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Comparative Determination of Saponins in Nerium Oleander Using Foam-Forming, Optical Activity, and Spectrophotometric Techniques

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Abstract

Nerium oleander L is a medicinal plant of significant ethnopharmacological importance, yet its well-documented toxicity necessitates rigorous phytochemical characterization and standardization. This study comprehensively evaluated the influence of eight extraction solvents of varying polarity (water, methanol, ethanol, acetone, dichloromethane, chloroform, ethyl acetate, and hexane) and three analytical methods (foam-forming, optical activity, and spectrophotometric vanillin-acetic acid) on saponin quantification in N. oleander leaf extracts. Plant material was collected, dried, pulverized, and extracted via maceration, with saponin content determined using the three analytical approaches. Data were analyzed using two-way ANOVA with replication and Tukey's HSD post-hoc testing. Results demonstrated statistically significant variation in saponin yield attributable to both extraction solvent ($F = 143.44$, $p < 0.001$) and analytical method ($F = 90.53$, $p < 0.001$), with a significant interaction effect ($F = 6.35$, $p < 0.001$). Polar solvents, particularly methanol (18.22 ± 0.57%) and ethanol (16.32 ± 3.94%), exhibited superior extraction efficiency, consistent with the glycosidic nature of saponins. Hexane yielded anomalously elevated content (18.00 ± 2.57%), potentially due to unique hydrogen-bonding interactions. The spectrophotometric method consistently produced the highest saponin values due to enhanced sensitivity, while the foam-forming method proved suitable only for preliminary screening, and optical activity lacked specificity due to interference from other chiral constituents. This study conclusively demonstrates that methodological selection critically influences saponin quantification, with significant implications for ethnopharmacological standardization and quality control. Based on these findings, methanol extraction followed by spectrophotometric vanillin-acetic acid analysis is recommended as the optimal protocol for routine quantitative analysis, providing the highest sensitivity, reproducibility, and practical feasibility. These findings provide an evidence-based framework for method selection and underscore the necessity of harmonized analytical protocols to ensure accuracy, reproducibility, and comparability in natural product research, thereby supporting the safe and effective development of N. oleander-based therapeutics and cosmetic formulations.

Keywords: Nerium Oleander, Saponins, Phytochemical Analysis, Spectrophotometry, Extraction Solvents, Ethnopharmacology

1. Introduction

Medicinal plants have served as a cornerstone of traditional healthcare systems for millennia, with approximately 80% of the global population relying on plant-based remedies for primary healthcare needs, particularly in developing regions [1]. The therapeutic potential of these botanical resources lies in their diverse array of secondary metabolites, which have garnered increasing scientific interest for pharmaceutical development and ethnopharmacological validation. Among the vast repertoire of medicinal flora, Nerium oleander L. (Apocynaceae) stands out as a species of considerable pharmacological significance and toxicological concern, having been utilized in traditional medicine

across various cultures for the treatment of conditions ranging from cardiac disorders to dermatological ailments [2,3]. In South Africa, N. oleander is widely cultivated as an ornamental species, and its traditional medicinal applications include the treatment of skin conditions, haemorrhoids, and as a cardiotonic, despite the inherent risks of poisoning [4].

The bioactive profile of N. oleander is characterized by a complex mixture of phytochemicals, most notably cardiac glycosides (oleandrin, neriifolin) and saponins, which collectively mediate its diverse biological activities [3]. Saponins, a structurally heterogeneous class of amphiphilic glycosides, have attracted substantial research attention due to their demonstrated antimicrobial, anti-inflammatory, immunomodulatory, and anticancer properties, as well as their surface-active characteristics that render them valuable in pharmaceutical and cosmetic formulations [5,6]. Saponins are classified into two major groups—triterpenoid and steroidal glycosides—based on their aglycone structure, with the former being more prevalent in dicotyledonous plants such as N. oleander (Apocynaceae). This structural diversity influences their physicochemical properties and, consequently, their extractability and detectability by various analytical methods [7].

However, the same glycosidic nature that underpins their therapeutic potential also contributes to their hemolytic activity and, in conjunction with cardiac glycosides, the well-documented toxicity of N. oleander, necessitating rigorous phytochemical characterization and standardization for safe and effective therapeutic application [8,9]. Despite the ethnopharmacological significance of N. oleander, the accurate quantification of its saponin content remains a formidable analytical challenge. Methodological inconsistencies arising from the selection of extraction solvents and analytical techniques have led to considerable variability in reported saponin yields, undermining the reproducibility and comparability of research findings [8,10]. The efficiency of saponin extraction is intrinsically linked to solvent polarity, given the amphiphilic nature of these compounds comprising hydrophilic sugar moieties and lipophilic aglycones [11].

Polar solvents such as methanol and aqueous ethanol are generally favored for their ability to disrupt hydrogen bonding and solubilize glycosidic compounds, whereas non-polar solvents may preferentially extract the aglycone fractions [6]. Concurrently, the choice of analytical methodology ranging from conventional gravimetric approaches (foam-forming, optical activity) to more sensitive spectrophotometric techniques profoundly influences detection limits, specificity, and quantitative accuracy [12,13]. The selected solvents in this study encompass a broad polarity range (water, polarity index 9.0; hexane, 0.1), enabling systematic evaluation of polarity-dependent extraction efficiency. Additionally, these solvents represent those commonly employed in phytochemical laboratories and industrial extraction processes, enhancing the practical relevance of findings. Extensive research has documented the inherent variability in phytochemical quantification arising from methodological differences across various plant species; however, limited systematic investigation has been directed toward evaluating how these factors specifically influence saponin determination in N. oleander [14,15].

For N. oleander, accurate saponin quantification is particularly critical given the species' narrow therapeutic index. Saponins contribute to both the therapeutic (anti-inflammatory, antimicrobial) and toxicological (haemolytic, membrane-disrupting) profiles of the plant. Without reliable quantification, the risk-benefit assessment of N. oleander-based preparations remains inherently compromised, potentially endangering consumers in traditional medicine settings where dosage standardization is lacking [9]. This knowledge gap is further compounded by the absence of standardized analytical protocols, which impedes the development of quality control measures for N. oleander-based phytopharmaceuticals and cosmetic products, potentially compromising consumer safety and product efficacy [6,8]. Regulatory frameworks, including the African Traditional Medicine (ATM) policy and the World Health Organization's guidelines on herbal quality control, emphasize the necessity of validated analytical protocols for ensuring product safety and efficacy [16].

However, the lack of harmonized methodologies for saponin quantification remains a significant barrier to regulatory compliance in developing countries. Therefore, the present study was undertaken to comparatively evaluate the influence of eight extraction solvents of varying polarity (water, methanol, ethanol, acetone, dichloromethane, chloroform, ethyl acetate, and hexane) and three analytical methods (foam-forming, optical activity, and spectrophotometric) on the quantification of saponins in N. oleander leaf extracts. By employing rigorous statistical analysis, including two-way ANOVA with replication and post-hoc Tukey's HSD testing, this study aims to delineate the methodological factors contributing to analytical variability and identify optimal protocols for reliable saponin quantification. The findings are intended to contribute to the standardization of phytochemical analysis protocols, enhance the reproducibility of ethnopharmacological data, and provide a robust framework for quality control in the development of plant-based therapeutics and cosmetic formulations derived from N. oleander.

2. Methodology, Materials and Methods

2.1. Plant Material Collection and Preparation

Fresh leaves of Nerium oleander L. were collected from a cultivated nursery in Vereeniging, Gauteng Province, South Africa (26°40′S, 27°55′E) during the flowering stage. The plant material was identified by a traditional healer and was purchased at Magic Lawns Nursery in Vereeniging. To comply with botanical documentation and alignment with scientific standards, the plant name was cross-checked and validated using internationally recognized taxonomic databases, including Plants of the World Online (POWO), World Flora Online (WFO), and the Medicinal Plant Names Services (MPNS). Leaves were thoroughly washed with distilled water, air-dried at ambient temperature (25 ± 2°C) for 14 days, and ground to a fine powder (particle size < 0.5 mm) using a laboratory mill. The powdered material was stored in

airtight containers at 4°C until further analysis.

2.2. Plant Material Extraction

Powdered leaf material (50 g) was extracted with 500 mL of each solvent (1:10 w/v ratio) by maceration at room temperature (25 ± 2°C) with intermittent shaking (150 rpm) for 72 hours. The solvents evaluated included water, methanol, ethanol, acetone, dichloromethane, chloroform, ethyl acetate, and hexane, selected to encompass a broad polarity range (polarity index: 0.1–9.0). The extract was filtered through Whatman No. 1 filter paper, and the residue was re-extracted twice with fresh solvent (2 × 250 mL) to ensure complete extraction. The combined filtrates were concentrated under reduced pressure at 40°C using a rotary evaporator (Büchi Rotavapor R-210) to obtain the crude extract, which was then dried to constant weight in a vacuum desiccator and stored at 4°C until analysis. All extractions were performed in triplicate, and results are expressed as mean ± standard deviation.

2.3. Determination of Saponins using Foam-forming Method

The foam-forming assay was performed according to the ASTM D1173 International method with modifications [17]. Filtrate obtained after extraction (5 mL) was transferred to a 100 mL graduated measuring cylinder and diluted with 10 mL of distilled water. The cylinder was stoppered and shaken vigorously for 30 seconds, then allowed to stand for 1 minute. Foam height (cm) was measured from the surface of the liquid to the top of the foam layer. Foam stability was recorded as the time taken for the foam to collapse completely. Each sample was analyzed in triplicate. A standard curve was prepared using a commercially available saponin standard (e.g., quillaja saponin) at concentrations ranging from 0.1–2.0 mg/mL. Saponin content was calculated using the equation derived from the standard curve and expressed as percentage of raw material used (w/w).

2.4. Determination of Saponins using Optical Activity Method

Optical rotation was measured using a polarimeter (Model [SAC-i Saccharimeter], Atago) at the sodium D line (589 nm) at 25 ± 0.5°C. Filtrates (1 mL) were placed in a 10 cm polarimeter tube, and the observed rotation (α) was recorded in triplicate. Specific rotation [α] was calculated using the equation:

$[\alpha]^{25} D = \alpha \times 100 / l \times c$

α = observed rotation

l = path length in decimetres

c = concentration in g/100 mL

A solvent blank was measured for each solvent system and subtracted from the sample readings to correct for any baseline optical activity. Saponin content was calculated by reference to a standard curve prepared using oleanolic acid, a triterpenoid sapogenin standard commonly employed for saponin quantification, and expressed as percentage of raw material used (w/w).

2.5. Spectrophotometric Determination of Saponins using a Sapogenin Standard

The spectrophotometric method was performed according to the modified vanillin-acetic acid method of Sharma et al [12]. Briefly, extract (1 mL) was transferred to a test tube and the solvent was evaporated at 60°C. To the dried residue, 0.5 mL of vanillin reagent (8% w/v vanillin in ethanol) and 5 mL of 72% (v/v) sulfuric acid were added. The mixture was heated in a water bath at 60°C for 15 minutes, cooled to room temperature, and absorbance was measured at 550 nm using a UV-Vis spectrophotometer (Model [Cary 60]). A calibration curve was prepared using oleanolic acid (sapogenin standard) at concentrations of 0.1–1.0 mg/mL. The colour was allowed to develop for 30 minutes and remained stable for at least 2 hours. All measurements were performed in triplicate, and saponin content was expressed as percentage of raw material used (w/w) calculated from the calibration curve equation.

2.6. Statistical Analysis

Statistical analysis was performed using Microsoft Data Analysis tools. All experiments were conducted with three biological replicates, each analysed in triplicate (technical replicates), and results are expressed as mean ± standard deviation. Q-Q plots. Two-way analysis of variance (ANOVA) with replication was employed to evaluate the effects of extraction solvent (8 levels) and analytical method (3 levels), as well as their interaction, on saponin content. Post-hoc comparisons were performed using Tukey's Honestly Significant Difference (HSD) test. Significance was set at $\alpha = 0.05$. The criteria for methodological evaluation included sensitivity (detection limit and quantification limit), linearity (coefficient of determination, R^2), and reproducibility (intra-day and inter-day precision).

3. Results

3.1. Comparative Analysis of Saponins in Nerium Oleander (N. oleander) Using Foam-Forming, Optical Activity, and Spectrophotometric Methods

3.1.1. Determination of Saponin Content Using Foam-Forming Method

Table 1 shows the results for the estimation of saponin content from the Foam-forming method.

Polarity	1	0,762	0,654	0,355
Polarity Index	9	5,1	5,2	5,1
Solvent	Water	Methanol	Ethanol	Acetone
Raw Material (g)	50,05	50,03	50,03	50,06
Extract (g)	12,31	10,41	8,98	10,51
Foam-forming:				
Filtrate	5			
Filtrate + Water (ml)	15			
Final volume (ml)	17	18	18	16
Foam volume (ml)	2	3	3	1
Foam stability (min)	>60	>120	<1	<1
Average Saponins (% of raw material used)	8,82	9,71	8,87	4,20
STDEV	0,25	0,92	0,17	0,45
Solvent	Dichloromethane	Chloroform	Ethyl Acetate	Hexane
Raw Material (g)	50,3	50,03	50,03	50,03
Extract (g)	4,25	3,93	3,68	14,09
Final volume (ml)	15	15	16	18
Foam volume (ml)	0	0	1	3
Foam stability (min)	<1	-	<1	>180
Saponins (% of raw material used)	0	0	1,38	14,24
STDEV	0	0	0	0,27

Table 1: Estimation of Saponin Content from *Nerium Oleander* Leaf Filtrates Using Foam-Forming Method

The most stable foam was produced by water, methanol, and hexane solvents with stability time of more than 60, 120, and 180 minutes, respectively. The highest saponin content of 14.24±0.27% of raw material used, was provided by hexane. Generally, highly polar solvents resulted in the highest saponin content.

3.1.2. Determination of Saponin Content Using Optical Activity Method

Table 2 shows the results for the estimation of saponin content using Optical Activity method;

Polarity Index	9	5,1	5,2	5,1
Solvent	Water	Methanol	Ethanol	Acetone
Raw Material (g)	50,05	50,03	50,03	50,06
Extract (g)	12,31	10,41	8,98	10,51
Optical activity				
AR	+45 to +225			
α (◦)	124,5452	188,2477	189,3909	107,9124
[α] (◦)	79,5452	143,2477	144,3909	62,9124
Average Saponins (% of raw material used)	8,70	13,25	11,5	5,87
STDEV	0,02	0,01	0,05	0,01
	Dichloromethane	Chloroform	Ethyl Acetate	Hexane
Raw Material (g)	50,3	50,03	50,03	50,03
Extract (g)	4,25	3,93	3,68	14,09
α (◦)	180,3143	100,7245	167,9023	183,0075

[α] (◦)	135,3143	55,7245	122,9023	138,0075
Average Saponins (% of raw material used)	5,08	1,95	4,02	17,28
STDEV	0	0,01	0,01	0,01

Table 2: Estimation of Saponin Content from *Nerium Oleander* Leaf Filtrates Using Optical Activity Method

The highest saponin content of 17.28±0.01% of raw material used, was also obtained by hexane. Generally, highly polar solvents resulted in the highest saponin content.

3.1.3. Determination of Saponin Content from Spectrophotometric Method Using Sapogenin Standard

Table 3 shows the results of the absorbance versus concentration for the sapogenin standard. Figure 1 is the calibration curve of the sapogenin standard obtained from Table 3, with a good correlation coefficient (R2 = 0.9457).

Concentration (mg/mℓ)	0,1	0,2	0,4	0,6	0,8	1
Absorbance	0,2751	0,4391	0,6302	0,674	0,7883	1,0947

Table 3: Estimation of Saponin Content from Nerium Oleander Leaf Filtrates Using Optical Activity Method

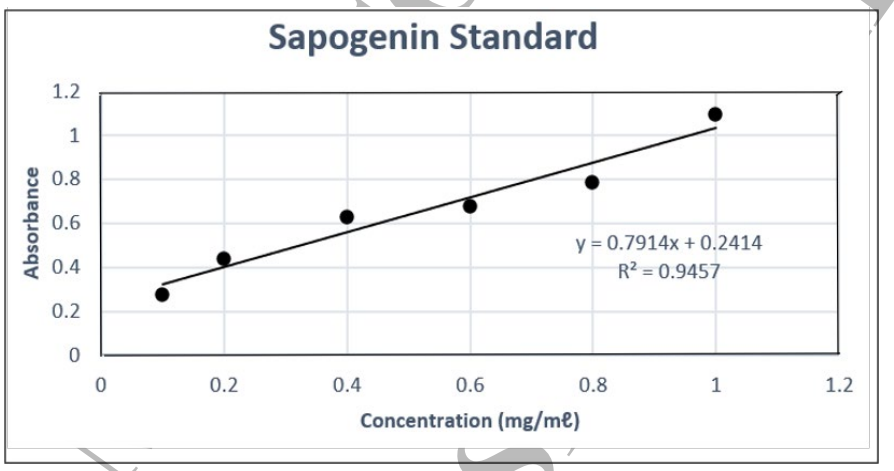

Figure 1: Sapogenin Standard Calibration Curve for Determination of Saponin Content

Table 4 shows the results for the estimation of saponin content using Spectrophotometric method. The highest saponin content of 18.22±0.57% was provided by methanol. Generally, highly polar solvents resulted in the highest saponin content.

Solvent	Water	Methanol	Ethanol	Acetone
Raw Material (g)	50,05	50,03	50,03	50,06
Extract (g)	12,31	10,41	8,98	10,51
Calibration equation	$x = \frac{Absorbance - 0{,}2414}{0{,}7914}$ $1\ mg\ extract\ dissolved\ in\ 1\ m\ell\ solvent$			
Absorbance	0,5986	0,9345	0,9609	0,7628
Average Saponins (% of raw material used)	11,1	18,22	16,32	13,83
STDEV	0,38	0,57	3,94	0,81
Solvent	Dichloromethane	Chloroform	Ethyl acetate	Hexane
Raw Material (g)	50,3	50,03	50,03	50,03
Extract (g)	4,25	3,93	3,68	14,09
Absorbance	0,6717	0,7698	0,6373	0,7472
Conc (% w/w of raw material)	4,6	5,25	3,68	18
STDEV	0,92	0,05	1,75	2

Table 4: Estimation of Saponin Content from Nerium Oleander Leaf Filtrates Using Spectrophotometric Method.

3.1.4. Comparative Analysis of The Saponin Content from Foam-Forming, Optical Activity, And Spectrophotometric Methods

Figure 2 is a bar chart illustrating the comparison of the saponin content of N. oleander from the foam-forming, optical activity and spectrophotometric methods.

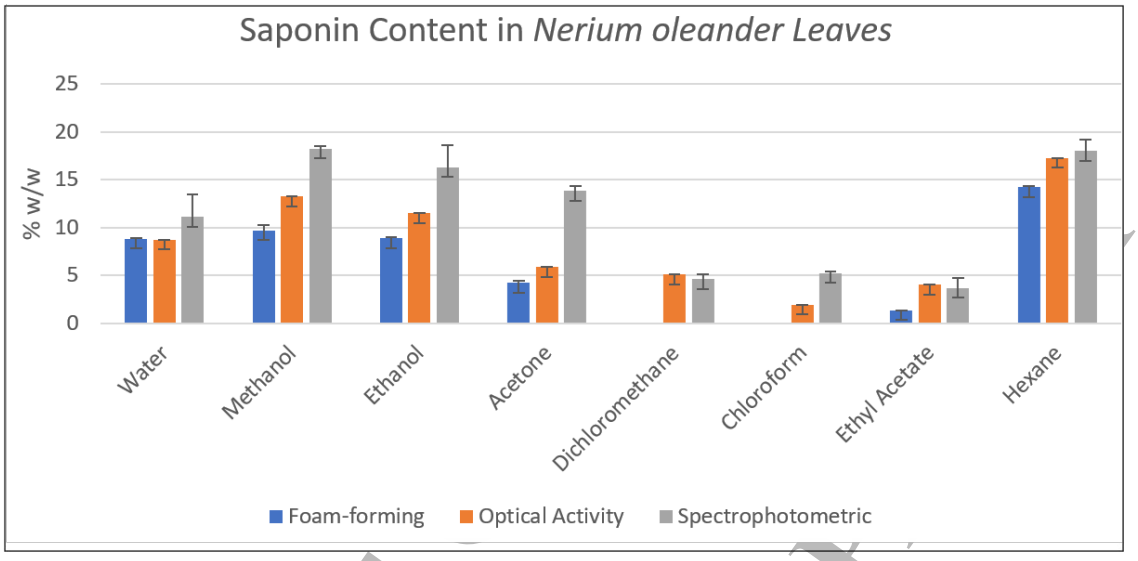

Figure 2: Bar Chart Comparing Average Saponin Content of *Nerium Oleander* Leaves Estimated from Foam-Forming, Optical Activity and Spectrophotometric Methods

Saponin content from spectrophotometric method decreased with decrease in polarity of the solvent from methanol (18.22 ± 0.57%) to dichloromethane (4.60 ± 0.92%). Foam-forming method showed a decrease in saponin content from methanol (9.71 ± 0.92%) to acetone (4.20± 0.45%). Optical Activity method decreased in saponin content from methanol (13.25 ± 0.012%) to acetone (1.95± 0.01%). Generally, spectrophotometric method produced higher saponin content. There were similarities in the saponin content produced by hexane from the optical activity (17.28±0.01%) and, respectively, to those produced by methanol and hexane from the spectrophotometric method, with saponin content of 18.22±0.57% and 18.00±2.00%, respectively. There seemed to be no significant difference between the saponin content between Foam-forming and Optical Activity methods or between Optical activity and Spectrophotometric methods.

3.1.5. Investigating Whether or Not the Saponin Content Between Any Two Methods Was Significant

Table 5 illustrates the descriptive statistics of the saponin data to evaluate if the data followed normal distribution.

normal distribution.	
Saponin content	
Mean	8,486806
Standard Error	1,173719
Median	8,715
Mode	0
Standard Deviation	5,750026
Sample Variance	33,0628
Kurtosis	-1,06284
Skewness	0,284217
Range	18,22333
Minimum	0
Maximum	18,22333
Sum	203,6833
Count	24

Table 5: Descriptive Statistics

Normal distribution is determined by the closeness of the mean to the median of the data points.

Both the mean and median, which are 8.49 and 8.71 respectively, are very close to each other. The other determining factor is the skewness of the data, which should be nearly zero. The skewness of the data was 0.28 which was closer to zero.

Figure 3 shows the Q-Q plot of the normal distribution curve for the data obtained from the saponin content derived from the foam-forming, optical activity and spectrophotometric methods. For data that comes from a normal distribution, most data points should lie on or near the straight line. The Q-Q plot of the data quantiles (z-score) versus the normal theoretical quantiles (z-score) showed that most data points lied on or near the straight line, which confirmed the data was normally distributed.

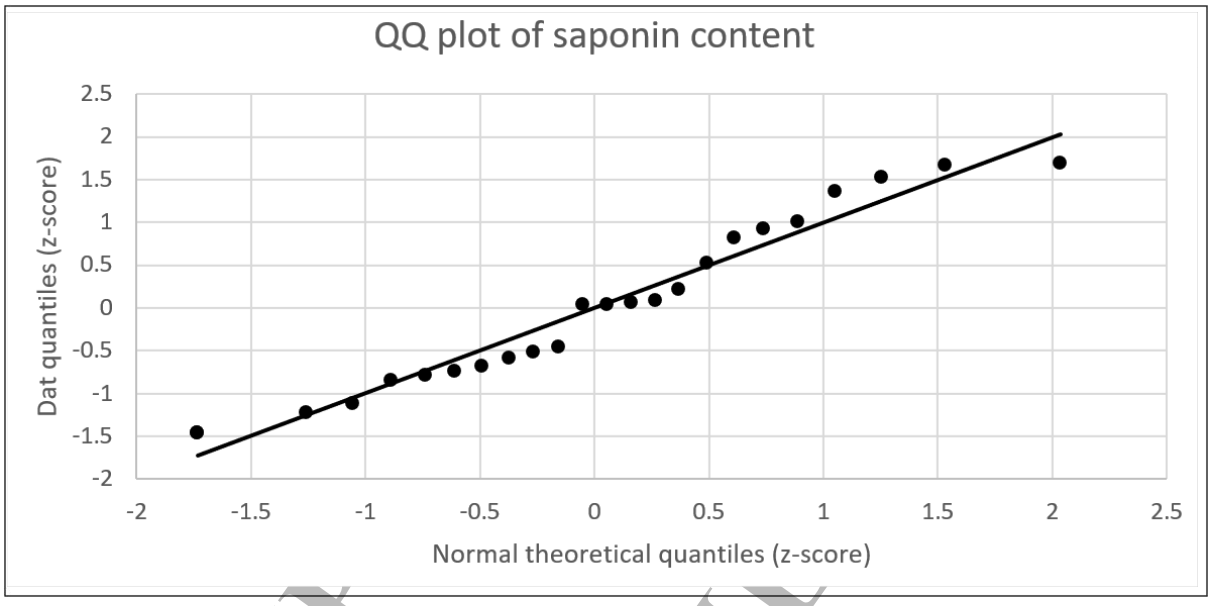

Figure 3: Normal Distribution Curve of The Data Points Obtained from Analysis of Saponin Content of *Nerium Oleander* Leaves Using

Table 6 shows the results obtained with the two-way ANOVA (with replication) to investigate whether or not the means of the three analysis methods were significantly different. Table 7 shows the mean saponin content from foam-forming, optical activity and spectrophotometric methods. Table 8 illustrates the post hoc Tukey's HSD test results.

Source of Variation	***df***	***F***	***P-value***	***F crit***	**Significance Difference**	
Solvent	7	143,437631	5,98642E-30	2,20743604	Significant	
Methods	2	90,5345497	5,13563E-17	3,190727336	Significant	
Interaction	14	6,34804385	6,09587E-07	1,903652567	Significant	
Within	48					
Total	71					

Table 6: Two-way ANOVA

Total	**Foam-forming**	**Optical activity**	**Spectrophotometric**
Count	24	24	24
Sum	141,67	202,98	266,44
Average	5,90	8,46	11,10
Variance	25,45	24,81	38,60
STDEV	5,05	4,98	6,21

Table 7: Mean Saponin Content

Group Pairs	Absolute Difference	Standard Error	q_tukey	q_critical	Result
Foam-forming vs Optical Activity	2,55	1,41	1,81	3,44	Not significant
Foam-forming vs Spectrophotometric	5,2	1,41	3,69	3,44	Significant
Optical Activity vs Spectrophotometric	2,64	1,41	1,87	3,44	Not significant

Table 8: Tukey's HSD Summary

The analysis from Table 6 showed a significant effect of extraction solvent ($F = 143.44$, $p < 0.001$), indicating that solvent type played a critical role in the extraction efficiency of saponins.

The analytical method also had a significant effect ($F = 90.53$, $p < 0.001$), demonstrating differences in sensitivity and quantification among the three methods used (Table 6). A significant interaction effect between extraction solvent and analytical method was observed ($F = 6.35$, $p < 0.001$), suggesting that the effectiveness of each method depends on the solvent employed (Table 6). This interaction was particularly evident for methanol extracts, where spectrophotometric analysis yielded significantly higher values than foam-forming (18.22% vs. 9.71%), while for hexane extracts, the difference was less pronounced (18.00% vs. 14.24%).

Post-hoc analysis using Tukey's HSD test indicated that the spectrophotometric method vs optical activity method produced moderate significant difference in some extracts ($p < 0.05$), while in others there were no significant difference ($p > 0.05$), as shown in Table 8.

Figure 4 is an account of the observed trends between the different methods as outlined in the post-hoc analysis

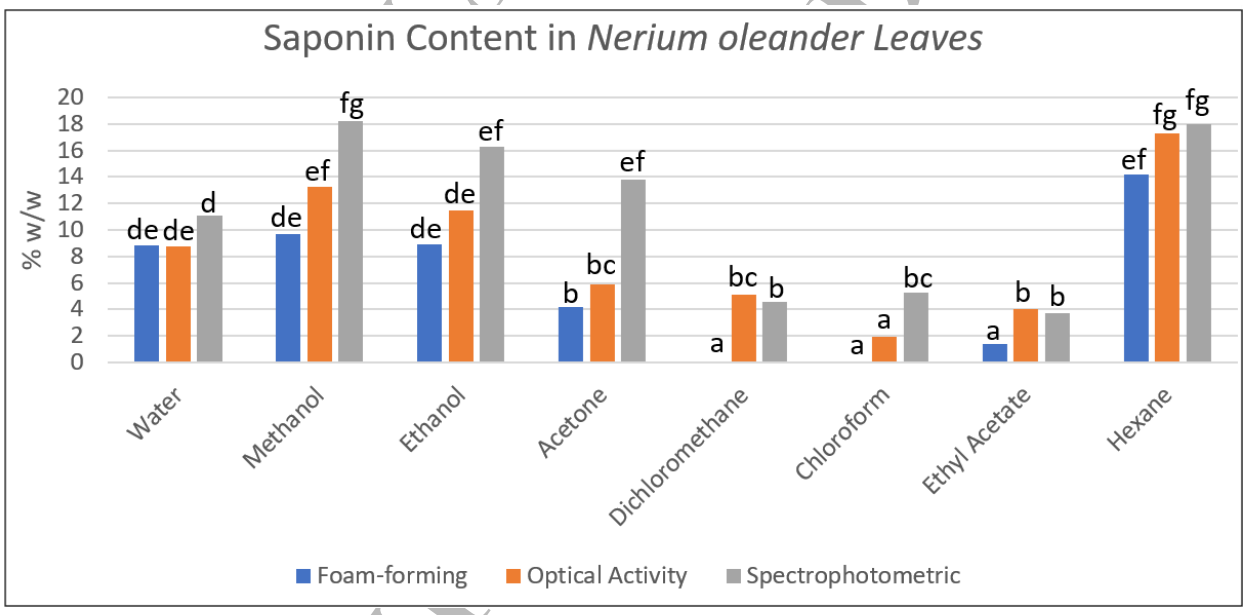

Figure 4: Illustration of Significant Differences Between the Foam-Forming, Optical Activity and Spectrophotometric Methods (Common or Overlapping Letters Indicate "No Significant Difference" In Yields)

Figure 4 demonstrated that methanol extracts analyzed by spectrophotometric method (letter designation: de and fg) and acetone extracts (letters: b and ef) showed significant differences compared to foam-forming method results. This is illustrated by the similar and overlapping letters shown by all the extracting solvents; Table 8 showed that there was no significant difference in the saponin content obtained between foam-forming and optical activity methods, as well as between optical and spectrophotometric methods. This is illustrated by the similar and overlapping letters shown by all the extracting solvents. The significant difference in the saponin content between foam-forming and spectrophotometric methods is illustrated by the methanol (de and fg) and acetone (b and ef) solvents.

4. Discussions

Polar solvents, particularly methanol (9.71 ± 0.92%) and water

(8.82 ± 0.25%), provided appreciable foam formation, consistent with the glycosidic nature of saponins and their affinity for polar media through hydrogen bonding [12]. The observed absence of foam formation in dichloromethane and chloroform extracts suggests that non-polar solvents failed to extract saponins effectively, likely due to the hydrophilic nature of the glycosidic moieties, which require polar environments for solubilization [6]. Contrariwise, hexane produced substantial foam formation (14.24 ± 0.27%) despite its low polarity (polarity index = 0.1), consistent with previously reported anomalous behaviour [18].

This unexpected result requires further investigation through complementary analytical techniques, such as thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC), to confirm whether the foam-active substances are indeed saponins or other surface-active compounds co-extracted by hexane. The foam stability data further supported these observations, with hexane extracts demonstrating exceptional foam stability (>180 minutes), followed by methanol (>120 minutes) and water (>60 minutes). In contrast, ethanol, acetone, dichloromethane, chloroform, and ethyl acetate extracts exhibited rapid foam collapse (<1 minute), indicating either low saponin content or the presence of foam-destabilizing compounds. The foam-forming method, while useful as a rapid preliminary screening tool, exhibited inherent limitations for precise quantitative analysis. The subjective nature of foam height measurement, the influence of factors such as temperature, pH, and the presence of other surface-active agents, and the lack of specificity (as other compounds may also produce foam) render this method suitable primarily for qualitative or semi-quantitative assessment rather than definitive quantification [15].

These limitations underscore the need for more sensitive and specific analytical approaches for accurate saponin determination. The relationship between optical rotation and saponin concentration is not strictly linear due to the potential contribution of other optically active compounds, including flavonoids, which were inconclusively detected in preliminary phytochemical screening [18]. This lack of specificity represents a significant limitation of the optical activity method, rendering it suitable primarily for preliminary screening rather than definitive quantification. Furthermore, the use of a fixed minimum angular rotation value of +45° lacks mechanistic justification and may introduce systematic bias, as baseline optical activity can vary depending on the solvent system and the presence of other chiral constituents. The method also assumes that all observed optical activity is attributable to saponins, an assumption that may not hold true for complex plant extracts containing multiple chiral secondary metabolites [5].

Despite these limitations, the optical activity method demonstrated greater sensitivity than the foam-forming method, as evidenced by the detection of saponin-related optical activity in all extracts, including those where foam formation was minimal or absent. This enhanced sensitivity, combined with the relative simplicity and speed of the measurement, positions the optical activity method as a useful complementary tool for preliminary screening. However, for accurate quantification, more specific techniques such as spectrophotometry or chromatography are essential. The spectrophotometric method produced the highest saponin values among the three analytical approaches, with methanol (18.22 ± 0.57%) and hexane (18.00 ± 2.57%) yielding the highest content, followed by ethanol (16.32 ± 3.94%) and acetone (13.83 ± 0.81%). The enhanced sensitivity of the spectrophotometric method is attributable to the chromogenic reaction between vanillin and the aglycone moiety of saponins in acidic conditions, which enables detection of saponin-related compounds across a broader concentration range, including those present at trace levels that may escape detection by gravimetric approaches [12,15].

The relatively high standard deviation observed for ethanol extracts (3.94%) may reflect incomplete extraction or batch-to-batch variability, emphasizing the need for rigorous quality control in routine applications. Similarly, the elevated standard deviation for hexane extracts (2.57%) suggests variability in the anomalous extraction behaviour discussed previously, further underscoring the need for method validation and optimization. These results are comparable with findings from other studies. For example, a saponin content of 20 ± 2.845% using the spectrophotometric method was obtained from Sapindus mukorossi Gaertn (pericarp of fruit) by Singh and co-workers using methanol: water (1:1) [13]. Similarly, studies on other saponin-rich plants have reported values ranging from 5–25% depending on the extraction solvent and analytical method employed [11,14]. It is important to note that the spectrophotometric method quantifies sapogenin equivalents rather than true saponin content, as the vanillin-acetic acid reaction primarily detects the aglycone moiety following acid hydrolysis of the glycosidic bonds.

This should be considered when interpreting results and comparing with studies using different standards (e.g. quillaja saponin, aescin). The spectrophotometric method is not without limitations, including potential interference from other chromogenic compounds (e.g., phenolic acids, flavonoids) and the requirement for a suitable reference standard. Future studies employing HPLC-MS would provide more specific saponin profiles and enable accurate quantification of individual saponin congeners. The observed differences between spectrophotometric and gravimetric methods align with previous reports indicating that spectrophotometric techniques provide greater sensitivity (detection limits typically 0.1–1.0 µg/mL versus mg/mL for gravimetric methods) and broader analyte coverage [14]. The foam-forming method, while rapid and cost-effective, is inherently semi-quantitative and influenced by factors such as temperature, pH, and the presence of other surface-active agents. The optical activity method offers intermediate performance but suffers from a lack of specificity, as previously discussed. From an ethnopharmacological perspective, reliable quantification of bioactive compounds such as saponins is essential for validating traditional medicinal uses and ensuring the safety and efficacy of plant-based therapies. Furthermore, the results have implications for the development of phytopharmaceuticals and cosmetic formulations, where consistent bioactive content is required for quality control.

5. Conclusions

This study provides a comprehensive comparative evaluation of extraction solvents and analytical methodologies for saponin quantification in Nerium oleander, revealing critical insights with significant implications for ethnopharmacological research, phytochemical standardization, and natural product quality control. The findings unequivocally demonstrate that both the choice of extraction solvent and analytical technique exert substantial and statistically significant influences on measured saponin content ($p < 0.001$), confirming that methodological selection is not merely a procedural consideration but a determinant factor affecting the reproducibility and comparability of phytochemical data. Among the solvents evaluated, polar solvents—particularly methanol consistently yielded the highest saponin recoveries across all analytical methods, attributable to their superior capacity to solubilize the glycosidic moieties of saponin molecules through hydrogen bonding and dipole-dipole interactions.

The comparative analytical evaluation demonstrated that the spectrophotometric vanillin-acetic acid method provided the highest sensitivity and broadest detection capability, yielding consistently higher saponin values than both the foam-forming and optical activity methods. The foam-forming method, while useful as a rapid preliminary screening tool, exhibited inherent limitations for precise quantitative analysis due to subjective endpoint determination and susceptibility to interference from other surface-active compounds. Similarly, the optical activity method, despite offering greater sensitivity than foam-forming, lacked sufficient specificity for reliable saponin quantification. Based on these findings, we recommend the use of methanol extraction followed by spectrophotometric vanillin-acetic acid analysis for routine saponin quantification in N. oleander, as this combination provides the highest sensitivity, reproducibility, and practical feasibility. However, for studies requiring definitive identification of saponin constituents, chromatographic techniques such as HPLC-MS should be employed as a confirmatory step.

The study has several limitations that should be acknowledged. First, the spectrophotometric method quantifies sapogenin equivalents rather than true saponin content, as the vanillin-acetic acid reaction primarily detects the aglycone moiety following acid hydrolysis. Second, the anomalous hexane extraction results require further investigation using complementary spectroscopic techniques to confirm the identity of the extracted compounds. Third, the study was conducted on plant material from a single geographical location and collection period, and seasonal or geographic variations in saponin content were not evaluated. Fourth, the lack of chromatographic confirmation limits the specificity of the saponin identification. From an ethnopharmacological perspective, reliable quantification of bioactive compounds such as saponins is essential for validating traditional medicinal uses and ensuring the safety and efficacy of plant-based therapies. For N. oleander, a species with a narrow therapeutic index, accurate saponin quantification is particularly critical for establishing safe dosage parameters and standardizing herbal preparations.

The demonstrated variability arising from methodological differences poses a significant challenge to regulatory compliance and may contribute to inconsistent clinical outcomes or adverse effects if not adequately addressed. The findings of this study provide an evidence-based framework for method selection and advocate for the adoption of harmonized analytical protocols to enhance data reliability and facilitate meaningful inter-study comparisons in natural product research, thereby supporting the safe and effective development of N. oleander-based therapeutics and cosmetic formulations. Based on the findings of this study and the identified methodological limitations, the following recommendations are proposed to advance phytochemical standardization, enhance analytical reliability, and support the safe development of N. oleander-based products.

The spectrophotometric method employed in this study quantifies total sapogenin equivalents rather than individual saponin congeners. Given the structural diversity of saponins in N. oleander and their differential biological activities, future studies should employ high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) or ultra-performance liquid chromatography (UPLC-MS) to achieve definitive identification and quantification of individual saponin constituents [19]. Such approaches would enable the development of saponin-specific fingerprints, facilitate structure-activity relationship studies, and improve the specificity of quality control protocols. Additionally, chromatographic methods can distinguish between triterpenoid and steroidal saponins, which may have different pharmacological and toxicological profiles [7].

The unexpectedly high saponin yield obtained with hexas (18.00-± 2.57%) represents a significant anomaly requiring further investigation. While hydrogen bonding interactions between hexane hydrogens and glycosidic hydroxyl groups have been proposed, this hypothesis requires verification through complementary spectroscopic techniques [18]. Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) spectroscopy should be employed to characterize the compounds responsible for the anomalous yield. Alternative explanations that warrant investigation include:

(i) The co-extraction of non-saponin surface-active compounds such as fatty acids or phospholipids that produce false-positive results in the foam-forming assay
(ii) The presence of saponin aglycones (sapogenins) rather than intact saponin glycosides, which would exhibit different polarity characteristics
(iii) The formation of reverse micelles that facilitate the extraction of hydrophilic saponins into the non-polar phase [20]. Resolution of this anomaly is essential for developing reliable and universally applicable extraction protocols.

Regulatory bodies, including national medicines regulatory authorities (NMRAs), the African Traditional Medicine (ATM) policy implementers, and the African Medicines Regulatory Harmonization (AMRH) initiative, should be encouraged to adopt

standardized protocols for saponin quantification in medicinal plants. Harmonized analytical standards would enhance consumer safety, facilitate cross-border trade of herbal products, and support the integration of traditional medicines into national healthcare systems. Such regulatory harmonization is particularly important in the context of the African Union's Agenda 2063 and the WHO's Traditional Medicine Strategy 2025-2035 [20,21].

Declarations

Ethical Approval (if applicable)

The name of the ethics committee was Vaal University of Technology's Faculty Research Ethics Committee and approval number was FACSREC-050325-302.

Author Contributions

Conceptualization, Majorobela Motaung and Fanyana Mtunzi; methodology, Majorobela Motaung and Fanyana Mtunzi; software, Majorobela Motaung; validation, Majorobela Motaung; formal analysis, Majorobela Motaung and Imelda Ledwaba; investigation, Majorobela Motaung; resources, Fanyana Mtunzi.; data curation, Majorobela Motaung; writing—original draft preparation, Majorobela Motaung; writing—review and editing, Qcobiza Manzane and Rosemary Montle; Michael Klink; visualization, Majorobela Motaung; supervision, Fanyana Mtunzi; project administration, Fanyana Mtunzi; funding acquisition, Fanyana Mtunzi.

Data Availability Statement

The data reported in this study was original.

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