Biological activities and GABA content of Ma-Lod (*Elaeagnus latifolia* Linn.)

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**Abstract.** *Elaeagnus latifolia* Linn. is a type of wild edible fruit belonging to the family Elaeagnaceae locally known as Ma-Lod in the Upper North of Thailand. In this study, the fruit of twelve Ma-Lod trees, namely ML-1, ML-2, ML-3, ML-4, ML-5, ML-6, ML-7, ML-12, ML-16, ML-17, ML-18, and ML-19, collected from Sangkhom District, Nong Khai Province were investigated for tyrosinase inhibitory activity, antioxidant activity, free radical scavenging activity, total phenolic content and GABA content. The results show that the reducing power of Ma-Lod flesh extracts highly correlated with their phenolic contents indicating phenolic compounds could be one of the main contributors to oxidant reducing power. Among twelve tested Ma-Lod trees, fruit flesh extracts of ML-1, ML-4, ML-5, and ML-17 were considered to be a promising source of biologically active compounds due to their high total phenolic contents, antioxidant activity and outstanding of tyrosinase inhibitory activity. GABA was also found in Ma-Lod flesh extract and the high recovery of GABA after passing through the Dowex 50W-X8 column was obtained. This wild and underutilized fruit exhibits many valuable characteristics which deserve conservation and utilization. It could be a potential source of natural biologically active compounds in the development of value-added products such as antioxidant-rich products and cosmetics. In addition, new investigation of the presence of GABA in Ma-Lod fruit might lead to the development of GABA food supplement in the future.

**Keywords:** γ-aminobutyric acid, biological activity, *Elaeagnus*, Ma-Lod, HPLC-ELSD, tyrosinase inhibition

1. Introduction

The genus *Elaeagnus* (silverberry or oleaster) contains about 50 species of flowering plants belonging to the family Elaeagnaceae. They are found around the world which are mainly distributed in subtropical regions of Asia, Europe and some parts of North America. *Elaeagnus latifolia* Linn. is a type of wild edible fruit distributed in Northeast India, Vietnam and Thailand. In Thailand, *Elaeagnus latifolia* Linn. is the commonly found species in the Upper North of Thailand. It is known with the Thai common name Ma-Lod. This deciduous shrub is mostly grown in the back yard garden of many families of the region and harvested during March and April in 3-4 picking. Fruit could be utilized for making jam, jelly, pickle and refreshing drinks. The fruit of many members of this genus including
Elaeagnus latifolia Linn., is a very rich source of vitamins, especially vitamins A, C and E, essential fatty acids, minerals, flavonoids, carotenoids and other bioactive compounds. Different parts of Ma-Lod plant, especially the fruits and flowers, have been used traditionally as an astringent, analgesic, antipyretic and laxative herbal medicine. The objective of this study was to determine the presence of γ-aminobutyric acid (GABA), total phenolics and biological activities, including the in vitro mushroom tyrosinase inhibitory activity, antioxidant activity and free radical scavenging activity in Ma-Lod flesh extracts.

2. Materials and methods

2.1 Materials
The fruits of twelve Ma-Lod trees, namely ML-1, ML-2, ML-3, ML-4, ML-5, ML-6, ML-7, ML-12, ML-16, ML-17, ML-18, and ML-19, were observed and collected from Sangkhom District, Nong Khai Province in March 2015. They were transported to the laboratory in cold conditions (4°C) and cleaned by rinsing under running water and pat them dry with paper towels. Maturation stages of Ma-Lod fruit were visually classified based on the color of the exocarp as follows: stage 1 greenish-yellow, stage 2 yellow, stage 3 yellowish-orange, stage 4 orange, stage 5 orangish-red, stage 6 red and stage 7 dark red. Stages 6 and 7 were chosen for this study. Pure Ma-Lod flesh samples were obtained by removing the seeds and the flesh was cut into small pieces, frozen in liquid nitrogen and kept at -80°C until use. GABA, tyrosinase from mushroom, L-3,4-dihydroxyphenylalanine (L-DOPA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylychranmon-2-carboxylic acid (Trolox), iron (III) chloride hexahydrate, gallic acid and kojic acid were purchased from Sigma-Aldrich (St. Louis, USA). HPLC-grade acetonitrile was purchased from BDH (Lutterworth, UK). Water was purified by a Milli-Q plus system from Millipore (Bedford, USA). Dowex 50W-X8 was purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu's reagent was purchased from Carlo Erba (France).

2.2 Preparation of Ma-Lod flesh extract
Frozen pieces of Ma-Lod flesh were ground into a fine powder using a mortar and pestle and liquid nitrogen. A 10 g sample of the finely ground Ma-Lod flesh was weighed accurately and placed into a 100-mL Erlenmeyer flask. Using a pipette, 100 mM potassium phosphate buffer (pH 7.8) was added to the flask at a ratio of 1 : 1 (w/v). The flask was then covered with aluminium foil and sealed with parafilm. The sample was shaken at 40°C at 150 rpm for 1 h in an orbital shaking incubator. After shaking, the sample was then sonicated in an ultrasonic bath for 30 min in order to enhance extraction efficiency. The sample was centrifuged at 8,000 x g for 15 min using a refrigerated centrifuge. The actual volume of supernatant was recorded. Triplicate 1-mL aliquots of the supernatant were evaporated using a centrifugal vacuum concentrator at 35°C. The extraction yield was 9.94±1.15% (w/w). Ma-Lod flesh extract was stored at -20°C until use.

2.3 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay
The DPPH radical scavenging assay was carried out according to the method of Yamasaki et al. [1]. The ability to scavenge DPPH radical (scavenging activity, %) was calculated by the following equation. All determinations were performed in triplicate.

\[
\text{Scavenging activity} \% = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \tag{1}
\]

where \(A_{\text{control}}\) is the absorbance of DPPH radical in ethanol at 517 nm; \(A_{\text{sample}}\) is the absorbance of DPPH radical solution mixed with Ma-Lod flesh extract solution at 517 nm. The free radical scavenging activity of the flesh extract was expressed as \(IC_{50}\). The half-maximal inhibitory concentration (\(IC_{50}\)) value, which is the sample concentration that inhibits the formation of DPPH radicals by 50%, was obtained by probit analysis [2].
2.4. **Ferric reducing antioxidant power (FRAP) assay**

The FRAP assay was carried out according to the method of Benzie and Strain [3] with minor modifications. The FRAP values were calculated and expressed as millimole Trolox equivalents (TE) per gram dry weight of sample based on the calibration curve plotted using Trolox as a standard at concentrations ranging from 0.01 to 0.15 mM. All determinations were performed in triplicate.

2.5. **Tyrosinase inhibitory activity assay**

The inhibitory activity of Ma-Lod flesh extract on mushroom tyrosinase was determined using the dopachrome method with L-DOPA as a substrate, according to the method of Jiménez et al. [4] with minor modifications. Kojic acid, a standard tyrosinase inhibitor, was used as a positive control in the assay. The percentage inhibition of the tyrosinase activity was calculated by the following equation. All determinations were performed in triplicate.

\[
\text{Tyrosinase inhibition (\%) = } \left\{ \frac{(A-B)-(C-D)}{(A-B)} \right\} \times 100
\]

where A is the absorbance at 475 nm with L-DOPA without sample, B is the absorbance at 475 nm without L-DOPA and sample, C is the absorbance at 475 nm with sample and L-DOPA, and D is the absorbance at 475 with sample and without L-DOPA.

2.6. **Determination of total phenolic content**

The amount of total phenolic content of Ma-Lod flesh extract was determined according to the Folin-Ciocalteu micro method [5]. The total phenolic contents were calculated based on the calibration curve plotted using gallic acid as a standard at concentrations ranging from 0.05 to 0.50 mg/mL and expressed as milligram gallic acid equivalents (GAE) per gram dry weight of sample. All determinations were performed in triplicate.

2.7. **Determination of the GABA content in aqueous extract of Ma-Lod flesh**

The fruit of ML-1 was chosen for evaluation of GABA contents at three different stages of maturation including stages 3, 5 and 7. The extraction and the analysis of GABA by high-performance liquid chromatography coupled with an evaporative light scattering detector (HPLC-ELSD) were carried out according to the method of Meeploy and Deewatthanawong [6].

2.8. **Separation of GABA from aqueous extract of Ma-Lod flesh**

The separation of GABA was carried out on the Dowex 50W-X8 column according to the method of Carmona et al. [7].

2.9. **Statistical analysis**

Samples were analysed in triplicate and the results were given as mean ± standard deviation (SD).

3. **Results and discussion**

3.1. **Tyrosinase inhibitory activity of Ma-Lod flesh extracts**

Tyrosinase is a copper monooxygenase widely distributed in animals, plants and microorganisms. It is responsible for the synthesis of melanin in animals and browning in plants. Therefore, finding an effective tyrosinase inhibitor is beneficial in antibrowning of foods, medicinal and cosmetic applications in relation to hyperpigmentation. The tyrosinase inhibitory activity of Ma-Lod flesh extracts at the final concentration of 6.00 mg/mL were determined using the dopachrome method. It was found that all the flesh extracts exhibited inhibitory activity against mushroom tyrosinase ranging from 72.41±2.42% to 15.99±3.29%. The fruit flesh extract of ML-17 possessed the highest inhibition of tyrosinase activity (figure 1). Meanwhile, the visual color change of a reaction mixture was also
observed, color alteration from colorless to reddish brown colored dopachrome was obviously reduced in the reaction mixture containing the fruit flesh extracts of ML-1, ML-4, ML-5, and ML-17. This finding was related to their high total phenolic contents (figure 2 and figure 5).

**Figure 1.** The tyrosinase inhibition of the fruit flesh extract of twelve Ma-Lod trees (*Elaeagnus latifolia* Linn.). Results represent the means ± SD of three replications.

**Figure 2.** The tyrosinase inhibition of the fruit flesh extract of twelve Ma-Lod trees (*Elaeagnus latifolia* Linn.) was determined using the dopachrome method with L-DOPA as a substrate. The assay was conducted in a 96-well microtiter plate in a total volume of 250 µl. The final concentration of Ma-Lod flesh extracts in the reaction mixture was 6.0 mg/mL. Color of dopachrome formation was observed after incubation in the dark at room temperature for up to 24 h. (A) control, (B) blank of control, (C) sample, and (D) blank of sample.

The inhibition of tyrosinase activity of Ma-Lod flesh extract could be caused by one of the following: 1) reducing agents such as ascorbic acid, which is used as a melanogenesis inhibitor because of its capacity to reduce back o-dopaquinone to DOPA, 2) alternative enzyme substrates such as some phenolic compounds, which have a good affinity for the enzyme prevent dopachrome formation, 3) specific tyrosinase inactivators, these inhibitors inhibit tyrosinase activity by inducing the enzyme catalyzing and 4) specific tyrosinase inhibitors, they reversibly bind to tyrosinase and reduce its catalytic capacity. *Elaeagnus latifolia* fruit contains at least three classes of compounds including ascorbic acid, carotenoids, and phenolic compounds, that can inhibit tyrosinase activity [8].
3.2. *Ferric reducing antioxidant power (FRAP)* of *Ma-Lod* flesh extracts

The antioxidant capacity of *Ma-Lod* flesh extracts was determined using FRAP assay. In this assay, ferric ions are reduced to ferrous ions in the presence of a water-soluble antioxidant (or a reducing agent) which form a blue-colored ferrous tripyridyltriazine complex (Fe$^{2+}$-TPTZ) at pH 3.6. The increase in absorbance at 593 nm is proportional to the total ferric reducing power of the tested sample. Among the *Ma-Lod* flesh extracts tested in this study, ML-1 possessed the highest antioxidant capacity followed by ML-17, ML-5, and ML-4 with the FRAP values of 169.45±1.16, 113.57±1.80, 96.29±5.92, and 79.64±3.49 mmole TE/g of dried *Ma-Lod* flesh extract, respectively. The fruit flesh extracts of ML-12 showed the lowest antioxidant capacity with the FRAP value of 12.79±0.77 mmole TE/g of dried *Ma-Lod* flesh extract (figure 3). The reducing power of *Ma-Lod* flesh extracts is probably due to the action of hydroxyl group of the phenolic compounds which might act as electron donors.

![Figure 3. Ferric reducing antioxidant power (FRAP) values of the fruit flesh extract of twelve *Ma-Lod* trees (*Elaeagnus latifolia* Linn.). Results represent the means ± SD of three replications.](image)

3.3. *DPPH radical scavenging activity* of *Ma-Lod* flesh extracts

In this study, all *Ma-Lod* flesh extracts exhibited the moderate activity on scavenging DPPH radicals. The fruit flesh extracts of ML-1, ML-2, ML-3, ML-4, ML-5, ML-6, and ML-7 had IC$_{50}$ values below 5.00 mg/mL, while the IC$_{50}$ values of ML-12, ML-16, ML-17, ML-18, and ML-19 ranged from 6.48 to 24.74 mg/mL (figure 4). The lower IC$_{50}$ value means the higher antioxidant activity. The DPPH radical scavenging of *Ma-Lod* flesh extracts is probably due to the action of phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to free radical. In addition, the structure of phenolic compounds have extended conjugated aromatic system to delocalize an unpaired electron. Total antioxidant capacity from both DPPH and FRAP assays does not show similar trend (figure 3 and figure 4), this could be due to different mechanism of assay method, structure of different phenolic compounds, the antioxidant protection mechanism exhibited by compounds and also the synergistic effects of different compounds [9].

3.4. *Total phenolic content* of *Ma-Lod* flesh extracts

Phenolics are widely distributed in the plant kingdom and are the most abundant secondary metabolites of plants with more than 8,000 phenolic structures currently known. Flavonoids are mainly present in fruits, their phenolic hydroxyl groups are able to chelate metals, reduce lipid peroxidation and have shown a high antioxidant and free radical scavenging activities [10].
In the present study, the amount of total phenolic content of Ma-Lod flesh extract was determined according to the Folin-Ciocalteu micro method. Among the Ma-Lod flesh extracts tested, ML-1 had the highest total phenolic content followed by ML-17, ML-5, and ML-4 which value of 31.49±0.34, 19.75±0.74, 16.66±0.80, and 14.99±0.21 mg GAE/g of dried Ma-Lod flesh extract respectively. The fruit flesh extracts of ML-12 showed the lowest total phenolic content which value of 3.56±0.41 mg GAE/g of dried Ma-Lod flesh extract (figure 5).

Figure 4. DPPH radical scavenging activity of the fruit flesh extract of twelve Ma-Lod trees (*Elaeagnus latifolia* Linn.). Results represent the means ± SD of three replications.

![DPPH radical scavenging activity](image)

In addition, we found that the inhibitory effects of the flesh extracts of ML-1, ML-4, ML-5, and ML-17, on mushroom tyrosinase are related to their total phenolic contents (figure 2 and figure 5) and FRAP values (figure 2 and figure 3). These results support the fact that polyphenols are highly potent...
Antioxidants and several polyphenols are accepted as tyrosinase inhibitors. Phenolic compounds such as catechin, epicatechin, kaempferol, quercetin, coumarines, caffeic acid, and stilbenes are found in *Elaeagnus angustifolia* Linn. fruit extract. These phenolic compounds showed monophenolase and/or diphenolase inhibitory activity toward mushroom tyrosinase [11].

![Figure 6](image1.png)

3.5. **GABA content**

In this study, the fruit of ML-1 was chosen for evaluation of GABA contents at three different stages of maturation including stages 3, 5, and 7. Chromatographic separation was carried out on the Inertsil®NH<sub>2</sub> HPLC column. GABA was separated with the retention time of 54.6500 min. It was found in stages 3, 5, and 7 with the amounts of GABA content at 0.1301±0.0054, 0.1640±0.0032, and 0.0913±0.0080 mg/g fresh weight, respectively (figure 7). The results show that the highest level of GABA content was detected in stage 5 and decline level of GABA content started from stage 5 to stage 7, indicating the beginning of fruit senescence. It decreased almost 2-fold for stage 7. Previous research studies have reported that GABA content in the fruit of cherry tomato peaked before starting maturation and then declined during ripening. A similar pattern was observed during fruit development in Micro-Tom. GABA accumulation in fruits at the early development stage might protect immature seed and the GABA levels decline during the ripening stage when seeds have already matured. In addition, the contents of GABA found in bitter melons, tomatoes, apples, and rambutans were 0.41-2.06 mg/g dry weight, 0.50-0.67, 0.007, and 0.48-0.94 mg/g fresh weight, respectively [6,12].

After extraction of Ma-Lod flesh with water, 5-mL of a filtered sample extract was passed through the Dowex 50W-X8 column. GABA was eluted with 2 N NH<sub>4</sub>OH at a flow rate of 0.8 mL/min. Of the 3 mL effluent and eluate fractions were collected. The HPLC-ELSD chromatogram of GABA in the 22nd fraction resulted in high GABA recovery of 98.08% obtained from the column (figure 8). From figure 7 and figure 8, the results show that numerous impurities in the aqueous extract of Ma-Lod flesh were removed after passing through the Dowex 50W-X8 column.
Figure 7. Typical HPLC-ELSD chromatogram of the aqueous extract of Ma-Lod flesh separating on the Inertsil® NH\textsubscript{2} HPLC column. GABA was separated with retention time of 54.6500 min.

Figure 8. HPLC-ELSD chromatogram of the eluate (the fraction 22) from the Dowex 50W-X8 column.

4. Conclusions
According to the results of this study, there was a high correlation between the FRAP value and total phenolic content, indicating phenolic compounds could be one of the main contributors to oxidant reducing power of Ma-Lod flesh extracts. The fruit flesh extracts of ML-1, ML-4, ML-5, and ML-17 could be the promising sources of biologically active compounds due to their high total phenolic contents, antioxidant activity and outstanding of tyrosinase inhibitory activity among twelve tested Ma-Lod trees. In addition, GABA was also found in Ma-Lod flesh extract and the high recovery of GABA was obtained after separation it by ion exchange chromatography. This study shows that this wild edible fruit could be considered as a potential source of natural biologically active compounds for application in food supplement, medicine, and cosmetics in the future.

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