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INTEGRATED TLC AND BIOAUTOGRAPHIC TECHNIQUES FOR COMPREHENSIVE METABOLITE PROFILING OF DEKOPON PEEL EXTRACTS

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ABSTRACT

Dekopon (*Citrus reticulata*) peel contains a wide range of secondary metabolites whose distribution varies according to solvent polarity. This study aimed to characterize the metabolite profiles of n-hexane, ethyl acetate, and 96 percent ethanol extracts using thin-layer chromatography and bioautography-based visualization. TLC analysis with two different mobile phases enabled clear separation of both polar and non-polar constituents, with terpenoids predominantly observed in the n-hexane extract and flavonoids and polyphenols more concentrated in the ethyl acetate and ethanol extracts. Derivatization with $AlCl_3$, $FeCl_3$, H_2SO_4, and vanillin–sulfuric acid confirmed the presence of major phytochemical classes and revealed solvent-dependent migration patterns across the chromatographic plates. Fluorescent bands under UV 254 nm and 366 nm further demonstrated the structural diversity of metabolites present in each extract. The use of multiple visualization reagents allowed effective identification of terpenoid-, flavonoid-, and phenolic-rich regions, highlighting distinct chemical characteristics among solvent fractions. The integration of TLC and bioautographic techniques provided a comprehensive overview of metabolite distribution and enhanced the resolution of compound class differentiation. Taken together, the findings demonstrate that dekopon peel is chemically rich in diverse secondary metabolites, and that TLC-based profiling is a valuable tool for rapid and systematic characterization of plant-derived compounds.

Keywords: Dekopon (*Citrus reticulata*); Natural Product; Thin-Layer Chromatography (TLC); TLC–Bioautography; Phytochemical Analysis.

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INTRODUCTION

Natural plant materials are known to contain a wide assortment of chemical constituents that contribute to their functional and biological properties. Among these, citrus fruits are recognized as one of the richest botanical sources of secondary metabolites, particularly in their outer peels.[1] Citrus peel biomass, which is often treated as a low-value by-product in the food industry, has gained attention due to its abundance of compounds such as flavonoids, phenolic acids, terpenoids, and volatile oils. These metabolites have been widely investigated for their potential applications in nutraceutical, cosmetic, and pharmaceutical formulations.[2] However, the composition of citrus peel metabolites varies across cultivars, making detailed profiling essential for understanding their chemical characteristics. Dekopon (*Citrus reticulata*), a hybrid citrus variety cultivated in several tropical regions, possesses a peel that has not been extensively examined compared with more common citrus species.[3] Its peel contains a complex mixture of compounds whose distribution depends on factors such as solvent polarity, extraction method, and environmental influences during fruit development. Because these metabolites differ in their physicochemical properties, systematic extraction using solvents of varying polarity can reveal distinctive chemical patterns.[4] Such an approach helps distinguish non-polar constituents like terpenoids from semi-polar and polar groups such as flavonoids and polyphenols. Analytical techniques such as thin-layer chromatography (TLC) are widely employed to visualize and classify plant metabolites due to their simplicity, rapid analysis time, and ability to separate compound mixtures effectively.[5] The use of specific

derivatization reagents can enhance the detection of particular metabolite groups, allowing for clearer interpretation of chemical diversity within extracts. When combined with different mobile phases, TLC provides a comprehensive overview of metabolite separation across polarity gradients.[6] Bioautographic plate-transfer assessments can further illustrate how compounds migrate or diffuse from chromatography media, adding another dimension to the characterization process.[7] In light of these considerations, this study focuses on evaluating the chemical composition of dekopon peel extracts obtained through n-hexane, ethyl acetate, and 96 percent ethanol. By applying TLC with multiple visualization reagents alongside bioautographic assessment, the research aims to generate a detailed chromatographic fingerprint of dekopon peel metabolites. The outcomes are expected to contribute valuable insight into the chemical potential of dekopon peel as a natural source of diverse bioactive compounds and to support future utilization of citrus peel by-products in a range of applications.

EXPERIMENTAL

Plant Material

The primary material used in this study was dekopon fruit (*Citrus reticulata*), obtained from the Jeruk Dekopon Green House located at Jl. Terusan Sersan Bajuri No. 50, Cihideung, Parongpong District, West Bandung Regency, West Java. The fruits were harvested at full maturity and transported in clean containers to the laboratory for further processing. Fresh peels were separated manually and air-dried under shade to avoid degradation of active constituents. The dried peels were then crushed using a blender into coarse powder and stored in an airtight container at room temperature. This powdered material was used as the sample for extraction. The selection of this plant was based on its traditional use and reported phytochemical content. Extraction solvents were selected based on polarity range to ensure comprehensive phytochemical coverage. The sample collection was conducted ethically and did not involve endangered or protected plant species. All experimental work followed safety and ethical research guidelines. Documentation and labelling of samples were maintained for traceability.

Plant Determination

The taxonomic determination of dekopon was conducted at Herbarium Bandungense, School of Life Sciences and Technology, Institut Teknologi Bandung (SITH ITB). A voucher specimen was prepared from the collected plant sample, and morphological characteristics were compared with reference herbarium collections. Determination was carried out by trained botanists to ensure taxonomic accuracy. The purpose of this step was to confirm that the raw material used in the study was botanically verified. Correct botanical identification is essential for reproducibility and validity in pharmacognostic research. Documentation of the determination was obtained as an official certificate, in which the sample was identified as *Citrus reticulata* (family *Rutaceae*) according to determination letter No. 1017/IT1.C11.2/TA.00/2025.[8] This step was completed before sample processing and extraction. The verification ensured that further experiments were conducted on the correct plant material.

Extraction Procedure

Extraction was performed using three solvents: n-hexane, ethyl acetate, and 96% ethanol. A total of 100 g of dried peel powder was macerated in 500 mL of each solvent separately for 72 hours with occasional stirring. The maceration process was repeated twice to ensure maximum yield. The filtrates were collected through Whatman No. 1 filter paper. The solvent from each extract was evaporated using a rotary evaporator at 40–45°C under reduced pressure. The resulting concentrates were weighed and stored at 4°C in amber bottles until further analysis. Different solvents were used to extract different polarities of compounds from the plant material.

The yield of each extract was calculated as a percentage of the initial dried weight. Each extract was subjected to further phytochemical and antimicrobial testing. The maceration method was chosen for its simplicity and suitability for heat-sensitive compounds. Solvent residues were minimised by drying the concentrated extracts under vacuum. Proper labelling and record-keeping were maintained throughout the process.[9]

Thin-Layer Chromatography (TLC)

TLC analysis was conducted on each extract to visualize the chemical composition and identify specific phytochemicals. Silica gel GF254 plates were used as the stationary phase. Two mobile phases were employed to optimize separation of non-polar and semi-polar compounds, namely n-hexane:ethyl acetate (9:1) and chloroform:methanol (9:1). The extracts were spotted using capillary tubes at 1 cm from the edge of the plate. The plates were developed in a TLC chamber pre-saturated with mobile phase vapours. After development, the plates were air-dried and visualized under UV light at 254 nm and 366 nm. Post-visualization was also carried out using spray reagents, including $AlCl_3$, $FeCl_3$, H_2SO_4, and vanillin-sulphuric acid. Retention factor (Rf) values were calculated for each visible spot. The colour and fluorescence were noted as indicative of specific compound classes. This procedure was essential in supporting the phytochemical screening results. Comparative analysis was done across all extracts.[10]

TLC–Bioautography Method

The TLC–bioautography method was carried out to visualize the distribution of compounds separated on the TLC plate. Silica gel TLC plates measuring 1 × 7 cm were activated at 105°C for 10 minutes and marked 0.5 cm from the edges to guide sample application and solvent migration. The selected extract was applied 0.5 cm above the lower edge using a capillary tube, allowed to dry, and then developed in a chromatography chamber containing the appropriate mobile phase. After development, the chromatograms were examined under UV light at 254 nm and 366 nm and sprayed with $FeCl_3$, H_2SO_4, vanillin–sulfuric acid, and $AlCl_3$ to reveal different groups of metabolites. The developed TLC plate was then placed onto the prepared agar surface for 30 minutes to facilitate compound transfer. Following contact, the plate was removed and the agar was incubated under controlled conditions. Zones appearing on the agar surface corresponded to areas where compounds diffused from the TLC plate. These regions were then compared with the derivatized TLC chromatograms to determine the types of metabolites associated with each Rf position.[11]

RESULTS AND DISCUSSION

Thin Layer Chromatography Analysis of Dekopon Peel Extracts

The thin-layer chromatography analysis of dekopon orange peel extracts revealed clear differences in chemical composition among the n-hexane, ethyl acetate, and 96% ethanol fractions. Distinct spot patterns under UV 254 nm, UV 366 nm, and after derivatization in Fig.-1 and Fig.-2 indicated a wide diversity of metabolites. The Rf values summarized in Table-1 and Table-2 confirmed that each solvent extracted compounds with different polarity characteristics. $AlCl_3$ 5% derivatization consistently yielded strong fluorescent bands across all extracts, indicating the presence of flavonoids abundant in citrus peels. These flavonoid-associated bands appeared over a broad Rf range, reflecting variations in polarity between flavonoid glycosides and aglycones. Ethyl acetate and ethanol extracts showed the highest fluorescence intensity, consistent with their high affinity for semi-polar phenolic compounds. UV 366 nm visualization further supported this trend by revealing multiple brightly fluorescent spots. H_2SO_4 10% derivatization produced numerous strong responses, suggesting the presence of acid-reactive metabolites including sterols and triterpenoids. The n-hexane extract exhibited especially intense H_2SO_4 signals at high Rf values, reflecting its enrichment in non-polar constituents. Ethyl acetate and ethanol extracts produced moderate responses, consistent with the presence of more polar compounds. The combination of UV and reagent-based visualization confirmed that flavonoids and general secondary metabolites are widely distributed across all extracts. Variations in spot patterns also illustrated the influence of solvent polarity on metabolite migration. These findings align with literature noting citrus peels as rich sources of polyphenols and terpenoids.[12] In general, the observed chromatographic features demonstrate the chemical complexity of dekopon peel extracts.

Vanillin–sulfuric acid derivatization revealed distinct terpenoid profiles, especially in the n-hexane and ethyl acetate extracts. Prominent vanillin-reactive spots at Rf values around 0.42, 0.87, and 0.90 indicated the presence of non-polar terpenoids characteristic of citrus peel essential oils. The ethyl acetate extract also produced several vanillin-positive bands, though weaker in intensity, reflecting semi-polar terpenoid derivatives.

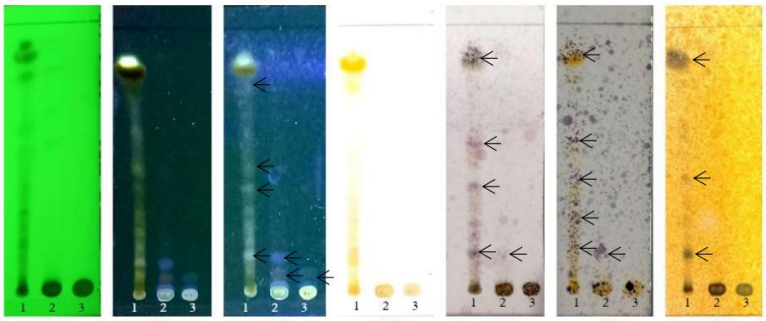

Fig.-1: Thin-Layer Chromatogram of Dekopon Orange Peel Extracts on Silica Gel GF254 using n-Hexane:Ethyl Acetate (9:1) as the Mobile Phase: (1) n-Hexane Extract; (2) Ethyl Acetate Extract; (3) 96% Ethanol Extract. (A) UV λ 254 nm; (B) UV λ 366 nm; (C) $AlCl_3$ 5% (λ 366 nm); (D) Visible Light; (E) H_2SO_4 10%; (F) Vanillin–Sulfuric Acid; (G) $FeCl_3$ 10%

Table-1: Rf Values of Dekopon Orange Peel Extracts using n-Hexane:Ethyl Acetate (9:1) Mobile Phase

Reagent	Rf Value of Dekopon Peel Extract		
	n-Hexane	Ethyl Acetate	Ethanol 96%
$AlCl_3$ 5%	0.13	0.08	0.06
	0.38	0.13	0.28
	0.48	-	-
	0.78	-	-
H_2SO_4 10%	0.13	0.12	-
	0.40	-	-
	0.55	-	-
	0.88	-	-
Vanilin-Sulfat	0.15	0.13	-
	0.27	-	-
	0.42	-	-
	0.57	-	-
	0.90	-	-
$FeCl_3$ 10%	0.13	-	-
	0.42	-	-
	0.87	-	-

In contrast, the ethanol extract exhibited minimal vanillin reactivity, indicating that polar solvents extract fewer terpenoids. $FeCl_3$ 10% derivatization confirmed the presence of phenolic compounds across all extracts, with stronger responses observed in the ethanol fraction. These strong $FeCl_3$-reactive bands reflect the high affinity of ethanol for polar phenolics. Ethyl acetate extract demonstrated moderate $FeCl_3$ responses, consistent with its intermediate polarity. Meanwhile, the n-hexane extract produced weaker $FeCl_3$-positive spots, in line with its limited ability to extract phenolic constituents. UV 254 nm detection also revealed more UV-absorbing compounds in the ethyl acetate and ethanol extracts. These combined observations clearly demonstrate a polarity-dependent distribution of flavonoids, terpenoids, and polyphenols. The dual responses from vanillin and $FeCl_3$ provided strong confirmation of compound class separation. Such distribution patterns correspond well with phytochemical characteristics reported for citrus species. The overall data indicate that terpenoids dominate the non-polar extract while phenolics and flavonoids dominate the polar fractions. These findings strengthen the classification of metabolite groups based on solvent extraction behaviour.

Comparison of the two mobile phases revealed important differences in their ability to separate polar and non-polar metabolites. The chloroform:methanol (9:1) mobile phase produced clearer separation of semi-polar flavonoids in the ethyl acetate and ethanol extracts, as reflected in more defined fluorescent bands. Meanwhile, the n-hexane:ethyl acetate (9:1) system effectively separated non-polar terpenoids in the n-

hexane extract. These differences in Rf values demonstrate the strong influence of solvent polarity on compound mobility.

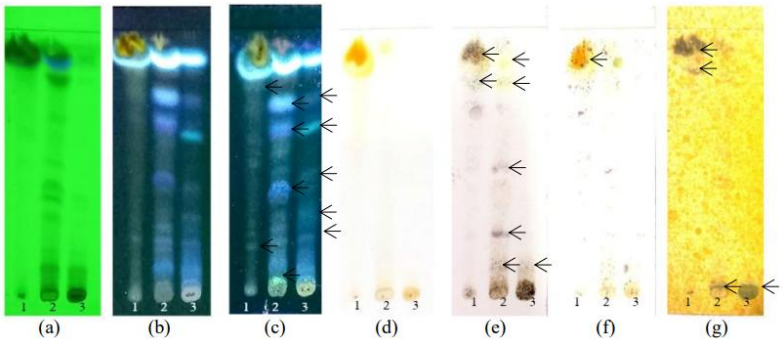

Fig.-2: Thin-Layer Chromatogram of Dekopon Orange Peel Extracts on Silica Gel GF254 using Chloroform:Methanol (9:1) as the Mobile Phase: (1) n-Hexane Extract; (2) Ethyl Acetate Extract; (3) 96% Ethanol Extract. (A) UV λ 254 nm; (B) UV λ 366 nm; (C) $AlCl_3$ 5% (λ 366 nm); (D) Visible Light; (E) H_2SO_4 10%; (F) Vanillin–Sulfuric Acid; (G) $FeCl_3$ 10%

Table-2: Rf Values of Dekopon Orange Peel Extracts using Chloroform:Methanol (9:1) Mobile Phase

Reagent	Rf Value of Dekopon Peel Extract		
	n-Hexane	Ethyl Acetate	Ethanol 96%
$AlCl_3$ 5%	0.17	0.05	0.22
	0.77	0.38	0.28
	-	0.43	0.43
	-	0.60	0.60
H_2SO_4 10%	0.77	0.10	0.20
	0.90	0.28	-
	-	0.47	-
	-	0.70	0.73
Vanilin-Sulfat	0.87	-	-
$FeCl_3$ 10%	0.77	0.05	0.08
	0.90	-	-

Flavonoids appeared consistently across all extracts and visualisation methods, confirming their abundance in dekopon peel. Strong terpenoid-associated vanillin bands in the n-hexane extract highlight the essential-oil characteristics of the citrus peel. $FeCl_3$ staining further validated the presence of polar phenolics in the ethanol and ethyl acetate extracts. Differences in spot intensity and migration suggest substantial structural diversity among extracted metabolites. The use of two mobile phases enabled detection of compounds spanning a broader polarity range. This dual-phase approach improved chromatographic resolution and strengthened qualitative interpretation of metabolite composition. The integration of UV detection and derivatization reagents allowed comprehensive profiling of chemical constituents. Furthermore, the TLC results demonstrate that dekopon peel contains a complex mixture of bioactive metabolites. These findings support its potential application in functional food, nutraceutical, and phytopharmaceutical research. Together, the results highlight the value of TLC as a rapid and effective tool for metabolite differentiation.

TLC–Bioautographic Screening of Dekopon Peel Extracts

The chromatographic profile of the n-hexane extract presented in Fig.-3 and summarized in Table-3 indicates a predominance of non-polar metabolites characterized by high Rf values. Distinct bands observed at Rf 0.75, 0.83, and 0.93 after vanillin–sulfuric acid and $FeCl_3$ derivatization are indicative of non-polar terpenoid-type constituents commonly associated with citrus peel essential oils. Several low-Rf spots detected under UV 254 nm, particularly at Rf 0.03 and 0.26, suggest the presence of minor aromatic or conjugated compounds with limited polarity. Weak fluorescence following $AlCl_3$ derivatization at Rf 0.88 indicates that only a small proportion of flavonoid aglycones is present in this fraction. Pronounced

darkened bands after H_2SO_4 treatment at Rf 0.42 and 0.90 further support the presence of acid-reactive metabolites such as triterpenoids or sterol-like compounds. $FeCl_3$ derivatization produced minimal responses, confirming the low abundance of phenolic compounds in the n-hexane extract. Moreover, the spot distribution demonstrates that n-hexane selectively extracts hydrophobic constituents that migrate toward the upper region of the chromatographic plate. Sharp and well-defined bands at high Rf values indicate reduced interaction with the polar silica stationary phase. Variations in band color and intensity suggest the coexistence of several subclasses of non-polar metabolites. The chloroform:methanol (9:1) mobile phase provided effective separation of these constituents, yielding a clear chromatographic pattern. This migration behavior is consistent with the expected profile of non-polar secondary metabolites derived from citrus peel matrices. Taken together, the TLC results confirm that the n-hexane extract is dominated by terpenoid-rich, non-polar compounds.

Table-3: Rf Values of Dekopon Orange Peel Extracts using Chloroform:Methanol (9:1) Mobile Phase

Reagent	Rf Value of Dekopon Peel Extract	
	n-Hexane	Ethyl Acetate
$AlCl_3$ 5%	0.03	0.27
	0.26	0.32
	0.88	0.38
	-	0.50
	-	0.77
H_2SO_4 10%	0.27	0.27
	0.42	0.38
	0.90	0.48
	-	0.65
Vanilin-Sulfat	0.20	0.67
	0.42	-
	0.75	-
	0.83	-
$FeCl_3$ 10%	0.08	0.08
	0.65	0.77
	0.70	-
	0.81	-
	0.93	-

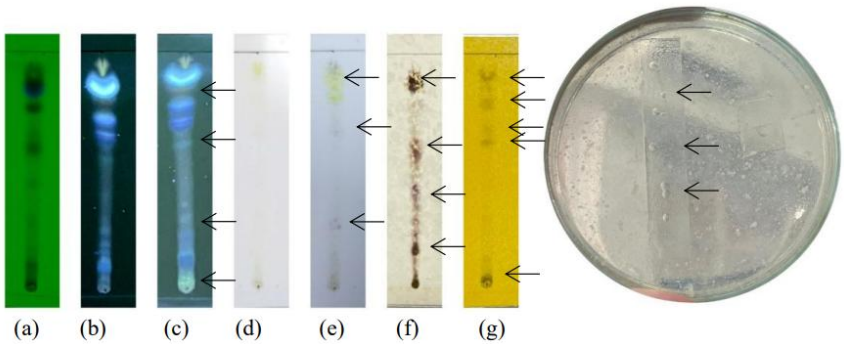

Fig.-3: Thin-Layer Chromatogram of Dekopon Orange Peel n-Hexane Extract on Silica Gel GF254 using Chloroform:Methanol (9:1) as the Mobile Phase: (A) UV λ 254 nm; (B) UV λ 366 nm; (C) $AlCl_3$ 5% (λ 366 nm); (D) Visible Light; (E) H_2SO_4 10%; (F) Vanillin–Sulfuric Acid; (G) $FeCl_3$ 10%

In contrast to the n-hexane fraction, the chromatographic profile of the ethyl acetate extract depicted in Fig.-4 shows a broader and more diverse distribution of semi-polar metabolites. Under UV 366 nm and following $AlCl_3$ derivatization, several strong fluorescent bands were observed at Rf 0.27, 0.32, 0.38, 0.50, and 0.77, confirming the presence of abundant flavonoid compounds characteristic of citrus peels. These

mid-range Rf values reflect the semi-polar nature of flavonoid glycosides and aglycones. $FeCl_3$ derivatization produced visible dark bands at Rf 0.08 and 0.77, indicating the presence of phenolic constituents with varying polarity.

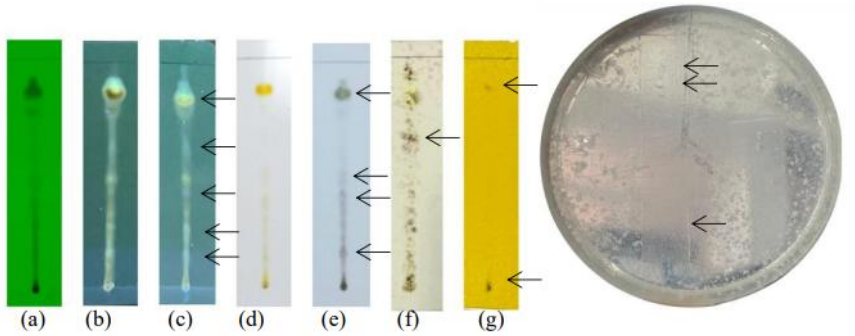

Fig.-4: Thin-Layer Chromatogram of Dekopon Orange Peel Ethyl Acetate Extract on Silica Gel GF254 using Chloroform:Methanol (9:1) as the Mobile Phase: (A) UV λ 254 nm; (B) UV λ 366 nm; (C) $AlCl_3$ 5% (λ 366 nm); (D) Visible Light; (E) H_2SO_4 10%; (F) Vanillin–Sulfuric Acid; (G) $FeCl_3$ 10%

Additional bands revealed after H_2SO_4 treatment at Rf 0.27, 0.38, 0.48, and 0.65 suggest the presence of structurally diverse carbon-based metabolites, including oxidized aromatic compounds. Vanillin–sulfuric acid staining showed limited but detectable responses at Rf 0.20 and 0.67, indicating that the ethyl acetate extract contains a smaller proportion of semi-polar terpenoid derivatives compared with the n-hexane extract. The greater number and diversity of detectable bands reflect a higher phytochemical complexity within this fraction. The predominance of mid-range Rf values indicates a balanced polarity profile consistent with the extraction capacity of ethyl acetate. Overlapping fluorescent bands further suggest the coexistence of multiple flavonoid subclasses with similar chromatographic behavior. Compared with the n-hexane extract, fewer high-Rf non-polar constituents were observed, while phenolic and flavonoid metabolites were more prominent. This separation pattern demonstrates that the chloroform:methanol (9:1) system effectively resolves semi-polar compounds.[13] Finally, the ethyl acetate extract represents a chemically rich fraction dominated by flavonoids and phenolics, clearly distinct from the terpenoid-rich n-hexane extract.

CONCLUSION

This study demonstrated that dekopon (*Citrus reticulata*) peel extracts contain a wide diversity of secondary metabolites that differ according to solvent polarity. The thin-layer chromatography results revealed clear variations in spot intensity, colour, and Rf values among the n-hexane, ethyl acetate, and ethanol fractions, indicating the presence of both polar and non-polar constituents. Multiple UV–visible responses and derivatization patterns confirmed the presence of flavonoids, terpenoids, polyphenols, and other phytochemical groups characteristic of citrus peels. The strong fluorescent bands observed after $AlCl_3$ derivatization highlighted the abundance of flavonoids, particularly in the ethyl acetate and ethanol extracts. Vanillin–sulfuric acid staining identified non-polar terpenoid-rich regions, especially in the n-hexane fraction, while $FeCl_3$ reactivity confirmed substantial phenolic content in the more polar extracts. Differences in Rf distribution across solvents and staining reagents further demonstrated the structural diversity of the metabolites extracted. The use of two mobile phase systems improved chromatographic resolution and enabled clearer differentiation of metabolite classes across polarity gradients. These complementary analytical outcomes emphasize the chemical complexity of dekopon peel and highlight its potential as a valuable natural source of bioactive compounds. In general, the study reinforces the utility of TLC-based profiling as an effective and rapid approach for characterizing metabolite groups in plant-derived extracts.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-Being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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