Synthesis of High-purity Pentagamavunon-0: Purification Improvement and Crystal Isolation from Rinse Solvent

Yance Anas[1,2], Nunung Yuniarti[3], Ronny Martien[4] and Ratna Asmah Susidarti[5]*

[1]Doctoral Program of Pharmacy Science, Faculty of Pharmacy, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia

[2]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Wahid Hasyim, 50236 Semarang, Indonesia

[3]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia

[4]Department of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia

[5]Department of Pharmacy Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, 55281 Yogyakarta, Indonesia

*Corresponding author (e-mail: ratna_asmah@ugm.ac.id)

Recent advancements in nanotechnology have led to a growing demand for pentagamavunon-0 (PGV-0) synthesis. PGV-0 is a curcumin analogue with various pharmacological effects, and its synthesis has been improved for consistency. However, the purity of PGV-0 remains unclear. This study aims to advance a high-purity PGV-0 synthesis method via purification, recrystallization, and isolation from the rinse solvent. The two-hour synthesis was conducted using vanillin and cyclopentanone. Crude PGV-0 was extracted with a 1:1 mixture of acetic acid and distilled water, followed by rinsing with ethanol and boiling distilled water, and recrystallization with hot ethanol and iced-cooled distilled water. The chemical structure was verified by ^{1}H-NMR spectra, while purity was examined using TLC, melting point and range, and HPLC. After rinsing and recrystallization, we acquired 9.497 g of synthesized PGV-0 with a purity of 99.29 %, a melting point of 204.0 - 205.5 °C, and a melting range of 1.3 ± 0.27 °C. The yield from the rinse solvent was 3.161 g, with a purity of 99.18 %. This study presents a novel method for isolating PGV-0 from the rinse solvent and establishes the foundation for future optimization of high-purity PGV-0 synthesis.

Keywords: High-purity PGV-0; pentagamavunon-0 synthesis; purification improvement; recrystallization; rinse solvent

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Pentagamavunon-0 or 2,5-bis-(4'-hydroxy 3'-methoxybenzylidine) cyclopentanone is a curcumin analogue with unique pharmacological properties. Studies have demonstrated its antioxidant and free radical scavenging abilities [1, 2], as well as its anti-inflammatory [3], selective COX-2 inhibitory [4], and cytotoxic effects against various cancer cells [5–8]. In addition, PGV-0 exhibits antibacterial[3], histamine-release inhibitory [9], antiviral [10], and hepato-protective activity [3]. Despite these promising findings, PGV-0 is still in the early stages of development. Researchers have assessed its biological effects through in vitro, in vivo, and cell-based assays using PGV-0 obtained from the National Molecule Team at Universitas Gadjah Mada. However, the purity data for the PGV-0 used in these studies was undisclosed, leading to some uncertainty regarding its reported pharmacological effects. In drug discovery, compound purity is crucial as it determines the drug's effects and stability. Researchers must use material of at minimum pharmaceutical-grade quality to ensure accurate results [11]. Any impurities present in the compound can significantly impact study results and even pose a risk to patients [12].

From 1997 to 2015, scientists explored the pharmacological effects of PGV-0. Despite initial motivation around PGV-0's potential as a drug candidate, its low solubility and bioavailability diminished this interest. To address these limitations, researchers have turned to nanotechnology-based drug delivery strategies [4,13] to improve PGV-0's bioavailability and investigate its pharmacokinetics. Consequently, there is a pressing need to synthesize high-purity PGV-0 to advance further research.

Sardjiman et al. were the first to report on PGV-0 synthesis [2]. They used vanillin and cyclopentanone as starting materials and hydrochloric acid as a catalyst in a 14-day reaction that resulted in 97 % crude PGV-0. Despite this early success, subsequent studies have shown that this synthesis method struggled to produce PGV-0 with consistent physicochemical properties [14]. To tackle this

challenge, researchers modified reaction conditions such as time, temperature, catalysts, and stirring techniques [10, 15, 16]. Recently, a study advanced the PGV-0 synthesis method by incorporating a constant stirring speed and alternative catalysts. The author rinsed crude PGV-0 with warm water and recrystallized it with hot ethanol, producing an orange-coloured PGV-0 [14]. However, the study did not report on the purity of the synthesized PGV-0.

In this study, we aimed to develop a purification procedure that would yield high-purity PGV-0. To achieve this, we applied both boiling distilled water (100 °C) and ethanol as rinsing solvents, with the latter chosen for its eco-friendly properties [17]. However, despite its benefits, ethanol also has the potential to dissolve PGV-0 [16]. Thus, our secondary objective was to isolate PGV-0 from the rinse solvent. We then recrystallized crude PGV-0 using a two-solvent approach with hot ethanol and ice-cooled distilled water. We confirmed the chemical structure of PGV-0 using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, and the findings were compared to prior literature. Finally, we evaluated the purity of the PGV-0 using three methods: thin layer chromatography (TLC), melting point analysis, and high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and Materials

This study used pro-analysis chemicals for synthesis, rinsing, recrystallization, and evaluation of PGV-0 purity. These included vanillin pro-synthesis, cyclopentanone, hydrochloric acid, acetic acid, acetonitrile, ethanol, methanol, ethyl acetate, and chloroform. All were sourced from Merck (Germany). In addition, we purchased other materials from registered suppliers, such as aquadest, or distilled water (Brataco chemika, Indonesia) and aquabidest, or double distilled water (IKA Pharmindo, Indonesia).

PGV-0 Synthesis

PGV-0 synthesis was implemented according to the improved technique [14] with modifications and was conducted on ten consecutive replicates. In brief, 7.4 g of vanillin (50.0 mmol) was mixed with 2.20 mL of cyclopentanone (25.0 mmol) in a 100.0 mL round-bottom flask. It was stirred using a Corning PC-420D hot plate magnetic stirrer at a speed of 600 rpm and a temperature of 75 °C. After two minutes, 1.0 mL of HCl was added, and stirring continued until solid formation occurred. The reaction was maintained for two hours. Next, crude PGV-0 was extracted with 30 mL of a 1:1 mixture of acetic acid and distilled water for 10 minutes. The solid was transferred to a mortar and ground for 15 minutes using another 30 mL of 1:1 acetic acid-distilled water. The crude PGV-0 was separated from unreacted material using a Buchner funnel connected to a vacuum pump (Rocker 300). The solid was washed on Whatman filter paper (GE Healthcare) with 50 mL of ethanol and 150 mL of boiling distilled water. Then, the crude PGV-0 was re-extracted with 60 mL of 1:1 acetic acid-distilled water and rinsed again with 50 mL of ethanol and 150 mL of boiling distilled water. The extraction and rinse procedure was repeated until the filtrate was clear. Finally, the crude PGV-0 was oven-dried at 76 ± 2 °C for three hours.

PGV-0 Isolation from the Rinse Solvent

We developed a procedure to isolate PGV-0 from the rinse solvent, which was conducted on the 14th day after the two-hour synthesis reaction of PGV-0. The separation of PGV-0 from impurities was initially achieved through filtration using a Buchner funnel and a vacuum pump. Next, the solid was transferred to a mortar and extracted with 60 mL of acetic acid-distilled water (1:1). Then, we rinsed it with 50 mL of ethanol and 100 mL of boiling distilled water. We repeated the extraction and rinsing procedure twice or more until the isolated PGV-0 turned yellow. The crude PGV-0 was then dried in an oven at 76 ± 2 °C for 3 hours and weighed.

PGV-0 Recrystallization and Identification of its Chemical Structure

In a 1.000 mL glass beaker, crude PGV-0 was mixed with 100 mL of ethanol. The mixture was stirred at 100 °C using a hot-plate magnetic stirrer (750 rpm) until the crude PGV-0 dissolved. The solution was then allowed to cool at room temperature. Next, ice-cooled distilled water was added as a second solvent in a 3:1 ratio with hot ethanol. The recrystallization process took 1-2 days. PGV-0 crystals were filtered and dried in an oven at 76 ± 2 °C. Finally, the crystals were weighed to determine the actual yield of PGV-0. In this synthesis, the theoretical yield of PGV-0 was 8.8 g, and the percentage yield was calculated using equation 1. The chemical structure of PGV-0 was confirmed using ^{1}H-NMR spectra on a JNM-ECZ500r/S1 spectrometer from JEOL Ltd.

$$\text{Yield (\%)} = \frac{\text{Actual yield (g)}}{\text{Theoretical yield (g)}} \text{ x } 100\% \quad \text{(Equation 1)}$$

Determination of PGV-0 Melting Point and TLC Profile

We used a melting point apparatus (Reichter, Austria) to measure the melting point and range of PGV-0. The temperature was recorded from when the sample started to liquefy until the entire specimen melted. To determine the TLC profile of PGV-0, we used a 5 cm x 1.5 cm silica gel 60 F254 plate (Merck, Germany) with ethyl acetate-chloroform (1:5) as the mobile phase. PGV-0 and vanillin were dissolved in methanol and then applied to the TLC plate, which had been

activated in an oven at 60 °C for 30 minutes. The plate was developed at 4.0 cm and the spots were visualized under 254 nm UV light.

PGV-0 Purity Analysis with HPLC

PGV-0 and vanillin (5.0 mg each) were carefully weighed and dissolved in methanol to achieve concentrations of 5 and 10 μg mL^{-1} in a 5.0 mL volumetric flask. The wavelengths of PGV-0 and vanillin were scanned between 250-500 nm using a UV-Vis spectrophotometer (Shimadzu UV-1700). To determine the purity of the synthesized and isolated PGV-0 from the rinse solvent, we conducted HPLC analysis using a Shimadzu LC-20AD/T instrument. PGV-0 and its impurities were separated using a LiChospher 100 RP-18 Encapped (5 mm) LiChroCART 250-4 column (Merck) with a mobile phase of acetonitrile-aquabidest (65:35) at a flow rate of 0.75 mL min^{-1}. We recorded a chromatogram at 7.5-8.5 min for the vanillin λ_{max} (365 nm). To determine the level of PGV-0 purity, we first assessed the chromatograms of methanol and vanillin at concentrations of 50, 250, and 500 ng mL^{-1}. We then assessed the chromatograms of synthesized and isolated PGV-0 from the rinse solvent at concentrations of 1, 10, and 100 μg mL^{-1}. Finally, we calculated the purity level of PGV-0 using the following equation:

$$\text{PGV} - 0 \text{ Purity } (\%) = \frac{\text{PGV}-0 \text{ peak area}}{\text{Total Peak Area}} \text{ x } 100\% \quad \text{(Equation 2)}$$

RESULTS AND DISCUSSION

Synthesis and Isolation of PGV-0 from the Rinse Solvent

The study presents a detailed procedure for rinsing and purifying high-purity PGV-0. A prior author published a method for crude PGV-0 washing and recrystallization but it lacked detail [14]. Here, we describe the extraction, rinse, and recrystallization steps involved in PGV-0 synthesis. We also introduce ethanol and boiling distilled water as solvents for the washing procedure to effectively flush the solid that adhered tightly to the Whatman filter paper. The results showed an impressive synthesized PGV-0 yield of 10.79 % in 2 hours (Figure 1b). This result is noteworthy compared to previous studies that reported PGV-0 yields ranging from 75 - 90 % with a 14-day reaction time [15]. While an improved technique in PGV-0 synthesis by Ritmaleni (2016) did not present the resulting PGV-0 yield data [14], the study conducted by Ramadhan et al. using these methods showed that the yield generated was 31.1 % [18]. By shortening the reaction time in this study, we aimed to improve the efficiency of PGV-0 synthesis and purification. This would also reduce production time and costs, making it a valuable contribution to the field. The techniques implemented in this study, such as boiling distilled water and ethanol for rinsing and recrystallization with ice-cold ethanol and distilled water, were responsible for the significantly improved yield and have potential applications in the pharmaceutical industry.

We used ethanol in our rinse procedure to dissolve synthesized PGV-0, and within 14 days, it crystallized in the rinse solvent (Figure 2a). We then isolated and purified it, yielding 3.161 g of PGV-0 (Figure 1a). The isolated PGV-0 exhibited the same bright yellow colour as the synthesized PGV-0 (Figure 2b and 2c). However, the textures of both products were different. The synthesized PGV-0 had a smooth surface and did not adhere to filter paper or glass containers, while the isolated PGV-0 exhibited slight adhesion. Other studies used hot distilled water as a solvent for washing crude PGV-0. They produced final products with different colours, such as yellow [16], orange [14], and pale orange [18].

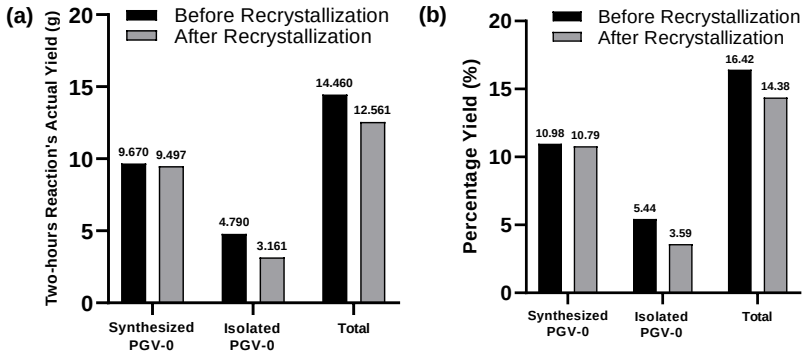

Figure 1. (a) Two-hour actual yield and (b) percentage yield of synthesized and isolated PGV-0 from the rinse solvent before and after recrystallization.

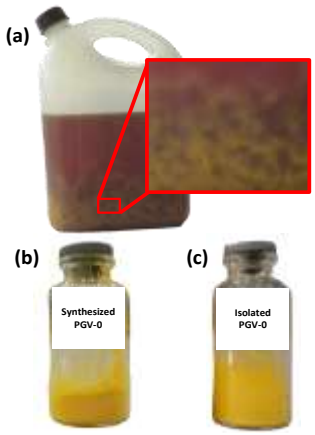

Figure 2. (a) PGV-0 in rinse solvent mixed with the impurities; (b) synthesized PGV-0 and (c) PGV-0 isolated from the rinse solvent.

We analyzed the PGV-0 ^{1}H-NMR spectrum to confirm its chemical structure (Figure 3). The spectra exhibited six proton signals characteristic of PGV-0, including chemical shifts (δ) at 3.121 (s, 4H, C**H**$_2$-C**H**$_2$ - 3,4), 3.918 (s, 6H, 2-OC**H**$_3$ - 3'), 6.889 (s, 1H, **H**-5'), 7.176 (dd, 2H, **H**-6'), 7.205 (d, 2H, **H**-2') and 7.457 (s, 2H, **H**-6 and **H**-7). The phenolic O-H proton does not appear in the ^{1}H-NMR spectrum because it is exchanged with deuterium atoms from the solvent used (CD$_3$-OD). Our results agreed with the ^{1}H-NMR spectra of PGV-0 when it was first reported [2], except for the proton signal of the hydroxy group. The earlier work mentioned that the OH signal of the PGV-0 was at δ 8.79 [2] and 9.60 [15]. In general, the OH signal attached to the aromatic ring was observed between δ 4.50 to δ 7.20 [19]. In addition, different solvents may affect the ^{1}H-NMR spectra. We used methanol (CD$_3$-OD) as a solvent to dissolve PGV-0 in the ^{1}H-NMR analysis. Methanol is a protic solvent and a potential donor for hydrogen bonding [20], especially in the ^{1}H-NMR OH chemical shift of phenol compounds via intermolecular hydrogen bonds [21]. In contrast, preceding studies employed a polar aprotic solvent, dimethyl sulfoxide [22], to dissolve PGV-0 [2, 15]. Based on the resulting ^{1}H-NMR spectra, our results confirmed the successful synthesis of PGV-0.

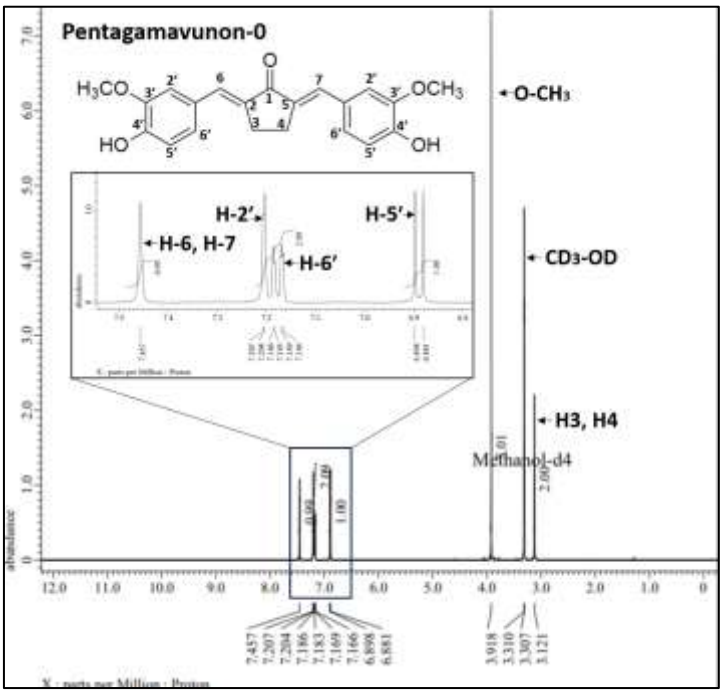

Figure 3. ^{1}H-NMR spectra of synthesized PGV-0 at 500 MHz.

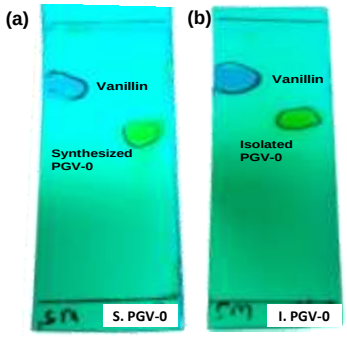

Figure 4. The TLC profile of (a) synthesized PGV-0 and (b) PGV-0 isolated from the rinse solvent.

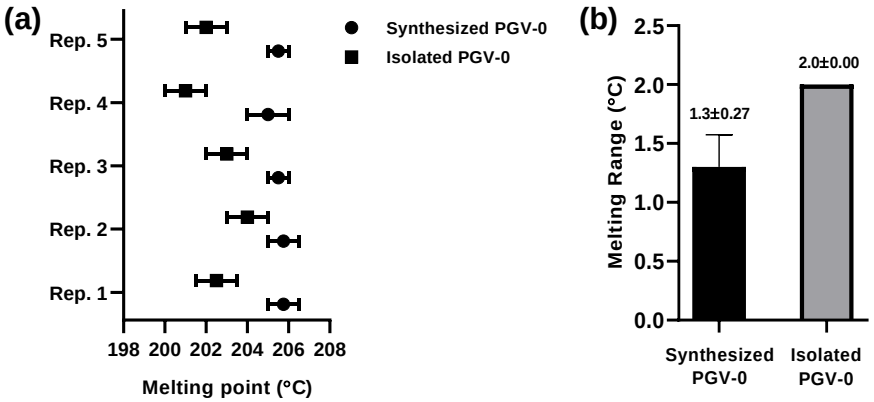

Figure 5. (a) Melting points and (b) melting ranges of synthesized PGV-0 and PGV-0 isolated from the rinse solvent.

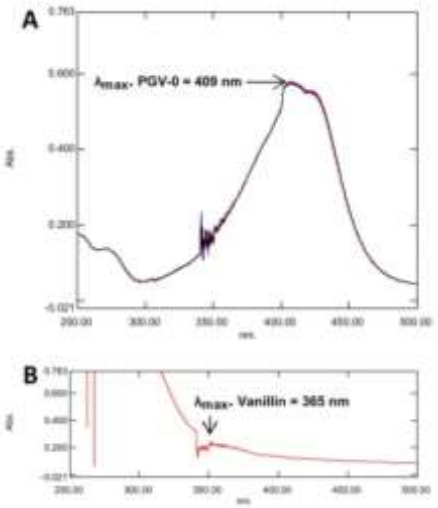

Figure 6. The UV-Vis absorbance profile of A. PGV-0 (5 μg mL^{-1}) and B. Vanillin (10 μg mL^{-1}).

PGV-0 Purity Analysis

Our work determined the purity of the synthesized and isolated PGV-0 samples using several methods, including TLC, melting point analysis, and HPLC. TLC analysis showed no significant differences between the synthesized and isolated PGV-0. We observed similar spots after eluting them with ethyl acetate-chloroform 1:5 (Figure 4a and 4b). The PGV-0 yellow spot was below the purple vanillin

spot, which served as the starting material. The synthesized PGV-0 had a melting point between 204.0 °C and 206.5 °C (Figure 5a) with a narrow range of 1.3 ± 0.27 °C (Figure 5b). In contrast, the melting point of PGV-0 isolated from the rinse solvent varied from 200.0 °C to 205.0 °C, with a melting range of 2.0 ± 0.00 °C. However, this was lower than the PGV-0 melting points reported in earlier studies, which were 212 - 214 °C [2], 222 - 224 °C [15]), and 210 - 212 °C [14]. Despite this, our rinse and recrystallization procedure yielded PGV-0 with a narrow melting point range of 1.0 - 2.0 °C. Therefore, this result suggests that our synthesized PGV-0 had high purity.

To assess the purity of PGV-0, we used a detection wavelength of 365 nm on the HPLC-UV detector to detect vanillin as an impurity, as vanillin was a starting material in PGV-0 synthesis and a potential source of impurity [23–25] in the final product. When scanned with a UV-Vis spectrophotometer, PGV-0 and vanillin showed maximal absorbance (λ_{max}) at 409 nm and 365 nm, respectively (Figure 6A and 6B). The λ_{max} of PGV-0 was consistent with previous reports [18].

We employed HPLC for the purity analysis of PGV-0. This method is widely used in the pharmaceutical industry to determine impurities in drug products [26–28]. Prior to this analysis, we conducted a series of preliminary assays to identify possible impurities. These assays involved using methanol as the solvent for PGV-0, and three different concentrations of vanillin (50, 250, and 500) ng ml^{-1} in both methanol and PGV-0 (1 and 100) µg ml^{-1}. The results showed that methanol produced two peaks with retention times of 2.65 and 2.89 min (Figure 7a), indicating impurities in the HPLC-grade methanol [29]. To identify these impurities, we ran the methanol sample through a HPLC column and examined the resulting peaks. These peaks could interfere with the assessment of PGV-0 purity. Vanillin yielded a peak at 3.29 min (Figure. 7b), and PGV-0 yielded a peak at 4.67 min (Figure 7a). Moreover, during the 100 µg ml^{-1} PGV-0 purity test, we identified several impurities besides vanillin at retention times of 1.88, 2.42 and 3.99 min (Figure 7c). Impurities may be derived from side products of the reaction or contained in vanillin, cyclopentanone, or solvents used in the extraction and washing procedure, such as acetic acid, distilled water, and ethanol. However, based on the HPLC analysis, the concentration of these impurities was less than 1.0 %. The purity of the synthesized and isolated PGV-0 was found to be 99.29 % and 99.18 %, respectively (Table 1). However, the number and quantity of impurities in the isolated PGV-0 from the rinse solvent was higher than in the synthesized PGV-0, suggesting that its purification procedure needed further development. Overall, the study findings suggest that the isolation, rinse, and recrystallization techniques used in the synthesis were successful in achieving high-purity PGV-0.

Our study presents a comprehensive method for synthesising, rinsing and purifying high-purity PGV-0, resulting in a two-hour actual yield of 9.497 %. The amount of trace impurities in the synthesized PGV-0 was less than 1.0 %. Additionally, this study offers, for the first time, a technique for the isolation of PGV-0 from rinse solvent, which can enhance the yield. This step has significant implications for PGV-0 synthesis. Notably, there is no PGV-0 standard available for bioanalytical methods. The PGV-0 produced in this study may serve as a standard due to its high purity (>99 %). These findings suggest that the PGV-0 obtained was a pharmaceutical-grade material and may be used in PGV-0 nanoparticle development and pharmacological-toxicological assays.

Table 1. Purity of synthesized PGV-0 and PGV-0 isolated from the rinse solvent (n = 3, mean value ± SD).

PGV-0	Conc. (µg mL^{-1})	Peak Area					PGV-0 Purity (%)
		PGV-0	Vanillin	impurity 2	impurity 3	impurity 4	
Synthesized	1	75,460 ± 1,932	0 ± 0	812 ± 111	0 ± 0	0 ± 0	**99.29 ± 0.057**
	10	704,940 ± 11,229	3,847 ± 338	0 ± 0	0 ± 0	0 ± 0	
	100	6,507,682 ± 19,687	25,281 ± 987	2,471 ± 356	5,560 ± 248	0 ± 0	
Isolated from the rinse solvent	1	72,335 ± 772	429 ± 412	0 ± 0	0 ± 0	181 ± 21	**99.18 ± 0.306**
	10	655,171 ± 12,534	5,487 ± 288	0 ± 0	0 ± 0	183 ± 12	
	100	6,134,299 ± 110,320	29,926 ± 524	8,140 ± 427	9,274 ± 372	174 ± 30	

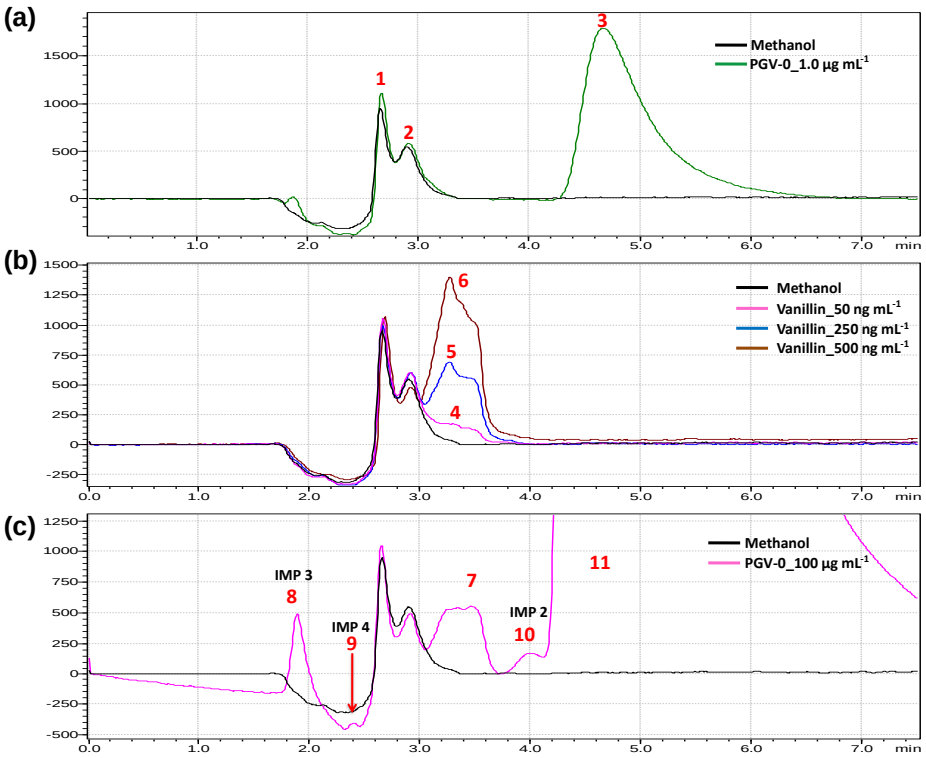

Figure 7. Overlaid chromatograms of PGV-0 purity analysis with HPLC (a) Impurities peaks in methanol as solvent (**1** and **2**) and peak of synthesized PGV-0 1.0 µg ml^{-1} in methanol (**3**); (b) Peak of vanillin 50 ng ml^{-1} (**4**); 250 ng ml^{-1} (**5**) and 500 ng ml^{-1} (**6**) in methanol; (c) Magnified image for detection of vanillin (**7**) and other impurity peaks (**8**, **9** and **10**) in isolated PGV-0 100 µg ml^{-1} in methanol (11)

The present study has several limitations. Firstly, the synthesis produced a low yield due to the limited reaction time. Another limitation was that the PGV-0 purity was solely analysed using HPLC, which may not be comprehensive. Therefore, future research is required to optimize reaction conditions and enhance the yield of high-quality PGV-0. This should include an investigation of the effects of reaction time, temperature, and reactant concentration on the yield of synthesized PGV-0. Additionally, alternative analytical methods, such as Liquid Chromatography-Mass Spectrometry (LC-MS), Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR), LC-NMR-Mass Spectrometry (LC-NMR-MS), and Gas Chromatography-Mass Spectrometry (GC-MS), should be considered for the assessment of impurities in PGV-0 [25].

CONCLUSION

Our study presents a comprehensive approach for high-purity PGV-0 synthesis, rinsing and purification. The results showed an actual yield of 9.497 % after a two-hour reaction with trace impurities of less than 1.0 %. Notably, this study developed a novel technique for isolation of PGV-0 from the rinse solvent, which has the potential to enhance the synthesis yield and has significant implications for future research designs for PGV-0 synthesis. In addition, the synthesized PGV-0 was of pharmaceutical grade and may be used as a standard due to its high purity (>99 %), as well as in PGV-0 nanoparticle development and pharmacological-toxicological assays. However, further study is required to optimize reaction conditions and find alternative methods for purity analysis.

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