
| 4 | Quercetin 3-rutinoside-3'-apioside | $C_{32}H_{38}O_{20}$ | 10.271 | 741.1871 |
| 5 | Kaempferol 3-(2"-rhamnosylgalactoside) 7-rhamnoside | $C_{33}H_{40}O_{19}$ | 10.522 | 739.2082 |
| 6 | Schaftoside 4'-glucoside | $C_{32}H_{38}O_{19}$ | 10.924 | 725.1928 |
| 7 | Luteolin 6-C-glucoside 8-C-arabinoside | $C_{27}H_{30}O_{16}$ | 11.175 | 609.1459 |
| 8 | Saponarin | $C_{27}H_{30}O_{15}$ | 11.978 | 593.151 |
| 9 | Isorhamnetin 3-O-[b-L-rhamnofuranosyl-(1->6)-D-glucopyranoside] | $C_{28}H_{32}O_{16}$ | 12.053 | 623.1601 |
| 10 | Niazicinin A | $C_{17}H_{23}NO_8$ | 12.129 | 368.1345 |
| 11 | 3',7-dimethoxy-4',5,8-trihydroxyflavone 8-glucoside | $C_{23}H_{24}O_{12}$ | 13.785 | 491.1179 |
| 12 | Vestitone 7-glucoside | $C_{22}H_{26}O_9$ | 13.886 | 433.1492 |
| 13 | 3-[3-carboxy-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-5-benzofuranyl]-2-propenoic acid | $C_{20}H_{18}O_8$ | 14.238 | 385.092 |
| 14 | Hieracin | $C_{15}H_{10}O_7$ | 16.196 | 301.0349 |
| 15 | Oxynarcotine | $C_{22}H_{25}NO_8$ | 17.551 | 430.1501 |
| 16 | Kaempferol | $C_{15}H_{10}O_6$ | 18.154 | 285.0402 |
| 17 | 1-(3,4-dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione | $C_{20}H_{18}O_6$ | 20.865 | 353.1027 |
| 18 | Myrsinone | $C_{17}H_{26}O_4$ | 22.07 | 293.1752 |
| 19 | Ventinone A | $C_{17}H_{14}O_6$ | 22.848 | 313.072 |
| 20 | Physcion | $C_{16}H_{12}O_5$ | 23.099 | 283.0606 |

**Table 3** Phenolic compounds in McW leaf extracts.

| Compounds | Bioactive constituents | Formula | Tr (min) | [m/z]⁻ |
|---|---|---|---|---|
| 1 | Dihydroferulic acid 4-O-glucuronide | $C_{16}H_{20}O_{10}$ | 3.428 | 371.0979 |
| 2 | 4-glucogallic acid | $C_{13}H_{16}O_{10}$ | 3.453 | 331.0665 |
| 3 | 3,3',5-Trihydroxy-4'-methoxy-6,7-methylenedioxyflavone 3-glucuronide | $C_{23}H_{20}O_{14}$ | 3.553 | 519.0802 |
| 4 | Flaviolin | $C_{10}H_6O_5$ | 4.056 | 205.0142 |
| 5 | 4,7-dihydroxy-2H-1-benzopyran-2-one | $C_9H_6O_4$ | 4.081 | 177.0194 |
| 6 | Caffeoyl C1-glucuronide | $C_{15}H_{16}O_{10}$ | 8.85 | 355.0666 |
| 7 | Isofraxidin | $C_{11}H_{10}O_5$ | 8.875 | 221.0453 |
| 8 | Gentisic acid | $C_7H_6O_4$ | 8.976 | 153.0191 |
| 9 | Trans-caffeic acid [apiosyl-(1->6)-glucosyl] ester | $C_{20}H_{26}O_{13}$ | 9.277 | 473.1293 |
| 10 | Esculetin | $C_9H_6O_4$ | 9.453 | 177.0191 |
| 11 | 1-O-2'-hydroxy-4'-methoxycinnamoyl-b-D-glucose | $C_{16}H_{20}O_9$ | 9.553 | 355.1028 |
| 12 | Verbasoside | $C_{20}H_{30}O_{12}$ | 10.156 | 461.1655 |
| 13 | Quercetin 3-rutinoside-3'-apioside | $C_{32}H_{38}O_{20}$ | 10.282 | 741.1877 |
| 14 | Kaempferol 3-(2"-rhamnosylgalactoside) 7-rhamnoside | $C_{33}H_{40}O_{19}$ | 10.482 | 739.2084 |
| 15 | Schaftoside 4'-glucoside | $C_{32}H_{38}O_{19}$ | 10.884 | 725.1927 |
| 16 | Rutin | $C_{27}H_{30}O_{16}$ | 11.148 | 609.1457 |
| 17 | Quercetin 3-xylosyl-(1->3)-rhanosyl-(1->6)-[apiosyl-(1->2)-galactoside] | $C_{37}H_{46}O_{24}$ | 11.16 | 873.2288 |
| 18 | Scutellarein 7,4'-dirhamnoside | $C_{27}H_{30}O_{14}$ | 11.236 | 577.1551 |
| 19 | Kaempferol 3-xylosylglucoside | $C_{26}H_{28}O_{15}$ | 11.662 | 579.1341 |
| 20 | Saponarin | $C_{27}H_{30}O_{15}$ | 11.989 | 593.1508 |
| 21 | Isorhamnetin 3-O-[b-L-rhamnofuranosyl-(1->6)-D-glucopyranoside] | $C_{28}H_{32}O_{16}$ | 12.064 | 623.1604 |
| 22 | Vestitone 7-glucoside | $C_{22}H_{26}O_9$ | 13.695 | 433.1495 |
| 23 | 3-[3-Carboxy-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-5-benzofuranyl]-2-propenoic acid | $C_{20}H_{18}O_8$ | 14.147 | 385.0924 |

## Conclusions

These results support the following Thai folk wisdom by which *M. citrifolia L.* leaves have been used to cure gout. *M. citrifolia L.* leaves possessed potent phenolic and nonphenolic XO inhibitors resulting in it could exhibit high XO inhibitory activity. Ultimately, *M. citrifolia* L. leaves may be used as an alternative pharmaceutical remedy for relieving hyperuricemia, preventing or slowing the gouty arthritis progression, and high uric acid-related diseases caused by XO activity.

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