RESEARCH ARTICLE

Polyherbal Formulation for Atherosclerosis: GC-MS Fingerprinting, *In-vivo* Antioxidant and Antibacterial Activities

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ABSTRACT

Atherosclerosis is a prolonged inflammatory disease affecting large and medium-sized arteries, requiring a phytotherapeutic approach that incorporates multiple bioactive elements and a multi-target strategy. A novel polyherbal formulation has been formulated to treat atherosclerosis. The methanolic extract of the formulation has been characterized using gas chromatography–mass spectrometry (GC-MS) fingerprinting analysis. The extract was further screened for antioxidant activity like DPPH radical scavenging assay, hydrogen peroxide scavenging activity, intracellular reactive oxygen species (ROS) activity and antibacterial activity like Resauzarin assay as most of the identified compounds are with antioxidant and antimicrobial properties. Upto 38 phytocompounds were tentatively identified by GC-MS characterization using NIST library. Scavenging activities were observed more in the DPPH assay, ranging from 33.33 to 86.68% than in the hydrogen peroxide scavenging assay which ranged from 31.05 to 81.66%. In the resauzarin assay, the plant extract displays substantial antibacterial activity compared to the positive control chloramphenicol. The DPPH and hydrogen peroxide scavenging activity results indicate that the extract has significant radical-scavenging properties compared to the positive control ascorbic acid. Based on MIC values in antibacterial assay, the extract exhibits potential antibacterial activity, stating its stability towards bacterial growth.

Keywords: Polyherbal formulation, GC-MS fingerprinting, Antioxidant assay, Intracellular ROS assay, Resauzarin assay.

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INTRODUCTION

The phrase "cardiovascular disease", sometimes shortened as CVD, encompasses a collection of medical diseases such as ischemic heart disease, strokes, and peripheral vascular disease. Atherosclerosis is a chronic inflammatory disease that affects big and medium-sized arteries.[1] Plaques caused by atherosclerosis have the potential to become unstable, burst, or erode, all of which may result in a significant adverse cardiovascular event.[2] There are a variety of allopathic hypolipidemic medications available on the market, which has serious side effects.[3] Herbal pharmaceuticals, also referred to as herbal medicines (HMs), has a rich historical background in their use as natural remedies for disease prevention because of their synergistic effects on the physiological systems.[4] According to the World Health Organization (WHO), traditional medicine is projected to maintain a significant role in the healthcare system of third-world nations since over 80% of their population relies on its use.[5] The use of fingerprint analysis to evaluate the quality of herbal medicinal ingredients and formulations to develop standardized herbal products has been more popular in recent years.[6] Gas chromatography–mass spectroscopy (GC–MS) method is one of these approaches that is often used for characterization and is a very helpful method in the identification of different phytocompounds on several herbal samples.[7,8]

When pathogenic events occur, the formation of tissue damage is significantly contributed by the action of free radicals. Antioxidants are chemical substances that have the potential to snuff out free radicals; as a result, they protect the human body against a wide range of ailments.[9] Plants are excellent sources of antioxidants because they include secondary metabolites with redox and metal-chelating capabilities. Oxidative stress with an excess of reactive oxygen species (ROS) has a major impact on the development of cardiovascular diseases. Studies involving the ablation of antioxidant systems in ApoE-KO mice reveal increased levels of mitochondrial ROS and accelerated atherosclerosis, indicating a potential involvement of mitochondrial ROS in the process of atherogenesis.[10] The research has chosen to conduct DPPH assay, hydrogen peroxide scavenging assay and a ROS

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assay in human leukemia monocytic cell lines (THP-1) to know the usage of antioxidants which might potentially be a rational therapy option to halt the progression of the disease condition.

Assays known as minimum inhibitory concentrations, or MICs, are often used to determine the lowest concentration of a chemical that can prevent the development of certain bacterial strains. Microdilution assays are the standard method for determining a drug's antibacterial activity. This methodology enables the investigation of the inhibitory effect on strain development in relation to different concentrations of the tested medication. In particular, it facilitates the analysis of a strain's growth inhibition curve, often referred to as GIC. To optimize the formulation's efficacy in preservation and shelf life extension, the phytocompounds within the formulation that function as natural antibacterial agents will be the preferred choice.[11]

The present study works on the GC- MS characterization, antioxidant and antibacterial properties, and identifying the capability of novel polyherbal formulations fighting against free radicals and developing microbes.

MATERIALS AND METHODS

Preparation of Polyherbal Formulation

The formulation is made up of ten plants: *Garcinia cambogia, Rubia cordifolia, Commiphora mukul, Vitis vinifera, Helianthus annus, Acacia catechu, Linum usitatissimum, Allium sativum, Acacia catechu, Terminalia arjuna,* and *Piper nigrum*. All of the chosen parts of the plant are collected, dried in the shade, and ground into a powder using a mixer. Fine powder that went through sieve no. 80 was mixed in equal amounts to make the polyherbal formulation (PHF).

Preparation of Extract and Isolation by Column Chromatography

About 50 grams of PHF was immersed in 500 mL of methanol and sonicated in ultrasonicator[12] for 4 hours, filtered, and the extract was subjected to isolation using column chromatography. Solvents such as n-hexane, chloroform and methanol were used for elution. The percentage yield of n-hexane (12%) and chloroform (13.5%) extracts were less compared to methanolic extract (56%). The methanolic extract was further evaporated using a rotary evaporator and dried.[13]

GC- MS Fingerprinting Analysis

GC-MS instrument equipped with an HP-5MS (5 percent phenyl methyl siloxane) column of 30 m length, 0.25 mm diameter, and 0.25 m film thickness was utilized to analyze the methanolic extract of PHF. Column ovens were meant to rise 2°C each minute from 50 to 250°C. The sample components were ionized using electron impact ionization at 70 eV and the injector temperature was maintained at 220°C. The 40 to 700 m/z mass range was scanned at a rate of 0.5 scans/s using helium (99.996% purity) at a flow rate of 1-mL min^{-1}. Using a Hamilton syringe, 2.0 μL of extract was manually injected into the GC-MS for total ion chromatographic analysis as part of the split injection method and running it for 46 minutes to complete. The relative fraction of each extract constituent was given as a percentage after peak area normalization. The components of methanol extract were identified using NIST version-2011 sources mass spectrum patterns and retention indices.[14]

Antioxidant Assays

Assays such as the DPPH, hydrogen peroxide radical scavenging assays and intracellular ROS activity were carried out to assess the extract's antioxidant capacity to neutralize free radicals. In the investigations, ascorbic acid served as a positive control. For the purpose of calculating the antioxidant activity, the following formula was used:

$$\text{Radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100\%$$

DPPH radical scavenging assay

The extract's capacity to scavenge free radicals was assessed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), which was administered in a colorimetric way and given some slight alterations. In simple terms, 100 µL of the sample extracts (20–120 µg/mL) was added to 100 µL of the DPPH solution, which had a concentration of 0.1 mM. Using a microplate reader, the absorbance (A) of the reaction mixture was detected at a wavelength of 517 nm. After a 30-minute incubation period at room temperature, the capacity of the extract to scavenge free radicals was evaluated using the formula mentioned before.[15]

Hydrogen peroxide scavenging assay

According to the technique developed by Ruch *et al.,* with minor modifications, the capacity of the extract to scavenge hydrogen peroxide (H_2O_2) was also evaluated. An aliquot of 10 µL of extracts with a concentration of 20–120 µg/mL were used for the study. About 60 µL of H_2O_2 solution (2 mM) was added and finally, after 10 minutes of reaction time, the absorbance of the mixture resulting from the reaction was measured at 230 nm. Hydrogen peroxide by itself is not a very reactive substance, yet, it may occasionally be hazardous to cells because it has the potential to generate hydroxyl radicals inside the cells. As a result, getting rid of H_2O_2 is a critical step in the antioxidant defense process.[16]

Intracellular ROS activity

The fluorescent dye DCFH-DA method was selected to identify the intracellular reactive oxygen species (ROS) using THP-1 cells using an established method with a few minor modifications.[17] The cells were treated at the IC_{50} concentration (3.99 µg/mL) of the extract after being treated with hydrogen peroxide. The H_2O_2 treatment alone was used as a positive control for the experiment. In this method, we used BD FACS calibrator with an excitation wavelength at 488 nm and emission wavelength at 530 nm to measure the fluorescence intensity using the BD Cell Quest Pro software.

Antibacterial Activity by Resauzarin Assay

According to Elshikh *et al.,* (2016), the standard broth dilution technique with some alterations was selected to

screen the antibacterial activity of the extract.[18-20] According to a micro broth dilution method, the activity of the extract was determined against *Escherichia coli* (35218 ATCC), *Pseudomonas aeruginosa* (1214 PTCC), *Staphylococcus aureus* (1189 ATCC), *Bacillus cereus* (CI 2106), *E. coli* (35218 ATCC), and *Klebsiella pneumoniae* (27736 ATCC). Chloramphenicol was used as a positive control. Microbe growth is indicated by the color shift from purple to pink. The maximum dilution at which there was no color change was chosen as the extract's MIC value, which was reported in mill molar (mM) units.

Statistical Analysis

Every measurement was done a total of three times and the results are interpreted as mean ± standard deviation (SD). Graph Pad Prism 9.0 software (USA) was used to conduct a one-way analysis of variance using Dunnett's multiple comparison tests in order to ascertain the statistical significance. Variations in each mean value that meet the statistical significance threshold of $p \leq 0.05$.

RESULTS

GC MS Fingerprinting Analysis

A majority of the 38 possible phytocompounds have been identified using GC- MS analysis. The methanolic extract of PHF included a significant number of peaks with a high area percentage, indicating the presence of core compounds such as 3-hydroxy-2,3-dihydromaltol, hydroxy methyl furfural, p-hydroxybenzoic acid methyl ester, dodecyl acrylate, methyl 3-butyl-4-nitro-4-pentenoate, n-hexadecanoic acid, trans-13-octadecenoic acid methyl ester, 17-octadecynoic acid, octadecanoic acid, 4H-1,3-benzodioxin-4-one, 2-(1,1-dimethylethyl) hexa hydro- [2S-(2α,4aβ,8aβ)], 2-tert-butyl hexa hydro-4H-1,3-benzodioxin-4-one. In Table 1, a list of identified constituents was presented. In order to determine the identities of the compounds found in the extract, the mass spectra of those chemicals were compared to the information included in the version 2011 MS data database maintained by the NIST (NIMC Database- Spectrum MS-NW-1684) (Figure 1).

Table 1: Tentative identification of chemical constituents by GC-MS in the methanolic extract of PHF[22]

S. No.	*Retention time (Rt)*	*Compound name*	*S. No.*	*Retention time (Rt)*	*Compound name*
1	9.508	Neopentyl glycol	20	32.983	9,12-Octadecadienoic acid, methyl ester
2	9.768	2,2,4- Trimethyl- 4-penten-1-ol	21	33.050	trans-13-Octadecenoic acid, methyl ester
3	16.149	3-Hydroxy-2,3-dihydromaltol	22	33.235	(E)-Phytol
4	20.414	Hydroxy-methyl furfural	23	33.354	Stearic acid, methyl ester (Methyl Stearate)
5	21.394	1- cyclohexene	24	33.421	1,2-Cyclohexanedicarboxylic acid, di(2-methylbutyl) ester
6	23.801	1-Undecanol	25	33.778	17-Octadecynoic acid
7	24.856	p-Hydroxybenzoic acid, methyl ester	26	34.008	Octadecanoic acid
8	25.718	Dodecanoic acid	27	35.717	2,3-dihydroxypropyl ester, (Z,Z,Z))
9	26.721	5-Hydroxymethylfurfural	28	36.385	4H-1,3-Benzodioxin-4-one, 2-(1,1-dimethylethyl) hexa hydro- [2S-(2α,4aβ,8aβ)]
10	27.426	Dodecyl acrylate	29	36.623	2-tert-Butyl hexa hydro-4H-1,3-benzodioxin-4-one
11	27.894	Myristic acid, methyl ester	30	37.366	Ethyl 2,3-dipropyl cyclo propane carboxylate
12	28.793	Methyl 3-butyl-4-nitro-4-pentenoate	31	37.585	Ethyl stearate, 9,12-diepoxy
13	29.179	1.4-Isopropyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-imidazol-1-ol 3-oxide	32	37.782	2-Methylhexadecan- 1-ol
14	30.205	(10E)-10-Henicosene	33	38.636	Phthalic acid, di(2-propylpentyl)ester
15	30.479	Caffeine	34	39.008	Ethyl 9,10,12,13-diepoxyoctadeanoate
16	30.769	Palmitic acid, methyl ester (Hexadecanoic acid, methyl ester)	35	39.223	1,4-Dimethyl-3-n-octadecylcyclohexane
17	31.245	1,1-Bis(dodecyloxy)hexadecane	36	41.259	Erythro-9,10-dihydroxyoctadecanoic acid
18	31.549	n-Hexadecanoic acid	37	41.608	Azelaaldehydic acid, butyl ester
19	32.871	Hexadecyl trichloroacetate	38	42.313	Squalene

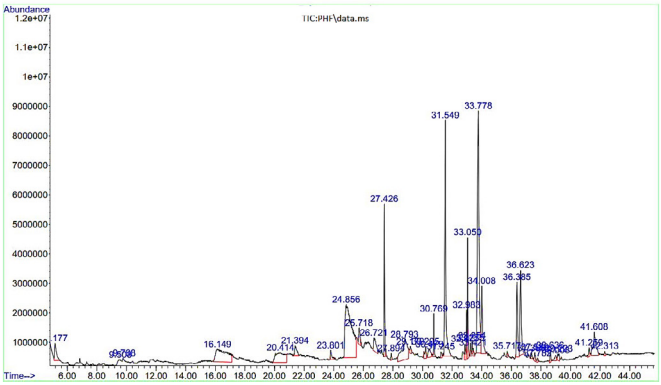

Figure 1: GC chromatogram of the methanolic extract of PHF[21]

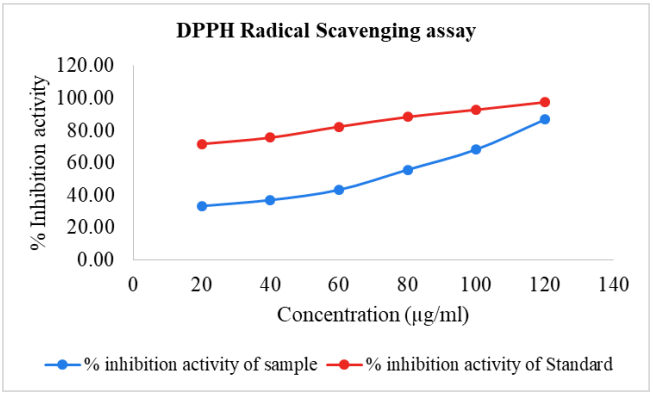

Figure 2: DPPH radical scavenging assay of PHF[23]

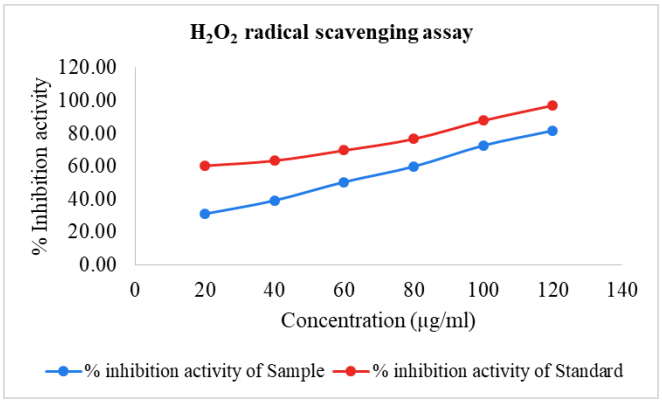

Figure 3: H_2O_2 radical scavenging activity of PHF[24]

Antioxidant Assays

DPPH radical scavenging assay

After being reduced, the color of DPPH shifts from violet to yellow, indicating that it is a stable nitrogen-centered free radical. The color shift that happens is determined by the capacity of antioxidant extracts or pure compounds to scavenge free radicals and reduce the production of the DPPH radical by hydrogen donation. A positive control was carried out using ascorbic acid. Scavenging activities were observed in the methanolic extract at levels ranging from 33.33 to 86.68% (Figure 2).

Hydrogen peroxide scavenging assay

Hydrogen peroxide (H_2O_2) scavenging activity of natural antioxidants contained in plant extracts has been tested

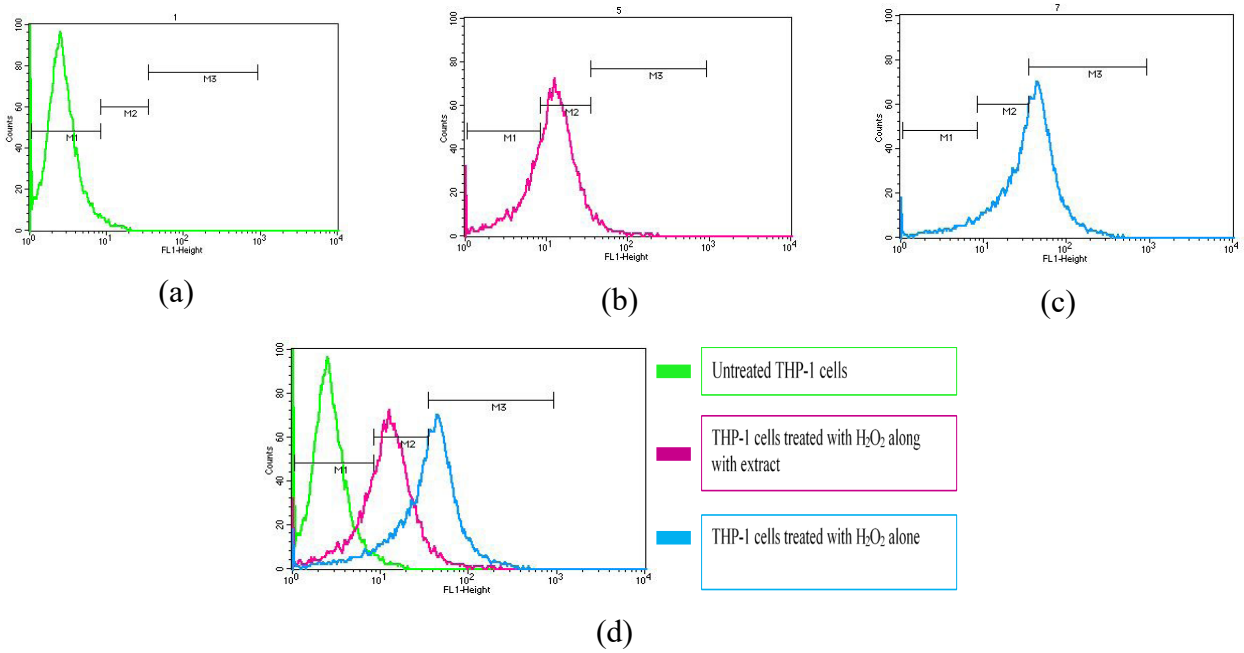

Figure 4: FACS images of PHF in THP- 1 cells to assess ROS activity (a) Untreated THP- 1 cells (b) THP- 1 cells treated with H_2O_2 along with extract(c) THP- 1 cells treated with H_2O_2 alone (d) Overlay spectrum of all groups[25]

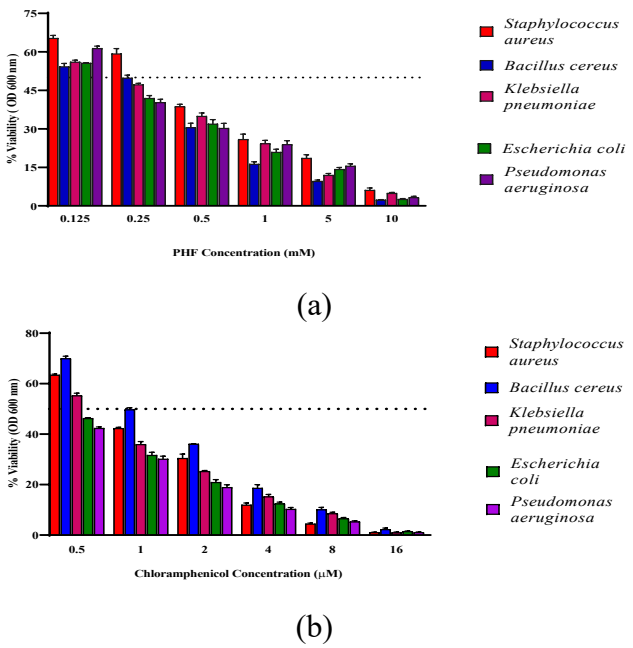

Figure 5: Resauzarin assay for evaluating antibacterial activity of (a) PHF extract (b) Chloramphenicol[26,27]

extensively by analysing the decrement of amount of H_2O_2 in the incubation system and detecting using the traditional UV technique at 230 nm. Figure 3 illustrates the extract's capacity to scavenge hydrogen peroxide free radicals. The extract had a scavenging activity on hydrogen peroxide that ranged from 31.05 to 81.66%.

Intracellular ROS activity

The cells marked with DCFDA underwent FACS assessment to understand the ability of extract on intracellular oxidative stress. The extract significantly reduced the quantity of free radical production at its IC_{50} concentration (3.99 µg/mL), based on the findings of the FACS investigation represented in Figure 4. THP-1 cells treated with both peroxide and an extract showed a significant reduction ($p < 0.001$) in fluorescence intensity compared to cells treated with peroxide alone.

Antibacterial Assay by Resauzarin Assay

The efficacy of the extract in the modified resazurin test was depicted in Figure 5a, as well as that of ciprofloxacin as the positive control in Figure 5b. Based on the MIC values observed in Figure 5, it scientifically validates that the methanolic extract has significant antibacterial properties.

DISCUSSION

Atherosclerosis is widely recognized as the primary etiology of cardiovascular disease (CVD), including conditions such as myocardial infarction (MI), heart failure, and stroke. Medicinal plants are significant sources of medications and now account for around 25% of prescriptions filled by pharmaceutical companies. The current investigation involves a unique herbal formulation of 10 medicinal herbs, namely *G. cambogia, R. cordifolia, C. mukul, V. vinifera, H. annus, A. catechu, L. usitatissimum, A. sativum, A. catechu, T. arjuna,* and *P. nigrum*, which is intended for the treatment of atherosclerosis. Gas chromatography–mass spectrometry characterization leads to identifying 38 distinct plant components and 11 core compounds reported in Table 1. Based on the DPPH radical scavenging assay results it demonstrates scavenging activity within a range of 33.33 to 86.68%. The compounds exhibited significant ($p < 0.01$) antioxidant activity that varied with concentration, as shown in Figure 2. The IC_{50} values were 12 µg/mL for the standard and 70 µg/mL for the the sample. The scavenging of H_2O_2 by extracts may be ascribed to the presence of phenolics, which have the ability to donate electrons to H_2O_2, resulting in its neutralization and conversion to water. The findings in this assay indicate that the extract exhibited significant H_2O_2 scavenging action shown in Figure 3 with a scavenging range of 31.05% to 81.66%. The IC_{50} values for the standard and the extract were found to be 15 and 60 µg/mL, respectively. In the intracellular ROS assay, the THP-1 cells treated with H_2O_2 and extract show better scavenging activity than those treated with H_2O_2 alone (Figure 4). When undergoing conversion by oxidoreductases found inside living cells, it transitions into a pink dye that exhibits fluorescence in resauzarin assay. Upon further reduction, the compound resorufin undergoes a transformation into hydro resorufin, resulting in a colorless and nonfluorescent substance. The antibiotic chloramphenicol inhibited the bacterial growth at the minimum specified dose, as seen in Figure 5b. The effectiveness of the extract was illustrated in Figure 5a. When compared to the positive control, this reveals that the plant extract displays substantial antibacterial activity. Hence, although the existing findings about the antioxidant and antibacterial activities show promising effects, more study is necessary to ascertain their potential benefits prior to its endorsement as conventional pharmacotherapy. Hence, more research is required to address the safety aspects of bioactive molecules derived from herbal medications.

CONCLUSION

Gas chromatography coupled with mass spectrometry was used in this investigation to determine the identities of various chemical components and ensure the formulation under investigation satisfied certain quality standards. Pharmacological effects, including antioxidant, and antimicrobial actions, have been attributed to the phytochemicals that have been identified by GC-MS analysis. According to the current research results, the polyherbal formulation has shown its ability to prevent the formation of free radicals and fight bacterial infections, proving significant antioxidant and antibacterial capabilities. Further quantification and evaluation of the identified phytomarkers is needed for future use.

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