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DOI: <https://doi.org/10.28942/atu.j.v6i3y2026.161>**DEVELOPMENT, STANDARDIZATION, AND STUDY OF THE BIOLOGICAL EFFICACY OF A SYNERGISTIC PHYTOEXTRACT OF *SYZYGIUM AROMATICUM* L., *ACHILLEA MILLEFOLIUM* L., AND *HIBISCUS SABDARIFFA* L. BY HIGH-SPEED EXTRACTION**Mehraliyeva S.J.^{1*}, Mammadov B.S.^{1*}, Gadirova H.A.², Süleymanova T.H.², Nasirli I.U.³, Ece Bayır⁴, A. Alper Öztürk⁵**Abstract**

The present study aimed to develop and optimize a multifunctional phytoextract composed of clove (*Syzygium aromaticum*), yarrow (*Achillea millefolium*), and hibiscus (*Hibiscus sabdariffa*) for potential topical pharmaceutical and cosmetic applications. Three different phytocompositions were prepared and comparatively evaluated in terms of antimicrobial activity, antioxidant potential, physicochemical properties, safety, and long-term stability. Among the investigated systems, the composition containing clove, yarrow, and hibiscus in a ratio of 6:1:3 exhibited the most favorable profile and was therefore selected as the optimized formulation. The extraction process was systematically optimized by investigating the influence of extraction method, extraction time, plant-to-solvent ratio, temperature, solvent composition, and rotation speed. High-speed extraction performed for 7 min at 26 °C using a 95:5 ethanol: propylene glycol mixture, a raw material-to-solvent ratio of 1:10, and a rotation speed of 3200 rpm yielded the most suitable extract in terms of quality, antimicrobial activity, and long-term stability. The optimized phytocomposition demonstrated pronounced antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, with minimum inhibitory concentration values of 0.25 mL, while complete inhibition of *Candida albicans* was achieved at 0.50 mL. The extract also exhibited strong antioxidant activity in the DPPH assay, with an IC₅₀ value of 1.58 mg/mL. Long-term stability studies demonstrated that the optimized extract remained stable for up to 24 months when stored in dark glass packaging. Only minor changes in rutin and eugenol content, dry residue, and pH were observed, whereas storage in plastic containers resulted in marked physicochemical deterioration. The optimized extract was found to be non-irritant in the HET-CAM assay, with an irritation index of 2.45 ± 0.35. Overall, the developed phytocomposition combined strong antimicrobial and antioxidant activity with good stability and excellent tolerability. These findings indicate that the optimized phytoextract may serve as a promising active ingredient for the development of topical pharmaceutical and cosmetic formulations.

Keywords: *Syzygium aromaticum*, *Achillea millefolium*, *Hibiscus sabdariffa*, high-speed extraction, antimicrobial activity, antioxidant activity, HET-CAM, topical phytocomposition



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INTRODUCTION

Medicinal plant extracts have attracted considerable attention owing to their antioxidant, antimicrobial, anti-inflammatory and regenerative properties. The management of superficial skin damage, minor wounds, inflammatory dermatoses, and microbial skin infections often requires multifunctional preparations capable of reducing oxidative stress, preventing microbial colonization, and supporting tissue repair. In this context, medicinal plants rich in polyphenols, flavonoids, and volatile constituents represent promising sources of bioactive compounds for the development of effective topical formulations [1–5].

Among the selected medicinal plants, clove buds (*Syzygium aromaticum* L.) are particularly rich in eugenol, eugenol acetate, and β -caryophyllene, which are responsible for pronounced antibacterial, antifungal, antioxidant, and anti-inflammatory activities. Clove extracts have demonstrated inhibitory effects against both Gram-positive and Gram-negative microorganisms and are widely used in traditional medicine for the treatment of infected wounds, inflammatory skin disorders, and superficial lesions [6,7]. Yarrow (*Achillea millefolium* L.) contains flavonoids, tannins,

sesquiterpene lactones, and phenolic acids, which contribute to its well-documented wound-healing, anti-inflammatory, antimicrobial, and regenerative properties. In addition, yarrow has been reported to promote tissue repair and reduce local irritation, making it a valuable component of topical herbal preparations [8,9]. Hibiscus flowers (*Hibiscus sabdariffa* L.) are characterized by high levels of anthocyanins, organic acids, and polyphenolic compounds, particularly gallic acid, quinic acid, quercetin, rutin, and related flavonoids. These constituents exhibit strong free radical scavenging capacity and may contribute to protection against oxidative stress and inflammation [10,11]. Therefore, the rational combination of clove, yarrow, and hibiscus extracts may provide broader and potentially synergistic biological activity than each extract used individually, owing to the complementary actions of volatile oils, flavonoids, tannins, and phenolic compounds. The use of multicomponent phytocompositions is especially attractive because plant-derived constituents may interact synergistically. A rational combination of plant extracts may improve antimicrobial activity through the simultaneous action of volatile oils, phenolic acids, and flavonoids on different microbial targets, while also enhancing antioxidant capacity and reducing irritation. However, the biological efficacy of combined herbal systems strongly depends on the ratio of the individual components [12–16]. For this reason, the optimization of the proportion between clove, yarrow, and hibiscus is essential to obtain a phytocomposition with maximum biological activity and acceptable physicochemical properties. Preliminary microbiological observations indicated that the 6:1:3 ratio provided superior antimicrobial activity and comparable antifungal activity compared with other tested combinations, suggesting that this composition may represent the most

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promising balance between the active plant constituents [17–19].

In addition to phytochemical composition, the extraction method plays a crucial role in determining the quality and activity of the obtained extract. Conventional methods such as maceration and percolation are widely used for the extraction of biologically active substances from plant materials; however, these methods are generally time-consuming and may provide limited extraction efficiency. In contrast, high-speed extraction based on cavitation and intense shear forces significantly accelerates mass transfer processes, increases the contact surface between plant material and solvent, and facilitates the release of intracellular compounds. High-speed dispersion technologies are also advantageous for the extraction of thermolabile and volatile constituents because they allow efficient extraction within a short period of time and under relatively mild conditions [18–24].

The use of rotor–stator homogenizers and high-speed dispersers has recently emerged as a promising strategy for the intensification of pharmaceutical and phytochemical processes. These systems generate turbulence, cavitation, and strong hydrodynamic forces, leading to rapid disruption of plant cell walls and enhanced release of active compounds. Compared with conventional extraction methods, high-speed extraction can reduce processing time several-fold while simultaneously improving extract yield and preserving biologically active constituents [25–29]. Nevertheless, despite the growing interest in these technologies, there is still limited information regarding their application to multicomponent phytocompositions intended for topical pharmaceutical use.

Furthermore, although the antioxidant, antimicrobial, and anti-inflammatory

properties of individual extracts obtained from clove buds (*S.aromaticum* L.), yarrow (*A.millefolium* L.), and hibiscus flowers (*H.sabdariffa* L.) have been reported previously, their combined use in a rationally optimized ratio has not been sufficiently investigated. There is limited information regarding the simultaneous optimization of plant ratio and extraction conditions, together with comprehensive evaluation of antioxidant activity, antimicrobial efficacy, irritation potential, and cytotoxicity. Moreover, no previous study has been identified in which a high-speed extracted phytocomposition consisting of clove, yarrow, and hibiscus was optimized according to extraction parameters and component ratio, chemically characterized by GC/MS and LC/MS, and biologically evaluated using antimicrobial, antioxidant, HET-CAM, and MTT assays.

Therefore, the aim of the present study was to develop and optimize a multicomponent phytocomposition based on clove buds, yarrow, and hibiscus flowers, and to determine the most suitable extraction conditions for obtaining a biologically active extract. Different extraction methods, extraction times, solvent systems, and plant ratios were systematically compared to identify the optimal phytocomposition. The resulting extracts were subsequently characterized by modern chromatographic techniques and evaluated in terms of their antimicrobial, antioxidant, irritation, and cytotoxic properties. To the best of our knowledge, this is the first study to report the comprehensive development and biological evaluation of a high-speed extracted phytocomposition composed of *S.aromaticum*, *A.millefolium*, and *H.sabdariffa* in an optimized 6:1:3 ratio for potential application in topical pharmaceutical preparations [18, 32].

High-Speed Extraction of Phytoextract from Clove, Hibiscus, and Achillea millefolium and Development of Phytoextract



MATERIALS and METHODS

Materials

Dried flower buds of *S. aromaticum* L. (Myrtaceae), aerial parts of *A. millefolium* L. (Asteraceae), and flowers of *H. sabdariffa* L. (Malvaceae) were used as plant materials. The botanical materials were cleaned, dried under ambient conditions, and pulverized prior to extraction. Ethanol (95%, v/v),

propylene glycol, and distilled water were used as extraction solvents. All other chemicals and reagents were of analytical grade. Preliminary studies were performed using different extraction methods, solvent systems, extraction times, and plant ratios to determine the optimum phytocomposition. Based on microbiological and physicochemical screening, the most active

phytochemical composition consisted of *S.aromaticum*, *A.millefolium*, and *H.sabdariffa* in a ratio of 6:1:3[18].

Preparation of Phytoextracts

The volume of extractant required for the extraction procedure was calculated according to the following equation (Eq. 1.):

$$V_{extractant} = V + mK$$

Eq.1.

where (V) is the required volume of extract (mL), (m) is the amount of plant material (g), and (K) is the water absorption coefficient of the plant material.

The amount of dry residue in the obtained extracts was determined using the following equation (Eq. 2.):

$$Dry\ residue = \frac{m_2 - m_1}{V} \times 100$$

Eq.2.

where (m_1) is the weight of the empty container (g), (m_2) is the weight of the container after evaporation of the sample (g), and (V) is the volume of extract used for the analysis (mL).

Three different extraction methods were compared: maceration (12–48 h), percolation (8–24 h), and high-speed extraction (1–10 min). The powdered plant materials were mixed with an ethanol: propylene glycol solvent system (95:5, v/v) and subjected to high-speed extraction using a Stegler DG-360 high-speed rotor–stator disperser operated at 3200 rpm, 50 Hz, and 360 W. The extraction process was carried out at 26 ± 1 °C to minimize thermal degradation of volatile and thermolabile constituents [18].

Different extraction times (1, 3, 5, 7, and 10 min) and plant ratios were evaluated. The optimum conditions were determined as a 6:1:3 ratio of *S.aromaticum*: *A.millefolium*: *H.sabdariffa*, extracted for 7 min using the ethanol: propylene glycol (95:5, v/v) solvent system[18].

Determination of Alcohol Content

The alcohol content of the obtained phytoextract was determined by pycnometric analysis after distillation. Briefly, 25 mL of

liquid extract was transferred into a 250 mL distillation flask and diluted with distilled water to a total volume of 75 mL Porcelain boiling chips were added and the sample was distilled. Approximately 48 mL of distillate was collected in a 50 mL receiving flask cooled in an ice-water bath. The distillate was adjusted to 20 °C, and its density was measured using a pycnometer. The alcohol concentration of the extract was found to be 95%.

Stability Evaluation of the Phytoextract

The stability of the optimized phytoextract was evaluated by determining its organoleptic and physicochemical properties, including appearance, color, odor, pH, density, dry residue, and extractive matter content. Samples were filled into dark glass and plastic containers and stored at 12–15 °C under long-term conditions. The influence of packaging material on the stability of the extract was assessed periodically during the storage period.

Determination of Antioxidant Activity

The antioxidant activity of the optimized phytoextract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay according to the method of Brand-Williams et al. with slight modifications. Briefly, different concentrations of the phytoextract were prepared in methanol and mixed with freshly prepared DPPH solution. The reaction mixtures were incubated in the dark at room temperature for 30 min, and the absorbance was measured at 517 nm using a UV–Vis spectrophotometer. Ascorbic acid was used as the reference antioxidant. The radical scavenging activity was calculated as the percentage inhibition of DPPH radicals, and the IC_{50} value (concentration required to inhibit 50% of DPPH radicals) was obtained from the concentration–response curve. All experiments were performed in triplicate, and the results are presented as mean $\pm$ SD.[30–33, 43–45].

The radical scavenging activity was calculated according to the following equation (Eq. 3.):

$$DPPH \text{ inhibition (\%)} = \frac{A_0 - A_s}{A_0} \times 100$$

Eq. 3.

where (A_0) is the absorbance of the control and (A_s) is the absorbance of the sample.

Microbiological Evaluation

The antimicrobial activity of the phytoextracts was investigated in the Department of Medical Immunology and Microbiology using both agar disk diffusion and serial dilution methods. The following test microorganisms were used: *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*.

For the disk diffusion assay, sterile paper disks were impregnated with the extracts and placed on inoculated agar plates. Following incubation, the diameters of the inhibition zones were measured and expressed in millimeters [32].

The minimum inhibitory concentration was further evaluated by serial dilution. Briefly, four serial dilutions of each extract (1:100, 1:200, 1:400, and 1:800) were prepared in sterile distilled water. A standardized microbial suspension containing approximately 5×10^8 CFU/mL was added to each tube. After exposure for 10, 20, 40, and 60 min, aliquots were inoculated onto agar media. Fungal cultures were incubated at 28 °C for 48 h, while bacterial cultures were incubated at 37 °C for 24 h. Among the investigated formulations, the composition containing *S.aromaticum*, *A.millefolium*, and *H.sabdariffa* showed the highest antimicrobial activity against both Gram-positive and Gram-negative microorganisms as well as *C.albicans* [17,32,42]:

HET-CAM Assay

The anti-inflammatory and irritation potential of the optimized extract was evaluated using the Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM). Fertilized chicken eggs were incubated for 9 days at 37.8 °C and

60% relative humidity. On day 9, the eggshell was carefully opened at the blunt end, and the inner membrane was removed to expose the chorioallantoic membrane [33, 34].

Aliquots of the test samples were applied directly onto the membrane surface. The occurrence of vascular hemorrhage, coagulation, and lysis was visually monitored for 5 min. Sodium lauryl sulfate was used as the positive control and physiological saline as the negative control.

The irritation score (IS) was calculated according to the Luepke method (Eq. 4.) [34]:

$$IS = \frac{(301 - H) \times (301 - L) \times 7 + (301 - C) \times 9}{300}$$

Eq. 4.

where (H), (L), and (C) represent the times (s) required for the appearance of hemorrhage, vascular lysis, and coagulation, respectively.

Cytotoxicity Analysis

The cytotoxicity of the optimized phytoextract was evaluated by the MTT assay. Cells were seeded into 96-well plates and incubated under standard culture conditions. After reaching appropriate confluence, the cells were treated with different concentrations of the extract samples for 24 h. Following incubation, MTT solution was added to each well and the plates were incubated for an additional 3–4 h. The resulting formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance was measured at 570 nm using a microplate reader [32,35].

Cell viability was calculated as follows (Eq. 5.):

$$Cell \text{ viability (\%)} = \frac{A_s}{A_c} \times 100$$

Eq. 5.

where (A_s) is the absorbance of the treated cells and (A_c) is the absorbance of the untreated control cells. The cytotoxicity results were interpreted together with the antimicrobial and HET-CAM findings to determine the suitability of the Composition II

for potential topical pharmaceutical applications.

Results and Discussion

Selection of the Optimal Phytocomposition and Synergistic Effect

Three phytocompositions composed of clove buds (*S.aromaticum*), yarrow herb (*A.millefolium*), and hibiscus flowers (*H.sabdariffa*) were prepared in different ratios to identify the most suitable composition for the development of a stable and biologically active topical formulation. The compositions differed markedly in both antimicrobial performance and physicochemical behavior. Composition I contained the plant materials at a ratio of 3:6:1, Composition II at 6:1:3, and Composition III at 1:3:6 [17, 36-40, 42].

The obtained results demonstrated that the biological activity of the compositions was strongly dependent on the relative amount of clove extract. Composition II, which contained the highest proportion of clove together with a moderate amount of hibiscus, exhibited the most pronounced antimicrobial and antioxidant activity. This finding may be attributed to the high content of eugenol and other phenolic derivatives originating from clove. Eugenol is known to disrupt bacterial cell membranes, increase membrane permeability, denature intracellular proteins, and induce leakage of cytoplasmic contents. In addition, the presence of hibiscus extract likely enhanced the overall activity due to its anthocyanin and organic acid content, while

yarrow contributed flavonoids and sesquiterpenes with additional anti-inflammatory and regenerative properties. Composition I demonstrated intermediate activity. Although the high proportion of yarrow improved the anti-inflammatory potential of the mixture, the lower amount of clove reduced the overall antibacterial effect. Furthermore, the lower concentration of hibiscus resulted in a less intense antioxidant activity. The composition remained physically acceptable during short-term storage; however, slight precipitation and gradual loss of color intensity were observed after prolonged storage. Composition III exhibited the least favorable profile. The low concentration of clove markedly reduced the antibacterial activity, whereas the high hibiscus content caused progressive precipitation, increased acidity, and intense color instability. The predominance of hydrophilic hibiscus constituents probably decreased the compatibility of the extract with the solvent system and reduced the stability of the final product [32, 36-40,42].

As summarized in **Table 1**, the tested phytocompositions exhibited distinct physicochemical and biological profiles depending on the relative proportions of *S.aromaticum*, *A.millefolium*, and *H.sabdariffa*. Among them, Composition II demonstrated the most favorable balance between antimicrobial activity, antioxidant potential, physicochemical stability, and overall suitability for further formulation development.

Table 1. Comparative physicochemical and biological evaluation of the tested phytocompositions

Parameter	Composition I (3:6:1)	Composition II (6:1:3)	Composition III (1:3:6)
Main dominant constituents	Flavonoids, sesquiterpenes	Eugenol, anthocyanins, flavonoids	Anthocyanins, organic acids
Antimicrobial activity	Moderate	Highest	Weak
Antioxidant activity	Moderate	High	Moderate–high

Physicochemical stability	Acceptable	Excellent	Poor
Precipitation tendency	Slight	None	Marked
Color stability	Moderate	Stable	Unstable
Overall suitability	Acceptable	Optimal	Unsuitable

The superior behavior of Composition II suggests that the simultaneous presence of eugenol-rich clove extract, anthocyanin-containing hibiscus, and anti-inflammatory yarrow creates a synergistic phytochemical system. The combination appears to provide

a balance between antimicrobial potency, antioxidant protection, and physicochemical stability. Therefore, Composition II was selected for all subsequent experiments (**Table 2**).

Table 2. Comparative evaluation of different plant ratios used for preparation of the phytoextract.

Ratio (Syzygium : Achillea : Hibiscus)	Appearance	Stability	Antimicrobial activity	Final assessment
3:6:1	Slight turbidity	Moderate	Lower	Not selected
6:1:3	Homogeneous, no sediment	Stable	Highest	Selected
1:3:6	Darkening / sedimentation	Poor	Lower	Not selected

The superior behavior of Composition II provides empirical validation of a true synergistic effect achieved by modulating the multi-component plant ratios. The baseline methodology and raw microbiological dynamics of this system have been fully validated and published in our previous study [18, 32, 42]. Our standardized serial dilution screening, referenced strictly to 0.5 McFarland (1.5×10^8 CFU/mL) for bacterial strains and 2.0 McFarland (6×10^8 CFU/mL) for *C. albicans*, revealed that the biological response is strictly dependent on the architectural alignment of the matrices. While Compositions I (3:6:1) and III (1:3:6) exhibited a standard baseline Minimum Inhibitory Concentration (MIC) of 0.5 mL against *St. aureus* and *E. coli*, Composition II (6:1:3) demonstrated a notable 2-fold increase in antibacterial potency, dropping the MIC threshold to just 0.25 mL.

This strict formulation synergy is further manifested in the significant enlargement of the zones of inhibition; for instance, the inhibition zone against encapsulated *Klebsiella pneumoniae* increased by over 50%, jumping from 14 ± 0.9 mm (standalone clove extract) to 22 ± 1.28 mm within the composite system. Crucially, this synergistic performance has been formally certified via an Official Act of Laboratory Evaluation/Implementation.

Mechanistically, this synergistic interaction is hypothetically attributed to multi-target biochemical pathways described in ethnopharmacological literature. It is proposed that the phenolics within *S. aromaticum*, particularly eugenol, disrupt bacterial outer cell wall integrity, induce intracellular lipid leakage, and alter cell membrane permeability [38, 39, 40].

This structural destabilization operates cooperatively, thereby facilitating the

accelerated intracellular influx and localized efficacy of the co-delivered *H.sabdariffa* organic acids (such as hibiscus and citric acids) and anthocyanins, which effectively lower internal pH [11, 36, 37], alongside *A.millefolium* flavonoids that inhibit essential bacterial enzyme synthesis [8, 9]. Consequently, this literature-supported molecular cooperation firmly positions Composition II as the optimal certified matrix for subsequent formulation development.

Antimicrobial Activity and MIC Determination

The antimicrobial activity of the prepared phytocompositions was evaluated against *St. aureus*, *E.coli*, and *C.albicans*, representing Gram-positive bacteria, Gram-negative bacteria, and fungi, respectively. The tested microorganisms were selected because they are commonly associated with infected skin lesions and impaired wound healing. The obtained results clearly demonstrated that

Composition II possessed the strongest antibacterial activity among all investigated samples. Against *S. aureus*, the MIC value of Composition II was 0.25 mL, whereas Compositions I and III required 0.50 mL to inhibit microbial growth. A similar trend was observed for *E. coli*. The lower MIC value of Composition II indicates a more potent antibacterial effect and suggests that the higher content of clove-derived phenolic compounds was sufficient to overcome both the thick peptidoglycan layer of Gram-positive bacteria and the more resistant outer membrane of Gram-negative bacteria.

The MIC values presented in **Table 3** clearly indicate that Composition II exhibited the strongest antimicrobial activity against both *St.aureus* and *E.coli*, whereas all tested phytocompositions showed comparable antifungal activity against *C.albicans* [17-18, 32-33, 42].

Table 3. Minimum inhibitory concentration values of the tested phytocompositions

Sample	<i>S. aureus</i> (mL)	<i>E. coli</i> (mL)	<i>C. albicans</i> (mL)
Composition I	0.50	0.50	0.50
Composition II	0.25	0.25	0.50
Composition III	0.50	0.50	0.50

The difference between the antibacterial activities against *S. aureus* and *E. coli* was relatively small, which indicates that the Composition II exerted a broad-spectrum antimicrobial effect. This broad activity may arise from the coexistence of several phytochemicals acting through different mechanisms. Eugenol is known to penetrate and disrupt lipid membranes, while hibiscus acids lower the local pH and thereby create an unfavorable environment for bacterial survival. Yarrow-derived flavonoids may further contribute by interfering with bacterial enzyme systems.

The antifungal activity against *C. albicans* was lower and more comparable among all compositions. Complete inhibition was

achieved at 0.50 mL for all samples. This finding suggests that the phytochemical composition mainly influenced the antibacterial rather than antifungal effect. Nevertheless, the observed antifungal activity remains important because *Candida* colonization is frequently associated with chronic skin lesions.

Overall, these findings indicate that Composition II was the most promising phytocomposition owing to its superior broad-spectrum antimicrobial activity. The optimized ratio of *S.aromaticum*, *A.millefolium*, and *H.sabdariffa* appeared to provide the most favorable balance between membrane-disrupting volatile compounds and biologically active phenolic constituents.

Therefore, Composition II was selected as the optimized extract for subsequent extraction optimization and stability studies [18, 32].

Optimization of the Extraction Process

The extraction process was systematically optimized to maximize the recovery of biologically active compounds while maintaining good physicochemical stability. The influence of extraction method, extraction time, plant-to-solvent ratio, temperature, extractant composition, and rotation speed was investigated [18, 43].

Effect of Extraction Method

Among the investigated techniques, high-speed extraction provided the highest dry residue value ($2.56 \pm 0.01\%$), followed by

maceration and percolation (**Table 4**). The superiority of high-speed extraction may be explained by the rapid mechanical destruction of plant tissues, which increases the contact area between the plant material and solvent. Intensive mixing likely enhanced the diffusion of eugenol, flavonoids, anthocyanins, and other phenolic constituents into the extractant. In addition, the relatively short duration of this method minimized the degradation of volatile and thermolabile compounds. By contrast, percolation and maceration required a longer extraction period and therefore probably resulted in greater losses of volatile compounds [18].

Table 4. *Effect of extraction method on dry residue content*

Method	Dry residue (%)
Percolation	2.01 ± 0.03
Maceration	2.13 ± 0.02
High-speed extraction	2.56 ± 0.01

Effect of Extraction Time

The amount of dry residue increased gradually with increasing extraction time and reached a maximum value after 7 min (**Table 5**). The sharp increase between 1 and 7 min indicates that most extractable constituents were released during the early stages of extraction. The subsequent decrease observed after 10 min most likely reflects the evaporation of essential oil components and oxidative degradation of phenolic compounds. Since eugenol and several anthocyanins are sensitive to prolonged exposure to oxygen and mechanical stress, excessive extraction time may reduce the quality of the extract despite a similar total dry residue. The extraction efficiency increased progressively with increasing processing time and reached its maximum at 7 min. This finding indicates that high-speed extraction

provided sufficient disruption of plant tissues and promoted rapid release of bioactive constituents within a relatively short period. However, extending the extraction time beyond 7 min resulted in a slight decrease in dry residue content. This reduction may be associated with the degradation or volatilization of thermolabile and volatile compounds, particularly eugenol and other aromatic constituents present in *S.aromaticum*. Prolonged processing may also promote oxidation of polyphenolic compounds and partial destruction of sensitive phytochemicals due to increased shear stress and cavitation intensity. Therefore, 7 min was considered the optimal extraction time, providing the best balance between extraction efficiency and preservation of biologically active compounds [18, 42-44].

Table 5. *Effect of extraction time on dry residue content*

Time (min)	Dry residue (%)
1	1.06 ± 0.00
3	2.21 ± 0.00
5	2.43 ± 0.00
7	2.58 ± 0.00
10	2.47 ± 0.00

Effect of Raw Material-to-Extractant Ratio

Although the 1:5 ratio produced the highest dry residue, the resulting extract was excessively viscous, difficult to filter, and contained a substantial amount of ballast material. In contrast, the 1:10 ratio provided a more homogeneous extract with lower

viscosity and superior physical stability. The lower number of insoluble substances obtained at 1:10 also facilitated subsequent incorporation into the final semisolid dosage form. Therefore, the ratio of 1:10 was considered optimal (**Table 6**).

Table 6. *Effect of raw material-to-extractant ratio on dry residue content*

Ratio	Dry residue (%)
1:5	3.16 ± 0.02
1:10	2.57 ± 0.03
1:15	2.25 ± 0.02
1:20	1.52 ± 0.01

Effect of Extraction Temperature

The highest extraction efficiency was obtained at 26 °C (**Table 7**). Temperatures above this value led to a gradual decrease in dry residue. This reduction probably resulted from the instability of thermolabile compounds such as anthocyanins and volatile terpenes.

Elevated temperatures may also accelerate oxidation reactions and evaporation of clove essential oil constituents. Consequently, the use of mild temperatures is advantageous for preserving the biological activity of the extract [18].

Table 7. *Effect of temperature on dry residue content*

Temperature (°C)	Dry residue (%)
22	2.41 ± 0.04
24	2.52 ± 0.03
26	2.79 ± 0.02
30	2.56 ± 0.01
38	2.42 ± 0.01

Effect of Ethanol: Propylene Glycol Ratio

The influence of the solvent composition was one of the most critical parameters affecting the quality of the obtained phytoextract (**Table 8**). Although the highest dry residue was obtained with the 80:20 ethanol:propylene glycol system, this solvent mixture also extracted a larger amount of undesired ballast

substances, including sugars, mucilage, and other high-molecular-weight constituents. As a consequence, the resulting extract exhibited higher viscosity, lower transparency, more difficult filtration, and reduced long-term stability. Increasing the proportion of propylene glycol above 5% also appeared to impair the efficiency of high-speed extraction

because the higher viscosity of the medium limited solvent penetration and mass transfer within the plant particles. Similar observations have been reported for plant extraction systems in which excessive amounts of propylene glycol decreased extraction selectivity and complicated subsequent processing. By contrast, the 95:5 ethanol:propylene glycol mixture yielded a lower total dry residue but produced a cleaner and more selective extract enriched in the desired phenolic and volatile compounds. This solvent system provided improved extract clarity, lower viscosity, easier filtration, better microbiological stability, and a more

reproducible composition. The lower proportion of propylene glycol was still sufficient to enhance the solubility of semi-polar constituents, while the high ethanol content favored the extraction of eugenol-rich and flavonoid-rich fractions [18-19, 32, 42]. Therefore, the final selection of the 95:5 ethanol:propylene glycol ratio was based not only on the total extraction yield but also on the superior qualitative composition and stability of the resulting extract. This finding indicates that the highest dry residue does not necessarily correspond to the most suitable phytochemical composition for further pharmaceutical development.

Table 8. *Effect of ethanol: propylene glycol ratio on dry residue content*

Ratio	Dry residue (%)
80:20	3.73 ± 0.03
90:10	3.46 ± 0.02
95:5	2.56 ± 0.04
60:40	2.48 ± 0.05
50:50	2.13 ± 0.02
40:60	1.68 ± 0.04
30:70	1.04 ± 0.04

Effect of Rotation Speed

Among the investigated rotation speeds, 3200 rpm provided the highest extraction efficiency and yielded the most suitable physicochemical characteristics of the phytoextract (**Table 9**). At lower rotation speeds, the mechanical force was insufficient to achieve complete disruption of the plant material, resulting in lower extraction efficiency. In contrast, higher rotation speeds negatively affected the extract quality, most

likely because of excessive aeration, foaming, and possible degradation of thermolabile constituents. Therefore, 3200 rpm was selected as the optimum rotation speed for further studies. Consequently, the final optimized extraction conditions consisted of high-speed extraction for 7 min at 26 °C using a 95:5 ethanol: propylene glycol mixture, a raw material-to-solvent ratio of 1:10, and a rotation speed of 3200 rpm [18].

Table 9. *Effect of rotation speed on dry residue content*

Rotation speed (rpm)	Dry residue (%)
2800	2.37 ± 0.04
3000	2.43 ± 0.02
3200	2.56 ± 0.04
3400	2.18 ± 0.01
3600	1.65 ± 0.05

Elemental Composition of the Optimized Extract

The macro- and microelement composition of the optimized extract (Composition II) was evaluated to assess both its potential biological benefits and its safety (**Table 10**). The relatively high calcium content may contribute to keratinocyte proliferation and epidermal regeneration, whereas magnesium and potassium are involved in cellular metabolism and osmotic balance. Copper is

of particular interest because it is closely associated with collagen synthesis and wound healing. Therefore, the elemental profile of the extract may additionally support its biological activity. Importantly, the heavy metal contents remained well below accepted pharmacopoeia and toxicological limits. This result confirms that the developed extract is suitable for topical use and that the selected raw plant materials were of adequate quality [18, 31-33, 42].

Table 10. *Elemental composition of the optimized phytoextract*

Element	Content
Ca (%)	0.23
P (%)	0.05
Mg (%)	0.0201
K (%)	0.02
Fe (%)	0.0012
Cu (mg/kg)	5.34
Ni (mg/kg)	1.62
Co (mg/kg)	0.22
Cr (mg/kg)	0.41
Sr (mg/kg)	11.4
Pb (mg/kg)	0.11
As (mg/kg)	0.07
Cd (mg/kg)	0.011
Hg (mg/kg)	0.003

Stability Study

The long-term stability study demonstrated that the packaging material had a pronounced effect on the quality of the optimized phytoextract. When stored in dark glass containers, the extract retained its original appearance, characteristic odor, pH, and phytochemical composition throughout the storage period. Only minor reductions in dry residue, extractive substances, rutin, and eugenol content were observed after 24 months, indicating gradual but limited degradation of the bioactive constituents (**Table 11**). In contrast, samples stored in

plastic containers underwent substantially greater changes during storage. Progressive darkening, marked reduction in odor intensity, lower pH values, and significant decreases in dry residue, extractives, rutin, and eugenol content were observed. These findings suggest that the permeability of plastic packaging to oxygen and volatile compounds accelerated the oxidation and loss of phenolic and aromatic constituents (**Table 12**).

Therefore, storage in dark glass containers at 12–15 °C should be recommended for the final phytoextract formulation.

Table 11. *Stability of the optimized phytoextract during storage in dark glass containers*

Storage period	Color	Odor	Dry residue (%)	Extractives (%)	Rutin (mg/mL)	Eugenol (%)	pH
Initial	Cherry red	Characteristic aromatic odor	2.56	32.5	26.90	95	4.5
6 months	Cherry red	No significant change	2.50	31.8	25.9	93	4.4
12 months	Slightly lighter red	Slight decrease	2.44	31.0	24.8	91	4.2
24 months	Slight fading	Slight decrease	2.30	29.8	20.82	89	3.9–5.1

Table 12. *Stability of the optimized phytoextract during storage in plastic containers*

Storage period	Color	Odor	Dry residue (%)	Extractives (%)	Rutin (mg/mL)	Eugenol (%)	pH
Initial	Cherry red	Characteristic aromatic odor	2.56	32.5	26.90	95	4.5
6 months	Dark red	Noticeable decrease	2.28	26.4	18.7	84	4.0
12 months	Brownish red	Markedly reduced	2.12	22.1	14.5	78	3.7
24 months	Brown	Marked decrease	2.00	19.3	10.0	73	3.5

Antioxidant Activity

The antioxidant activity of the optimized extract (Composition II) was assessed by the DPPH method (**Table 13**). The extract exhibited strong concentration-dependent free radical scavenging activity. The low IC₅₀ value indicates that only a relatively small amount of extract was required to neutralize 50% of the DPPH radicals. This pronounced activity is likely related to the high content of

eugenol, flavonoids, and anthocyanins present in the optimized composition. The strong antioxidant capacity is particularly important for the intended topical application because oxidative stress is one of the major factors responsible for delayed wound healing and chronic inflammation. Therefore, the developed extract may contribute not only to microbial control but also to tissue regeneration[30,32].

Table 13. *Antioxidant activity of the optimized extract determined by DPPH assay*

Parameter	Value
IC ₅₀ (mg/mL)	1.58
R ² (Coefficient of Determination)	0.998

3.7. HET-CAM Assay and Irritation Potential

The irritation potential of the optimized phytoextract (Composition II) was evaluated using the HET-CAM assay, and the obtained

irritation scores are presented in **Table 14**. As expected, 0.9% NaCl produced no irritation, whereas sodium lauryl sulfate (SLS) caused a severe irritant response with an irritation index of 18.0 ± 1.15 . In contrast, Composition

II exhibited a markedly lower irritation index (2.45 ± 0.35) and was therefore classified as non-irritant [59]. The low irritation potential may be attributed to the balanced phytochemical composition of the extract. Although the formulation contains phenolic compounds with strong antimicrobial activity, the presence of yarrow- and hibiscus-derived constituents may contribute to reducing local

irritation. The observed result is particularly important because the intended application involves damaged or sensitive skin. Taken together with its pronounced antimicrobial and antioxidant properties, the very low irritation potential indicates that the optimized phytoextract is suitable for further development as a topical pharmaceutical or cosmetic ingredient.

Table 14. HET-CAM irritation scores of the optimized extract.

Group	Irritation index	Classification
0.9% NaCl	0.00 ± 0.00	Non-irritant
SLS	18.0 ± 1.15	Severe irritant
Composition II	2.45 ± 0.35	Non-irritant

Overall, the study demonstrated that the biological and physicochemical properties of the investigated phytocompositions depended strongly on the ratio of the three plant extracts and on the selected extraction conditions. The optimized 6:1:3 composition provided the most favorable combination of antimicrobial efficacy, antioxidant activity, physicochemical stability, and safety. The results further showed that obtaining the highest extraction yield is not necessarily equivalent to obtaining the highest quality extract. Several conditions, such as the 80:20 solvent system and the 1:5 plant-to-solvent ratio, produced higher dry residue values but also extracted undesirable ballast substances and reduced the stability of the final product. Therefore, the selected extraction parameters represent a compromise between quantitative yield and qualitative composition. The final optimized extract (Composition II) remained stable for 24 months in dark glass packaging, demonstrated significant antioxidant activity, exhibited broad-spectrum antimicrobial activity, and showed no irritation in the HET-CAM assay. These findings support the potential use of the developed phytocomposition as a safe and effective topical ingredient for pharmaceutical or cosmetic applications.

CONCLUSION

In the present study, a multifunctional phytoextract based on *Syzygium aromaticum*, *Achillea millefolium*, and *Hibiscus sabdariffa* was successfully developed and optimized for potential topical pharmaceutical and cosmetic applications. Among the investigated phytocompositions, the 6:1:3 ratio exhibited the most favorable combination of physicochemical properties, antimicrobial activity, antioxidant potential, and tolerability. The extraction procedure was successfully optimized using high-speed extraction, and the most suitable conditions were identified as 7 min extraction time, 26 °C, a 95:5 ethanol: propylene glycol solvent system, a raw material-to-solvent ratio of 1:10, and a rotation speed of 3200 rpm. Under these conditions, the optimized extract (Composition II) demonstrated strong inhibitory activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*, together with pronounced antioxidant activity.

Long-term stability studies further demonstrated that the Composition II retained its quality for up to 24 months when stored in dark glass containers, whereas marked deterioration occurred in plastic packaging. In addition, the extract was found to be non-irritant in the HET-CAM assay. Overall, the

results obtained indicate that the optimized phytoextract is a promising natural active ingredient for the preparation of stable, effective, and well-tolerated topical formulations (ointment, cream, nanogel with Ag/Au nanoparticles) possessing high prospective potential for the management of small skin lesions, inflammatory dermatoses, and microbially contaminated skin conditions.

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YÜKSƏKSÜRƏTLİ EKSTRAKSİYA ÜSULU İLƏ SYZYGIUM AROMATICUM L., ACHILLEA MILLEFOLIUM L. VƏ HIBISCUS SABDARİFFA L. BİTKİLƏRİNDƏN SİNƏRGİSTİK FİTOEKSTRAKTIN İŞLƏNİB HAZIRLANMASI, STANDARTLAŞDIRILMASI VƏ BİOLOJİ EFFEKTİVLİYİNİN TƏDQIQI

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Xülasə

Bu tədqiqatın məqsədi potensial topikal əczaçılıq və kosmetik tətbiqlər üçün mixək (*Syzygium aromaticum*), boymadərən (*Achillea millefolium*) və hibiskusdan (*Hibiscus sabdariffa*) ibarət çoxfunksiyalı fitoekstrakt hazırlamaq və optimallaşdırmaqdır. Üç fərqli fitokompozisiya hazırlanmış və antimikrob aktivliyi, antioksidant potensialı, fiziki-kimyəvi xüsusiyyətləri, təhlükəsizliyi və uzunmüddətli stabilliyi baxımından müqayisəli şəkildə qiymətləndirilmişdir. Tədqiqat sistemləri arasında mixək, boymadərən və hibiskusdan ibarət tərkib 6:1:3 nisbətində ən əlverişli profil nümayiş etdirmiş və buna görə də optimallaşdırılmış formulasiya kimi seçilmişdir. Ekstraksiya prosesi ekstraksiya metodunun, ekstraksiya müddətinin, bitki-həlləddici nisbətinin, temperaturun, həlləddici tərkibinin və fırlanma sürətinin təsirini araşdırmaqla sisteməlik şəkildə optimallaşdırılmışdır. 95:5 etanol: propilen qlitol qarışığı, xammal-həlləddici nisbəti 1:10 və fırlanma sürəti 3200 dövr/dəq istifadə edilərək 26 °C-də 7 dəqiqə ərzində yüksək sürətli ekstraksiya keyfiyyət, antimikrob aktivliyi və uzunmüddətli stabillik baxımından ən uyğun ekstraktı verdi. Optimallaşdırılmış fitokompozisiya, minimum inhibitor konsentrasiyası 0,25 ml olmaqla *Staphylococcus aureus* və *Escherichia coli*-yə qarşı açıq antimikrob aktivliyi nümayiş

etdirdi, *Candida albicans*-ın tam inhibisiyonu isə 0,50 ml-də əldə edildi. Ekstrakt həmçinin DPPH analizində 1,58 mq/ml IC50 dəyəri ilə güclü antioksidan aktivlik nümayiş etdirdi. Uzunmüddətli stabillik tədqiqatları göstərdi ki, optimallaşdırılmış ekstrakt tünd şüşə qablaşdırmada saxlanıldıqda 24 aya qədər sabit qalıb. Rutin və evgenol tərkibində, quru qalıqda və pH-da yalnız kiçik dəyişikliklər müşahidə edildi, plastik qablarda saxlama isə nəzərəcarpacaq dərəcədə fiziki-kimyəvi pisləşməyə səbəb oldu. Optimallaşdırılmış ekstraktın HET-CAM analizində qıcıqlanmayan olduğu və qıcıqlanma indeksinin $2,45 \pm 0,35$ olduğu aşkar edilmişdir. Ümumilikdə, hazırlanmış fitokompozisiya güclü antimikrob və antioksidant aktivliyi yaxşı stabillik və əla tolerantlıqla birləşdirmişdir. Bu tapıntılar optimallaşdırılmış fitoekstraksın topikal əczaçılıq və kosmetik formulaların hazırlanması üçün perspektivli aktiv tərkib hissəsi kimi xidmət edə biləcəyini göstərir.

Açar sözlər: *Syzygium aromaticum*, *Achillea millefolium*, *Hibiscus sabdariffa*, yüksək sürətli ekstraksiya, antimikrob aktivlik, antioksidan aktivlik, HET-CAM, topikal fitokompozisiya

РАЗРАБОТКА, СТАНДАРТИЗАЦИЯ И ИССЛЕДОВАНИЕ БИОЛОГИЧЕСКОЙ ЭФФЕКТИВНОСТИ СИНЕРГЕТИЧЕСКОГО ФИТОЭКСТРАКТА *SYZYGIUM AROMATICUM* L., *ACHILLEA MILLEFOLIUM* L. И *HIBISCUS SABDARIFFA* L. МЕТОДОМ ВЫСОКОСКОРОСТНОЙ ЭКСТРАКЦИИ

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Резюме

Целью настоящего исследования было разработать и оптимизировать многофункциональный фитоэкстракт, состоящий из гвоздики (*Syzygium aromaticum*), тысячелистника (*Achillea millefolium*) и гибискуса (*Hibiscus sabdariffa*), для потенциального применения в фармацевтике и косметике. Были приготовлены три различных фитокомпозиции, которые были сравнительно оценены с точки зрения антимикробной активности, антиоксидантного потенциала, физико-химических свойств, безопасности и долговременной стабильности. Среди исследованных систем композиция, содержащая гвоздику, тысячелистник и гибискус в соотношении 6:1:3, показала наиболее благоприятный профиль и поэтому была выбрана в качестве оптимизированной рецептуры. Процесс экстракции был систематически оптимизирован путем исследования влияния метода экстракции, времени экстракции, соотношения растения и растворителя, температуры, состава растворителя и скорости вращения. Высокоскоростная экстракция, проведенная в течение 7 минут при 26 °C с использованием смеси этанола и пропиленгликоля в соотношении 95:5, соотношения сырья к растворителю 1:10 и скорости вращения 3200 об/мин, позволила получить наиболее подходящий экстракт с точки зрения качества, антимикробной активности и долговременной стабильности. Оптимизированный

фитокомполит продемонстрировал выраженную антимикробную активность против *Staphylococcus aureus* и *Escherichia coli* с минимальной ингибирующей концентрацией 0,25 мл, в то время как полное ингибирование *Candida albicans* было достигнуто при 0,50 мл. Экстракт также проявил сильную антиоксидантную активность в тесте DPPH со значением IC50 1,58 мг/мл. Исследования долговременной стабильности показали, что оптимизированный экстракт оставался стабильным до 24 месяцев при хранении в темной стеклянной упаковке. Наблюдались лишь незначительные изменения содержания рутина и эвгенола, сухого остатка и pH, тогда как хранение в пластиковых контейнерах привело к заметному ухудшению физико-химических свойств. Оптимизированный экстракт оказался не вызывающим раздражения в тесте HET-CAM, с индексом раздражения $2,45 \pm 0,35$. В целом, разработанная фитокомпозиция сочетает в себе сильную антимикробную и антиоксидантную активность с хорошей стабильностью и отличной переносимостью. Эти результаты показывают, что оптимизированный фитоэкстракт может служить перспективным активным ингредиентом для разработки лекарственных препаратов для местного применения и косметических средств.

Ключевые слова: *Syzygium aromaticum*, *Achillea millefolium*, *Hibiscus sabdariffa*, высокоскоростная экстракция, антимикробная активность, антиоксидантная активность, HET-CAM, фитокомпозиция для местного применения