# ENZYME-ASSISTED EXTRACTION OF TRITERPENOID AND PHENOLIC COMPOUNDS FROM *Rubus alceaefolius* Poir LEAVES

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## ABSTRACT

The leaves of *Rubus alceaefolius* Poir primarily consist of phenolic and triterpenoid compounds, which possess a variety of biological activities beneficial to human health. This research aimed to explore the impact of enzyme-assisted extraction conditions on the recovery of triterpenoid and phenolic compounds from *Rubus alceaefolius* Poir leaves. Factors considered in the enzymatic treatment process with Viscozyme L cellulase included the solid-to-water ratio (1:15-1:45 w/v), enzyme concentration (0.5-3.5%), temperature (40-60 °C), and extraction duration (30-180 min). The results suggested that the optimal conditions for the enzymatic extraction of triterpenoid and phenolic compounds were a 2.5% enzyme concentration, a 1:35 (w/v) solid-to-solvent ratio, an incubation temperature of 50 °C, and an extraction time of 120 minutes. Under these conditions, the highest amounts of total triterpenoids and phenolics obtained were 231.45 mg UA/g and 9.13 mg GAE/g, respectively. GC-MS analysis identified 17 constituents that potentially contribute to antioxidant potency. Furthermore, the findings showed that the enzyme-assisted extraction (EAE) technique was more efficient in extracting total phenolics and triterpenoids compared to conventional maceration extraction.

*Keywords:* Raspberry leaves, enzyme Viscozyme L cellulase, biological compositions, triterpenoid extraction, polyphenol extraction, antioxidant activity.

## 1. INTRODUCTION

*Rubus alceaefolius* Poir, known as bush raspberry, is a species of flowering plant in the family Rosaceae. It is indigenous to Southeast Asia, with occurrences in countries such as Thailand, Malaysia, and Vietnam [1, 2]. *Rubus alceaefolius* Poir is a perennial plant that tends to thrive in humid and shady environments. It has woody stems that can reach heights of up to 10 feet, and its leaves are simple, alternate, and toothed. The fruit of the *Rubus alceaefolius* Poir is a red or purple drupe, similar in appearance to a raspberry or blackberry, and has a sweet and tangy flavor [1, 2]. These fruits are celebrated for their high vitamin C and phenolic compound content [1-3]. Conversely, the leaves of *Rubus alceaefolius* Poir are rich in elements such as phenolics, flavonoids, and triterpenoids [3-5]. These constituents have impressive antioxidant properties [6] and anti-inflammatory activities [6-8]. Furthermore, they help

reduce the risk of fatty liver disease and other chronic conditions [7]. As a result, these plants have gained significant attention as a source of phenolics and triterpenoids.

The extraction method is crucial to ensure the quality of the resulting extract. Soxhlet extraction and maceration are the most common techniques for extracting phenolic and triterpenoid compounds from plants [8, 9]. However, these methods are time-consuming and have low extraction efficiency [10]. In contrast, enzyme-assisted extraction technique (overcomes these disadvantages, making the process more economical and environmentally friendly [11]. This approach uses less solvent and energy than traditional methods [12, 13] while preserving the activity of bioactive compounds because of their gentle operating conditions [12-14]. Various enzymes, such as alcalase, neutrase, protamex, pectinase, and cellulase have been used to extract bioactive compounds from natural sources. Enzymes can degrade or disrupt cell walls and membranes as well as weaken or break down phenol-polysaccharide links, facilitating the release of bioactive compounds into the extracts [15]. Nonetheless, our current understanding of cellulase-assisted extraction for the recovery of triterpenoids and phenolic compounds from *Rubus alceaefolius* Poir leaves remains limited.

Therefore, the objectives of this study were: (i) to investigate the effects of enzyme-assisted extraction under different extraction conditions for maximum recovery of phenolics and triterpenoids from *Rubus alceaefolius* Poir leaves, and (ii) to compare the extraction efficiency of enzyme-assisted extraction with that of the conventional extraction method.

## 2. MATERIALS AND METHODS

### 2.1. Materials

#### *2.1.1. Plant material*

*Rubus alceaefolius* Poir leaves were collected from Song Hinh district, Phu Yen province. Fresh leaves were washed, chopped, and dried in a Memmert UN110 oven (Germany) at 40 °C to achieve 10% moisture content. Following this, the dried leaves were ground to a fine powder, which was then sifted through a steel mesh sieve with a pore size of 1 mm. The samples were stored at 4 °C until used for further analysis.

#### *2.1.2. Chemicals*

Trolox (97%), Ursolic acid (97%), Ethanol (99.5%), Vanillin (99%), Acid acetic (99.5%), Galic acid (97%), and perchloric acid (99.99%) were procured from Anpha Chemika – India. Other reagents, such as 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH). 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and Folin - Ciocalteu were from Sigma (St. Louis, MO, U.S.A.). Additionally, the enzyme Viscozyme L cellulase (enzyme activity = 100 FBGU/g, pH 5.0 - 5.5) was purchased from Novozyme. All the solvents and reagents used in this study were of analytical pure grade.

### 2.2. Research methods

#### *2.2.1. Effects of enzymatic extraction conditions on bioactive compounds*

The study investigated the impact of enzyme-assisted extraction on triterpenoids and phenolic content. A 20 g sample of *Rubus alceaefolius* Poir leaf powder was dispersed in distilled water inside conical flasks, utilizing a powder-to-water ratio ranging from 1:15 to 1:45 (w:v). Following this, varying quantities of the enzyme Viscozyme L cellulase (0 to 3.5%, v/w) were added to the mixture. The blend was subsequently incubated in a temperature-regulated bath set to desired levels (40, 45, 50, and 60 °C) for specific incubation times (30,

60, 90, 120, 150, and 180 min). Throughout the extraction procedure, the mixture's pH was adjusted to 5.5. The extract was obtained using a vacuum filtration system and vacuum evaporation at 40 °C before analysis.

*2.2.2. Conventional extraction*

For ethanol maceration (ME), 10 g leaf powder was extracted with 100 mL ethanol 70% for 24 hours. The mixture was vigorously shaken in a water bath set to 200 rpm and a temperature of 30 ± 0.5 °C [16]. Subsequently, the solution was filtered through a Whatman No. 1 filter paper and the solvent was removed using a rotary evaporator (Buchi R210, Flawil Switzerland) under the vacuum pressure of 500 mmHg at 50 °C. The liquid extracts were used for further analysis. The experiments were conducted in triplicate.

*2.2.3. Determination of total triterpenoid content (TTC)*

The determination of total triterpenoids was carried out using the method of Hadidi et al. (2020), with a few slight adjustments [12]. In particular, a 0.16 mL extract was combined with 0.4 mL of 5% vanillin/glacial acetic acid (w/v) in a screw cap test tube. Following this, 1.0 mL of perchloric acid solution was introduced and incubated at 60 °C for 30 minutes utilizing a water bath (Memmert WNB, GmbH & Co. KG, Germany). The mixture was then rapidly cooled, 5.0 mL of glacial acetic acid was added, and the absorbance was measured at 573 nm using a UV spectrophotometer/NIR (Shimazu, UV-2600, Japan). For triterpenoid evaluation, ursolic acid served as the standard solution. In order to establish the calibration curves, triterpenoid standard solutions (0.1–1.0 g/100 mL in methanol) were employed. The findings were expressed in mg of ursolic acid equivalents per gram of dry matter (mgUA/g dm).

*2.2.4. Determination of total polyphenol content (TPC)*

The total polyphenol content was determined according to the method of Vuong et al (2013) with slight modifications [13]. A 40 µL sample of the extract was diluted in 1560 µL of water and combined with 100 µL of Folin-Ciocalteu reagent. Subsequently, 300 µL of 10% (w/v) sodium carbonate was added, and the mixture was incubated in darkness for 2 hours. The solution's optical density was measured at a 765 nm wavelength. A standard curve was devised using gallic acid (GAE) with concentrations varying between 0.004 and 0.5 mM, which facilitated the determination of the sample's total polyphenol content (TPC). The TPC is expressed as milligrams of gallic acid equivalents per gram of dry matter (mg GAE/g dm).

*2.2.5. Determination of antioxidant capacity*

In brief, a 35 µL extract was combined with a 7 mM ABTS radical solution (265 µL) in a screw-cap test tube [17]. Subsequently, the tubes were shaken in the dark for 10 minutes before measuring the absorbance at 734 nm using a microplate reader (PR2100, Bio-Rad, USA). Trolox served as the standard solution (0.1-1.0 g/100 mL in methanol) for the ABTS assessment. The results are expressed in milligrams of Trolox equivalents per gram of dry matter (mg TE/g d.m.).

*2.2.6. Determination of some chemical components of extracts by GS - MS method*

The chemical composition of the extract was analyzed using gas chromatography-mass spectrometry (GC-MS), incorporating modifications from the study by Mohd F. A. Bakar et al. [18]. The samples, dissolved in ethanol, were injected into an Agilent 7890A GC system coupled with an MS (Agilent Technologies). Sample separation occurred on a DB-5MS column (30 m length × 0.25 mm diameter × 0.25 µm film thickness). A 2 µL sample was injected in splitless mode, employing helium as the carrier gas at a flow rate of 1 mL/min. The GC-MS system operated under the following conditions: a temperature increase from 50-260°C at a

rate of 10 °C/minute, followed by a 10-minute isothermal phase. Compounds identified via GC-MS were cross-referenced with those in the NIST database.

*2.2.7. Statistical analysis*

In this study, each experiment was repeated 3 times, the results are presented as mean ± standard deviation. To assess the distinctions between treatments at a 5% significance level, a single-factor analysis of variance and the LSD test was used, utilizing the Minitab Statistical Software for assistance.

## 3. RESULTS AND DISCUSSIONS

### 3.1. Effect of ratio of enzyme Viscozyme L cellulase concentration on the extraction of total triterpenoid (TTC) and total phenolic compounds (TPC)

Fig. 1 illustrates the impact of enzyme Viscozyme L cellulase concentration on the extraction of total triterpenoid (TTC) and total phenolic compounds (TPC) from *Rubus alceaefolius* Poir leaf powder. As shown in Fig 1, it can be seen that the TTC and TPC in the extracts increased significantly when enzyme concentrations ranged from 0.5% v/w to 3.5% v/w. The highest TTC (219.43 mg UA/g dm) and TPC (8.49 mg GAE/g dm) were achieved at an enzyme concentration of 2.5%, approximately twice as high as the extracted sample at 0.5% (v/w) enzyme. A higher enzyme concentration enhances the enzyme's ability to break down cell wall structures, thereby promoting the release of bioactive compounds from the complex form [9]. Similar behavior has been referenced previously by Barzana et al. (2002) for the carotenoid extraction from marigold flowers [16]. However, further increases in enzyme concentration had no significant effect on the extraction yield of phenolics and triterpenoids. Consequently, a 2.5% (v/w) enzyme concentration was chosen for subsequent investigations.

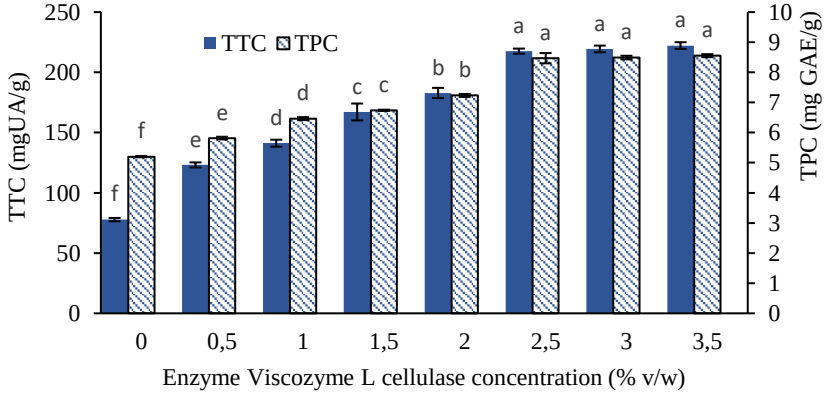


*Fig. 1.* Effect of enzyme Viscozyme L cellulase concentration (%v/w) on the TTC and TPC at a given solid-to-water ratio (1/40 w/v), temperature process (50 °C), and extraction time (150 min)

### 3.2. Effect of material and water ratio on the extraction of TPC and TTC

Fig. 2 demonstrates the impact of the powder-to-water ratio on the TTC and TPC of *Rubus alceaefolius* Poir leaf extracts. In this figure, the TTC and TPC notably increased as the powder-to-water ratio increased from 1:15 to 1:45 (w/v). The maximum values of TTC (234.44 mg UA/g) and TPC (9.13 mg GAE/g) were attained at a 1:35 (w/v) powder-to-water ratio. Similar findings regarding the impact of the solid-to-water ratio on the extraction yield of

phenolic compounds have been reported previously by other researchers [19, 20]. Our results are consistent with the principles of mass transfer. The driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater when a higher solvent-to-solid ratio is employed [19]. Nevertheless, when the water was added to a certain ratio (1/40-1/45 w/v in this experiment), it resulted in a decrease in the substrate concentration, leading to a limit on the enzyme's ability to act and reducing extraction efficiency.

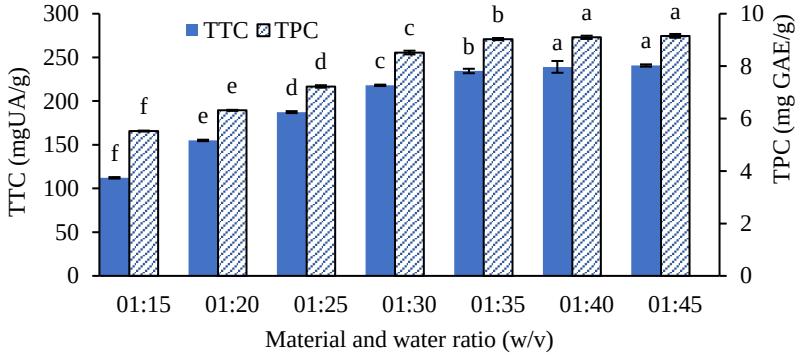


*Fig. 2.* Effect of ratio of material to water ratio on the TPC and TTC at given enzyme Viscozyme L cellulase concentration (2.5% v/w), temperature process (50 °C), and extraction time (150 min)

### 3.3. Effect of temperature on the extraction of TPC and TTC

Fig. 3 demonstrates the impact of extraction temperature on the TTC and TPC. As the temperature escalated from 30 °C to 50 °C, both TTC and TPC increased. The peak values for TTC and TPC were observed at 50 °C, reaching 233.14 mg UA/g and 9.01 mg GAE/g, respectively. However, as the extraction temperature increased from 50 to 60 °C, both TTC and TPC declined by a factor of 2.0. It could be attributed to the fact that higher temperatures during extraction might cause a change in the enzyme's three-dimensional structure, resulting in low extraction efficiency. In addition, an increase in temperature process (over 50 °C) enhanced the degradation of phenolic and triterpenoid constituents [21-22]. Furthermore, elevating the extraction temperature also enhanced solvent evaporation, thereby lessening the diffusivity of the solutes to be extracted. Consequently, an extraction temperature of 50 °C was chosen for the subsequent extraction of phenolic compounds from *Rubus alceaefolius* Poir leaves.

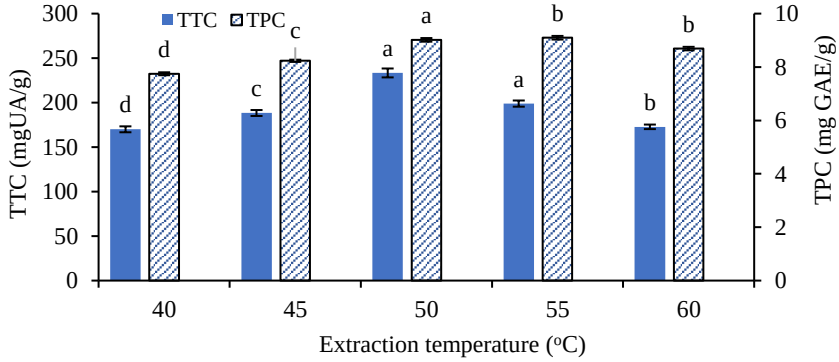


*Fig. 3.* Effect of treatment temperature on the TTC and TPC at given enzyme Viscozyme L cellulase concentration (2.5% v/w), solid-to-water ratio (1/35 w/v), and extraction time (150 min)

### 3.4. Effect of incubation time on the extraction of TPC and TTC

The incubation period during enzymatic extraction is regarded as a crucial factor that can impact the yield of extracted bioactive compounds. As depicted in Fig. 4, the TTC and TPC of *Rubus alceaefolius* Poir leaf extract exhibited a direct correlation with the extraction incubation time, which ranged from 0 to 120 min. Subsequently, a minor decline was noted when the duration was extended to 180 min. This trend may be attributed to the fact that longer extraction times facilitate the release of more bioactive compounds into the solution. Nevertheless, a further extension of extraction time may result in the degradation or oxidation of these compounds, thereby reducing the bioactive compound content in the final extract. A similar result was reported by Sara Martillanes et al. (2021) for extracting phenolic compounds from rice bran using cellulase treatment [23]. Consequently, after considering experimental and economic factors, an incubation period of 120 minutes was selected for subsequent research.

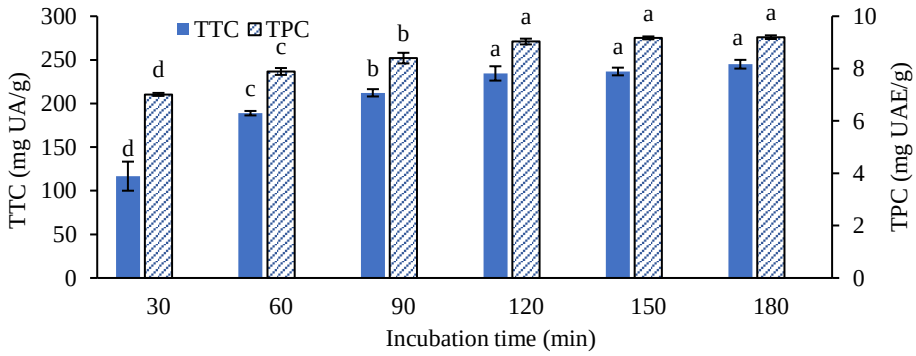


*Fig. 4.* Effect of treatment time on the TTC and TPC at given enzyme Viscozyme L cellulase concentration (2.5% v/w), solid-to-water ratio (1/35 w/v), and temperature process (50 °C)

### 3.5. Comparison of aqueous enzymatic extraction with conventional extraction

In this study, the recovery of bioactive compounds (TTC, TPC) and antioxidant activity (ABTS) extracted at the optimal enzyme-assisted extraction method (EAE) was compared to the maceration method (ME). The results indicate that EAE significantly enhanced the extraction efficiency and antioxidant activity, as compared to the ME. This improvement can be attributed to the observed changes in the plant's cell walls, as seen through scanning electron microscopy (SEM). As shown in Fig. 5A, the cell surface of *Rubus alceaefolius* Poir leaf powder appeared smooth, without any noticeable deformation of the cell wall. In contrast, the plant cells in the ME sample displayed a few broken cells visible (Fig. 5B). On the other hand, the EAE-extracted leaf powder was severely damaged, with small fragments present under optimal or extreme extraction conditions. Additionally, the EAE method substantially reduced solvent consumption and extraction time compared to ME. Thus, the EAE technique presents a promising new alternative for extracting phenolic compounds from *Rubus alceaefolius* Poir leaves.

*Table 1.* Comparison of the extraction efficiency of EAE with ME method

| Extraction method | TTC (mg UA/g dm) | TPC (mg GAE/g dm) | ABTS (mg TE/g dm) |
|---|---|---|---|
| EAE | $231.45^{a} \pm 1.95$ | $10.45^{a} \pm 0.61$ | $16.01^{a} \pm 0.88$ |
| ME | $165.47^{b} \pm 1.50$ | $6.45^{b} \pm 0.50$ | $10.14^{b} \pm 0.96$ |

*Different letters in the same column indicate statistically significant differences between treatments ($p<0.05$). The values are mean ±SD of duplicate runs.*

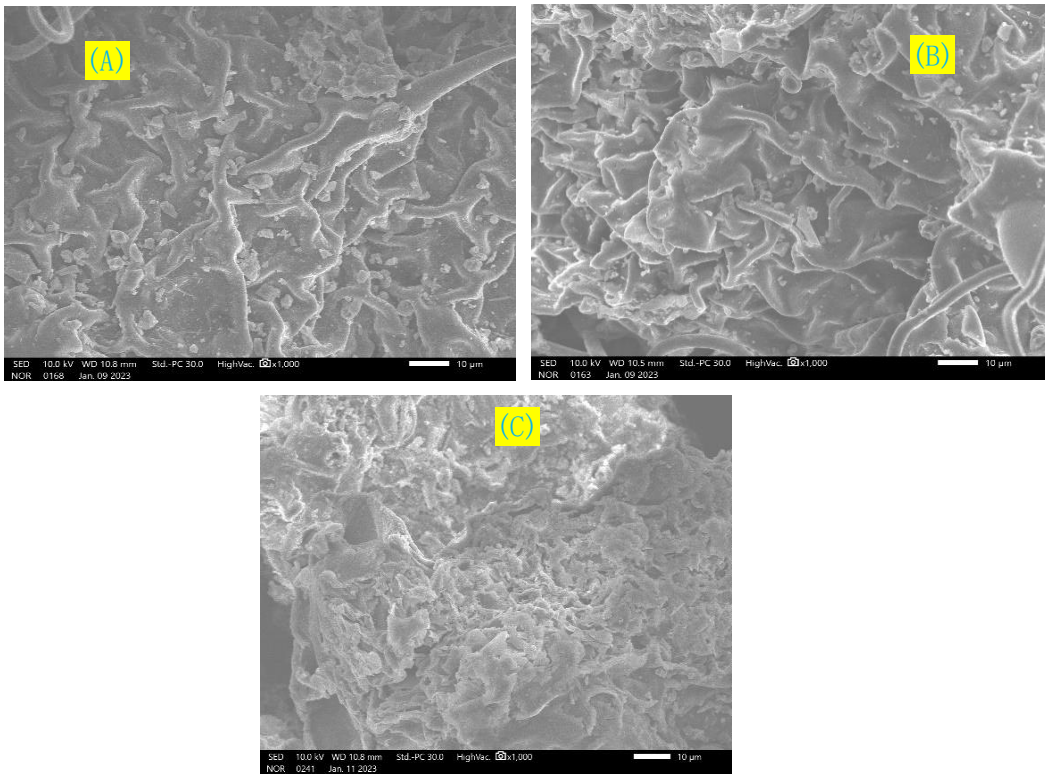


*Fig. 5*. SEM images of untreated *Rubus alceaefolius* Poir sample (A), ME treated sample (B), EAE-treated sample (C) (× 1000).

### 3.6. Bioactive compounds in *Rubus alceaefolius* Poir leaf extract

The analysis of triterpenoids and phenolic compounds found in *Rubus alceaefolius* Poir leaf extracts was conducted using GC-MS (Table 2 and Fig. 6). In total, 17 compounds were detected at various retention times (RT). The most abundant compounds identified within the *Rubus alceaefolius* Poir leaf extract were 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (RT=18.11 min), pyrogallol (RT=22.71 min), and D-allose (RT=25.13 min), followed by caffeic acid (RT=24.07 min). Conversely, the least abundant compounds were 2(3H)-Furanone, dihydro (RT=12.03 min), and Paromomycin (RT=24.90 min). Prior research has indicated that pyruvic acid, 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 3-dihydro-benzofuran, tetraacetyl-d-xylonic nitrile, pyrogallol, caffeic acid, paromomycin, and D-allose exhibit strong antioxidant capacities [24-26]. Additionally, previous studies have demonstrated the antitumor and antioxidant properties of tetra-acetyl-D-xylonic nitrile and paromomycin [27]. Caffeic acid, which belongs to the hydroxycinnamic acid class, could potentially affect the synthesis of noradrenaline and serotonin. Moreover, it has been found to exhibit anticancer properties [28]. In 2019, Seemaisamy revealed that pyrogallol has a wide range of pharmacological activities, such as antimicrobial and anti-cancer effects. Based on the findings, the identified compounds could be responsible for the ABTS radical scavenging activity of *Rubus alceaefolius* Poir leaf extract.

*Table 2*. Composition of chemical compounds of *Rubus alceaefolius* Poir leaf extract

| Peak | Compounds | Time (min) | Formula | Peak Area (%) |
|---|---|---|---|---|
| 1 | Pyruvic acid | 4.87 | $C_3H_4O_3$ | 5.24 |
| 2 | 2(3H)-Furanone, dihydro | 12.03 | $C_4H_6O_2$ | 2.81 |
| 3 | Glycerol | 13.45 | $C_3H_8O_3$ | 4.28 |
| 4 | Phenyl acetate | 13.80 | $C_8H_8O_2$ | 3.96 |
| 5 | Oxanamide | 15.66 | $C_8H_{15}NO_2$ | 3.00 |
| 6 | Tetraacetyl-d-xylonic nitrile | 16.75 | $C_{14}H_{17}NO_9$ | 4.98 |
| 7 | 4-Hydroxydihydro-2(3H)-furanone | 17.97 | $C_4H_6O_3$ | 3.58 |
| 8 | 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one | 18.11 | $C_6H_8O_{11}$ | 17.25 |
| 9 | Tetraacetyl-d-xylonic nitrile | 18.43 | $C_{14}H_{17}NO_9$ | 3.26 |
| 10 | Pyrocatechol | 18.96 | $C_6H_6O_2$ | 4.73 |
| 11 | 2,3-Dihydro-benzofuran | 19.59 | $C_8H_8O$ | 3.32 |
| 12 | 5-Hydroxymethylfurfural | 19.70 | $C_6H_6O_3$ | 6.13 |
| 13 | 1,4-Benzenediol | 20.57 | $C_6H_6O_2$ | 3.77 |
| 14 | Pyrogallol | 22.71 | $C_6H_6O_3$ | 11.12 |
| 15 | Caffeic acid | 24.07 | $C_9H_8O_4$ | 7.09 |
| 16 | Paromomycin | 24.90 | $C_{23}H_{45}N_5O_{14}$ | 2.88 |
| 17 | D-allose | 25.13 | $C_6H_{12}O_6$ | 8.12 |

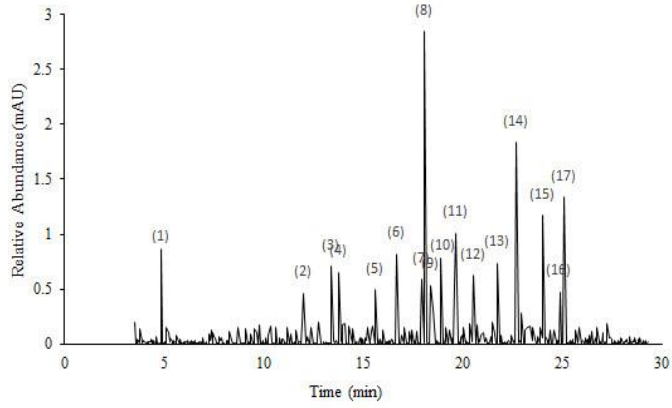


*Fig. 6.* GC - MS of *Rubus alceaefolius* Poir leaf extract

## 4. CONCLUSION

The research examined how variables such as the concentration of *Viscozyme L cellulase* enzyme, the ratio of powder to water, the temperature, and the incubation period affects the extraction of triterpenoids and phenolics from *Rubus alceaefolius* Poir leaf powder. The results indicated a significant correlation between these factors and the level of bioactive compounds in the *Rubus alceaefolius* Poir leaf extract. The extraction conditions were optimally found to be a 2.5% v/w enzyme concentration, a 1/35 w/v powder-to-water ratio, a temperature of 50 °C, and an incubation period of 120 minutes. In these conditions, the highest content levels

for TTC (231.45 mg UA/g dry matter) and TPC (9.13 mg GAE/g dry matter) were noted. The findings also showed that the enzyme-assisted extraction (EAE) method was doubly successful in obtaining phenolics and triterpenoids with strong antioxidant capacities (measured using ABTS) when compared to maceration extraction (ME). Additionally, GC-MS analysis revealed the presence of bioactive compounds with impressive antioxidant properties in the *Rubus alceaefolius* Poir leaf extract. Therefore, the EAE method can be considered an innovative and environmentally friendly approach to extract bioactive compounds.

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## TÓM TẮT

### TRÍCH LY THU NHẬN TRITERPENOID VÀ CÁC HỢP CHẤT PHENOLIC TỪ LÁ MÂM XÔI *Rubus alceaefolius* Poir VỚI SỰ HỖ TRỢ CỦA ENZYME

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Lá Mâm xôi (*Rubus alceaefolius* Poir) chủ yếu gồm các hợp chất polyphenol và triterpenoid, có nhiều hoạt tính sinh học có lợi cho sức khỏe con người. Nghiên cứu này nhằm mục đích khảo sát ảnh hưởng của điều kiện trích ly với sự hỗ trợ của enzyme *Viscozyme L cellulase* đến khả năng thu hồi các hợp chất sinh học từ lá Mâm xôi. Các yếu tố được khảo sát trong quá trình trích ly gồm tỷ lệ nguyên liệu:dung môi (1:15-1:45 w:v), nồng độ enzyme (0,5-3,5%), nhiệt độ (40-60 °C) và thời gian trích ly (30-180 phút). Kết quả thu được cho thấy điều kiện phù hợp để trích ly các hợp chất sinh học với sự hỗ trợ của enzyme là nồng độ enzyme 2,5%, tỷ lệ nguyên liệu:dung môi 1:35 w:v, nhiệt độ ủ 50 °C và thời gian trích ly 120 phút. Tại điều kiện khảo sát này, hàm lượng triterpenoid tổng và polyphenol tổng cao nhất thu được lần lượt là 231,45 mg UA/g và 9,13 mg GAE/g. Phân tích GC-MS cũng xác định được 17 hợp chất, trong đó một số có khả năng chống oxy hóa. Ngoài ra, nghiên cứu cũng cho thấy phương pháp trích ly với sự hỗ trợ của enzyme đem lại hiệu quả hơn trong việc thu nhận các hợp chất sinh học so với phương pháp trích ly thông thường.

*Từ khóa:* Lá Mâm xôi, Enzyme Viscozyme L cellulase, hợp chất sinh học, trích ly triterpenoid, trích ly polyphenol, kháng oxy hóa.