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Bioconversion of Gentisein to Norathyriol by *Mangifera indica* L. Leaf Cell-Free Extract

Jatupong Sitsutheechananon, Anuwatchakij Klamrak, Natsajee Nualkaew*

Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand

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ABSTRACT

Mangifera indica (mango) leaves are a source of bioactive xanthones—including mangiferin, gentisein, and norathyriol—that possess hypoglycemic, anti-cancer, and antioxidant activities. The co-occurrence of these structurally related compounds suggests the presence of enzymes involved in their biosynthesis in the mango leaves. For the synthesis of bioactive compounds using cell-free extracts, the catalytic activities of the enzymes involved in the proposed biosynthetic pathway should be determined. This study aimed to investigate the hydroxylase activity that converts gentisein to norathyriol, and the *C*-glycosidase activity that cleaves mangiferin to norathyriol in the mango leaf cell-free extract. The enzymatic products were detected via high-performance liquid chromatography. The results indicated the presence of norathyriol with an enzymatic activity of 3.89 µmoles/min/mg protein (64.83 nkat/mg protein) using gentisein as a substrate. The change in the peak area for mangiferin in the reaction was also presented. These results demonstrated that the enzymatic functions in the mango leaves extract catalyzed gentisein to norathyriol. Consequently, a potential strategy can be inferred for developing a biocatalytic process of norathyriol production.

Keywords: *Mangifera indica*, Mango, Gentisein, Norathyriol, Mangiferin, Cell-free extract.

Introduction

Mangifera indica is a plant belonging to the Anacardiaceae family. It contains bioactive xanthones, mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), as well as the intermediates of the xanthone biosynthetic pathway, 1,3,7-trihydroxyxanthone (gentisein), and 1,3,6,7-tetrahydroxyxanthone (norathyriol). These substances exhibit a wide range of biological activities—including antioxidant, anti-diabetic, and anti-cancer properties.[1-6]

Norathyriol—an aglycone and a metabolite of mangiferin—exhibits several activities and has great potential for use in health products.[7] It is a potent α-glucosidase inhibitor that reduces postprandial blood glucose with an IC_{50} of 3.12 µM, which is more effective than mangiferin (IC_{50} = 58.54 µM).[5,8] It also possesses anti-oxidant, anticancer, and anti-aging activities.[2,9] Additionally, it is used as a biomarker to monitor the pharmacokinetics of mangiferin in food supplements.[10,11] Norathyriol can serve as a lead compound for the production of prenylated xanthones such as α-mangostin, and glycoside derivatives such as norathyriol 6-O-glucoside,[12] which possess various pharmacological activities. However, norathyriol is very expensive as it is only present in trace amounts in plants. Purification from 25 kg dried mango leaf yielded 18 mg norathyriol,[13] whereas 1.8 kg dry weight of the stem bark and twig of *Hypericum lanceolatum* Lam. provided 4.2 mg norathyriol.[14]

*Corresponding author. E mail: nnatsa@kku.ac.th
Tel.: +66 43202178

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Similarly, only 5.3 mg norathyriol was purified from 2.4 kg dry weight of *Garcinia parvifolia*.[15] Gentisein is converted to norathyriol via xanthone 6-hydroxylase in the microsomal fraction of *Hypericum androsaemum* cell cultures, followed by further prenylation to yield prenylated xanthones.[16] In contrast, mangiferin—a *C*-glycoside of norathyriol—is not directly produced from the addition of sugar to norathyriol in the mango leaf.[17] Although cleaving the sugar moiety of mangiferin yields norathyriol, the *C*-glycosidic bond is difficult to break. The *C*-deglycosylation enzymes from plants have primarily been reported for flavonoid *C*-glycosides.[18] Amongst these, the enzymes that convert mangiferin to norathyriol in the mouse intestinal bacterium and the human gut microbiota have been identified.[7,19]

As mango leaves contain gentisein, norathyriol and mangiferin, they should presumably also include enzymes involved in the production of those compounds such as hydroxylase, *C*-glucosyltransferase, and *C*-glycosidase. However, although the biosynthetic pathway of mangiferin has been proposed, the bioconversion of gentisein and mangiferin to norathyriol by crude enzyme extracts of mango leaves has not been experimentally demonstrated to date. The proposed biotransformation has been depicted in Figure 1. Enzymes are biological catalysts that play essential roles in the synthesis of bioactive compounds and industrially valuable compounds, such as drugs and foods.[20] Crude enzymes can be used as biocatalysts to perform cell-free reactions, thereby yielding bioactive substances. This enzymatic method has advantages over the chemical synthesis method because enzymes exhibit substrate specificity, produce enzymatic products with specific stereochemistry, and target particular positions on functional groups. This method is also environmentally friendly, as it reduces waste from organic solvents. Using crude enzymes can save costs when compared to purified enzymes by skipping the enzyme purification step.[21] The use of crude enzyme extracts as a catalyst, such as the protein extract from recombinant *Escherichia coli* to produce chalcone glycosides.[22] The development of crude enzymes as biocatalysts for the biosynthesis of bioactive compounds should involve investigating the targeted enzymatic activities within the biosynthetic pathway. These crude enzyme extracts could be appropriately prepared to remove the low molecular weight compounds that might interfere with the enzymatic reaction.

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Hence, this study aimed to verify the formation of norathyriol by the forward reaction of gentisein, catalyzed by the xanthone hydroxylase, as well as the reverse reaction, the *C*-glycoside cleavage of mangiferin to norathyriol. The cell-free extract reaction of the crude enzyme from *Mangifera indica* L. leaves, which utilized the direct addition of gentisein and mangiferin has not been previously reported. As xanthone-6-hydroxylase, *C*-glucosyltransferase, and *C*-glycosidase are membrane-bound enzymes, and the biosynthesis might be occurred in specific compartments, those enzymatic activities were investigated in 2 separated fractions of crude enzyme extract, which were the supernatant part (containing soluble enzymes and microsomes) and the pellet (containing chloroplast, mangiferin, and the enzymes in chloroplast). The enzymatic products were evaluated via high-performance liquid chromatography (HPLC). The results demonstrated the presence of enzymatic activities within the proposed biosynthetic pathway, and suggested a potential strategy for developing an enzymatic system for the production of norathyriol.

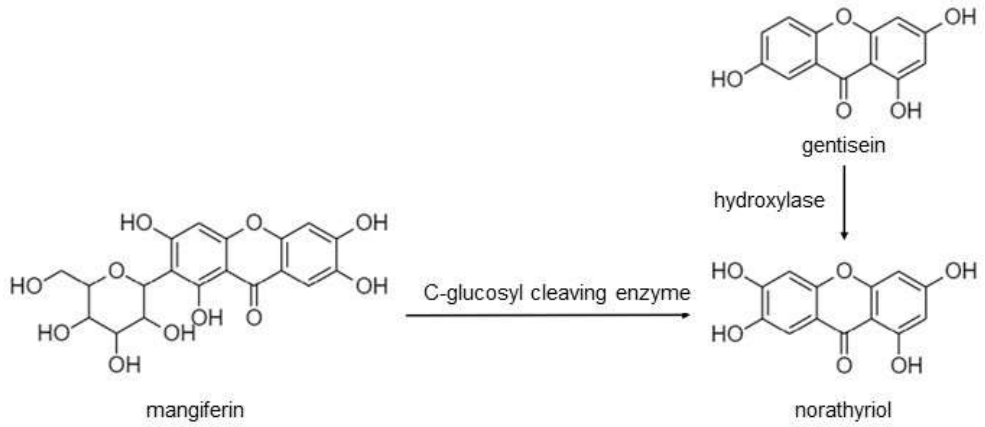

Figure 1: Proposed biosynthetic pathway related to the presence of gentisein, norathyriol, and mangiferin, by a combination of enzymes in mango leaves

Materials and Methods

Chemicals

All general solvents used in this study were of analytical grade. Solvents for HPLC analysis were HPLC grade. Standard compounds 1,3,7-trihydroxyxanthone (gentisein, %purity ≥95%) was purchased from Cayman (Michigan, USA); 1,3,6,7-tetrahydroxyxanthone (norathyriol, % purity 98%) was purchased from Hotspot Biotechnology (Weifang, China); and mangiferin (% purity >95%) was purchased from Biopurify (Sichuan, China).

Plant materials

Fresh young leaves of *Mangifera indica* (Figure 1S) were collected from an open field (Latitude 16.602239, Longitude 102.635293) in Khon Kaen province, Thailand. It was identified by Associate Prof. Dr. Somsak Nualkaew from Mahasarakham University, Mahasarakham Province, Thailand. The voucher specimen was deposited in the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen Province, Thailand.

Enzyme extraction

The fresh young mango leaves were washed and ground using liquid nitrogen in a mortar and pestle. Subsequently, they were homogenized in the ice bath for 15 min with the extraction buffer comprising 50 mM sodium phosphate buffer (pH 7.3), supplemented with 5 mM β-mercaptoethanol, and 10 mM EDTA, in a ratio of 1 g leaf powder to 5 mL of buffer. Thereafter, it was filtered through a cheesecloth, followed by centrifugation at 3,000 × *g*, 4 °C, for 10 min to remove cell debris. The supernatant was centrifuged at 15,000 × *g*, 4 °C for 20 min to yield the supernatant (S15000) and pellet (P15000). S15000 was concentrated using Vivaspin 20 (MWCO 10 kDa, GE HealthCare, Illinois, USA) and passed through a PD-10 column (GE Healthcare, Buckinghamshire, UK) to remove the low molecular weight substances. Subsequently, it was freeze-dried and stored at -20 °C until use. Dried S15000 was redissolved with 1 mL extraction buffer prior to use. Similarly, P15000 was redissolved in 1 mL of the extraction buffer containing 0.1% Triton X-100. The protein concentration was determined via the Bradford method[23] and calculated from the BSA standard graph $y = 0.0168x + 0.132,\ R^2 = 0.984$.

Enzyme assay

The enzymatic reaction (100 µL) consisted of 10 mM sodium phosphate buffer (pH 7.3), 1 mM β-mercaptoethanol, 20 mM $MnCl_2$, 1 mM substrate (gentisein or mangiferin), and 30 µL enzyme extract. The reaction was incubated at 37 °C, stopped by adding 10 µL of 20% HCl, and was partitioned with 500 µL EtOAc. Subsequently, the EtOAc layer was collected and dried using a SpeedVac concentrator (Savant, Thermo Fisher Scientific, USA). The residues were re-dissolved with MeOH prior to HPLC analysis. The boiled enzyme was prepared by heating the enzyme in a water bath at 100 °C for 30 min. The specific enzyme activity was calculated from the amount of an enzymatic product (µmol) observed per minute per mg protein in 1 mL of the reaction.

HPLC analysis

An HPLC system (Agilent 1260, Agilent Technology, CA, USA) was used for the quantitative analysis of gentisein, norathyriol and mangiferin. A 20 µL aliquot of the sample was applied to the RP-18 column (VDSpher PUR 100 C18-E, 4.6 x 250 mm, 5 µm, VDS optilab, Berlin, Germany). The mobile phase used in this study was modified,[21] wherein it consisted of solvent A (45% MeOH in 20 mM KH_2PO_4 and adjusted pH to 3.19 with *O*-phosphoric acid) and solvent B (100% MeOH). The column was equilibrated with solvent A, and gradient-eluted as follows: 0–5 min, 100%–90% A; 5–30 min, 90%–87.5% A, and washed with 0% A. The flow rate was set at 1.3 mL/min, the column temperature was 27 °C, and the peak was monitored by a UV detector at a wavelength of 310 nm. The peaks for gentisein, norathyriol and mangiferin were identified by their retention times, and confirmed by comparison with the UV spectra of the standards.

Additionally, the comparative contents of gentisein, norathyriol, and mangiferin were determined from the standard graphs plotted between the concentration (µg/mL) versus the peak area (Figure 2S). The formulas were $y = 88.013x - 0.0826, R^2 = 0.9998$ for gentisein in a concentration range 0.04–1.25 µg/mL; $y = 96.229x - 1.5708, R^2 = 0.9994$ (concentration range of 0.08–1.25 µg/mL) for norathyriol, and $y = 20.476x - 7.941, R^2 = 1.000$ in a concentration range of 0.08–640 µg/mL for mangiferin. Only the peak area within the linearity range was calculated for the concentration.

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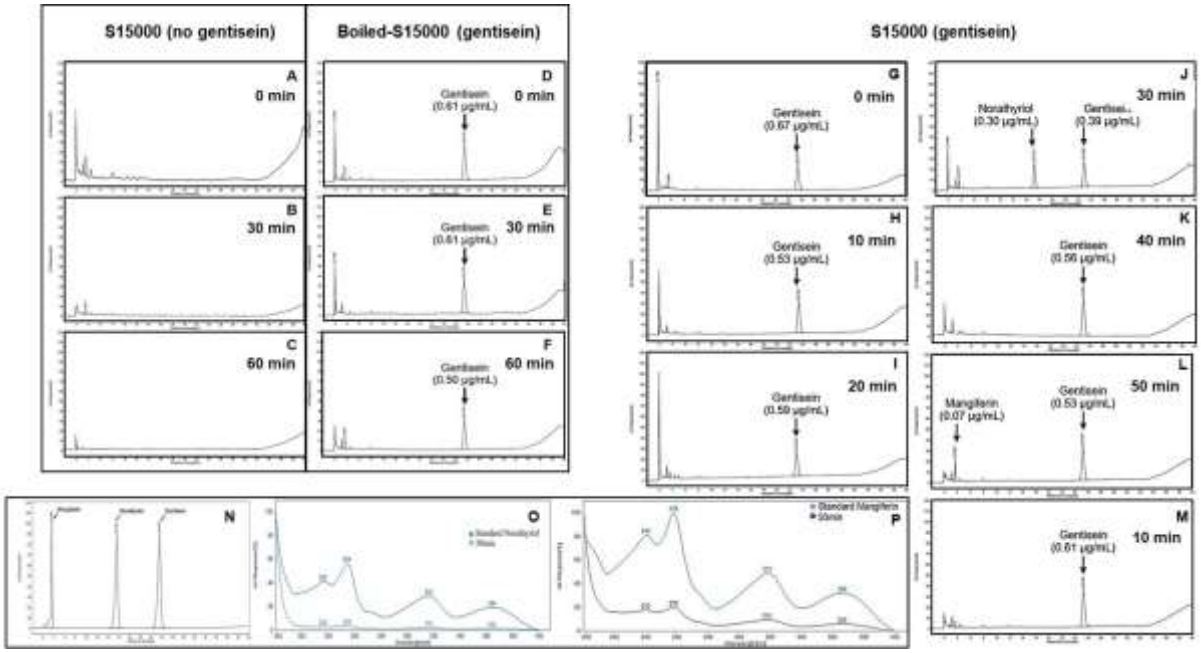

Figure 2: HPLC chromatogram of the enzymatic products with various incubation times. (A–C) Incubation of S15000 without gentisein; (D–F) Boiled S15000 incubated with gentisein; (G–M) Reactions between S15000 and gentisein at 0–60 min incubation times; (N) Mixture of gentisein (RT 23.468 min), norathyriol (RT 15.582 min), and mangiferin (RT 3.594 min); (O) Comparison between the UV spectrum of norathyriol from the enzyme reaction at 30 min and that of standard norathyriol; (P) Comparison between the UV spectrum of mangiferin from the enzyme reaction at 50 min and that of standard mangiferin.

Results and Discussion

Norathyriol (1,3,6,7-tetrahydroxyxanthone) is a bioactive compound that is an intermediate in the xanthone biosynthetic pathway in plants. It is formed from the hydroxylation of 1,3,7-trihydroxyxanthone (gentisein).[16] Mango leaves contain abundant mangiferin, as well as lesser quantities of gentisein, and a trace amount of norathyriol. In humans, the conversion of mangiferin to norathyriol occurs through the activity of gut microbiota.[19] Furthermore, based on the biosynthetic pathway, norathyriol is an essential intermediate of α-mangostin. The prenylation of C2 and C8 of norathyriol generates γ-mangostin, which further undergoes O-methylation at the hydroxy group of C7 to yield α-mangostin.[12] Although xanthone-6-hydroxylase activity has been observed in plants such as *Hypericum androsaemum* cell cultures,[16] its activity in enzyme extracts from mango leaves has not been reported. Consequently, this study investigated the transformation of gentisein to norathyriol and the *C*-glycoside cleaving activity of mangiferin to norathyriol in mango leaves using the supernatant fraction (S15000), which contained microsomal and membrane-bound enzymes, as well as the pellet fraction (P15000), comprising chloroplasts and mangiferin.

Enzyme extracts and detection of mangiferin, norathyriol and gentisein by HPLC

S15000 and P15000 fractions were obtained via centrifugation of the crude enzyme extract. P15000 was composed of chloroplasts, which accumulated mangiferin, as reported in the *Coffea pseudozanguebariae* leaf.[24] The protein contents in the supernatant and pellet fraction had a concentration of 3.29 µg/mL, and 39.00 µg/mL, respectively.

The HPLC retention time (RT) of mangiferin, norathyriol, and gentisein was 3.594, 15.582, and 23.468 min, respectively (Figure 2N). However, no mangiferin, norathyriol, or gentisein was detected in S15000 (Figure 2A). This was because they were removed by the gel filtration column (PD-10 column), whereas the P15000 fraction contained of mangiferin (Figure 4A).

Transformation of gentisein into norathyriol

Boiled enzyme S15000 was incubated with gentisein and did not exhibit any enzymatic activity until 60 min (Figure 2D–F). The time-course reaction between S15000 and gentisein demonstrated the formation of norathyriol 0.30 µg/mL after a 30-min reaction period (peak at RT 15.197 min) (Figure 2J). The peak for norathyriol was confirmed via UV spectrum analysis, whereby the absorption peaks corresponded to those obtained for the standard norathyriol at 254, 236, 316, and 364 nm (Figure 2O), as per the previous report.[11] Furthermore, the specific activity of the enzyme was calculated from the formation of norathyriol (0.30 µg/mL) after 30 min of incubation with 3.29 µg/mL of enzyme in a 30 µL reaction volume of 0.1 mL, corresponding to 3.89 µmoles/min/mg protein or 64.83 nkat/mg protein.

The results confirmed xanthone hydroxylase activity in S15000, which catalyzed gentisein to norathyriol. This enzyme has been known as xanthone 6-hydroxylase (X6H), which is classified as a cytochrome P450 monooxygenase due to its NADPH-dependent hydroxylase activity. X6H is also found distributed in the microsomal fraction of *Hypericum androsaemum* cell cultures and cell cultures of *Centaurium erythraea* RAFN.[16] Although X6H function required NADPH (the electron donor), the hydroxylation of gentisein to form norathyriol in the absence of NADPH was observed in this study. This may be attributed to an NADPH-non-dependent reaction or by a reduction in catalytic hydroxylase activity. Similarly, the hydroxylation of geranylgeraniol in *Croton stellatopilosus* in the absence of NADPH yields plaunotol, with NADPH-dependent hydroxylase activity estimated at approximately 54%.[25] Thus, the characterization of the enzyme activity in S15000 needs to be further investigated.

The peak for mangiferin appeared at 50 min of reaction time, as confirmed via the UV spectrum of standard mangiferin, which exhibited absorption peaks at 258, 240, 318, and 366 nm (Figure 2P), in accordance with the findings of Souza et al.[11]

Longer incubation times for S15000 and substrates gentisein or mangiferin for 2 h and 24 h, did not yield any norathyriol (Figure 3B). However, a small peak for mangiferin was detected in the reaction using gentisein as a substrate (Figure 3A,B).

Transformation of mangiferin by crude enzyme extracts

Norathyriol is an aglycone derivative of mangiferin. Therefore, the conversion of mangiferin backwards into norathyriol by the crude enzyme from mango leaf was investigated in the present study. The reaction mixture containing S15000 and mangiferin; and the incubation of P15000 containing mangiferin, were performed with or without the addition of gentisein.

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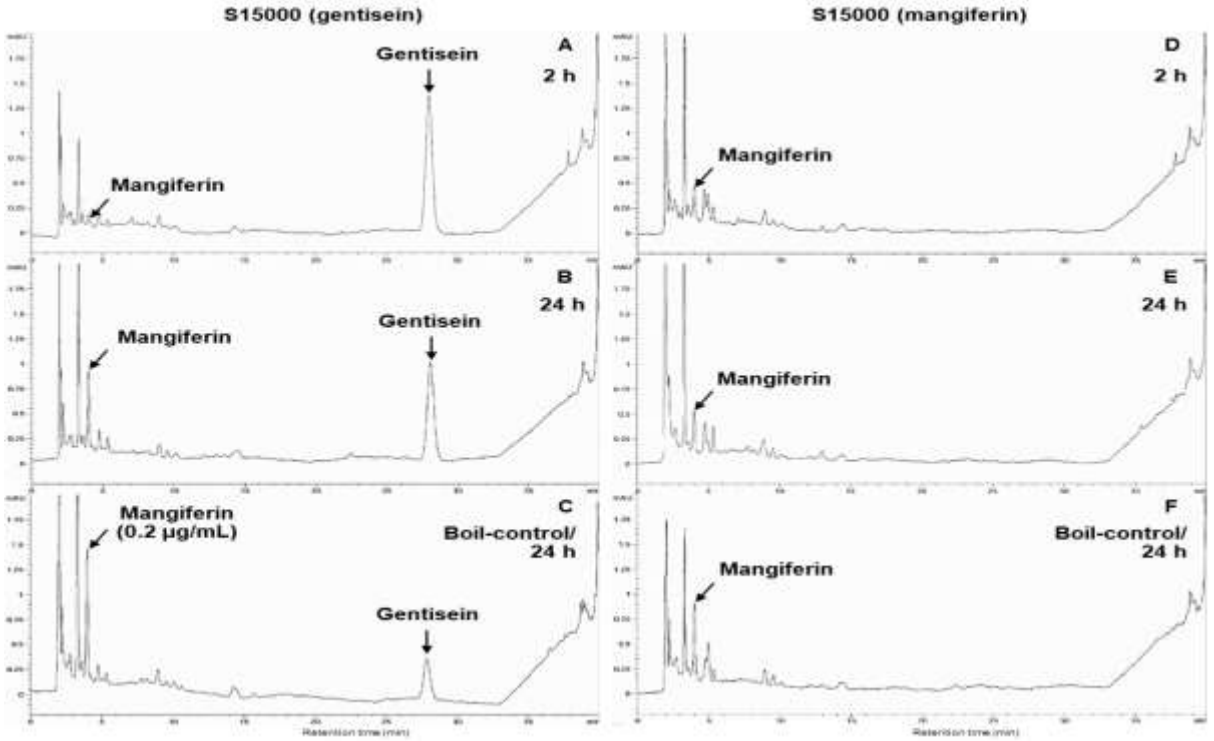

Figure 3: HPLC chromatogram of the enzymatic reaction extracts at 2 and 24 h of incubation. (A–B) The reaction between S15000 and gentisein; (C) The reaction between boiled S15000 and gentisein; (D–E) The reaction between S15000 and mangiferin; (F) The reaction between boiled-S15000 and mangiferin.

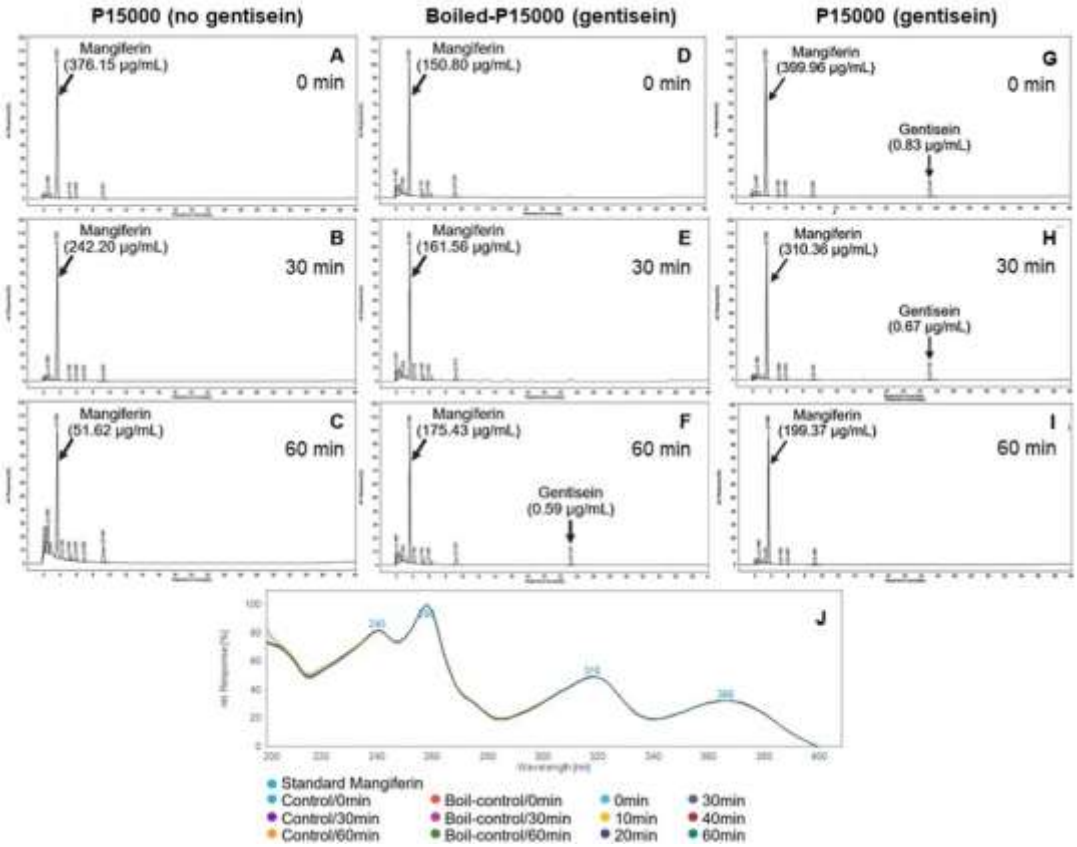

Figure 4: HPLC chromatogram of the reaction between P15000 and gentisein at 0, 30, and 60 min. (A–C) Incubation of P15000 without gentisein; (D–F) Incubation of boiled P15000 with gentisein; (G–I) Incubation of P15000 with gentisein; (J) Comparison of the UV spectrum of mangiferin from the enzyme reactions between P15000 and gentisein with that of standard mangiferin. The concentrations of mangiferin, and gentisein were calculated from the standard graph.

The results did not demonstrate any production of norathyriol from the reactions of S15000 and P15000, regardless of whether gentisein or mangiferin was used as the substrate (Figure 2–4). The non-formation of norathyriol indicated the absence of *C*-glucosyl-cleaving enzyme activity that cleaves the glucose moiety of mangiferin, despite using optimal conditions for this enzyme (including Mn^{2+} as a cofactor and a pH of 7.3).[26]

The peak area of mangiferin was altered after the enzymatic reaction (Figure 2–4). Additionally, incubation of P15000 with and without gentisein led to a decrease in mangiferin, which might be due to its transformation. In contrast, the reaction of boiled P15000 with gentisein increased mangiferin levels.

Although the boiled enzyme has been commonly used as a negative control, in some cases, it still retains enzyme activity. For instance, boiling the cell-free extract of *Croton stellatopilusus* for 30 min enhanced GGOH-18-hydroxylase activity.[25] Furthermore, the enzymatic product may also be produced via a non-enzymatic effect. *C*-glycosylation of flavan-3-ols has been observed to originate via non-enzymatic reactions with oligo- and polysaccharides.[27]

The results also indicated a higher peak area for mangiferin from the reaction of P15000 with gentisein (Figure 4G–I) than from the reaction without gentisein (Figure 4A–C) at the same incubation time. The concentrations for the reactions with and without gentisein were 399 and 376 µg/mL, 310 and 242 µg/mL, as well as 199 and 51 µg/mL, respectively, at 0, 30, and 60 min thus indicating the formation of mangiferin from gentisein. The increase in mangiferin could be explained on the basis of its biosynthetic pathway. It is known that norathyriol—the aglycone derivative of mangiferin—is not a precursor in the biosynthesis of mangiferin.[17,28,29] Therefore, the peak for mangiferin observed in the reaction using gentisein as a substrate in this study might not have resulted from the glycosylation of norathyriol. *Mangifera indica* leaf contains benzophenone *C*-glycosyltransferase (*Mi*CGT), which is capable of converting maclurin to mangiferin.[29] It also includes norathyriol, which provides tripteroside, a norathyriol 6-*O*-glucoside catalyzed by norathyriol 6-O-glucosyltransferase (StrGT9).[30]

As the low molecular weight substances—including maclurin in S15000—were removed using a PD-10 column, the detected peak was unlikely to be mangiferin. From the previous study, norathyriol 4-C-glycosyltransferase (N4CGT1) has been previously identified in *Hypericum perforatum*. This enzyme utilizes norathyriol as a substrate to form isomangiferin.[31] Mangiferin and isomangiferin are isomers with similar properties such as molecular weight, UV spectrum, and polarity.[32] Therefore, the HPLC peak identified as mangiferin in this study could potentially have been isomangiferin (1,3,6,7-tetrahydroxyxanthone 4-*C*-glucoside) instead.

This corresponded to the previous findings that gentisein is a substrate of norathyriol, and is further glycosylated by N4CGT1 to form isomangiferin.[31] Therefore in this study, it could be explained that norathyriol was formed via the enzymatic reaction of enzymes in the mango leaf that utilized gentisein as a substrate, and the resulting norathyriol was presumably transformed to isomangiferin. Further experiments should be conducted to confirm this observation, and the condition of the reaction should be improved to get a higher yield of norathyriol.

In summary, the microsomal-containing enzyme extract of mango leaf catalyzed gentisein to norathyriol at an optimal incubation time of 30 min. In contrast, the catalytic activity of the *C*-glycosidic cleaving enzyme that cleaved the glucose moiety from mangiferin to norathyriol could not be detected. The results suggested the possible utilization of gentisein as a substrate for the mango leaf crude enzyme extract to produce norathyriol.

Although mangiferin is cheaper than gentisein, it could not produce norathyriol under the conditions of the enzymatic reaction in this study. The preparation of norathyriol from mangiferin using a *C*-glycosidase might be developed from other sources, such as *Bacillus* sp. KM7-1 from mouse intestine or human colon bacteria.[7,19]

The two main drawbacks of using immobilized NADH-dependent cytochrome P450 have been its intrinsic low stability and the high cost of NADPH.[33] Our reaction condition demonstrated the potential in utilizing crude enzyme extract from mango leaf to transform gentisein to norathyriol in the absence of NADPH. For the supply of gentisein as a substrate, the development of a process for its production by recombinant *E. coli* is ongoing.

Conclusion

Norathyriol was formed via an enzymatic reaction between a cell-free mango leaf extract and gentisein. No enzymatic activity of *C*-glycoside was detected in the cleaving of mangiferin. The results suggest the potential use of the crude enzyme extract from mango leaves for the preparation of norathyriol using gentisein as a substrate. Since gentisein is not a cheap substrate, it is possible to generate it from recombinant *E. coli* carrying the biosynthetic pathway for gentisein. Moreover, the immobilized enzyme from mango leaves will be further developed into a cost-effective, recyclable cell-free synthetic biology system.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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