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Original Article

THE DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR EVALUATING GALLIC ACID IN ETHYL ACETATE FRACTION (EAF) OF SNEDDS FORMULATION: QUANTITATIVE ANALYSIS WITH *IN VITRO* ASSAY

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ABSTRACT

Objective: This study aimed to develop a simple, accurate, precise, sensitive, robust, and stable analytical method for the evaluation of gallic acid in Self-Nanoemulsifying Drug Delivery System (SNEDDS) incorporating ethyl acetate fraction (EAF) of *Melastoma malabathricum* leaves in combination with Gentamicin.

Methods: Validation process followed ICH guidelines and applied a reverse phase HPLC method with a mobile phase of acetonitrile-phosphoric buffer at pH 3.03 (20:80 v/v). The stationary phase consisted of a VP-ODS shim-pack C-18 column (250x4.6 mm) with a flow rate of 0.2 ml/min and detection at 263 nm using an Ultraviolet detector. Additionally, antioxidant activity was assessed through the DPPH and FRAP methods, and SPF value was determined with a UV/Vis spectrophotometer in the 290-390 nm wavelength range.

Results: The results showed that the retention time of quercetin was 16.648 min with a tailing factor of 1.623. The regression equation (y=224689x-989000) had a concentration range of 10-55 µg/ml and a correlation value of 0.9920. Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be 2.394±0.086 and 7.254±0.260 µg/ml, respectively. Method accuracy, determined by recovery values at concentrations of 50%, 100%, and 150%, ranged from 91.18% to 109.49%. Repeatability inter-day variations were expressed as %RSD values of 1.027-1.963% for AUC and 0.150-0.145 for RT. Moreover, the applied method showed stability within a temperature range of 14 °C–35 °C. Analysis showed gallic acid content of 1.773±0.049 mg/g in SNEDDS EAF formulation. Antioxidant activity measured through the DPPH and FRAP methods yielded IC_{50} values of 4.167±0.552 µg/ml and 20.253±0.619 µg/ml, respectively, while SPF value at SNEDDS concentration of 150 µg/ml was 36.993±0.183.

Conclusion: This study successfully developed a precise, accurate, specific, and stable method for quantifying gallic acid levels in SNEDDS EAF of *Melastoma malabathricum* leaves in combination with Gentamicin. Therefore, SNEDDS EAF formulation exhibited an effective wound-healing potential, supported by a robust quality control process.

Keywords: Quantitative analysis ethyl acetate fraction (EAF) of *Melastoma malabathricum* leaves in combination with gentamicin in SNEDDS formulation, Development and Validation,, *In vitro* assay

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INTRODUCTION

Gallic acid (GA), chemically referred to as 3,4,5-trihydroxybenzoic acid, is one of the phenolic compounds in existence. Furthermore, its phenolic structure serves as a source of hydrogen atoms capable of generating free radicals with delocalized electrons within the phenol group [1]. The results reported by Nayeem *et al.*, 2016 showed that gallic acid could exhibit various pharmacological activities, including anticancer, antioxidant, and neuroprotective effects, while Agrawal *et al.*, 2021 discovered its anti-inflammatory and analgesic properties. Yang *et al.*, 2016 investigated the potential of this compound as a wound healer, with good antioxidant activity, found to stimulate fibroblast cell migration and activate kinase enzymes. Gallic acid can promote wound healing in animal models of both type 1 and type 2 diabetes, particularly in chronic cases [1-3].

Gallic acid can be naturally obtained from plants such as *Allan blackia floribunda*, *Garcinia densivenia*, *Caesalpinia sappan*, *Diospyros cinnabarina*, *Paratecoma peroba*, *Psidium guajava*, *Phyllanthus emblica*, *Terminalia bellirica*, *Toona sinensis*, *Oenothera bienni*, *Rubus suavissimus*, *Punica granatum*, *T. bellerica,* and *Melastoma malabathricum* [3-7]. Apridamayanti *et al.*, 2022 conducted fractionation of *Melastoma malabathricum* leaves and reported gallic acid content of 49.29±0.49 mg/gram when using ethyl acetate as the solvent. Furthermore, the resulting ethyl acetate fraction (EAF) exhibited antioxidant activity against DPPH free radicals, with a value of 1.9±0.12 µg/ml, identical to the ascorbic acid activity of 1.9±0.2 µg/ml. Hainil *et al.*, 2023 detected that EAF showed an inhibitory effect of 92%, compared to the 96% observed in ascorbic acid. In a separate study by Apridamayanti *et al.*, 2022, the preparation of nanocream containing EAF indicated an antioxidant capacity of 1444.157±15.538 µg/ml as measured by the FRAP method [5, 8, 9].

The potential of gallic acid in wound healing has been explored through various formulations. Gan and Chin., 2021 incorporated this compound into a hydrocolloid film for wound dressing. Stefanov *et al.*, 2018 showed the effectiveness of a hydrogel containing gallic acid and chitosan as a means to control oxidative stress and bacterial contamination in chronic wounds and foot ulcers. Seulgi *et al.*, 2021 developed a chitosan and gallic acid-based synthetic tissue adhesive, which significantly improved wound closure and tissue regeneration through enhanced fibroblast cell production compared to the control group [10-12].

Pratiwi *et al.*, 2021 created a nanoemulsion formulation within a Self-Nanoemulsifying Drug Delivery System (SNEDDS), combining EAF of *Melastoma malabathricum* leaves with the antibiotic gentamicin for the treatment of diabetic foot ulcers (DFU). This study reinforced the use of plants containing gallic acid in formulation development, specifically focusing *Melastoma malabathricum* [13]. Sari *et al.*, 2022 showed the synergistic effects of EAF and Gentamicin combination, which acted as an effective antibiotic against *Bacillus cereus*, *Escherichia coli*, and *Staphylococcus aureus*. Therefore, this current study aims to develop and validate an analytical method for quantifying gallic acid levels within SNEDDS

formulation, a combination of *Melastoma malabathricum* leaves EAF and Gentamicin, which is promising for the effective treatment of chronic wounds such as DFU. The identification of active compounds (markers), particularly gallic acid in SNEDDS preparations, contributes scientifically to understanding pharmacological activity of the developed formulation. In this study, antioxidant activity tests through the DPPH and FRAP methods and the measurement of the Sun Protection Factor (SPF) value of formulation will be conducted. The development and validation of analytical method are expected to follow the ICH guidelines, using a reverse phase High-Performance Liquid Chromatography (HPLC) instrument to obtain reproducible and consistent measurement results.

MATERIALS AND METHODS

Materials

The materials used in this study included gallic acid standard (Sigma-HPLC grade), acetonitrile (Merck-HPLC grade), NaH_2PO_4 (Merck), $NaHPO_4$ (Merck), Double distilled water, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), $FeCl_3$ (Merck), TPTZ reagent (Sigma-aldrich), Citric acid (Merck), hydrochloric acid (Merck), soybean oil (Mazola®), VCO (Bagoes®), olive oil (bratachem), sunflower oil (Mazola®), Tween 20 (bratachem), Cremophor EL (bratachem), pH meter (Hanna), sonicator (Bronson), HPLC instrument (Shimadzu), UV/Vis spectrophotometer (Shimadzu), and Elisa reader (Thermo).

Instrumentation

Instrumentation for the RP-HPLC included a Shimadzu Corporation UFLC system, which featured a reservoir tray, Prominence Degasser (DGU-20A5), Prominence Liquid Chromatography (LC-20AD), Prominence Communication Bus Mobile (CBM-20A), Prominence UV Detector (SPD-20A), and Column Oven (CTO-20 A).

Preparation of mobile phase

The mobile phase used in this study comprised a 20:80 ratio of acetonitrile and phosphate buffer (pH 3.08), which was prepared by diluting a 0.008% H_3PO_4 solution to a final volume of 0.5L with double-distilled water.

Sample preparation

The sample used was SNEDDS loaded with EAF of *Melastoma malabathricum,* dissolved in acetonitrile. The botanical identity of *Melastoma malabathricum* has voucer specimen number is 021/AL/lB/FMIPA/UNTAN/2023. EAF was obtained by constituting the maceration of *Melastoma malabathricum* leaves with ethanol 80%. Subsequently, the fractionation process applied n-hexane, chloroform, ethyl acetate, methanol, and water as solvents. Sample in this study, namely EAF (Ethyl Acetate Fraction), it was incorporated into SNEDDS formulation.

Preparation of SNEDDS EAF combination with gentamicin

Up to 10 mg of *Melastoma malabathricum* EAF and 10 mg of gentamicin were added to 10 ml soybean oil, VCO, olive oil, sunflower oil, Tween 20, and Cremophor EL. SNEDDS was prepared using a combination of Tween 80, propylene glycol, and soybean oil, to which EAF was added. The mixture was conditioned in a water bath at 40 °C for 10 min, then the lipid and water phases were blended at 70 °C and homogenized with a homogenizer at 750 rpm for 10 min [13].

Validated analytical method

RP-HPLC instrumentation for gallic acid analysis

The sample analyzed in this study was an SNEDDS-loaded EAF of *Melastoma malabathricum* with gentamicin (SNEDDS EAF), dissolved in acetonitrile. Furthermore, it was filtered through a 0.2 µm membrane filter (Whatman) before injection into the HPLC system. A standard solution of gallic acid was prepared by dissolving 25 mg of the compound in 25 ml of acetonitrile, and different concentrations, ranging from µg/ml 10-55 µg/ml, were produced as a calibration curve. All the standard solutions were filtered through a 0.2 µm membrane filter (Whatman) before being transferred into the HPLC system [9].

Chromatography condition in RP-HPLC

The chromatography system applied was a modification of the method described by Apridamayanti *et al.*, 2023. This used a VP-ODS shim-pack C-18 column (250x4.6 mm), with a reverse phase column packed with 4.5 µm diameter particles. The mobile phase consisted of acetonitrile-phosphoric acid with pH 3.03 (20:80, v/v), filtered through a 0.2 µm membrane filter (Whatman) and de-aerated ultrasonically before use. The temperature was maintained at 30 °C, with an injection volume of 20 µl and a flow rate of 0.2 ml/min. The wavelength for gallic acid detection was set at 263 nm using a UV detector [9].

Assay validation

The RP-HPLC method for quantifying gallic acid was validated according to the International Council for Harmonisation (ICH) guidelines. Validation procedure included assessing linearity, range, precision, accuracy, sensitivity, and robustness [9, 15].

Stability of analytical solution

The strength of analytical solutions was determined by examining the standard preparations at-14 °C (in a refrigerator), room temperature (30 °C), and 35 °C under 24 h. Five injections drawn from each solution were analyzed, and the peak and the RSD average were calculated.

Linearity and range

A calibration curve was prepared by diluting 25 mg gallic acid with acetonitrile as a standard stock solution to produce concentrations ranging from 10 µg/ml-55 µg/ml. Three injections from each concentration were analyzed under the same condition. Furthermore, linear regression analysis was used to evaluate the curve Linearity through the least square linear regression method (R^2).

Sensitivity

The determination of sensitivity included calculating the limit of Detection (LOD)/limit of Quantitation (LOQ) of gallic acid solution by analyzing different solutions and measuring the signal-to-noise ratio. LOD was the concentration that produced a signal-to-noise ratio of approximately 3:1, while LOQ generated a signal-to-noise ratio of 10:1.

Accuracy

The accuracy of the analytical method was assessed through recovery studies at three concentration levels of gallic acid (50%, 100%, and 150%, i.e., 0.7 mg, 1.5 mg, and 2.2 mg). Three samples from each concentration were injected into the HPLC system, and the percentage recovery of added gallic acid and their RSD was calculated.

Precision

The system and method precision (repeatability) was determined by several measurements of sample solution (n=5) and standard solution (n=9) through intraday tests.

Robustness

The robustness test was carried out by introducing minor and deliberate changes into the chromatography system to evaluate their influences. This included using a column temperature of±3 °C, flow rate of±0.05 ml/min, wavelength of±5 nm, and mobile phase and organic composition (Buffer phosphate: Acetonitrile) at±5%. Moreover, five injections from each solution were analyzed, and the peak and the RSD average were calculated. In this study, column efficiency (N) and tailing factor (TF) parameters were found to be within the acceptable limit.

Quantitative analysis of gallic acid in SNEDDS EAF (Marker study in formulation)

A total of 1 ml of SNEDDS EAF preparation was dissolved in acetonitrile and then homogenized. The sample solution was passed through a 0.2 µm membrane filter (Whatman) before injection into the HPLC system.

Pharmacological study of formulation with *in vitro* assay

Antioxidant activities

Antioxidant activity was assessed using the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical assay to determine free-radical-scavenging activity (DPPH and FRAP assay). The method followed the protocol described by Apridamayanti *et al.*, 2022, with minor modifications. Up to 3 ml of each sample, with concentrations ranging from 1 µg/ml–6 µg/ml, was added to 3 ml of 1 mmol DPPH and incubated in the dark at room temperature for 30 min. Absorbance was measured at 515.5 nm with a Spectrophotometer UV/Vis for the DPPH assay. This study was conducted with experiments in triplicate measurement for each test [5]. The Ferric Reduction Antioxidant Power (FRAP) assay was also conducted according to [5] with slight modifications. A total of 30 µl of each sample at different concentrations (5 µg/ml to 30 µg/ml) was added to 30 µl $FeCl_3$ solution (3 mmol in 5 mmol citric acid) and 240 µl TPTZ reagent (1 mmol in 0.05 M HCL) in a 96-well microplate. After incubation at room temperature for 15-30 min, absorbance was measured at 615 nm using an Elisa Reader.

Identification of SPF

SNEDDS EAF formulation was dissolved in ethanol, and different concentrations were prepared at 50 µg/ml, 100 µg/ml, and 150 µg/ml. The solution was scanned in the UV range of 290 to 320 nm (at 5 nm intervals) using a spectrophotometer (n=3). The identification of sun protection activity was determined based on the equation proposed by Mansur [9].

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Note: CF represents the correction factor, EE is the Eritema effect in the spectrum (nm), I denotes light intensity (nm), and Abs signifies the absorbance sample. During the experimental process, the absorbance sample was measured three times and used for SPF calculation.

Statistical analysis

Statistical analysis for validation and *in vitro* studies was conducted using Microsoft Office Excel 365 for Windows and SPSS software, respectively. Parameters such as average, sum, Standard Deviation (SD), Regression (RSQ), and scattered charts were used for linearity assessment. Moreover, ANOVA was applied for the *in vitro* studies, with a significance level of $p<0.05$ considered significant.

RESULTS AND DISCUSSION

The objective of this study was to develop and validate analytical method for quantifying gallic acid content in SNEDDS EAF formulation containing EAF of *Melastoma malabathricum* leaves. Analysis of marker compounds could provide scientific information related to the activity of formulation.

Method validation

The determination of gallic acid levels was conducted through spectroscopic and chromatographic techniques. In this study, an HPLC method was developed for quantifying gallic acid, a marker compound present in EAF of nanoemulsion preparations. The development of RP-HPLC aimed to ensure precision, thoroughness, sensitivity, and specificity in quantifying this marker compound in natural sources, following ICH guidelines for method validation. The results obtained were documented in the form of Peak Area, Retention Time (TR), Asymmetry Factor (TF), and Column Efficiency (N) within predetermined limits. Moreover, the asymmetry factor is often applied to characterize a chromatographic system, with increased asymmetry leading to decreased separation and measurement result repeatability. Column efficiency evaluates the performance and effectiveness of column in relation to the length used in the system [20].

Chromatographic system

In this study, the chromatographic system was used to assess the solubility of the marker compound, gallic acid, and the suitability of the mobile phase composition under several design variations to achieve effective compound separation and column efficiency. The details of the chromatography system are presented in table 1.

Stability testing of SNEDDS EAF formulation and gallic acid

Stability testing aims to assess the stability of the compound under test temperature conditions and during storage. The strength of the standard solution was evaluated under temperature variations of 30 °C and 35 °C, with storage times of 7 d at 30 °C and 24 h at 35 °C and 14 °C. Meanwhile, the sample solution was stored for 24 h at 35 °C. The test parameters examined included RSD values for Peak area (AUC) and retention time, as well as the TF and N values, which were compared against regulatory limits, and the results can be seen in table 2.

Linearity and range

The linearity test of the calibration curve for the standard gallic acid solutions was conducted by repeating the experiment three times (n=3), resulting in slope, intercept, and regression values (R^2). The range in this study was found to be 10-55 µg/ml, as shown in Tables 3 and 4.

Table 1: RP-HPLC chromatographic system for gallic acid compounds

Mobile phase	Acetonitrile: water (20:80)
Stationary phase	VP-ODS shim-pack C-18 (250x4.6 mm), reverse phase column packed with 4.5 µm diameter particles
Wavelength (nm) maximum	263
Flow rate (ml/min)	0.2
pH with O-phosphoric acid	3.03
Injection volume (µl)	20
Run time (min)	20
Temperature	30 °C
Mobile Phase Mode	Gradient

Table 2: Stability test of SNEDDS EAF formulation and gallic acid

Parameter	AUC (%RSD)	RT (%RSD)	TF	N
After 24 h at 30 °C	1.353	0.10	1.859	7340.7
After 72 h at 30 °C	1.627	0.098	1.822	8017.1
After 168 h at 30 °C	1.562	0.028	1.983	5970.2
After 24 h at 35 °C	1.856	0.175	1.934	8670.2
After 24 h at-14 °C	1.689	0.052	1.923	8424.8

*Number of experiments, n=3

Table 3: Linearity of gallic acid standard solutions

Concentration (µg/ml)	RT (min)			AUC		
10	16.530	16.684	16.606	1676884	1814005.2	1776611.6
15	16.536	16.678	16.589	2117307.2	2079488.8	2236413
25	16.528	16.688	16.569	4175879.3	4712339.8	4205776.2
35	16.529	16.689	16.587	7238727.2	7401046.1	6594192.1
45	16.528	16.675	16.554	9141185.1	9307283.9	9196770.9
55	16.568	16.661	16.574	10948362.2	11112716.4	11623785.6

LOD and LOQ

LOD and LOQ were determined using SD formula of the analyte response and slope value as follows:

$$\text{LOD} = 3.3 \times \frac{\sigma}{\text{slope}} \text{ and LOQ} = 10 \times \frac{\sigma}{\text{slope}}$$

Information: σ = SD

The measured LOD and LOQ values are presented in table 4.

Precision

Precision testing constituted repeatability assessments of SNEDDS samples and gallic acid standard solution on days 1, 2, and 3. The %RSD value was examined, with a criterion of <2% and the test results can be seen in tables 5 and 6.

Accuracy

The accuracy test assessed the performance of the analytical method using the accuracy value expressed as a percentage of recovery. This study included adding the standard solutions at concentrations of 50%, 100%, and 150%, and the results are presented in table 7.

Strength testing for analysis method (Robustness)

The strength of the developed HPLC system was evaluated by analyzing changes made to various parameters, including column temperature, maximum wavelength, mobile phase composition, and flow rate within the HPLC system. Additionally, this condition was observed from the %RSD value<2%, TF, and N for each parameter change made to the AUC and RT. The results can be seen in table 8.

Table 4: Linearity, LOD, and LOQ test results of gallic acid solution

Parameters	Gallic acid
Linearity range (µg/ml)	10–55
Regression equation	224689x–989000
Correlation (r^2) coefficient	0,9920
Slope	220534±1.649
Intercept	835421±18.437
LOD (µg/ml)	2.394±0.086
LOQ (µg/ml)	7.254±0.260

*Values are expressed as mean±SD, Number of experiments, n=3

Table 5: Repeatability test for SNEDDS EAF of *Melastoma malabathricum* solution (n=5)

Replicated Number	AUC	RT
1	23358636	16.659
2	23253129	16.655
3	25150449	16.665
4	24606766	16.656
5	23551001	16.644
Averange	23983996	16.66
%RSD	3.53	0.05

Values are expressed as Mean and % RSD, Number of experiments, n=5

Table 6: Repeatability test of gallic acid solution on different days 1, 2, and 3 (n=9)

Replicated number	Intra-day					
	AUC			RT		
	Day-1	Day-2	Day-3	Day-1	Day-2	Day-3
1	5116059.8	5278190.1	5058116.3	16.673	16.655	16.637
2	5273940.9	5232745	5239643.4	16.709	16.659	16.629
3	5302301	5256787.4	5104652.9	16.695	16.664	16.644
4	5347227.5	5248185.2	5052308.7	16.663	16.693	16.628
5	5215010.5	5209942.1	5196298.1	16.705	16.674	16.633
6	5335218.6	5181547	5297182.1	16.695	16.677	16.661
7	5342826.9	5196323.8	5306210.5	16.727	16.695	16.663
8	5342679.5	5301196.3	5279978.7	16.700	16.697	16.648
9	5251981.4	5351739.8	5260215.3	16.741	16.701	16.679
Averange	5280805.1	5250740	5199401	16.701	16.679	16.647
%RSD	1.465	1.027	1.963	0.1445	0.106	0.105

Values are expressed as %RSD, Number of experiments, n=9

Table 7: The percentage recovery of SNEDDS EAF of *Melastoma malabathricum*

No	Level of concentration (%)	(%) Recovery		
		1	2	3
1	50	101.96	97.48	102.59
2	100	99.69	100.02	91.18
3	150	107.66	109.49	100.32

*Number of experiments, n=3

Table 8: Robustness data of gallic acid in the RP-HPLC system

Parameter		AUC (%RSD)	RT (%RSD)	TF	N
Column Temperature	27 °C	2.532	0.180	-	13566.2
	30 °C (Normal)	1.141	0.059	1.826	13746.7
	33 °C	0.954	0.023	1.669	13026.1
Wavelength	258 nm	1.458	0.089	1.883	15168.9
	263 nm (Normal)	0.919	0.298	2.070	16119
	268 nm	1.400	0.101	1.922	15944.5
Mobile Phase Composition	+5% acetonitrile	1.597	0.145	1.728	13739.6
	Normal	1.141	0.059	1.826	13746.7
	-5% acetonitrile	1.766	0.134	-	22769.4
Flow Rate	0.15 ml/min	2.532	0.152	1.495	13449.8
	0.20 ml/min (Normal)	2.004	0.123	1.802	11604.9
	0.25 ml/min	3.544	0.106	-	16539.5

Number of experiments, n=5, data given as % RSD

Quantification of Gallic Acid in SNEDDS EAFAnalysis of gallic acid, serving as a marker compound in SNEDDS EAF formulation, was conducted using a validated method. RP-HPLC was the instrumental method used for this purpose, and gallic acid content determined was 1.773±0.049 mg/g, with an RT of 16.648 min.

***In vitro* assay**

Antioxidant activity

The assessment of antioxidant activity in the preparation aimed to elucidate the *in vitro* pharmacological activity associated with the benefits provided by SNEDDS EAF formulation. Antioxidant activity was determined by reacting SNEDDS EAF formulation with DPPH solution and FRAP reagent. Therefore, the ability of the preparation to stabilize oxidant compounds, often indicated by the IC_{50} value, signifies its potential pharmacological activity. The results of this assay are presented in Tables 10(A) and 10(B).

SPF test results

SPF test aimed to evaluate the effectiveness of SNEDDS EAF of *Melastoma malabathricum* formulation. Three different concentration levels were tested, and the test results are presented in table 11. SPF value obtained showed the effectiveness of the sample concentration in protecting against UV rays. The maximum SPF value observed was 36.993±0.183 at a concentration level of 150 µg/ml.

Table 9: Quantification of gallic acid in SNEDDS EAF of *Melastoma malabathricum*

Name of sample	Retention time	Gallic acid (mg/g)
SNEDDS EAF	16.648	1.773±0.049

*Values are expressed as mean±SD, Number of experiments, n=3

Table 10: Antioxidant activity test of SNEDDS EAF of *Melastoma malabathricum* using DPPH method and FRAP method

DPPH Method

No	Concentration (µg/ml)	% Inhibition	% Inhibition	% Inhibition	
1	1	42.851	41.368	41.722	y = 2.0039x+40.675
2	2	44.624	46.459	45.176	R^2 = 0.9892
3	3	46.314	48.769	46.479	IC_{50} = 4.167±0.552 µg/ml
4	4	48.590	51.632	49.151	
5	5	51.385	54.598	52.507	
6	6	52.366	56.026	53.661	

*IC_{50} Values was expressed asmean±SD, Number of experiments, n=3

FRAP Method

No	Concentration (µg/ml)	% Inhibition	% Inhibition	% Inhibition	
1	5	21.744	21.744	21.447	y = 1.5728x+19.178
2	10	36.701	38.305	38.351	R^2 = 0.9389
3	15	44.171	45.242	45.815	IC_{50} = 20.253±0.619 µg/ml
4	20	52.114	51.723	53.355	
5	25	55.535	57.234	58.099	
6	30	60.366	60.668	63.137	

*IC_{50} Values was expressed as mean±SD, Number of experiments, n=3

Table 11: Measurement of photoprotector activity on SNEDDS EAF of *Melastoma malabathricum*

No	Concentration (µg/ml)	SPF value
1	50	20.517±0.518[a]
2	100	32.039±0.01[b]
3	150	36.993±0.183[c]

*a,b,c have a significant difference value of P<0.05, *Values are expressed as mean±SD, Number of experiments, n=3

DISCUSSION

The developed analytical method was designed as a preliminary step in the pharmaceutical formulation process [16], specifically for evaluating SNEDDS EAF formulation derived from *Melastoma malabathricum* leaves extract combined with Gentamicin. During the initial stages of method development, significant optimization efforts were made to ensure the solubility of the marker compound and the sample solution within the mobile phase of the applied RP-HPLC system used. The details of the developed chromatography system are provided in table 1. In this study, tests were carried out on SNEDDS formulation to determine the content of marker compounds present in the sample.

Arbianto *et al.*, 2019 discovered that HPLC analysis at 44%, as recommended by the US Pharmacopeia, offered selective and accurate results for quantifying chemical compounds in formulated products. The presence of excipients in formulated products often disrupts the selectivity of analytical method. Therefore, the development of a particular analytical method tailored to the formulated products is needed to separate the analytes from excipients and ensure their accurate, specific, and sensitive quantification using HPLC [16].

To ensure the selectivity of the chromatography system, tests were performed on both standard gallic acid and sample solutions, as represented in fig. 1 a, b, and c.

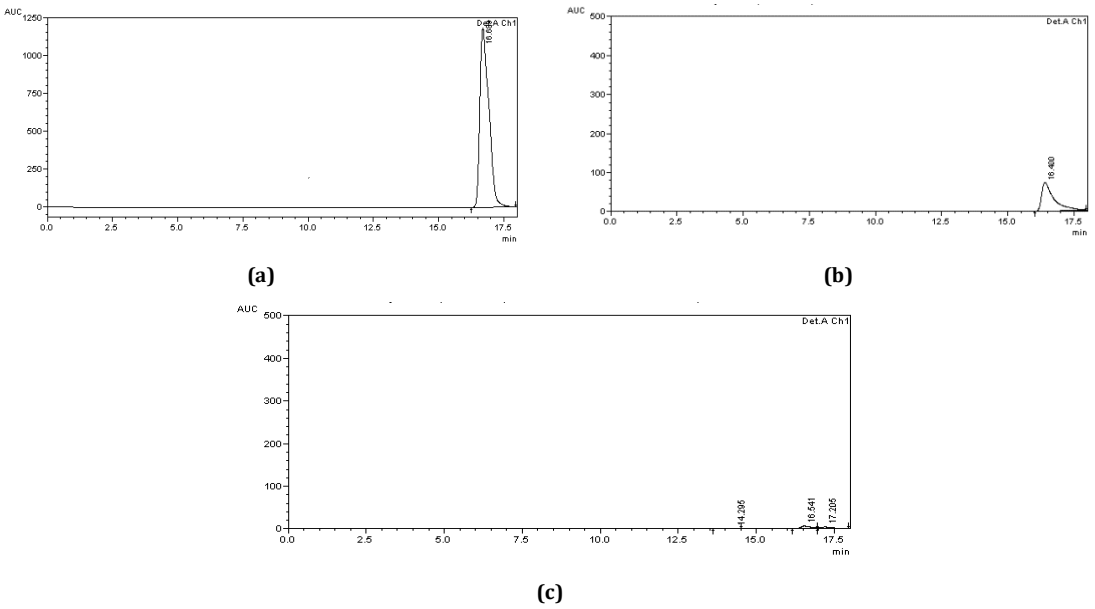


Fig. 1: Specificity test chromatography system (a) Gallic Acid; (b) SNEDDS EAF of *Melastoma malabathricum*; (c) Basis SNEDDS

Stability testing of both gallic acid standard and sample solutions aimed to evaluate the effect of storage temperature and the test temperature on the measurement results. The test focused on parameters such as the repeatability values of the peak area and retention time with a %RSD value<2%, Asymmetry Factor (TF)<2, and Column Efficiency (N)>2000 [18-20]. According to table 2, gallic acid and sample solutions exhibited stability under the specified temperature variations during analysis period, ensuring reliable results.

The development of an analytical method for measuring gallic acid present in SNEDDS EAF formulation is instrumental for quality control and assurance of compound content in pharmaceutical production. Therefore, validation of the analytical method was conducted following the guidelines set by ICH.

Linearity, expressed through a regression line based on a mathematical equation, establishes the relationship between analyte response and concentration variations, often referred to as the correlation value (R^2) in the linear regression equation. Additionally, Range is expressed in the interval of the largest and smallest concentration values in the test performed. In this study, an R-value of 0.9920 was obtained in the standard gallic acid solution concentration range of 10–55 (µg/ml), as presented in table 4.

LOD and LOQ indicate the minimum detectable and quantifiable levels of analytes measured statistically, precisely, and accurately by HPLC, and are often obtained through a linear regression line on a standard curve. Their values in this study were determined to be 2.394±0.086 and 7.254±0.260, respectively as shown in table 4. According to Patil, 2017, LOQ value determination is valuable for detecting low levels of analytes contained in matrix samples and evaluating impurities and degradation products [18].

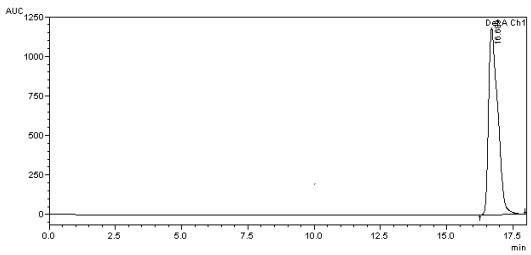
Precision is the estimate of the variation in measurement results from individual tests on the sample collected from a homogeneous mixture, expressed as the relative standard deviation (% RSD) value [21, 22]. Repeatability tests were conducted five times on the sample solution and nine times on the standard solution over three days. The %RSD values for the peak area and retention time are presented in Tables 5 and 6. These were found to be 2.289±0.828 in the sample solution, while in gallic acid solution, the values ranged from 1.027±0.106–1.963±0.105. In accordance with Patil, 2017 and Snyder *et al.*, 2010, the acceptable %RSD value for 100% analyte content (standard solution)

is<2%, and for 1% analyte content (sample solution), it is stipulated as<2.7%.

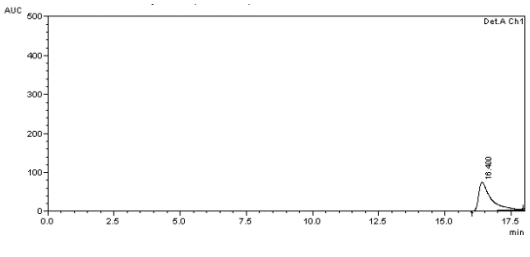
Accuracy reflects the closeness of measured analyte levels to the actual values and is expressed as %Recovery. The accuracy test was conducted using the addition method, where predetermined quantities of gallic acid were mixed with SNEDDS (placebo) formulation, and the resulting amounts of gallic acid were compared with the actual levels. The %Recovery values obtained in this study were compared with the applicable regulatory value and ranged from 91.18% to 109.49%, as presented in table 7.

Robustness is a validation parameter used to assess the resilience and responses of analytical method to small changes in various HPLC system parameters, such as maximum wavelength, column temperature, flow rate, and mobile phase composition (ratio). This is often evaluated by observing the peak area, retention time, asymmetry factor (TF), and column efficiency (N). The test results are presented in table 8, and according to [18-20], TF value is <2% and N is>2000 (plate count).

The quantification of gallic acid levels contained in the samples using the RP-HPLC system is presented in table 1. Measurements were conducted on the models and repeated three times (n=3). Gallic acid levels in the samples were found to be 1.773±0.049 mg/g with a retention time of 16.648 min. Fig. 2 shows a chromatogram of gallic acid standard solution and SNEDDS EAF.



(a)



(b)

Chromatgram	RT	AUC	TF	HETP
A	1.689	20389837	1.623	9850.20
B	1.648	4090311	2.857	8478.45

Fig. 2: Chromatogram results of gallic acid solution (a) and SNEDDS EAF of *Melastoma malabathricum* (b)

Antioxidants are chemical compounds capable of donating electrons to other molecules, such as Reactive Oxygen Species (ROS), one of the protective mechanisms within cells for neutralizing oxidants [24, 25]. Similarly to Reactive Nitrogen Species (RNS), ROS induced by ultraviolet radiation participate in wound healing [26]. According to Gulumian *et al.*, 2018, during the inflammatory phase, ROS plays a crucial role in facilitating the movement of neutrophils and monocytes in the blood vessels surrounding the wound [24]. Dunnil *et al.*, 2015 also stated that ROS contributes to infection control, as well as the proliferation of keratinocytes, endothelial cells, and fibroblasts, increasing angiogenesis and collagen deposition. However, uncontrolled ROS production can elevate oxidative stress, leading to wound severity and delayed healing. To maintain ROS levels within the range required by the body, antioxidants are used to stabilize ROS by donating electrons [24, 27, 28].

Regulating the amount of ROS content in the body during inflammation can serve as therapy, hence, stabilizing these oxidants within the inflammatory phase in wound tissue improves the healing process [25,27]. Gallic acid, a phenolic compound, is known to have good antioxidant activity. Studies by Monteiro *et al.*, 2017; Yang *et al.*, 2016 and Pressi *et al.*, 2022 showed that gallic acid could inhibit ROS and nitric oxide production, proinflammatory cytokine release, and phagocyte-induced lymphocyte proliferation in human peripheral blood mononuclear cells [29-31]. The topical application of this compound had been found to exhibit anti-inflammatory effects by increasing the expression of the enzymes such as catalase, SOD 2, and GPX-1 [31]. Pal *et al.*, 2018 also reported the inhibition of p65-NF-kB and IL-6/p-STAT3Y705 expression, further showing the anti-inflammatory properties. Yang *et al.*, 2016 stated the ability of gallic acid to induce wound healing agents such as adhesion kinases (FAK), c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (Erk) under normal and hyperglycemic conditions [32, 30].

SNEDDS is an isotropic mixture consisting of oil, surfactant, solvent, and co-solvent, with the addition of active pharmaceutical ingredients. SNEDDS can form stable solutions and emulsions with droplet sizes ranging from 10 to 200 nm [33]. This lipid-based system helps protect drug compounds, slow down degradation, and enhance drug delivery to target tissues, minimizing side effects. Moreover, the lipids in nanoemulsion preparations are suitable carriers for increasing the bioavailability of drugs at their target points [34]. SNEDDS have been shown to significantly boost drug release by 89% compared to 24% for traditional formulation [35]. Literature studies conducted on nano-sized lipid systems have shown their potential to effectively encapsulate antioxidant compounds. For instance, nanoencapsulation using green tea and peppermint oil phases resulted in an IC_{50} value of 116.07 mg/ml for the antioxidant. Additionally, SNES formulation from olive, pomegranate, and grape seed oil had IC_{50} values of 0.49%, 0.18%, and 0.8%, respectively. The development of SNEDDS from spirulina powder and combination of *spirulina* powder, fish oil, and *Tribulus terrestis* yielded IC_{50} values of 1606 and 1122 mg/ml [36-38].

In this study, the protective effect against oxidative stress was assessed using the DPPH and FRAP methods. The results in table 10 showed that the optimal protective concentration against free radicals was 50%, corresponding to IC50 values of 4.167±0.552 µg/ml and 20.253±0.619 µg/ml. The photoprotective effect against UV radiation, as indicated in table 11, was most potent at a concentration of 150 µg/ml, with SPF value of 36.993±0.183. Other investigations have reported some similar results; for example, [39] discovered that SNEDDS from ginger extract (*Zingiber officinale*) and eel fish bone oil (*Anguilla* spp) exhibited antioxidant activity with an IC_{50} value of 428.4 mg/ml. According to Kholieqoh *et al.*, SNEDDS formulation of Pandanus tectorius fruit extract showed an antioxidant activity 1.6 times higher than the non-formulated extract. Additionally, Lestari *et al.*, 2023 documented an antioxidant activity of 56.74±1.04% from SNEDDS formulation containing *Allium sativum* extract [40].

Phenolic compounds derived from natural sources have the potential to protect against the negative impacts of UV radiation. According to Hettihewa *et al.*, 2020, flavonoids and polyphenols present in fruit, flower, and vegetable samples have shown significant UV protection and antioxidant activity [26]. This observation was supported by the study of Frei *et al.*, 2023, which indicated SNEDDS containing curcumin extract to be capable of improving carrageenan and UV-induced inflammation in Mice [42]. The mechanism of action of antioxidant activity inhibition and UV

protection constitutes the disruption of ROS activity in cells. Moreover, in a test conducted on human fibroblast cells, Liu *et al.*, 2018 stated that the concentration of curcumin needed for UV protection was 5 µg/ml [43]. To mitigate the inflammatory process associated with wounds and generally accelerate healing, designing a formulation with antioxidant activity and UV protection is essential.

CONCLUSION

In conclusion, this study successfully developed a precise, accurate, specific,and stable analytical method for quantifying gallic acid levels contained in SNEDDS EAF of *Melastoma malabathricum* leaves combined with Gentamicin. The prepared SNEDDS formulation exhibited promising pharmacological activity, including potent antioxidant properties with IC50 values of 4.167±0.552 µg/ml and 20.253±0.619 µg/ml, assessed using the DPPH and FRAP methods, respectively. Moreover, it showed significant protection against UV radiation, as indicated by SPF value of 36.993±0.183 at a concentration of 150 µg/ml. These results showed the potential of SNEDDS formulation as an effective wound-healing agent, supported by a robust quality control process.

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AUTHORS CONTRIBUTIONS

All authors contributed equally to this study.

CONFLICTS OF INTERESTS

The author declares that there are no conflicts of interest.

REFERENCES

1. Yang DJ, Moh SH, Son DH, You S, Kinyua AW, Ko CM. Gallic acid promotes wound healing in normal and hyperglucidic conditions. Molecules. 2016;21(7):1-15. doi: 10.3390/molecules21070899, PMID 27399667.
2. Nayeem N, Smb A. Gallic acid: a promising lead molecule for drug development. J App Pharm 2016;8(2):2-4. doi: 10.4172/1920-4159.1000213.
3. Agrawal R, Ganeshpurkar A, Verma M. Evaluation of *in vitro* anti-inflammatory activity of gallic acid. Int J Res Rev. 2021;8(12):323-7. doi: 10.52403/ijrr.20211240.
4. Singh MP, Gupta A, Sisodia SS. Wound healing activity of *Terminalia bellerica* Roxb. and gallic acid in experimentally induced diabetic animals. J Complement Integr Med. 2019;17(2):20190133. doi: 10.1515/jcim-2019-0133, PMID 31494631.
5. Apridamayanti P, Sari R, Pratiwi L. Quantitative analysis study on marker compound in crude extract and polar fraction of *Melastoma malabathricum* L. with reversed-phase high-performance liquid chromatography and determination of antioxidant activity and sun protection factor value as natural resources for cosmetics. J App Pharm Sci. 2022. doi: 10.7324/JAPS.2022.121105.
6. Kardani K, Gurav N, Solanki B, Patel P, Patel B. RP-HPLC method development and validation of gallic acid in polyherbal tablet formulation. J Appl Pharm Sci. 2013;3(05):37-42. doi: 10.7324/JAPS.2013.3508.
7. Karupiah S, Ismail Z. Antioxidative effect of *Melastoma malabathticum* L. extract and determination of its bioactive flavonoids from various location in Malaysia by RP-HPLC with diode array detection. J Appl Pharm Sci. 2013;3(2):19-24.
8. Hainil S, Mayefis D, Wahyuni R. Antioxidant activity of ethanol extract and ethyl acetate fraction daun senduduk (*Melastoma malabathricum* L.) metode DPPH(2,2-diphenyl-1-picrylhydrazyl). J Public Health. 2023;2(1):35-42. doi: 10.55123/sehatmas.v2i1.981.
9. Apridamayanti P, Sari R, Pratiwi L. Development validation of quercetin compounds using Rp-HPLC and *in vitro* activity studies on *Melastoma malabathricum* leaf nanocream foundation preparations. Int J App Pharm. 2023;15(5):317-24. doi: 10.22159/ijap.2023v15i5.48297.
10. Gan JE, Chin CY. Formulation and characterisation of alginate hydrocolloid film dressing loaded with gallic acid for potential chronic wound healing. F1000Res. 2021;10:451. doi: 10.12688/f1000research.52528.1, PMID 34249341.
11. Stefanov I, Hinojosa Caballero D, Maspoch S, Hoyo J, Tzanov T. Enzymatic synthesis of a thiolated chitosan-based wound dressing crosslinked with chicoric acid. J Mater Chem B. 2018;6(47):7943-53. doi: 10.1039/C8TB02483A, PMID 32255040.
12. Seulgi P, Mei-Xian LMX, Chod WK, Joung YK, Huhs KM. Chitosan hexanoyl glycol conjugated with thermosensitive gallic acid as a biomaterial healing of new wounds. Carbohydr Polym. 2021:26(2021):117808. doi: 10.1016/j.carbpol.2021.117808.
13. Pratiwi L, Sari R, Apridamayanti P. Design and characterization of nanospray with self-nanoemulsifying drug delivery system using Sinergistic combination of *Melastoma malabathricum* L. Fraction and gentamicin. Int J App Pharm. 2021;13(2):254-63. doi: 10.22159/ijap.2021v13i2.40094.
14. Sari R, Pratiwi L, Apridamayanti P. The highest dosage combination activity screening from the leaf fraction of *Melastoma malabathricum* with antibiotic gentamicin and ciprofloxacin. J Pharmacopuncture. 2022;25(2):101-5. doi: 10.3831/KPI.2022.25.2.101, PMID 35837148.
15. Naseef H, Moqadi R, Qurt M. Development and validation of an HPLC method for determination of antidiabetic drug alogliptin benzoate in bulk and tablets. J Anal Methods Chem. 2018;2018:1902510. doi: 10.1155/2018/1902510, PMID 30345140.
16. Sharma S, Goyal S, Chauhan K. A review on analytical method development and validation. Int J App Pharm. 2018;10(6):8-15. doi: 10.22159/ijap.2018v10i6.28279.
17. Ppis P. Front Cover. PPIS;2019:c1. doi: 10.31153/ppis.2019.41.
18. Patil PN. HPLC method development-a review. SGVU J Pharm Res. 2017.
19. Bose A. HPLC calibration process parameters in terms of system suitability test. Austin Chromatogr. 2014;1:4.
20. Ravisankar P, Anusha S, Supriya K, Kumar UA. Fundamental chromatographic parameters. Int J Pharm Sci Rev Res. 2019;55(2):46-50.
21. Shaikh S, Jain V. Development and validation of a Rp-HPLC method for the simultaneous determination of quercetin, ellagic acid and Rutin in hydroalcoholic extract of *Triphala Churna*. Int J App Pharm. 2018;10(3):169-74. doi: 10.22159/ijap.2018v10i3.25860.
22. Saputri FA, Muchtaridi M. Analytical method development and validation for the determination of caffeine in green coffee beans (*Coffea Arabica L.*) from three districts of west java, Indonesia by high-performance liquid chromatography. Int J App Pharm. 2018;10(6). doi: 10.22159/ijap.2018v10i6.28551.
23. Snyder L, Kirkland J, Dolan J. Introduction to modern liquid. Chromatography. 3rd ed, John. NJ: Wileyandsons inc.; 2010.
24. Gulumian M, Yahaya ES, Steenkamp V. African herbal remedies with antioxidant activity: A potential resource base for wound treatment. Evid Based Complement Alternat Med. 2018. doi: 10.1155/2018/4089541, PMID 30595712.
25. Comino Sanz IM, Lopez Franco MD, Castro B, Pancorbo Hidalgo PL. The role of antioxidants on wound healing: a review of the current evidence. J Clin Med. 2021;10(16):3558. doi: 10.3390/jcm10163558, PMID 34441854.
26. Hettihewa SK, Opatha SAT. A comparative study on sunscreening and radical scavenging *in vitro* activities of solvent extracts obtained from dried *canna* (red) flowers grown in Sri Lanka. World J Pharm Res. 2020;9(4):94-104. doi: 10.20959/wjpr20204-16606.
27. Dunnill C, Patton T, Brennan J, Barrett J, Dryden M, Cooke J. Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process. Int Wound J. 2017;14(1):89-96. doi: 10.1111/iwj.12557, PMID 26688157.

28. Kurahashi T, Fujii J. Roles of antioxidative enzymes in wound healing. JDB. 2015;3(2):57-70. doi: 10.3390/jdb3020057.
29. Monteiro E Silva SA, Calixto GMF, Cajado J, De Carvalho PCA, Rodero CF, Chorilli M. Gallic acid-loaded gel formulation combats skin oxidative stress: development, characterization and ex vivo biological assays. Polymers. 2017;9(9):391-401. doi: 10.3390/polym9090391, PMID 30965694.
30. Yang K, Zhang L, Liao P, Xiao Z, Zhang F, Sindaye D. Impact of gallic acid on gut health: focus on the gut microbiome, immune response, and mechanisms of action. Front Immunol. 2020;11:580208. doi: 10.3389/fimmu.2020.580208, PMID 33042163.
31. Pressi G, Bertaiola O, Guarnerio C, Barbieri E, Rigillo G, Governa P. *In vitro* cell culture of rhus coriaria L.: A standardized phytocomplex rich of gallic acid derivatives with antioxidant and skin repair activity. Cosmetics. 2022;9(1):12. doi: 10.3390/cosmetics9010012.
32. Pal SM, Avneet G, Siddhraj SS. Gallic acid: pharmacogical promising lead molecule: a review. Int J Pharmacogn Phytochem Res. 2018;10(4):132-8. doi: 10.25258/phyto.10.4.2.
33. Alwadei M, Kazi M, Alanazi FK. Novel oral dosage regimen based on self-nanoemulsifying drug delivery systems for codelivery of phytochemicals–curcumin and thymoquinone. Saudi Pharm J. 2019;27(6):866-76. doi: 10.1016/j.jsps.2019.05.008, PMID 31516329.
34. Khairnar A, Shelke S, Rathod V, Kalawane Y, Jagtap A. Review on anti hyperlipidemia lipophilic drugs and their novel formulation approaches. Int J Pharm Pharm Sci. 2017;9(9). doi: 10.22159/ijpps.2017v9i9.19301.
35. Gohel M, Purohit AA, Patel A, Hingorani L. Optimization of bacoside a loaded snedds using d-optimal mixture design for enhancement insolubility and bioavailability. Int J Pharm Pharm Sci. 2016;8(12). doi: 10.22159/ijpps.2016v8i12.13488.
36. Shetta A, Kegere J, Mamdouh W. Comparative study of encapsulated peppermint and green tea essential oils in chitosan nanoparticles: Encapsulation, thermal stability, in-vitro release, antioxidant and antibacterial activities. Int J Biol Macromol. 2019;126:731-42. doi: 10.1016/j.ijbiomac.2018.12.161, PMID 30593811.
37. Lu L, Yang P, Chen T, Shen Y, Yao Q, Yan J. Changes in biological activities after olive oil, pomegranate seed oil, and grape seed oil were formulated into self-nanoemulsifying systems. J Oleo Sci. 2020;69(2):161-6. doi: 10.5650/jos.ess19255, PMID 31941867.
38. Abdel-All SR, Shakour ZTA, Abouhussein DMN, Reda E, Sallam TF, El-Hefnawy HM. Phytochemical and biological evaluation of a newly designed nutraceutical self-nanoemulsifying self-nanosuspension for protection and treatment of cisplatin-induced testicular toxicity in male rats. Molecules. 2021;26(2):408. doi: 10.3390/molecules26020408, PMID 33466804.
39. Wijayanti II, Budiharjo A, Pangastuti A, Prihapsara F, Artanti AN. Total phenolic content and antioxidant activity of ginger extract and SNEDDS with eel fish bone oil (Anguilla spp.). Nusantara Biosci. 2023;10(3):164-9. doi: 10.13057/nusbiosci/n100306.
40. Kholieqoh AH, Muhammad TST, Mohamad H, Choukaife H, Seyam S, Alfatama M. Formulation and characterization of SNEDDS of pandanus tectorius fruit extract and *in vitro* antioxidant activity. Orient J Chem. 2022;38(4):855-64. doi: 10.13005/ojc/380404.
41. Lestari SR, Prastita N, Maslikah Sl, Sunaryono S, Gofur A, Fajaroh F. Development of self nanoemulsifying drug delivery system (SNEDDS) to improve antioxidant activity of single garlic extract (*Allium sativum* L.). AIP Conf Proc. 2023;2634:020003. doi: 10.1063/5.0111654.
42. Frei G, Haimhoffer A, Csapo E, Bodnar K, Vasvari G, Nemes D. *In vitro* and *in vivo* efficacy of topical dosage forms containing self-nanoemulsifying drug delivery system loaded with curcumin. Pharmaceutics. 2023;15(8):2054. doi: 10.3390/pharmaceutics15082054, PMID 37631267.
43. Liu X, Zhang R, Shi H, Li X, Li Y, Taha A. Protective effect of curcumin against ultraviolet A irradiation-induced photoaging in human dermal fibroblasts. Mol Med Rep. 2018;17(5):7227-37. doi: 10.3892/mmr.2018.8791, PMID 29568864.