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Original Article

DESIGN AND CHARACTERIZATION OF OPTIMIZED POLYHERBAL NANOENCAPSULATION: *IN VITRO* ANTIOXIDANT ACTIVITY TARGETING NRF2 AND GST PATHWAYS

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ABSTRACT

Objective: Antioxidants are compounds that can stop oxidation, protecting cells from the harmful effects of free radicals produced by the body's metabolism and some external sources. Flavonoid and phenolic compounds are secondary metabolites of plants that have high antioxidant activity. The secondary metabolites found in *Nigella sativa* seeds contain terpenoid and phenolic compounds, while the leaves of *Moringa oleifera* and *Centella asiatica* plants contain flavonoids, alkaloids, tannins, saponins, and steroids. The problem is that the extracts from *Moringa oleifera* and *Centella asiatica* have large particle sizes and poor solubility, while *Nigella sativa* oil contains volatile and easily oxidizable chemicals. Therefore, it is very important to create a drug delivery system that can enhance solubility and reduce particle size, one of which is nanoencapsulation. The research the aim is to determine the optimum formula in the polyherbal (*Moringa oleifera, Centella asiatica* and *Nigella sativa*) nanoencapsulation preparation as a method of drug delivery and antioxidant activity targeting Nrf2 and GST pathways using PBMC.

Methods: Nanoencapsulation of polyherbal was carried out using chitosan and sodium tripolyphosphate polymers as crosslinkers and then characterizes. Optimization formulation by design expert (factorial design). In vitro analysis as an antioxidant in the regulation of Nrf2 and GST using PBMC with the flow cytometry method.

Results: The particle size, PDI and zeta potential from the optimum formula values were found 115.463±9.563 nm; 0.49±0.050, and-29.737±1.665mV. The Nrf2 and GST enhancement activity, it is evident that the nanopolyherbal preparations with concentrations of 3% and 1% have higher Nrf2 expression values compared to the control.

Conclusion: Nanoencapsulation of polyherbal (*Moringa oleifera, Centella asiatica* and *Nigella sativa*) have good antioxidant effect by Nrf2 and GST regulation, so it can be used as drug delivery system method.

Keywords: Antioxidant activity, Chitosan-sodium tripolyphosphate, Formula optimization, *In vitro* method, Nanoparticle

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INTRODUCTION

The variety of activities that activities, air pollution, and unhealthy lifestyles cause the body to be exposed to free radical compounds continuously [1]. The compounds needed to neutralize and prevent free radical damage are antioxidants. Antioxidants can complement the lack of electrons needed by free radicals and prevent the chain reaction of free radicals chain reaction of free radicals [2]. Generally, individuals consider antioxidants as chemicals that can stop oxidation, shielding cells from the harmful effects of free radicals produced by the body's metabolism and some outside sources. Free radicals, which are produced by the body's metabolism and certain outside sources, are not dangerous to cells. Chemically speaking, phenolic chemicals, including flavonoids (quercetin), hydroxamate derivatives, and vitamin C, are the primary source of natural antioxidants found in plants and foods [3]. Inhibitors that prevent autooxidation are called antioxidants. By creating a relatively steady and unreactive free radical reaction, free radical inhibitors block the action of free radicals. Plants are one source of naturally occurring antioxidants. The main regulator of the antioxidant response is nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), which is activated by ROS The enormous effect found in antioxidant response created by Nrf2 activation has prompted several research to better understand the processes involved in their generation [4]. Impaired Nrf2 pathway can lead to aging, inflammation, organ and tissue damage, and chronic disease development [5].

The defective Nrf2 pathway is linked to the activation of the Nrf2/ARE signalling pathway, which suppresses the activation of proinflammatory genes and antiinflammatory pathways. Cytoprotector genes like GPx and TXN have a role in regulating inflammation. Overexpression of HO-1 suppresses activator protein-1 (AP-1)-binding DNA, resulting in reduced TNF-α and IL-3-induced inflammation [6]. HO-1 inhibits the inflammatory response caused by lipopolysaccharide (LPS), lowering TNF-α and IL-B levels [7]. Nrf2 inhibits LPS-induced transcriptional regulation of proinflammatory cytokines IL-6 and IL-β in macrophages, preventing RNA polymerase II recruitment via binding to the genes [8].

Sources of antioxidants from plants are secondary metabolite compounds. Groups of secondary metabolites that can act as antioxidants by reducing free radicals include flavonoids and terpenoids [9]. Flavonoids and terpenoids belong to the category of very strong antioxidants [10]. Flavonoid molecules are among the substances found in plants that have antioxidant activity [11]. Among the polyphenolic chemicals with antioxidant capabilities are flavonoids. Antioxidants work by preventing oxygen or oxidized cells by transferring donor electrons or hydrogen atoms from antioxidant components to free radicals or reactive oxygen (hydroxyl, superoxide, and peroxy radicals) [12]. The secondary metabolite compounds found in the seeds of the *Nigella Sativa* plant contain terpenoid compounds [13] and the leaves of the *Moringa oleifera,* particularly their leaves, contain high antioxidants, including crucial phenolic bioactive compounds like flavonoids (quercetin, kaempferol, isorhamnetin, and apigenin) [14] and *Centella asiatica* plants contain triterpenoid glycosides like asiatic acid, asiaticosides, madecassoside acid, madecassoside, sitosterol, kaempferol, and flavonoids compounds [15]. In addition topic, *Moringa* leaf ethanol extract has strong antioxidant activity due to the presence of compounds including flavonoids, tannins, terpenoids, alkaloids, and saponins with an IC_{50} value of 50.595 ppm so that it has the potential for developing topical formulations in the form of lotions [16]. According to numerous studies, flavonoid compounds possess the ability to act as antioxidants due to their aromatic ring-associated hydroxyl groups, which enable them to

absorb free radicals produced by peroxidation reactions [17]. These three plants have different but complementary bioactive compounds, such as *Moringa oleifera*, which is rich in quercetin and chlorogenic acid as strong antioxidant [18]. *Centella asiatica* contains triterpenoids such as asiaticoside, which act as anti-inflammatory and antioxidant agents [19]. The content of *Nigella sativa* is rich in thymoquinone [20], which is a phenolic compound with antioxidant and immunomodulatory activities. The combination of the three has not been extensively explored in a single formulation system to investigate the synergy of various bioactive compounds that work through different antioxidant mechanisms (scavenging, chelating, enzyme regulation).

The combination of *Moringa oleifera*, *Centella asiatica*, and *Nigella sativa* in a nanoencapsulation system aims to enhance the synergy of antioxidant activity through modulation of the Nrf2/ARE pathway and phase II detoxification enzymes such as glutathione S-transferase (GST). This approach utilizes the main phytochemicals from each plant, namely thymoquinone from *Nigella sativa*, flavonoids from *Moringa oleifera* and *Centella asiatica*, as well as the synergistic potential in enhancing cellular antioxidant response [21]. Activation of the Nrf2/ARE pathway is an important mechanism in the cellular response to oxidative stress. Some of the key phytochemicals from the mentioned plants could activate this pathway: Thymoquinone: The active component of *Nigella sativa* can induce the expression of heme oxygenase-1 (HO-1) through the activation of Nrf2. Research shows that thymoquinone enhances the nuclear localization of Nrf2 and ARE reporter activity, as well as induces HO-1 expression in a concentration-and time-dependent manner in HaCaT cells. This activation is mediated by the generated ROS, which subsequently triggers the phosphorylation of Akt and AMPKα, two upstream pathways crucial in Nrf2 activation [22]. Flavonoids, such as luteolin, baicalein, myricetin, quercetin, and genistein, are known to enhance the nuclear translocation of Nrf2 and the expression of phase II target genes such as HO-1, NQO1, SOD, and CAT. For example, genistein activates the ERK1/2 and PKC pathways, which contribute to the activation of Nrf2 and the expression of HO-1 and GCLC in Caco-2 cells [23].

The issue is that extracts from *Moringa oleifera* and *Centella asiatica* have large particle sizes and poor solubility [24], whereas *Nigella sativa* oil contains volatile and readily oxidized chemicals [25]. Therefore, it is essential to create a medication delivery system that can boost the compound's ability by making it more soluble and reducing the size of its particles. One possible technological use is nanoencapsulation, a product design that uses a blend of polyherbal as antioxidant agents to create nanoparticles. The polyherbal combination may begin more quickly thanks to the benefits of the nanoencapsulation design.

Nanoencapsulation is a strategy to enhance the stability, solubility, and bioavailability of phytochemicals in the body. Several studies have explored the nano formulation of the active components of this plant: Nanoencapsulation is a strategy to enhance the stability, solubility, and bioavailability of phytochemicals in the body. Several studies have explored the nano formulation of the active components of this plant. Thymoquinone: nano formulations such as lipid nanoparticles (NLC) have been developed to enhance the stability and anticancer activity of thymoquinone. Studies show that NLC enhances the therapeutic efficacy of thymoquinone in the Hep3B liver cancer model integrated with the hepatitis B genome [26]. Nano formulations of flavonoids such as quercetin and luteolin have shown increased antioxidant activity and bioavailability. For example, quercetin nano formulation increases stability and antioxidant activity compared to its free form [27, 28]. The aim of this research is to determine the antioxidant activity of nanoencapsulation through the Nrf2 and GST pathways using the flow cytometry method.

MATERIALS AND METHODS

Preparation of sample and analysis of bioactive compound

The samples used were concentrated extracts of *Moringa oleifera* and *Centella asiatica* with the maceration method, where the dry extract was added to a 96% ethanol solvent and rotated with an ultra-turrax at a speed of 1000 rpm for 2 h, followed by maceration for 24 h. The first and second filtrates obtained were evaporated using a rotary vacuum evaporator at a temperature of 45 °C under vacuum pressure. Pour the obtained extract into a porcelain dish and evaporate the remaining solvent over a water bath, then weigh the obtained extract. For *Nigella sativa* oil, it is obtained through the distillation method.

Analysis of bioactive compounds of *Moringa oleifera* and *Centella asiatica* extracts using LCHRMS (Liquid Chromatography-High Resolution Mass Spectrometry) conducted at the National Research and Innovation Agency. Compounds phytochemicals were identified with LCHRMS using the ESI Positive and Negative Ion, with methanol solvent. Mass identification using Compound Discoverer™ 3.2 software with peak extraction filter and using the database MzCloud and Chemspider with mass annotations ranging from-5 ppm to 5 ppm. *Nigella Sativa* oil using GCMS (Gas Chromatography-Mass Spectrometry) at the Analysis Laboratory of Universitas Ahmad Dahlan. Sampel oil is placed into a micro tube and 1.5 ml of methanol solvent is added, then vortexed for 1 minute, then centrifuged for 3 min at a speed of 9000 rpm. The formed supernatant is continued for GCMS testing. The time is set for 60 min with an injector temperature of 260 °C, a detector temperature of 250 °C, and column 325 °C. The carrier gas used is helium gas as a carrier with a constant flow rate of 1 ml/minute. The identification process using the instrument GCMS produces several bioactive compounds. Bioactive compounds can be seen from the peaks of the chromatogram as the identification of chromatography and mass spectrometry (MS) viewed from the spectrum mass with their respective molecular weights bioactive compounds.

Preparation of nanoencapsulation polyherbal

The encapsulation solution (chitosan-NaTPP) used is based on the results of formula orientation with a 2:1 ratio, with total volume 20 ml. Whereas for surfactant orientation, it is based on the ratio of surfactants used to achieve the ideal HLB value. The ratio of surfactants, polysorbate 20 and polysorbate 80 is 6:4, which results in an HLB value of 16. Chitosan 200 mg was dissolved in 1% acetic acid that had been diluted in 100 ml of distilled water. Sodium-TPP 100 mg was dissolved in 100 ml of distilled water. Thick extracts of *Moringa oleifera* and *Centella asiatica* were mixed with *Nigella sativa* oil then polysorbate 80 and 20 surfactants were added until dissolved in a mortar. Then transfer to beaker glass and stirred at 1200 rpm for 10 min. Slowly pour 0.2% chitosan solution and continued to stirrer for 15 min. Pour drop by drop 0.1% sodium TPP solution and stirred for 60 min.

Characteristic of nanoencapsulation polyherbal

Examination of particle size and distribution using Particle Size Analyzer (PSA). Analysis of particle size analysis was carried out using Dynamic Light Scattering (DLS) technique using the Malvern device. Before measurement, 100 µl** of sample nano emulsion was diluted with 10 ml of distilled water to produce the appropriate scattering intensity. DLS produces the diameter and polydispersity index as benchmarks in nanoparticle size distribution in nanoparticle size distribution. Diameter and polydispersity index of the sample were obtained from three 173° angle measurements in a plastic polystyrene cell, all experiments were conducted at 25 °C. Characterization of zeta potential using zeta sizer (Malvern type 1203893) to determine the electrical charge parameters of a nanoencapsulation. Zeta potential can describe the stability of a dispersion system. Examination of zeta potential is determined by electrophoretic mobility measurement. A certain volume was put in a cuvette that electrodes (100 µl** of sample dissolved in 10 ml of distilled water).

Optimization of the formula using factorial design

Optimization in this formulation uses a design of experimental approach. The design and processing of data using Design-Expert software. The design chosen is part of the factorial design using $2^3$is used because this research will test 3 variables with each factor tested at 2 levels (low level and high level). The surfactant ratios used and the ratio of chitosan: sodium-TPP as factors, while the responses are particle size, polydispersity index and zeta potential.

This efficiency allows researchers to optimize the formula with a limited amount of data by identifying the main effects of each factor on the response variables (particle size, polydispersity index, and zeta potential).

In vitro as antioxidant effect

Peripheral blood mononuclear cells (PBMC) used were obtained from stored whole blood of patients suffering from cardiovascular diseases. Blood cell separation was performed using density centrifugation. After isolation, the PBMC were washed three times in phosphate-buffered saline with 1 µM EDTA. Cells were cultured for 48 h in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mmol glutamine, and 0.1% gentamicin (50 mg/ml) in a humidified environment containing 5% CO2. PBMC were produced fresh for each of the three tests, which were conducted in duplicate. The next step is the permeabilization of the cell membrane using a 0.03% saponin solution for 15 min at room temperature, to allow antibodies to penetrate the cell and bind to intracellular proteins. After that, staining of the intracellular phosphoprotein Nrf2 was performed using anti-Nrf2 antibodies and GST using anti-GST antibodies conjugated with the fluorochrome PE. ELISA Kit Nrf2 and GST by BioVendor. This staining was performed for 60 min at room temperature in the dark. To identify the cell nucleus and eliminate dead cells, 0.25 µg/10^6 cells of 7-AAD DNA dye was added and incubated for 10 min. After all staining stages are complete; the cells are washed again and prepared for analysis using a flow cytometer.

The analysis was conducted using the CellQuest Pro™ software, recording 10,000 events per sample. This procedure is replicated to ensure consistent results and reduce variation among individuals under the same culture conditions. With this technique, the translocation of Nrf2 and GST from the cytoplasm to the nucleus in PBMC subpopulations can be quantitatively and specifically measured, thereby providing important insights into the activation of immune signalling pathways in both physiological and pathological contexts.

RESULTS AND DISCUSSION

Analysis of bioactive compound

The results of GCMS analysis showed that there were 13 peaks representing 13 compounds identified in Nigella sativa oil. These compounds include (listed in table 1). Based on the results of the chromatogram analysis, three most dominant compound peaks were obtained, with area percentage values of 23.14%, 20.67% and 16.39%, respectively. Each of these compounds is the compound thymoquinone with a retention time of 10,668 min; compound 9,12-Octadecadienoyl chloride with a retention time of 16,066 min and Benzene compound, 1-ethyl-2,4-dimethyl, with a retention time of 7,882 min. Compound of Nigella sativa with GCMS analysis results are shown in table 1.

Table 1: List of phytochemicals identified of *Nigella sativa* oil by GCMS analysis

Peak	Retention time	Name of compound	Chemical formula	Molecular weight (g/mol)	Peak area (%)
1	6.019	Alpha Thujene	$C_{10}H_{16}$	136	8.53
2	6.450	Trans-Ocimene	$C_{10}H_{16}$	136	0.80
3	6.922	Bicylo (3.1.1) Heptane, 6,6-Dimethyl-2-metylene	$C_{10}H_{16}$	136	2.16
4	7.260	Alpha-pinene	$C_{10}H_{16}$	136	0.25
5	7.850	Benzene, 1-ethyl-2,4-dimethyl	$C_{10}H_{14}$	134	19.63
6	8.685	Bicyclo (4.1.0) Heptan-3-OI, 4,7,7-trimethyl	$C_{10}H_{18}O$	154	2.56
7	8.969	Bicyclo (4.1.10) heptan-3-ol,4,7,7-trimethyl	$C_{10}H_{18}O$	154	5.13
8	10.681	Thymoquinone	$C_{10}H_{12}O_2$	164	24.64
9	11.541	Humulen	$C_{15}H_{24}$	204	3.25
10	11.910	phenol	$C_{10}H_{14}O$	150	2.37
11	13.575	5-hepten-3-yn-2-ol	$C_{11}H_{18}O$	166	-1.48
12	15.077	Octadecanoic acid	$C_{18}H_{36}O_2$	284	7.32
13	15.540	d-nerolidol	$C_{15}H_{26}O$	222	1.00
14	15.724	2-hexadecen-1-ol	$C_{20}H_{40}O$	296	2.94
15	16.113	13-oxabicyclo (10.1.0) tridecane	$C_{12}H_{22}O$	182	16.51
16	16.830	1,6,10-dodecatrien-3-ol	$C_{15}H_{26}O$	222	1.44

The retention time refers to the time a compound takes to travel through the chromatography column. Peak area (%) indicates the relative abundance of each compound detected. Molecular weight is expressed in g per mole (g/mol).

In accordance with the working process of the GCMS instrument, by using retention time in the analyte identification diagram, it is possible to compare unknown compounds with reference compounds, thereby determining the compound content in the sample [29]. α-Thujene is a natural monoterpene having the chemical formula $C_{10}H_{16}$. This chemical is present in essential oils. Chemical modifications, such as the addition of hydroxyl or methoxy groups, transform monoterpene molecules into monoterpene derivatives. This alteration may increase its biological activity, particularly antioxidant function [30]. α-pinene is a natural monoterpene which is commonly used as a chemical, pharmaceutical and fragrance [31] and has antioxidant content with treatment caused increases in TAC (total antioxidant capacity) level at 25 and 50 mg/l and decreases in TOC (total oxidative stress) level at 200 mg/l on human lymphocytes [32]. The compound thymoquinone (fourth peak) is one of the main active components in *Nigella sativa* oil and has antioxidant activity. This compound has the IUPAC name 2-methyl-5-propan-2-ylcyclohexa-2,5-diene1,4-dione [29].

Flavonoid chemicals from the plants *Moringa oleifera* and *Centella asiatica* were detected using LCHRMS analysis. Identification of the flavonoid content of the *Centella asiatica* and *Moringa oleifera* plants. As secondary antioxidants, flavonoids work by either capturing or interrupting the chain oxidation reaction of free radicals. The flavonoid metabolites discovered in *Moringa oleifera* and *Centella asiatica*, including molecular formula, LCHRMS research revealed the distribution of several useful compounds, such as kaempferol, quercetin, and other phytochemicals. It discovered that *Centella asiatica* included 76 metabolites and *Moringa oleifera* contained 152 metabolites, while the current study only looked at flavonoid components. Flavonoid content in *Moringa oleifera,* such as quercetin, trifolin, rutin, and kaempferol, is also found in *Centella asiatica*. Flavonoids function as secondary antioxidants by either preventing or absorbing the free radical chain oxidation process. The mechanism of flavonoids as secondary antioxidants is by breaking the chain oxidation reaction of free radicals or capturing them [33]. By adding one hydrogen atom to the oxidant component, antioxidants can neutralize free radicals and make the oxidant compound more stable [34].

Quantitative phytochemical screening

Quantitative analysis of flavonoids in a sample is measured using UV-Vis spectrophotometry. The presence of aromatic systems conjugated with flavonoid structures will show strong absorption bands in the ultraviolet wavelength region and the visible light spectrum region. The principle of quantitative testing of total flavonoids using $AlCl_3$ is the formation of stable complexes of C-4

keto groups, C-3, or C-5 hydroxyl groups from flavones and flavonols. Because quercetin belongs to the flavonoid group, it will form a stable complex with the orthohydroxyl group in the flavonoid structure due to the addition of $AlCl_3$ or aluminum cations [35]. The flavonoid, alkaloid and thymoquinone content test of nanopolyherbal results are shown in table 2.

Table 2: Flavonoid content of nanopolyherbal

Compound	Result
Flavonoid	3.913±0.038 mg QE/gram Extract
Alkaloid	60,418±0,522 ppm
Thymoquinone	14,93±0,353 mg/l

QE: Quercetin Equivalent. Results are expressed as mean±standard deviation (SD) (n=3). ppm: parts per million. mg/l: milligrams per liter.

Optimization design using factorial design and characteristic nanoencapsulation polyherbal

In this study, the preparation of chitosan nanoparticles using ionic gelation method, with as a crosslinking agent, use sodium tripolyphosphate (TPP). Following the analysis of the eight formulae (table 3), the response data was imported into the Design Expert® 13 software, allowing the highest desirability value to determine which formula was best. The encapsulation solution (chitosan-NaTPP) used is based on the results of formula orientation with a 2:1 ratio. Whereas for surfactant orientation, it is based on the ratio of surfactants used to achieve the ideal HLB value. The ratio of surfactants, polysorbate 20 and polysorbate 80 is 6:4, which results in an HLB value of 16 [36].

The program's ability to create the intended outcome more precisely is shown by a desirability value that approaches 1. Based on the target response analysis, which includes the polydispersity index, zeta potential, and particle size, the ideal formula is determined. The most optimal formula with the largest desirability value of 1 was obtained based on the factorial design analysis results of the Design Expert® 13 program, and the requirements included the prediction of the three answers' outcomes. Desirability value shows how well the program can satisfy users' needs in relation to the standards established for the finished output [37].

The optimum formula response analysis to be carried out is the same as the eight formula response analyses, namely particle size, polydispersity index, and zeta potential. The results of the optimum formula response analysis are then compared with the prediction results in the Expert® 13 Design program to ensure that the optimum formula proposed by Expert® 13 Design meets the desired nanoparticle preparation characteristics. The design chosen is part of the factorial design using 2^3. It is more effective because it allows researchers to explore the main effects of two factors and their interactions using a minimal number of experiments. Through experimentation with the optimum condition, the found optimum formula was further confirmed. It is estimated that the ideal formula will yield polyherbal nanoparticles with a zeta potential of -34.06, a PDI of 0.388, and a particle size of 112.397 nm. Prediction and verification value of each response (particle size, PDI and zeta potential) results are shown in table 5.

Table 3: Composition of nanopolyherbal and experimental measured input responses

Run	Factor			Response		
	Polysorbate 20	Polysorbate 80	Encapsulation	Particle size	PDI	Zeta potential
1	5.4	1.2	20	161.00±4.62	0,67±0.09	-29.05±1.38
2	5.4	3.6	20	194.03±6.70	0.50±0.07	-34.87±0.45
3	1.8	3.6	10	235.20±8.72	0.95±0.04	-27.43±0.53
4	1.8	1.2	10	227.30±1.60	0.84±0.14	-31.11±1.46
5	1.8	1.2	20	262.40±3.04	0.53±0.02	-24.94±0.46
6	1.8	3.6	20	186.10±6.76	0.64±0.09	-27.94±1.25
7	5.4	3.6	10	255.90±2.72	0.63±0.06	-35.19±1.59
8	5.4	1.2	10	121.50±4.94	0.46±0.05	-32.68±0.32

PDI: Polydispersity Index, Particle size and Zeta Potential (n=3).

The prediction of the optimum formula of nano polyherbal was carried out using Design Expert software, namely Factorial Design. Results of statistical analysis from Factorial Design on overall response presented in table 4.

Table 4: Results of statistical analysis from factorial design on overall response

Response	Parameter						
	SD	Mean	CV %	R^2	Adjusted R^2	Predicted R^2	Adequate rescission
Particle Size	4.78	205.43	2.32	0.9973	0.9904	0.9562	34.0701
PDI	0.0247	0.7012	3.53	0.9935	0.9771	0.8955	21.8955
Zeta Potential	0.9153	-30.44	3.01	0.9820	0.9371	0.7126	11.9727

SD: Standard Deviation; CV%: Coefficient of Variation; R^2: Coefficient of Determination; Adjusted R^2: Adjusted R-squared; Predicted R^2: Predicted R-squared; Adequate Precision: Signal to noise ratio (a value>4 indicates an adequate model).

Interpretation of particle size parameter data, namely low standard deviation and CV, indicates very small data variation. R^2 is very high (0.9973), indicating that the model is very accurate in explaining data variation. Predicted R^2 (0.9562) is close to Adjusted R^2, indicating that the model has very good predictive ability. Adequate Precision is well above 4, indicating a very good signal and the model is highly suitable for design navigation. Interpretation of the PDI parameter data indicates that the model shows a very high fit with an R^2 value of 0.9935. High predicted R^2 (0.8955) indicates strong predictive capability. High Adequate Precision indicates that it can be used in the optimization process. Interpretation of the zeta potential data indicates that the model shows a very good fit (R^2 = 0.9820). Although the Predicted R^2 (0.7126) is lower than the Adjusted R^2, its value is still considered good. Adequate Precision exceeds 4, indicating that the model has a strong and valid signal for use in experiments.

Factorial design is used by a design professional to formulate optimization. Regression equations are used in factorial design to create a model that depicts the connection between a response variable and one or more independent variables. The most popular kind of design for process optimization is factorial. The factorial method is employed in research to quantify the interactions within a study and determine the impact of different conditions on its findings [38]. Tablets, syrups, and emulsions are examples of traditional dose formulations that can be used with Design-Expert

software. Numerous studies have been conducted on the creation of medication delivery systems with the use of Design-Expert software. Drug delivery systems are ways to formulate medications or devices such that the pace, location, and duration of drug release within the body can be controlled, thereby improving the medication's efficacy and safety. In this work, the medication delivery method employs nanoparticle technology. Colloidal formations with sizes ranging from 10 to 1000 nm are known as nanoparticles [39]. Because it can affect the nanoparticle system's in vivo distribution, toxicity, drug release, and targeting capability, particle size is the most crucial property [40]. The obtained particle size was 115.463±9.563 nm. These findings suggest that the formula's size has satisfied the necessary nanoparticle range. Size distribution can have an impact on drug release, delivery, and nanoparticle stability in addition to size, is shown by the value of the polydispersity index (PDI). For monodispersed particles, the PDI ranges from 0.01 to 0.5–0.7, and an index greater than 0.7 denotes a system of nanoparticles with a highly broad particle size distribution [41]. In this investigation, the nano polyherbal PDI was 0.490±0.050. This number satisfies the conditions needed to declare that the ideal formula chosen for this investigation has a uniform particle size distribution, or monodispersed distribution. The particle size of 115.463±9.563 nm is highly relevant in the context of bioavailability because it falls within the optimal range for cellular absorption through endocytosis, which is one of the main mechanisms by which cells take up particles from the external environment. The size less than 200 nm nanoparticles plays an optimal role in the endocytosis process, especially receptor-mediated endocytosis and pinocytosis, which is the process of absorbing fluids and small particles [42].

The zeta potential value indicates the stability of a colloidal system, and it is necessary to understand the characteristics of the surface charge properties of nanoparticles to demonstrate this. A steady suspension has often been seen for nanoparticles with a zeta potential of ±30 mV (33). Zeta potential measurement results are-29.737±1.665 mV is very close to the limit or requirement ±30 mV, which means the system can be considered quite stable, but it is at a critical threshold. This means that the formed colloid is likely stable in the short term, but for longer storage periods, sedimentation may occur, especially if the storage conditions change (pH, temperature, ions). Practical implications require the addition of stabilizers such as polymers or surfactants to enhance stability and storage under controlled conditions.

Table 5: Prediction and verification value of particle size, PDI, and zeta potential

Response	Prediction	Verification	*p*-value
Particle size	112.396	115.463±9.563	0.403
PDI	0.388	0.490±0.050	0.101
Zeta Potential	-34.060	-29.737±1.665	0.076

Prediction: Value predicted by the model; Verification: Experimental value with standard deviation; p-value: Probability value from statistical comparison between predicted and verified data (p>0.05 indicates no significant difference).

Table 5 shows the confirmed outcome of the optimal formula. This result suggests that the formula that the Design Expert program used and recommended is adequate. The one-sample t-test was then employed in SPSS to validate the verification. Next, perform a correlation study to ascertain the degree of the linear relationship between two or more variables. The value of maximum desirability is one. The closer to one, the better the desirability value. The effect between responses is tested using the probability value, or *p*-value. A significant association between responses is indicated by a p-value of less than 0.05, whereas no significant interaction between variables is shown by a *p*-value greater than 0.05 [43]. The expert design equation is correct, while there is not a significant distinction between the research results and the expert design outcomes, as indicated by the *p*-values of each parameter, particle size, PDI, and zeta potential, all of which are greater than 0.05. The regression model is valid and can be used for prediction, especially for particle size. However, the predictions for PDI and Zeta potential are slightly too low. The zeta potential results have a p-value of 0.076, which is close to the significance threshold (0.05), indicating that the model's accuracy for zeta potential is slightly lower compared to other parameters. It is recommended to refit the model with additional midpoints because zeta accuracy is a priority. The zeta potential is an important metric in the characterizations of nanoparticles since it indicates their surface charge and stability in colloidal solutions [44].

Transmission electron microscopy (TEM) is one of the properties of the formula that was done, along with particle size values, polydispersity index (PDI), and zeta potential. A technique for examining the distribution, form, and composition of solid pores is TEM. The working principle of TEM is that electrons are transmitted into the object of observation, and the results can be observed through the screen. The size distribution of nanoparticles and other small particles in any optical and electron microscope picture can be quickly and accurately analysed using this method. The TEM image of Nanopolyherbal is showing in fig. 1.

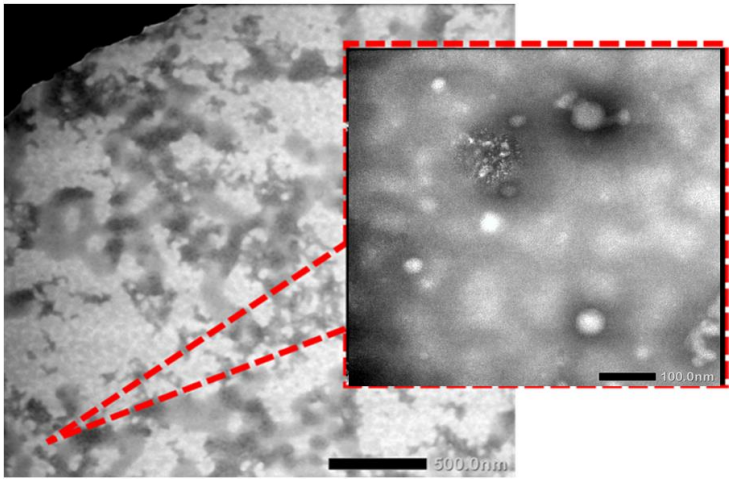

Fig. 1: TEM image of nanopolyherbal (ma. 80,000x)

Morphological testing of chitosan naringenin nanoparticles in this study produced nanoparticles with a relatively spherical shape (fig. 1**).** The shape and surface condition of nanoparticles are important to know because they can be used to determine the drug release properties. Nanoparticles with a spherical shape have a greater uptake than rod-shaped ones [39]. The results of the morphological observation of chitosan-sodium TPP nanoparticles using TEM at a magnification of 80,000x with particle size 100 nm.

FTIR testing on nanoemulsion preparations is also conducted as one of the characteristics of nanoemulsion preparations. FTIR is used to determine chemical interactions, drug-excipient compatibility studies between active substances and other materials used in the formulation. The procedure carried out was to dilute the Nanopolyherbal sample with water and centrifuge it at 15,000 rpm for 40 min. The residue is separately transferred to a glass plate and stored at 50 °C overnight to remove the aqueous phase. The samples were then used for FTIR analysis, subsequently scanned over the wavelength range of 4000 to 500 cm^{-1} at a resolution of 4 cm^{-1} in an FTIR instrument equipped with software. The sample is placed on the sample stage and the same force (100 N force gauge) is always applied to ensure reproducible contact between the sample and the crystal for scanning.

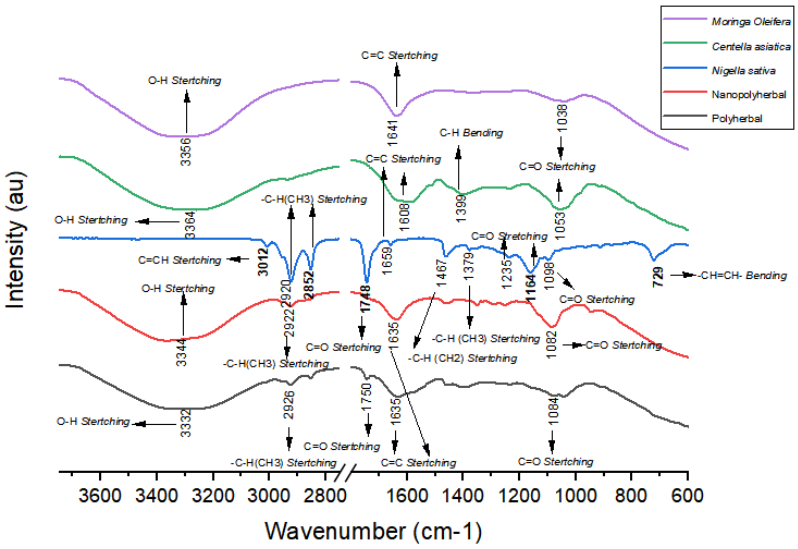

Fig. 2: FTIR results spectrum

Based on fig. 2, various absorption peaks are observed. The absorption peaks are the absorption groups in the sample that characterize the presence of molecular vibrations in the sample. In FTIR, there are vibrations or shifts, namely stretching and bending. Stretching is the movement of bonds forward-backward and/or up-side, while bending is the movement of bonds up and down.

The spectra that appear from each sample differ from one another, indicating that the components constituting each herbal compound are different. The FTIR spectrum is used to observe the success of functional group modification by examining the appearance of the resulting spectral pattern [45]. The spectrum of the *Nigella sativa* seed oil sample shows absorption peaks at wavenumbers 129, 1098, 1164, 1235, 1467, 1659, 1748, 2852, 2920, and 3012 cm^{-1}, which correspond to the functional groups carboxylic acid, ester, and alkene with bending and stretching vibrations [46]. The spectrum from the *Centella asiatica* sample shows spectral peaks at wave numbers 1053, 1399, 1608, and 3364 cm^{-1}, which contain the functional groups carboxylic acid, alkene, ether, and alkyl halides [47], with bending and stretching vibrations. In the spectrum of the Moringa oleifera sample, all wave number peaks exhibit stretching vibrations with absorption peaks at 1038, 1641, and 3356 cm^{-1}, which contain the functional groups carboxylic acid, alkene, and ether [48].

Table 6: Sample absorption area

Sample	Absorption area (cm^{-1})	Functional group	Vibration
Nigella sativa oil	729	-CH=CH-	*Bending*
	1098	C=O	*Stretching*
	1164-1235	C=O	*Stretching*
	1379	-C-H (CH3)	*Bending*
	1467	-C-H (CH2)	*Bending*
	1659	C=C	*Stretching*
	1748	C=O	*Stretching*
	2852-2920	-C-H (CH3)	*Stretching*
	3012	C=CH	*Stretching*
Centella asiatica	1053	C=O	*Stretching*
	1399	C-H	*Bending*
	1608	C=C	*Stretching*
	3364	O-H	*Stretching*
Moringa oleifera	1038	C=O	*Stretching*
	1641	C=C	*Stretching*
	3356	O-H	*Stretching*

The absorption area (cm^{-1}) corresponds to the infrared wavelengths at which specific functional groups absorb, indicating molecular vibrations. Bending refers to angular deformation, while stretching refers to linear displacement of bonds.

In the spectrum results of polyherbal nanoparticles, there are differences or shifts in the absorption regions, especially for the *Nigella sativa* seed oil sample. Differences are observed at wave numbers 129 cm^{-1}, 1467 cm^{-1}, 1748 cm^{-1}, 2852 cm^{-1}, and 3012 cm^{-1}, which are only present in *Nigella sativa* oil and not in the other two extracts. Where the spectrum at wave numbers 129 cm^{-1} and 1467 cm^{-1} is characterized by the bending vibrations of the-CH=CH-and-C-H (CH_2) groups, and the spectrum at wave numbers 1748 cm^{-1}, 2852 cm^{-1}, and 3012 cm^{-1} shows the stretching vibrations of the C=O,-C-H (CH_3), and C=CH groups (table 6), which are common indicators of phenolic compounds [49]. FTIR analysis revealed the carboxyl (-C=O), hydroxyl (-OH), and amine (N-H) groups, which are primarily involved in antioxidant activity [50]. The spectrum of *Nigella sativa* seed oil at those wavenumbers is not found in the spectrum of *Centella asiatica* and *Moringa oleifera* extracts and is observed in the mixture or combination of herbs. This is because the herbal combination is produced by the overlapping spectra of each herbal. Meanwhile, for the spectrum at the wavenumber 1748 cm^{-1}, it is not visible in the spectrum of the nanopolyherbal. This serves as evidence that there is a change in particle size. The particle size significantly affects the intensity and area of the infrared bands in the sample spectrum [51].

Antioxidant effect by expression Nrf2 and GST

Main phytochemicals such as thymoquinone from *Nigella sativa* and flavonoids from *Moringa oleifera* and *Centella asiatica* play an important role in activating the Nrf2 (Nuclear factor erythroid 2-related factor 2) pathway through the dissociation of Keap1 (Kelch-like ECH-associated protein 1) and nuclear translocation of Nrf2. The activation of this pathway contributes to the increased expression of phase II detoxification enzymes, including glutathione S-transferase (GST), which play a role in cellular detoxification mechanisms [52]. Activation of Nrf2 and GST can be an indicator that the active ingredients (for example, from herbal extracts) are available in bioactive form and reach cellular targets. This suggests that the delivery system is quite effective at penetrating biological barriers, supporting bioavailability.

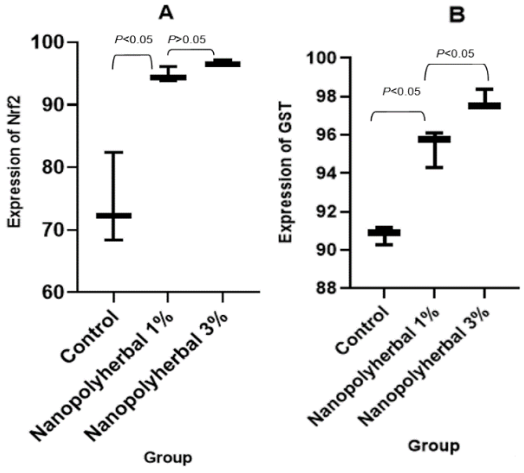

Fig. 3: Expression results of Nrf2 (A) and GST (B)

Activation of the Nrf2 pathway by thymoquinone

Thymoquinone is one of the main bioactive components in Nigella sativa oil identified in the GCMS profile (peak area: 24.64%), likely contributing to the increased expression of Nrf2 and GST studied. Research shows that thymoquinone enhances the nuclear localization of Nrf2 and ARE reporter activity, as well as induces HO-1 expression in a concentration-and time-dependent manner in HaCaT cells. This activation is mediated by the generated ROS, which subsequently triggers the phosphorylation of Akt and AMPKα, two upstream pathways crucial in Nrf2 activation. This activation is mediated by the ROS produced, which in turn triggers the phosphorylation of Akt and AMPKα, two important upstream pathways in the activation of Nrf2 [53].

Activation of the Nrf2 pathway by flavonoids

Flavonoids are a group of phytochemical compounds known for their ability to activate the Nrf_2 (Nuclear factor erythroid 2-related factor 2) pathway, which plays an important role in maintaining cellular redox homeostasis and detoxification mechanisms. Activation of this pathway can increase the expression of phase II detoxification enzymes, including glutathione S-transferase (GST), which contribute to the conjugation and elimination of reactive oxygen species (ROS) byproducts [54]. Flavonoids, such as quercetin, are known to enhance the nuclear translocation of Nrf2 and the expression of phase II target genes such as HO-1, NQO1, SOD, and CATs [55].

The decrease in antioxidants is largely caused by the disruption of the activation of the nuclear-erythroid-2 related factor 2 (Nrf2), a transcription factor that regulates genes encoding antioxidants and detoxifying enzymes. Under physiological conditions, oxidative stress triggers the endogenous regulation of antioxidants and cytoprotective proteins to prevent or limit tissue damage. Nrf2 activation increases the transcription rate of several genes [56]. Oxidative stress occurs when the formation of ROS exceeds the capacity of the antioxidant defense system. This can occur due to increased ROS production or decreased antioxidant defenses. This imbalance causes biomolecules to oxidize, and the structure and function of the molecules change. The body produces superoxide anion (O_2), hydroxyl (OH-), and hydrogen peroxide (H_2O_2) [57]. The increased production of ROS causes tissue damage and dysfunction by attacking, denaturing, and altering the structure and function of molecules, as well as activating transcription factors and redox-sensitive signaling pathways. This causes necrosis, apoptosis, inflammation, and fibrosis. Tissues will be protected from damage caused by ROS through a redox system consisting of antioxidant enzymes and phase 2 detoxification enzymes. Nrf2 plays an important role in basal activity and induces genes that encode several antioxidants and phase 2 detoxification enzymes [58]. The Nrf2 expression results between the control group and the 1% and 3% nanopolyherbal groups showed a p-value<0.05, indicating a significant difference between the test groups for the comparison between the 1% and 3% nanopolyherbal groups, the p-value>0.05 indicates that the results between these groups are not significantly different. The results of the increased NRf2 expression activity are presented in the following fig. 3.

Nrf2 is a highly important cytoprotective transcription component. Nrf2 is activated by oxidants, electrophiles, or endoplasmic reticulum stress. In Nrf2, there are six domains. The Neh1 domain contains a structure necessary for binding to DNA and forming dimers, the Neh2 domain interacts with Keap1, and the Neh6 domain is responsible for negative regulation. The role of Nrf2 in the inflammatory process is to inhibit the transcription factor NF-κB, which leads to a reduction in the inflammatory process. If Nrf2 is damaged, the inhibition of the NF-κB transcription factor decreases, leading to an increased expression of genes encoding inflammatory mediators [23]. Oxidative stress and inflammation play a role in the pathogenesis of various diseases. Oxidative stress triggers inflammation, which produces free radicals. If free radicals are present, the body will produce antioxidants. The production of these antioxidants is controlled by the Nrf2 transcription factor, which can enhance the production of endogenous antioxidants. However, oxidative stress is not followed by an increase in the body's antioxidant defense system. This is because the activation of the Nrf2 transcription factor is disrupted [4].

From the results of the Nrf2 enhancement activity, it is evident that the nanopolyherbal preparations with concentrations of 3% and 1% have higher Nrf2 expression values compared to the control. This means that the nanopolyherbal formulation is capable of significantly increasing Nrf2 expression. Thus, it is expected to reduce the occurrence of cardiovascular disease complications due to the presence of oxidants and inflammation.

Regulation of GST by Nrf2

Activation of Nrf2 through Keap1 dissociation and nuclear translocation increases the expression of phase II detoxification enzymes, including GST. Quercetin, one of the flavonoids frequently

used in research, is known to enhance GST activity through the activation of the Nrf2/ARE pathway [59].

Glutathione S-transferase (GST) is a multifunctional enzyme that plays an important role in protecting cells from damage by toxic chemicals. This enzyme plays a role in the conversion of one of the cytokines (leukotriene A4) into a product of arachidonic acid oxidation, one of which is prostaglandin through the lipoxygenase pathway [59]. The coagulation cascade, arachidonic acid pathway, as well as the formation of growth factors and cytokines, simultaneously work together to initiate and sustain the inflammatory phase [60]. The GST expression results among groups showed a p-value<0.05, indicating a significant difference among the test groups. The results of GST expression in this study are presented in fig. 3.

Prostaglandins are formed from the process of oxygenation and transformation of arachidonic acid and unsaturated fatty acids. Cyclooxygenase-2 (COX-2) is an enzyme that can convert arachidonic acid into pro-inflammatory prostaglandins, which are the main target of anti-inflammatory drugs. The use of *Centella asiatica* is as an anti-inflammatory, so the 40% and 50% concentrations of *Centella asiatica* extract in this study can be used in the treatment of gingivitis by inhibiting the transformation of arachidonic acid into prostaglandin [61].

From the results of the GST enhancement activity, it is evident that the nanopolyherbal preparations with concentrations of 3% and 1% have higher GST expression values compared to the control. This means that the nanopolyherbal preparation is capable of significantly increasing GST expression. Thus, it is expected to reduce the occurrence of cardiovascular disease complications due to the presence of oxidants and inflammation.

CONCLUSION

Based on the results obtained in this study, it can be concluded that nano polyherbal preparations from *Moringa oleifera* extract, *Centella asiatica* extract and *Nigella sativa* oil showed the Nrf2 and GST enhancement activity, it is evident that the nanopolyherbal preparations with concentrations of 3% and 1% have higher GST expression values compared to the control. This is due to the presence of several antioxidant agents, including phenolic and flavonoid groups such as thymoquinone, alpha pinene, beta pinene, trifolin, quercetin, kaempferol, and rutin. Furthermore, these findings provide strong evidence of the potential application of herbal combinations as a source of bioactive compounds made in the form of nanoencapsulation preparations as one of the drug delivery techniques. The limitations of this study include the lack of in vivo research and the still-limited stability data. so that in the future, research can be conducted focusing on *in vivo* efficacy testing and toxicity testing to further establish the safety and therapeutic potential of this formulation.

ETHICAL APPROVAL

This research has received ethical approval from the Research Ethics Committee of Universitas Ahmad Dahlan with the number: 012407208.

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AUTHORS CONTRIBUTIONS

Rafiastiana Capritasari, Akrom, Arif Budi Setianto: Conceived, analysis tools or data and drafting or critically revised for important intellectual content, Rafiastiana Capritasari, Ichwan Ridwan Rais, Sapto Yuliani: Analyzed, interpreted the data and drafting or critically revised for important intellectual content.

CONFLICTS OF INTERESTS

The authors report no financial or any other conflicts of interest in this work.

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