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Pharmacologically active herbal remedies against atherosclerosis, characterization and DoE based marker quantification by densitometry, and cell based assays on THP-1 cell lines

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ABSTRACT

Atherosclerosis is a complex condition that develops at varying rates in multiple configurations and blood vessels. The primary cause of morbidity and mortality worldwide, particularly in the industrialized nations, continues to be atherosclerosis. Ayurveda, Siddha, and Unani systems of medicine, among other traditional medical systems, utilize polyherbal compositions. The treatment of atherosclerosis has been improved with a novel multibotanical combination. In this study, we sought to formulate, characterize, and standardize a polyherbal formulation based on design of experiments (DoE), densitometric studies and to predict for antioxidant activity using molecular docking analysis based on LC- MS identified phytomarkers. In addition we have assessed its cell viability by MTT assay along with Ao/EtBr staining technique and intracellular ROS assay using THP-1 cell lines. Reported findings showed that the HPTLC based quantified components of selected multiherbals has the ability to treat for atherosclerosis. This document could be used to quickly authenticate the formulation as the method optimized was based on CCD design which shows desirability of 0.962 and 0.839. Cell based assays scientifically proves that the formulation was not toxic based on MTT assay along with AO/EtBr staining technique and has excellent antioxidant activities based on intracellular ROS assay using THP-1 cell lines. The observed findings would be crucial for future clinical aspects since the bioactive molecules contained in the extracts may have anticipated effects with other compounds and show a superior therapeutic potential. As a result, this study offers standardized and potentially therapeutic information about effective polyherbal formulation for atherosclerosis.

1. Introduction

Cardiovascular disease, which includes ischemic heart disease, strokes, and peripheral vascular disease, is caused by atherosclerosis, a long-term inflammatory condition of the major and medium-sized arteries (CVD). The accumulation of lipid-rich macrophages and calcification in the area of the arterial wall underneath the endothelium is a sign of atherosclerosis. The progression of disease may follow some series of events like increase in the level of LDL particles which accumulates into arterial intima, peroxidation of lipids, leukocyte recruitment, foam cells formation, Calcification of fatty substances, narrowing of blood vessels, Plaque growth. These events will altogether leads to

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Table 1
Factors and responses selected in CCD design for eight markers.

Run	F-1	F- 2	F- 3	R-1	R- 2	R-3	R-4	R-5	R- 6	R-7	R-8
	A:Toluene percentage	B:Saturation time Minutes	C:Developing distance Centimeters	Rf - Qr	Rf - Km	Rf- Tf	Rf- Ga	Rf- Rr	Rf- Pi	Rf-Vn	Rf- Ca
1	8	15	70	0.73	0.78	0.83	0.19	0.66	0.86	0.88	0.93
2	6	20	75	0.47	0.63	0.5	0.21	0.53	0.58	0.6	0.67
3	2.64	20	75	0.29	0.32	0.32	0.001	0.26	0.31	0.33	0.41
4	6	20	66.59	0.43	0.61	0.36	0.17	0.39	0.43	0.45	0.5
5	6	11.59	75	0.41	0.58	0.48	0.2	0.49	0.56	0.58	0.64
6	6	28.41	75	0.46	0.61	0.42	0.13	0.44	0.53	0.56	0.61
7	8	25	70	0.67	0.72	0.75	0.18	0.75	0.77	0.8	0.82
8	6	20	83.41	0.48	0.57	0.39	0.15	0.47	0.54	0.57	0.62
9	4	15	80	0.14	0.16	0.36	0.012	0.38	0.38	0.4	0.43
10	9.36	20	75	0.8	0.82	0.86	0.36	0.88	0.92	0.93	0.98
11	6	20	75	0.47	0.63	0.5	0.21	0.53	0.58	0.6	0.67
12	6	20	75	0.47	0.63	0.5	0.21	0.53	0.58	0.6	0.67
13	4	25	70	0.11	0.13	0.29	0.036	0.32	0.35	0.36	0.42
14	6	20	75	0.47	0.63	0.5	0.21	0.53	0.58	0.6	0.67
15	6	20	75	0.47	0.63	0.5	0.21	0.53	0.58	0.6	0.67
16	6	20	75	0.47	0.63	0.5	0.21	0.53	0.58	0.6	0.67
17	4	25	80	0.14	0.17	0.26	0.022	0.28	0.31	0.33	0.37
18	4	15	70	0.36	0.56	0.6	0.031	0.62	0.65	0.66	0.71
19	8	25	80	0.56	0.65	0.68	0.26	0.7	0.73	0.76	0.79
20	8	15	80	0.61	0.69	0.51	0.29	0.51	0.79	0.83	0.85

*F: Factor; R: Response; Rf: retention factor; Qr: Quercetin; Km: Kaempferol; Tf: Trans ferulic acid; Ga: Gallic acid; Rr: Resveratrol; Pi: Piperine; Vn: Vanillin; Ca: Caffeine.

Table 2
Factors and responses selected in CCD design for three markers.

Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
	A:Methanol percentage %	B:Saturation time Minutes	C:Developing distance Centimeters	Rf of Rubiadin	Rf of Catechin	Rf of Ellagic acid
1	1.5	25	70	0.35	0.31	0.6
2	2.5	25	70	0.43	0.54	0.57
3	2	20	66.591	0.37	0.57	0.63
4	1.5	15	80	0.41	0.42	0.56
5	1.1591	20	75	0.4	0.45	0.58
6	1.5	15	70	0.36	0.38	0.59
7	2.5	15	80	0.46	0.51	0.6
8	2	28.409	75	0.38	0.51	0.6
9	2	20	75	0.43	0.53	0.61
10	2	20	75	0.43	0.53	0.61
11	2.5	15	70	0.32	0.57	0.58
12	2	11.591	75	0.31	0.55	0.62
13	2.8409	20	75	0.48	0.58	0.66
14	2	20	75	0.43	0.53	0.61
15	1.5	25	80	0.41	0.41	0.51
16	2	20	75	0.43	0.53	0.61
17	2	20	83.409	0.34	0.49	0.55
18	2	20	75	0.43	0.53	0.61
19	2	20	75	0.43	0.53	0.61
20	2.5	25	80	0.45	0.52	0.57

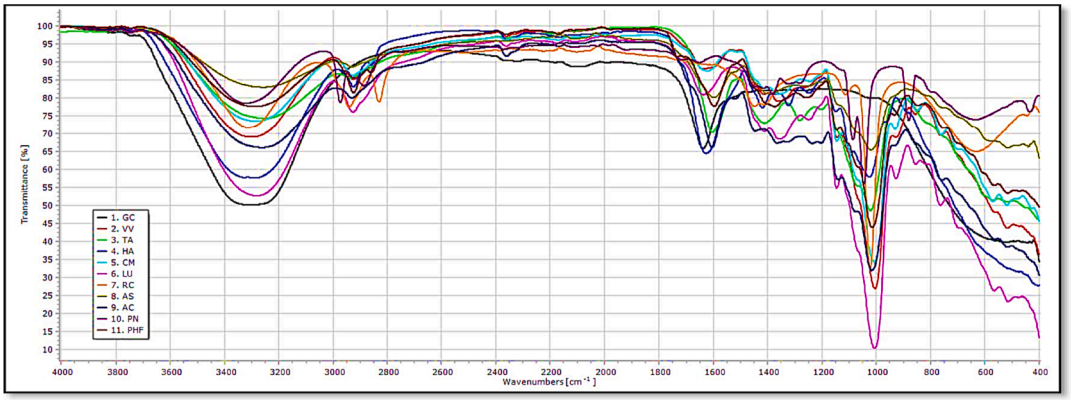


Fig. 1. Overlay FTIR spectrum of all individual plant extracts and PHF of the multibotanical preparation.

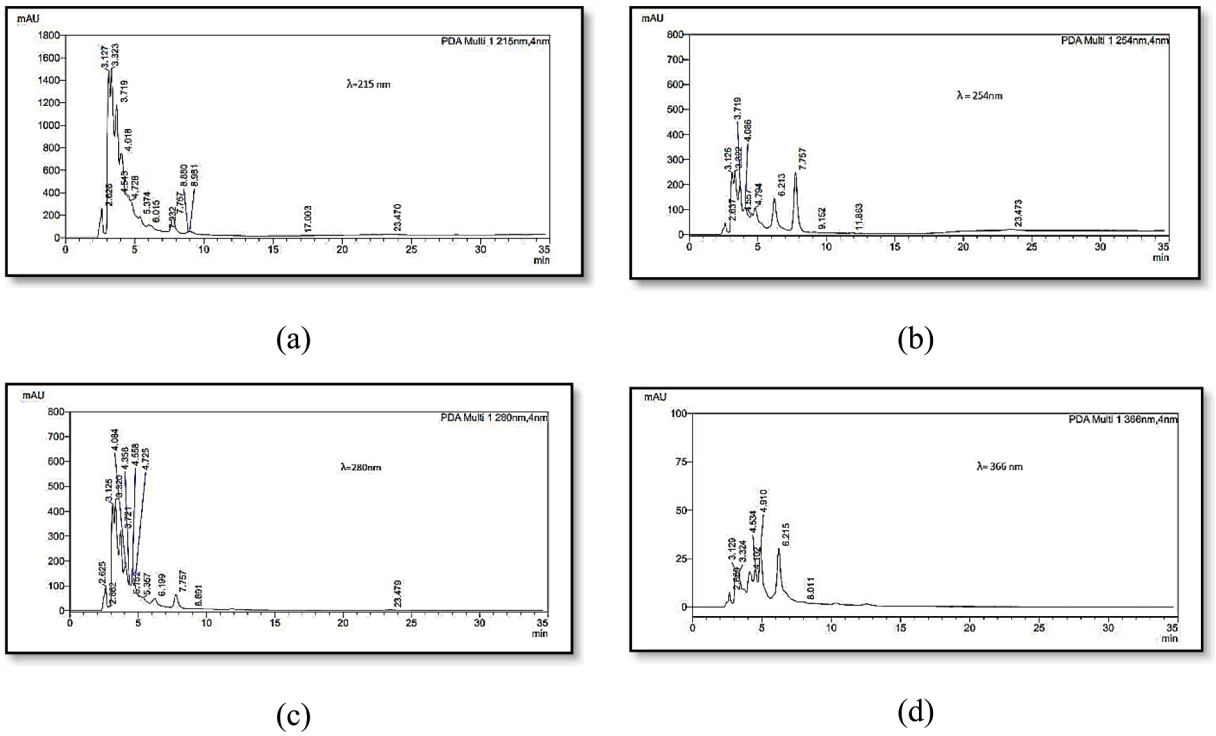


Fig. 2. HPLC Chromatograms of methanolic extract of the formulation at four different wavelengths (215, 254, 280, and 366 nm).

Table 3
LC-MS analysis of identified compounds in methanolic extract of PHF.

Retention time	Identified Compound name	Formula	Mass value *m/z*	Fragmentation mode	Biological activity
3.4	Gallic acid	$C_7H_6O_5$	170.12	Negative ionization	Antioxidant,anti-inflammatory, Antihyperlipidemic activities
20.53	Alliin	$C_6H_{11}NO_3S$	177.90	Positive ionization	Antihypertensive, Cholesterol lowering property
20.85	Citric acid	$C_6H_8O_7$	192.20	Negative ionization	Antioxidant, Cardioprotective effect
5.0	Hydroxy citric acid	$C_6H_8O_8$	208.50	Negative ionization	Antilipolytic, Antiobesity, Antihyperlipidemic activities
10.5	Resveratrol	$C_{14}H_{12}O_3$	228.40	Negative ionization	Anti-inflammatory, antioxidant, cardioprotectiveproperties
4.22	Rubiadin	$C_{15}H_{10}O_4$	254.75	Negative ionization	Antioxidant, anti inflammatory, antidiabetic, hepatoprotective properties
5.8	Kaempferol	$C_{15}H_{10}O_6$	286.05	Negative ionization	Antioxidant, anti- inflammatory, cardioprotective, neuroprotective activities
24.93	Catechin	$C_{15}H_{14}O_6$	290.45	Positive ionization	Antioxidant, anti- inflammatory
8.22	Quercetin	$C_{15}H_{10}O_7$	301.05	Positive ionization	Antioxidant, anti- inflammatory, Cardioprotective effects
13.4	Ellagic acid	$C_{14}H_6O_8$	302.75	Positive ionization	Antioxidant, anti- inflammatory activities

severe complications to heart and stays the reason for death worldwide (Jebari-Benslaiman et al., 2022).

Estimates indicate that 17.9 million deaths occurred as a result of CVDs in 2019 (Lusis, 2000; Moore et al., 2013). 80 % of heart strokes are accounted for CVD deaths. Low and middle income countries estimated to have death rate as more than 22.2 million people per year by 2030 (Ray & Saini, 2021). Since herbal formulations have considered to be safe when compared to synthetic medicines an approach of developing a multibotanical formulation has been encouraged in this study as usage of multiple herbs are preferred over single herbs according to Ayurveda systems of medicine. A number of cutting-edge techniques, including chromatographic and spectrophotometric approaches, are presently used to standardize herbal medicines (Nicoletti, 2011).

A physicochemical analytical method known as Fourier transform infrared spectroscopy may be used to determine the structure of an unknown composition as well as the intensity of the absorption spectra that are related to the molecular composition or the presence of specific chemical functional groups. This FTIR method can be used to characterize the complicated structures of plant secondary metabolites (Sravan Kumar et al., 2015).

Polyherbal formulations may be ensured to some degree by phytochemical standardization using multiple marker based fingerprint profiling and preliminary screening of marker chemicals. In this study we could able to fingerprint some of the marker compounds using hyphenated analytical techniques like LC-MS. High-performance thin-layer chromatography (HPTLC) is an important analytical tool method that is often used to standardize drugs and evaluate their quality(P. Mukherjee, 2019). Due to its ability to examine several simultaneously changing features, experimental design technique has shown to be an effective tool for validating HPTLC procedure. In this study, the central composite design (CCD) is used to optimize the developed method which helps to measure the content of biomarkers by comparing their R_f values to those of the standard markers.

Toxic effects of the herbal drugs is the another important area found to be documented in recent profile, the formulated drugs should be checked for their toxicity (Bhope et al., 2011). In High-throughput screening, cell-based bioassays are a fundamental strategy for identifying the functional changes of target proteins in response to a drug

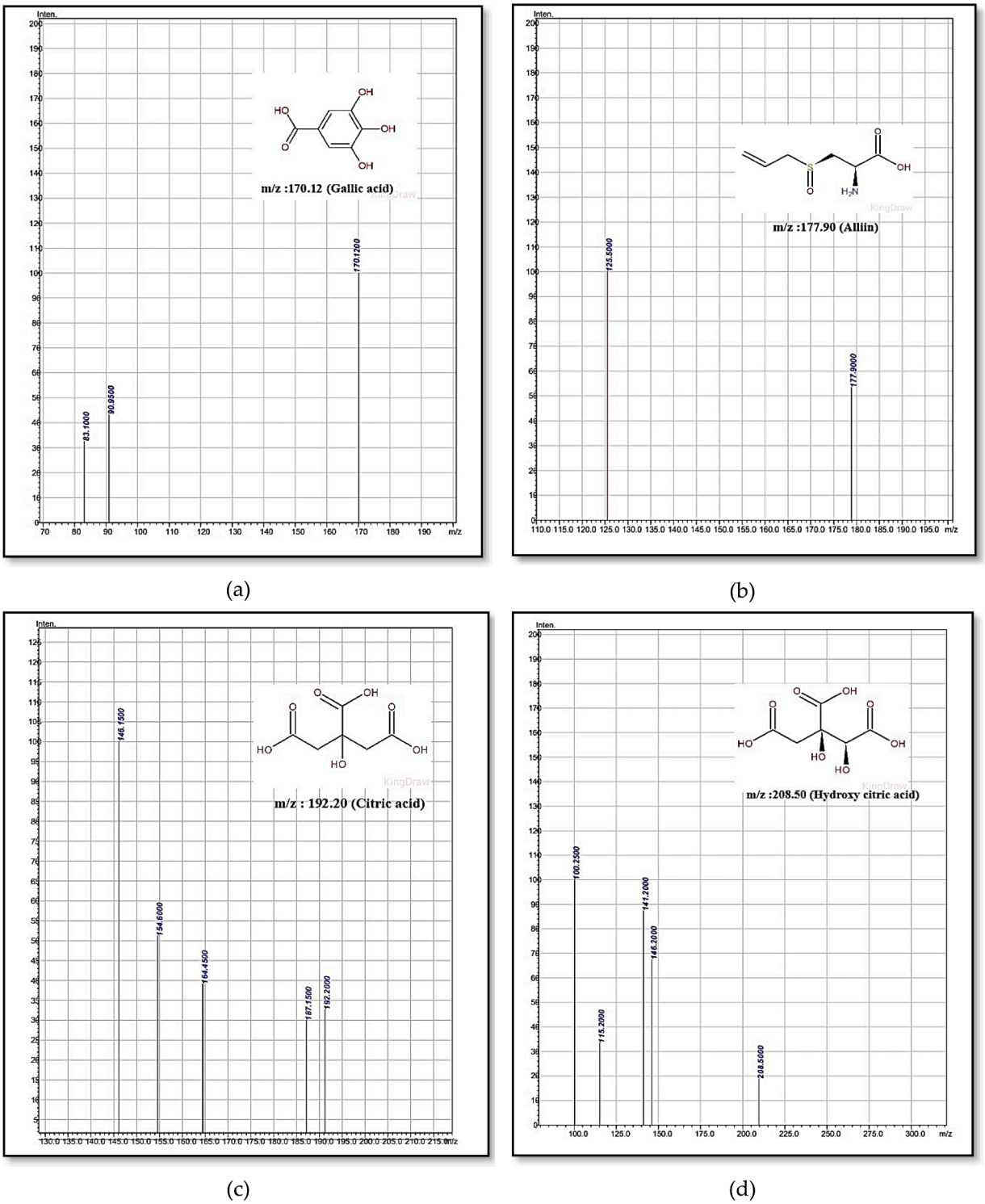
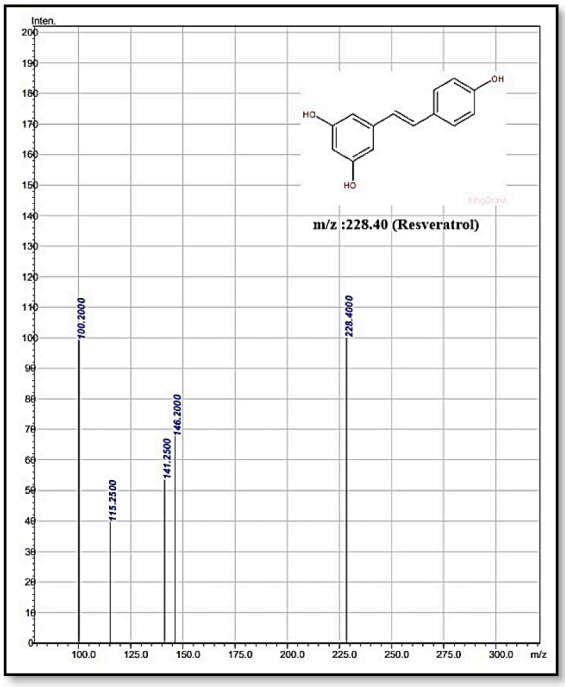


Fig. 3. Mass spectra of identified compounds present in methanolic extract of multiherbals.

molecule. A cell viability test is conducted based on the ratio of living to nonliving cells. This assay is based on an analysis of cell viability in cell culture and is used to evaluate in vitro drug effects in cell-mediated cytotoxicity assays and to monitor cell proliferation. In the present investigation, the highly sensitive MTT assay was chosen to determine the percentage of viable cells and also further distinguished with acridine orange/ethidium bromide (AO/EtBr) fluorescent staining, visualized under a fluorescent microscope(P. K. Mukherjee, 2019; Liu et al., 2015).

The development of cardiovascular disease associated with atherosclerosis is significantly influenced by oxidative stress, which is characterized by the overproduction of reactive oxygen species (ROS) and oxidized low-density lipoprotein (Ox-LDL). The primary cause of oxidative stress is an imbalance between radical production (formation of reactive oxygen and/or nitrogen species) and radical scavenging mechanisms (the antioxidant defense system). Experiments including



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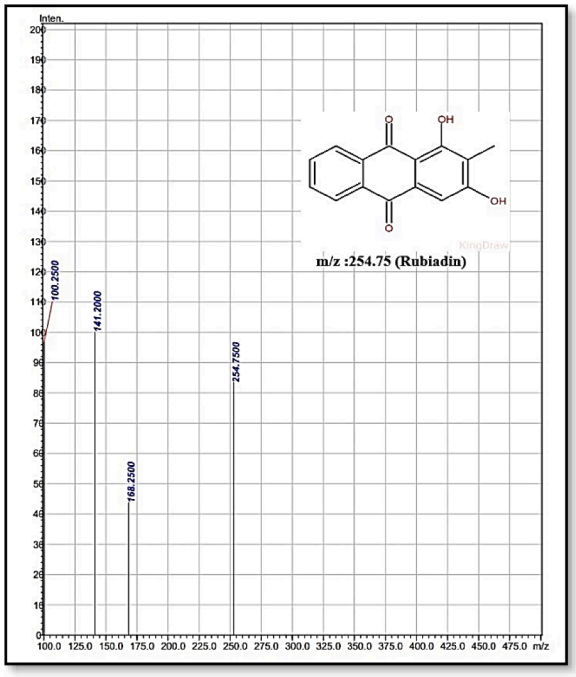

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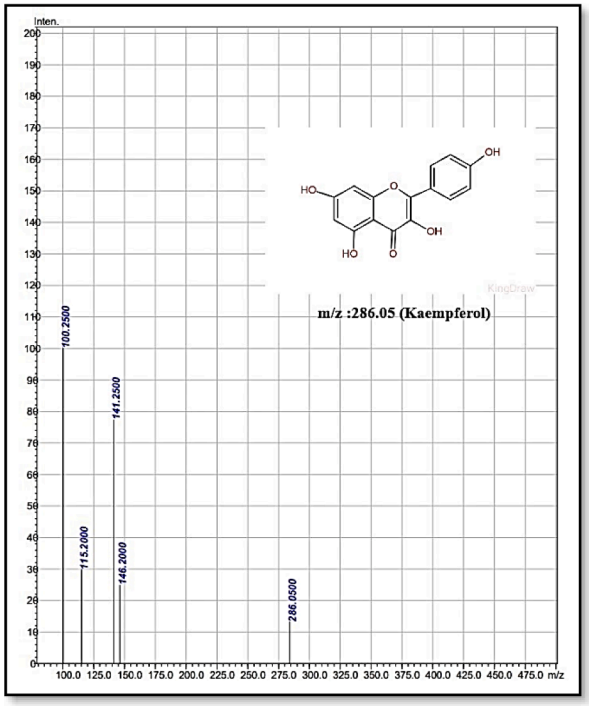
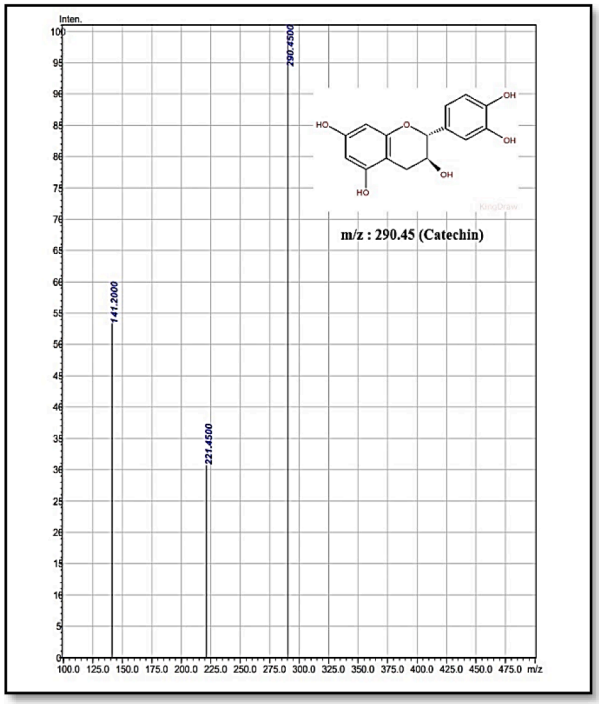


(g)

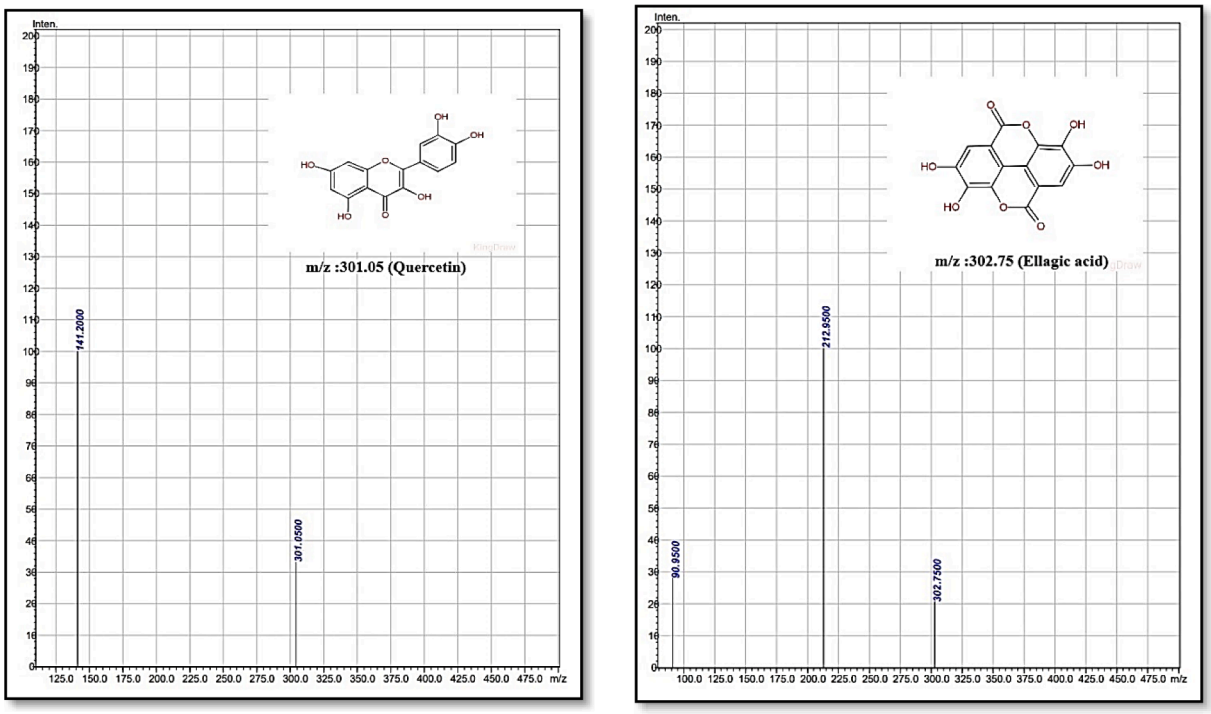


(h)

Fig. 3. (*continued*).

the ablation of anti-oxidant mechanisms in ApoE-KO mice show accelerated atherosclerosis and higher mitochondrial ROS, suggesting a role for mitochondrial ROS in atherogenesis (Kattoor et al., 2017). Intracellular ROS assay in THP-1 cell lines has been selected for the study as the use of antioxidants may well represent a logical treatment approach that will prevent the advancement of the problem.

The purpose of this study is to characterize the formulated multibotanicals by FTIR, LC-MS, insilico models and quantify the potential biomarkers using DoE based HPTLC method optimization studies in order to verify its identity, quality, and purity and to assess its cell viability by MTT assay along with Ao/EtBr staining technique, antioxidant activity by intracellular ROS assay using THP-1 cell lines for the polyherbal formulation.



(i) (j)

Fig. 3. (*continued*).

2. Materials and methods

2.1. Plant materials

Garcinia cambogia, Vitis vinifera, Terminalia arjuna, Helianthus annus, Commiphora mukul, Linum usitatissimum, Rubia cordifolia, Allium sativum, Acacia catechu, and *Piper nigrum* are among the herbs included in the composition. All of the components came from the local Tamilnadu market in India, and Dr. K Madhav Chetty, Professor of the department of Botany at SV University in Tirupati, Andhra Pradesh, India, verified their authenticity with voucher numbers *Garcinia cambogia* – (V. No.: 0517), *Vitis vinifera* –(V. No.: 0407), *Terminalia arjuna* –(V. No.: 0701), *Helianthus annus* –(V. No.: 0496), *Commiphora mukul* –(V. No.: 0599), *Linum usitatissimum* –(V. No.: 0616), *Rubia cordifolia* –(V. No.: 0778), *Allium sativum* –(V. No.: 0972), *Acacia catechu* –(V. No.: 0782), *Piper nigrum* – (V. No.: 0813) and submitted in herbaria.

2.2. Preparation of formulation

The procedure outlined in the Ayurvedic Formulary of India was followed in the preparation of the polyherbal formulation. According to the literature, each component was individually shade dried, ground, and sieved through a #80 mesh before being combined in equal parts to generate powder (Pattanayak et al., 2011).

2.3. Preparation of extracts

Fine powder (80 mesh size), was treated with methanol by ultrasonicator extraction for up to one hour. The solution was filtered and dried at a low temperature using rotary evaporator (Abubakar & Haque, 2020).

2.4. Materials and reagents

3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Quercetin, Kaempferol, Gallic acid, Trans ferulic acid, Resveratrol, Piperine, Vanillin and Caffeine were purchased from Natural remedies. RPMI-1640, Fetal Bovine Serum (FBS), Penicillin, Streptomycin, Dimethylsulfoxide (DMSO), and 2, 7-dichlorofluorescein diacetate (DCFDA) was purchased from Sigma Aldrich. Other chemicals and solvents are purchased from Merck, analytical grade.

2.5. Cell culture of THP-1 cell lines

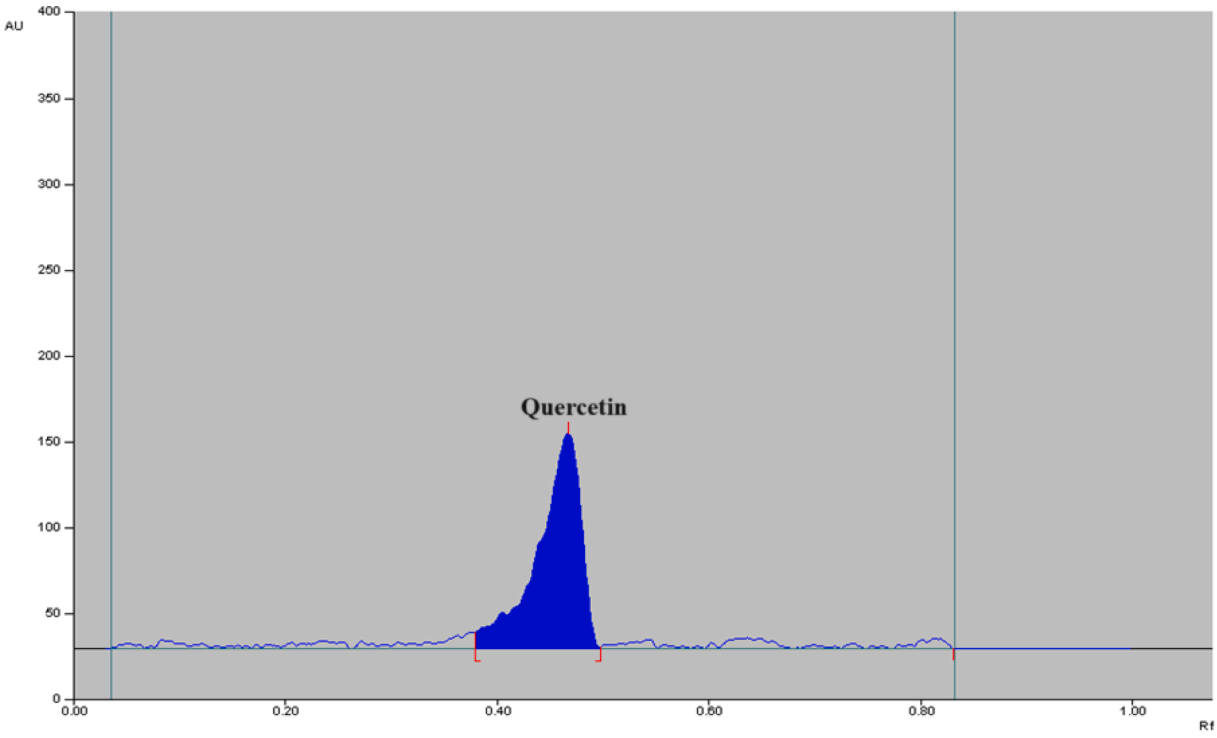
The THP-1 cell line, derived from human acute monocytic leukemia, was acquired from the National Centre for Cell Science (NCCS) in Pune. The cell line was grown in RPMI-1640 medium, which was supplemented with 10 % Fetal Bovine Serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml Streptomycin. The culture was maintained in a humidified environment with 5 % CO^2 at a temperature of 37 °C. When the cell culture reaches a confluency level of 70–80 %, a suspension containing 5×10^4 cells is introduced into each well of a 96-well microtiter plate, followed by incubation for the subsequent experiments.

2.6. Characterisation by FTIR

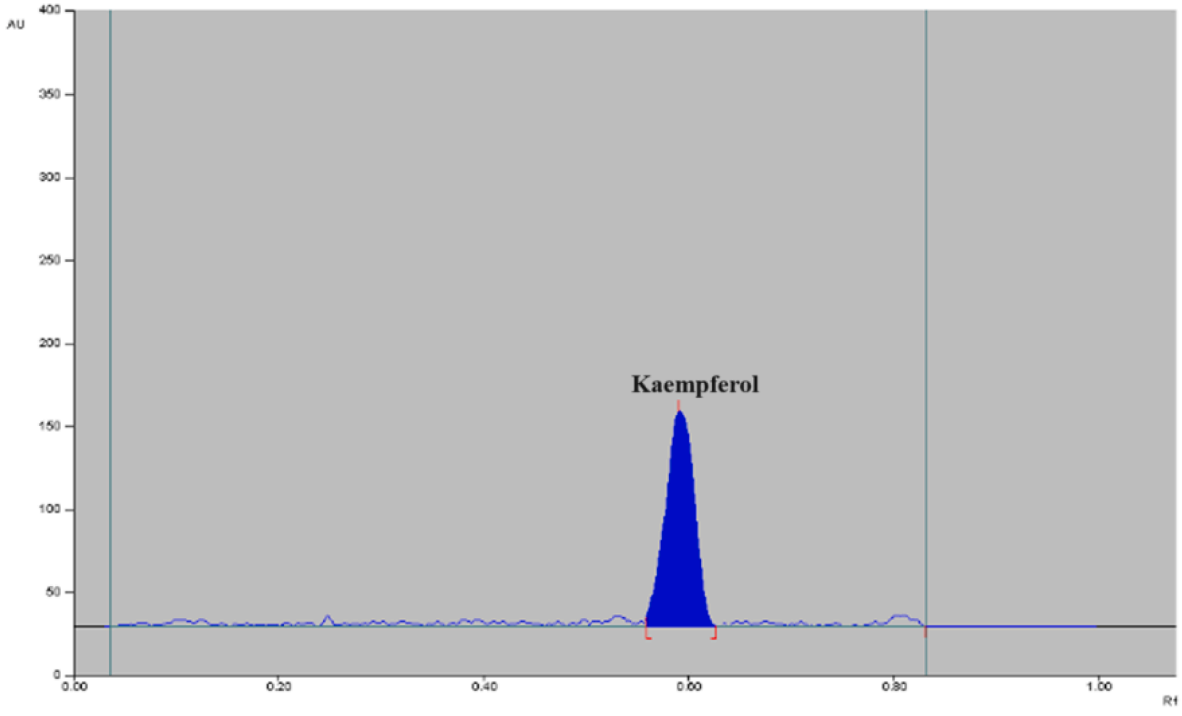
Functional groups in the methanolic extract of the formulation was characterized by Fourier transform infrared (FTIR) analysis. The individual plant extracts and polyherbal formulation (PHF) was mixed with KBR separately and made pellets which are subjected to analysis using Bruker alpha E & T instrument (Lab India). The samples were scanned from 4000 to 500 cm^{-1} and the % of transmittance versus wavenumber were recorded (Sultan et al., 2023).

2.7. LC-MS condition

"LC-MS 2020 system equipped with LC10ADVP binary pump (Shimadzu, Japan)" was used for LC- MS analysis. For sample separation Phenomenex column (250 x 4.6 mm, 5 µm) was used by running the instrument with mobile phase solvents in the ratio of 40 (Methanol):15



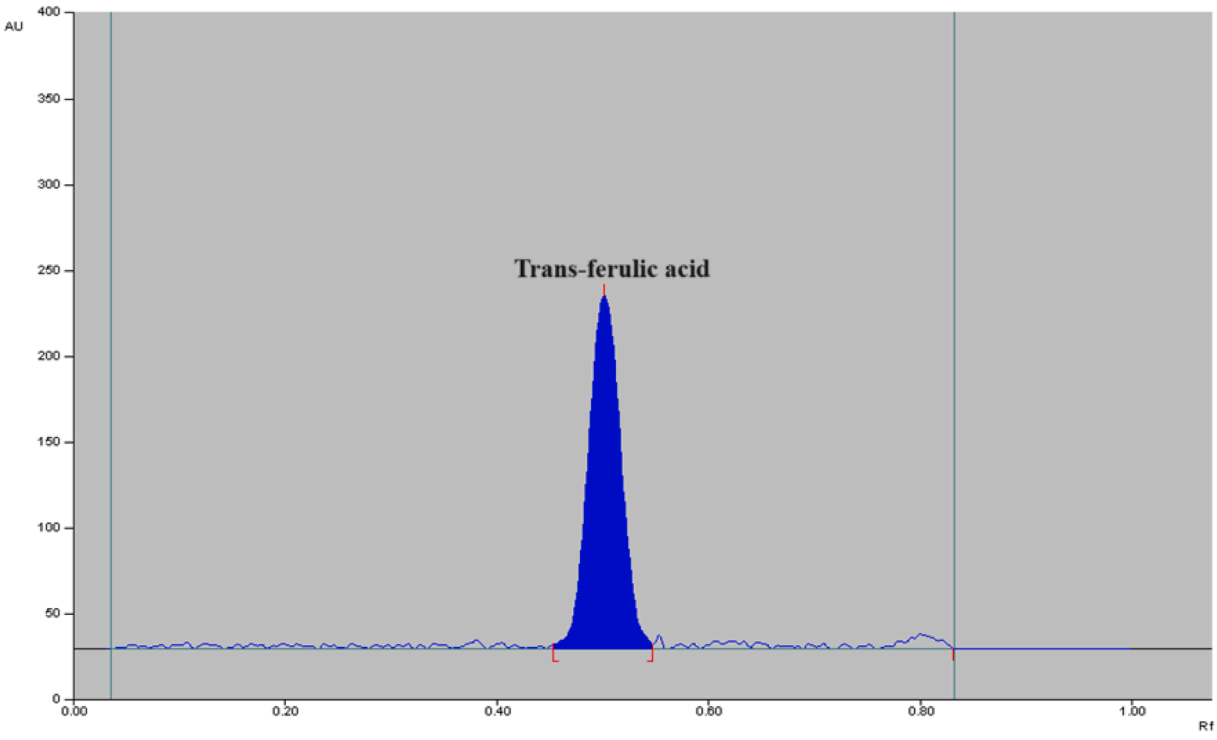
(a)



(b)

Fig. 4. HPTLC chromatograms of standards and sample under UV 254 nm (**a**) Quercetin (**b**) Kaempferol (**c**) Trans ferulic acid (**d**) Gallic acid (**e**) Resveratrol (**f**) Piperine (**g**) Vanillin (**h**) Caffeine (**i**) PHF Sample.

(Acetonitrile): 45 (0.1 % formic acid water) with a flow rate of 0.5 mL/min. The injection volume was 20 µL. Detection was done by a PDA detector at multiple wavelengths (λ) of 215, 254, 280, 366 nm, with a run time of 35 min. The electrospray ionization (ESI) technique was equipped in the Mass (MS) unit having a single quadrupole mass spectrometer with a source, and nitrogen gas with a flow rate of 1.5 L/min



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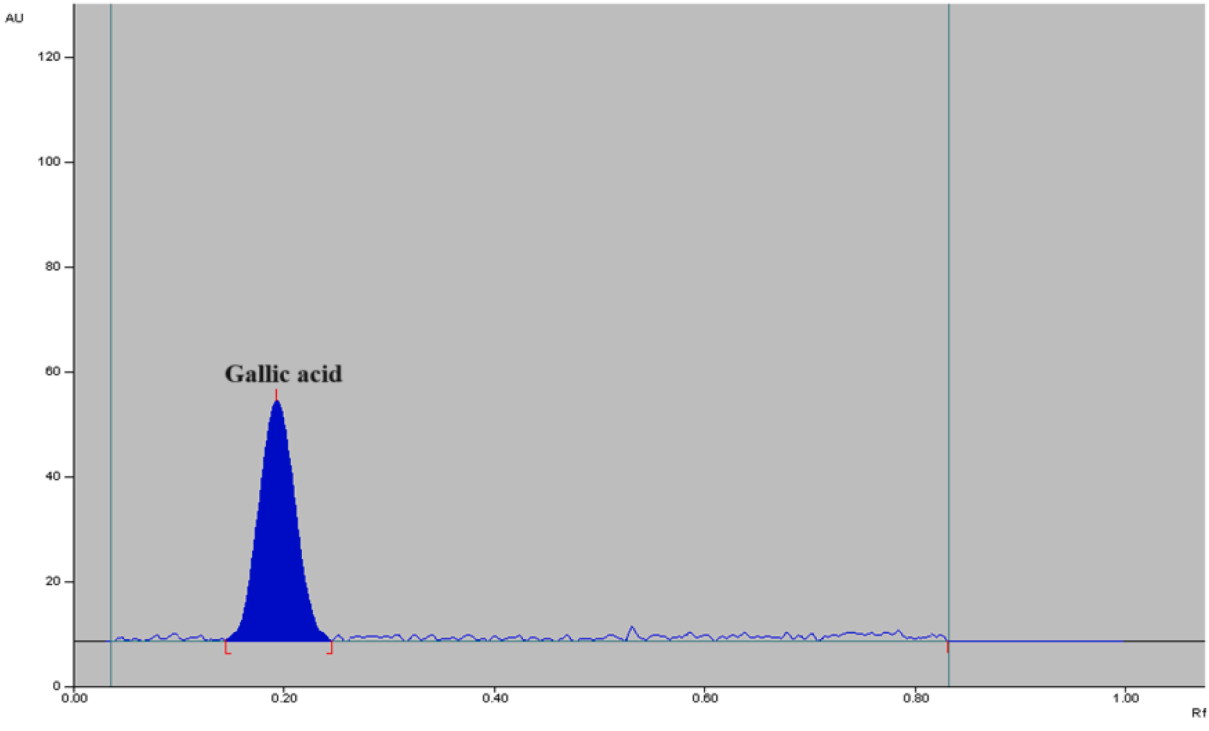
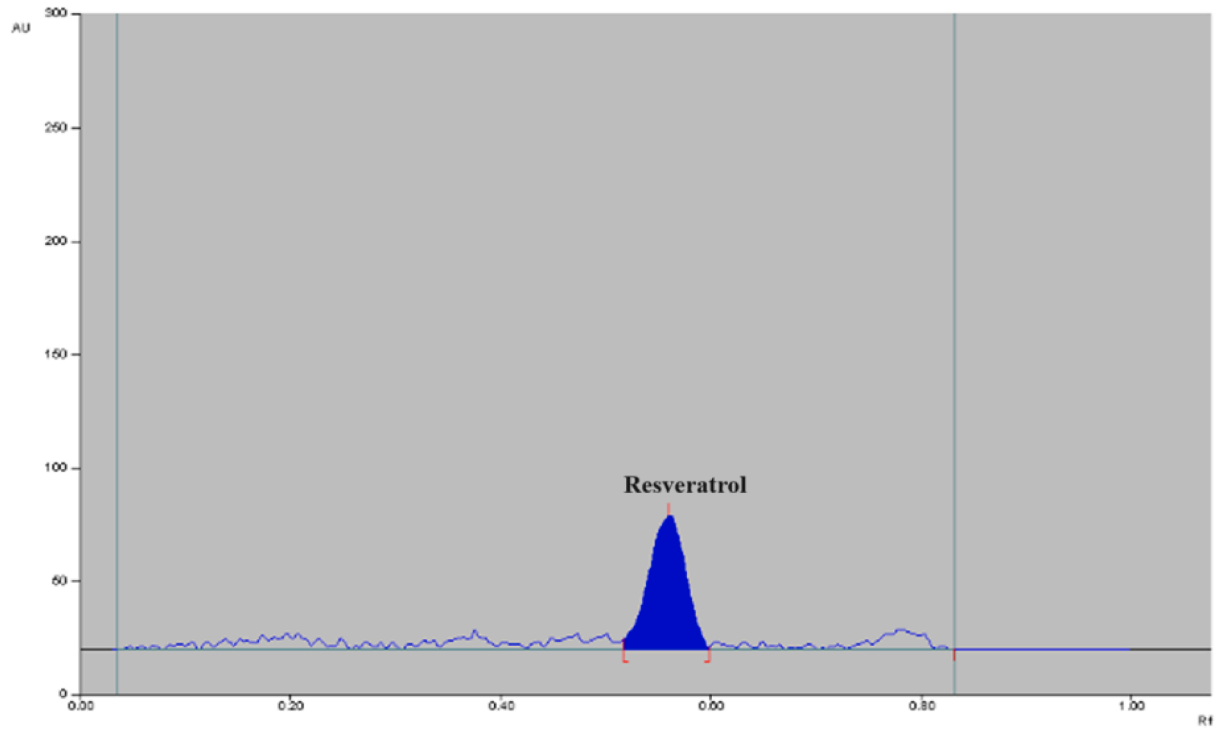


(d)

Fig. 4. (*continued*).

was used to assist nebulization. The temperature was set for a curved desolvation line (CDL) and heat block at 250 °C and 280 °C. Lab Solution Software was used for data collection and processing (Xiaohui et al., 2006).



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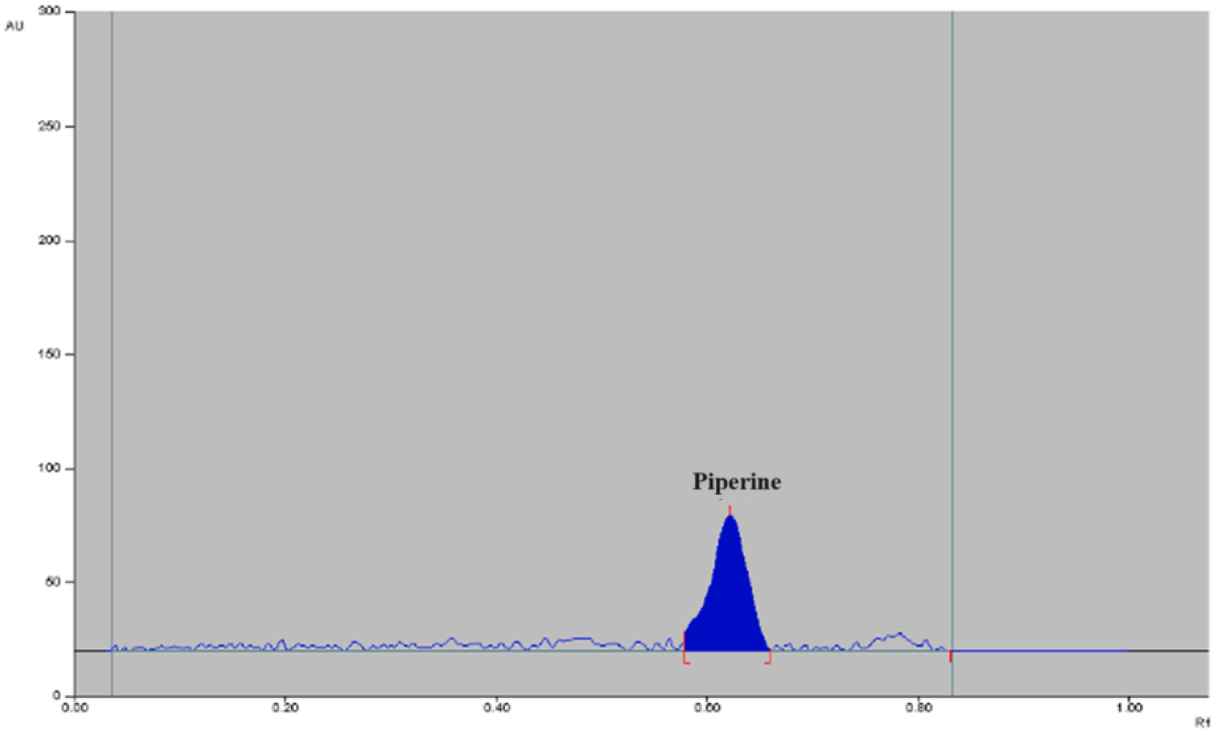
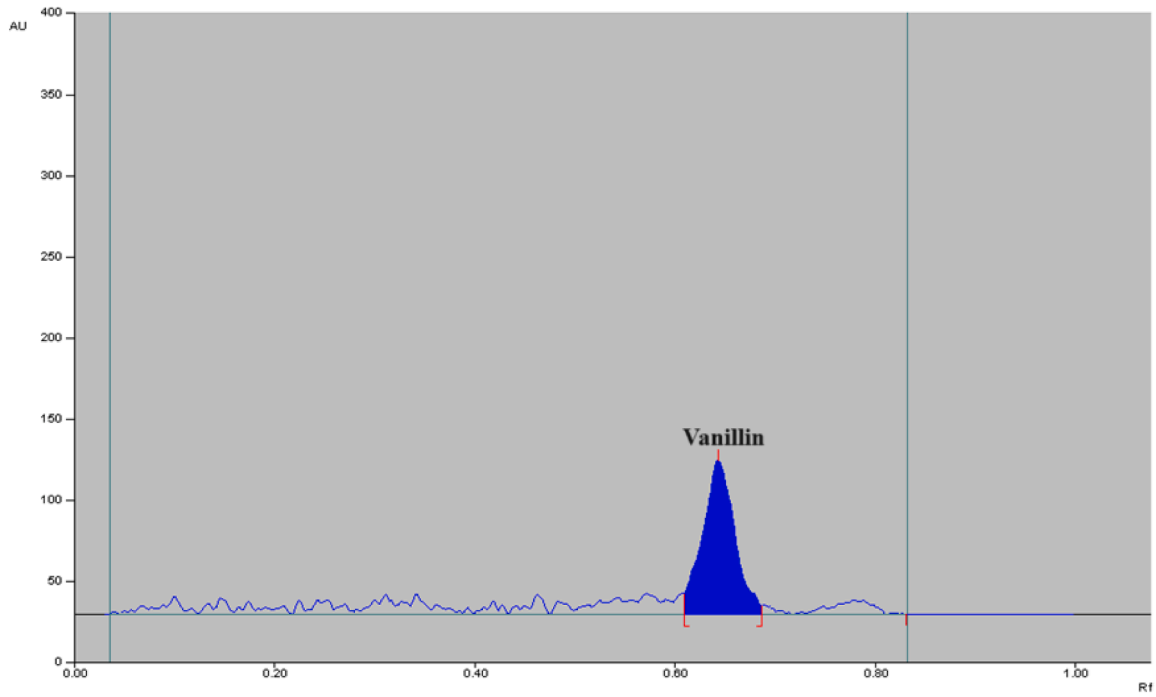


(f)

Fig. 4. (*continued*).

2.8. Simultaneous quantification of potential biomarkers by HPTLC

A high-performance thin-layer chromatography (HPTLC) technique was devised to concurrently determine the levels of quercetin, kaempferol, *trans*-ferulic acid, gallic acid, resveratrol, piperine, vanillin, and caffeine in the methanolic extract of a polyherbal formulation. The aliquots of the standard stock solution (100 mcg/mL) and the prepared sample (10 mg/mL) were dispensed using a Camag Linomat V sample applicator, manufactured in Muttenz, Switzerland. The plates underwent saturation inside a twin trough chamber. The slit dimensions were



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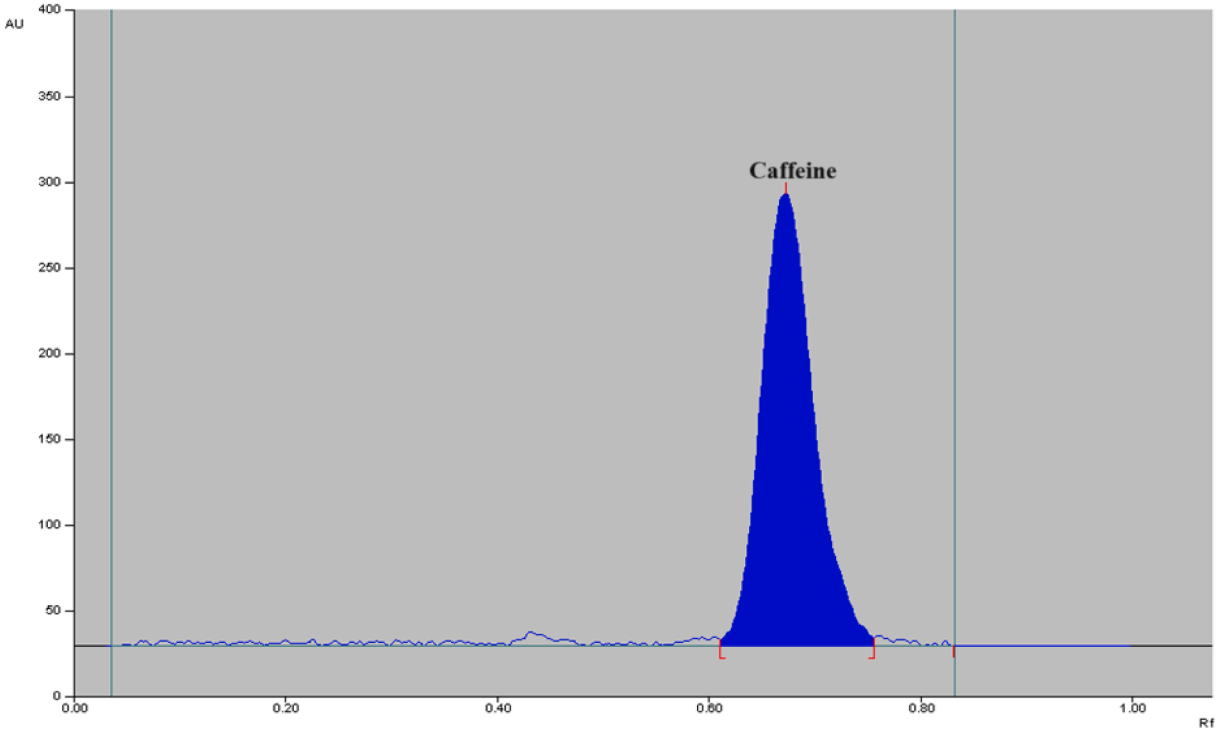
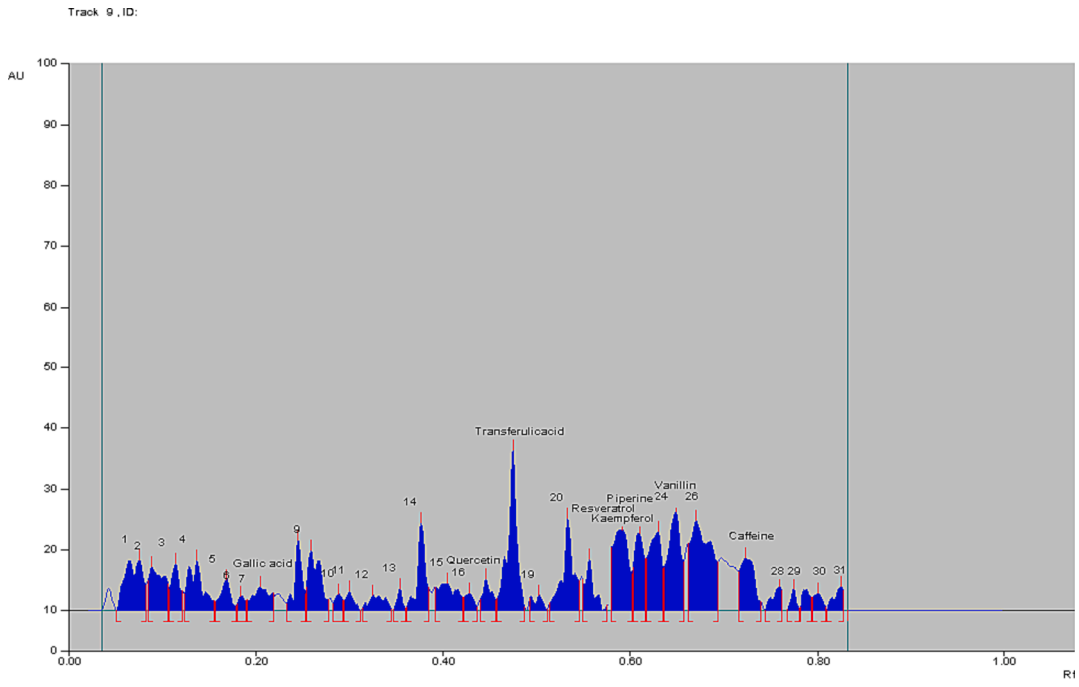


(h)

Fig. 4. (*continued*).

adjusted to a length of 4 mm and a width of 0.3 mm. The monochromator had a bandwidth of 20 nm and a scanning rate of 20 mm/s. The spraying rate was 10 s/µL, and the data resolution was 100 µm/step. The quantification of zones was conducted using the Camag TLC Scanner III densitometer, which was operated by Win CATS version 1.4.3.6336 software. The absorption mode was used, using a deuterium source and a filter with a wavelength of 254 nm. The chromatographic plates used in this study were aluminum plates that had been pre-coated with Silica Gel 60 F254 (20 cm × 20 cm, 0.25 mm) obtained from E. Merck in Darmstadt, Germany. The plates were developed by applying a mixture of toluene, ethyl acetate, methanol, and formic acid (in a volumetric ratio of 6:3:0.8:0.2) as mobile phase after allowing for a



(i)

Fig. 4. (*continued*).

saturation period of 20 min(Panchal et al., 2017). In the similar way using same mobile phase with different ratios 4:3:2:1 was selected for the determination of rubiadin, catechin, and ellagic acid at a detection wavelength of 366 nm. The optimized methods were validated according to ICH guidelines. Table 1 and 2 shows the model selected for central composite design in which both the experiments twenty runs were selected.

2.9. Software and statistics

The response surface optimization, utilizing analysis of variance (ANOVA) through Centre composite design (CCD), was conducted using Design Expert software version 12. Additionally, 3D response surface plots were generated, and the desirability function D was determined.

2.10. Molecular docking

Based on the LC-MS approach, the identified phytochemicals were docked against transferrin, an enzyme with significant antioxidant qualities because they prevent iron-dependent free radical generation and the subsequent cell damage which was one of the pathway for treating atherosclerosis (Halliwell & Gutteridge, 1990; Stocks et al., 1974; Gutteridge & Quinlan, 1993; Kibel et al., 2008).

2.10.1. Protein preparation

The Human Serum Transferrin x-ray crystal structures, with PDB IDs of 1D3K, 1D4N, and 1 N84 and resolutions of 1.80, 2.00, and 2.05, respectively, were obtained from the Protein Data Bank (https://www.rcsb.org) and imported into Autodock 1.4.6.

2.10.2. Ligand preparation

Using chemdraw Ultra (14.0), the active phytoconstituents discovered during LC-MS fingerprinting analysis were visualized. To create 3D structures, ligands' 2D chemdraw structures were imported. After that, the 3D ligands were exported to pdb format so that they could be molecularly docked with human serum transferrin protein.

2.10.3. Molecular recognition protein- ligand by molecular modelling

Molecular docking was used to assess protein ligand interactions and develop the scoring function in order to estimate the binding affinity and activity of the ligand molecule. The Autodock® 1.4.6 Program was used to dock the ligands into bioactive binding poses in the active sites of 1D3K, 1D4N, and 1 N84. The protein coordinates of the bound ligands 1D3K, 1D4N, and 1 N84 were used to determine the binding site. The Lamarckian genetic process was then used to produce the score function. For docking calculations, the target protein was centered on the grid map. Using Biovia Discovery Studio 2021, hydrogen and hydrophobic interactions at the inhibitor sites of 1D3K, 1D4N, and 1 N84 were modelled. Using Pymol, docking interactions were found.

2.11. Cell viability activity by MTT assay

MTT assay using methanolic extract of the formulation was performed on Human THP-1 cell lines for evaluating cell viability using standard protocol with slight modifications. The formulation at concentrations 5 – 0.15 μg/ml were used. The proportion of treated cells to untreated (control) cells was used to calculate the percentage of viable cells(Musthafa et al., 2021).

2.11.1. Evaluation of cytotoxicity using acridine orange (AO)/ ethidium bromide (EtBr) staining

Acridine orange (AO)/Ethidium Bromide (EtBr) staining of THP-1 cells was used to measure the cytotoxicity/ cell viability of PHF with the use of CLSM, Zeiss, Germany. THP-1 cells (20,000 cells/mL) were added into 1.5-ml Eppendorf tubes and treated with PHF at their IC_{50} concentration for 24 h. The cells were centrifuged, pelleted, and the supernatant was discarded before the cells were resuspended in Phosphate-buffered saline (PBS) after 24 h. The control cells were those left without the sample. The live and dead cell populations were estimated at a 40x magnification using CLSM (Zeiss LSM 700) after the cells were treated with PBS containing 5 μL of both Acridine orange (AO) and Ethidium bromide (EtBr) (60:100 μg/mL of AO: EtBr) for 20 min in the dark (Kasibhatla et al., 2006).

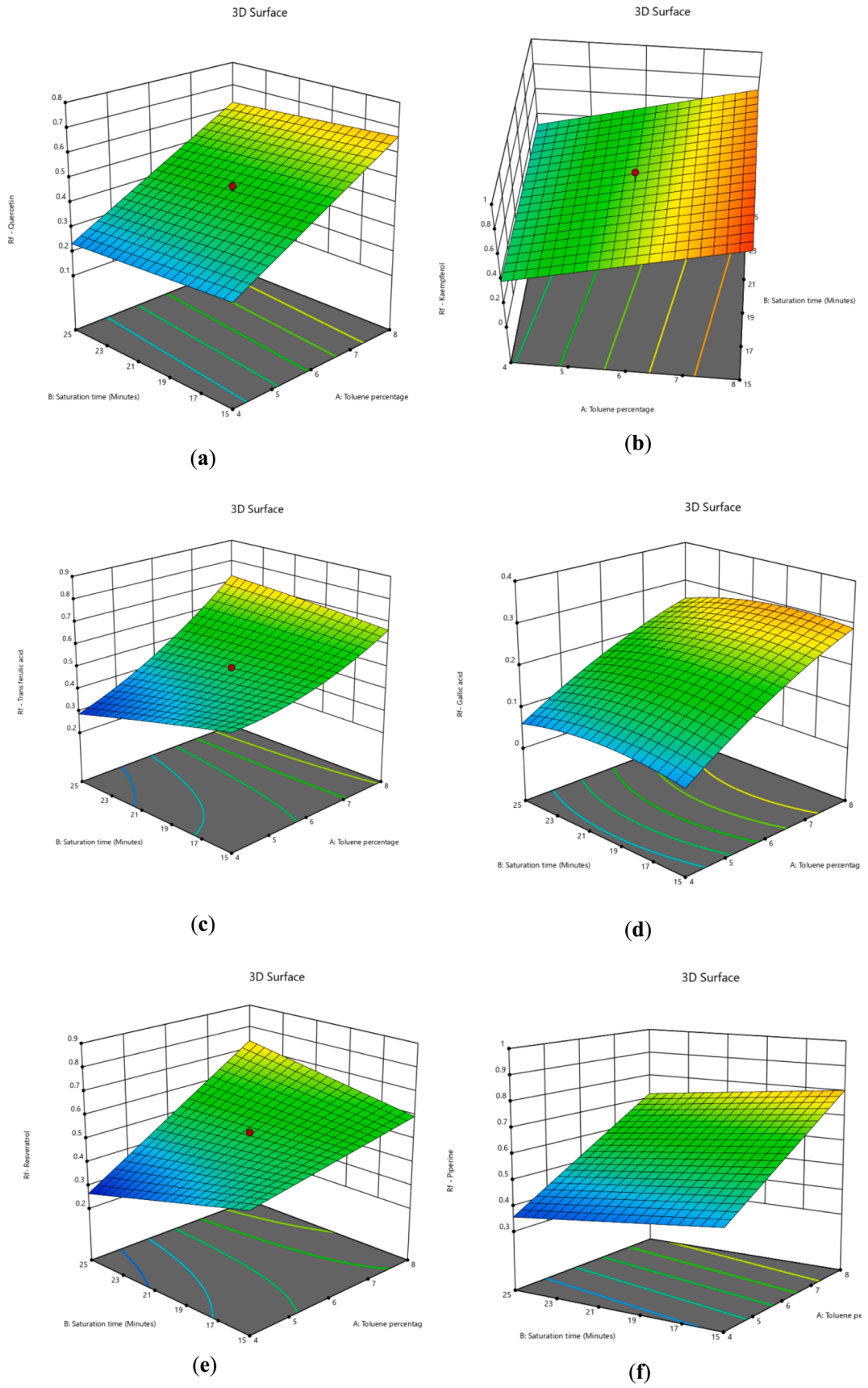


Fig. 5. 3D response surface plots of (**a**) Quercetin (**b**) Kaempferol (**c**) Trans ferulic acid (**d**) Gallic acid (**e**) Resveratrol (**f**) Piperine (**g**) Vanillin (**h**) Caffeine (**i**) Desirability.

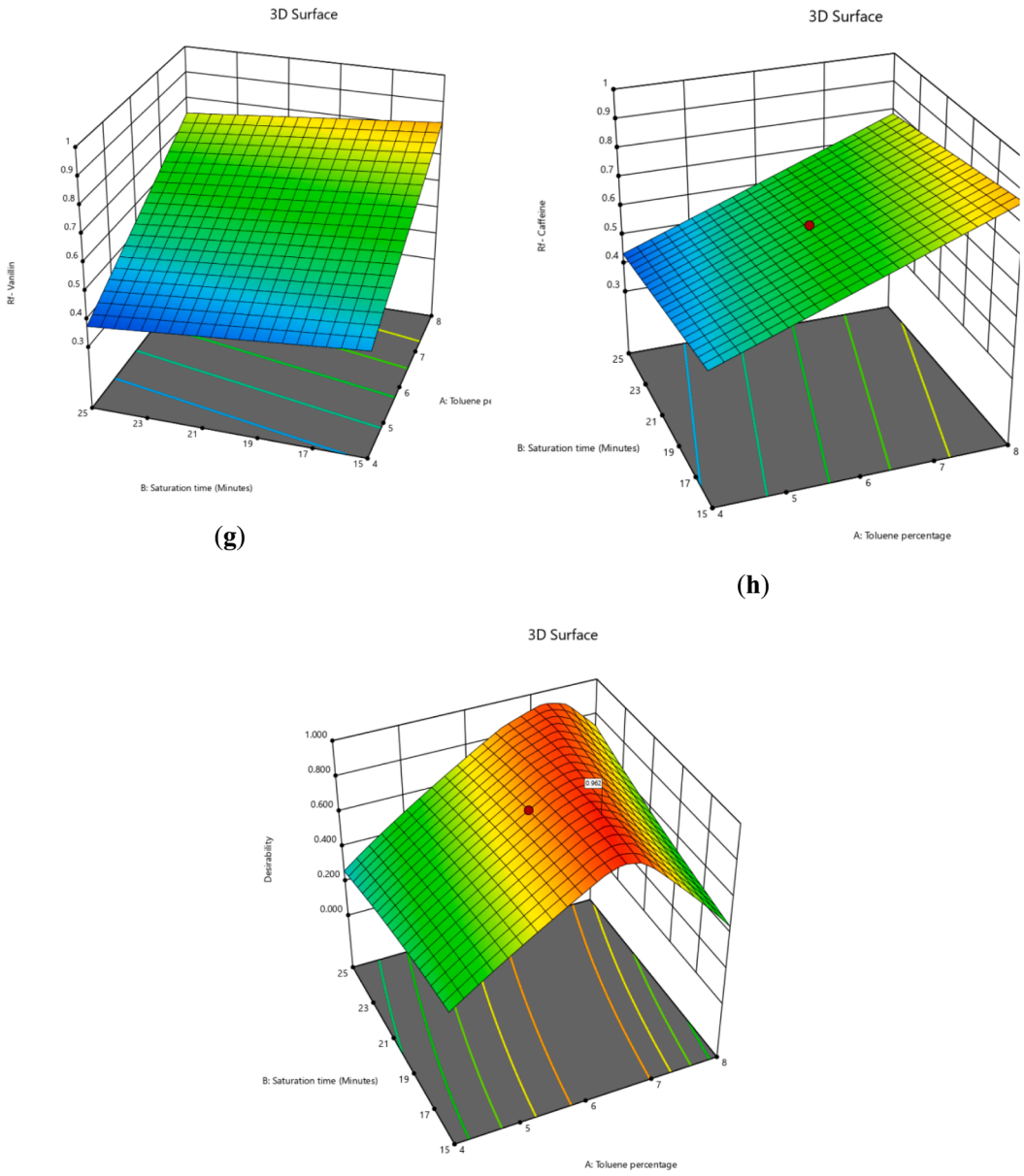


Fig. 5. (*continued*).

2.11.2. Intracellular ROS scavenging activity

Using THP-1 cells and a standardized approach with some minor adjustments(Ramachandran et al., 2018), intracellular reactive oxygen species (ROS) were detected with the help of the fluorescent dye DCFH-DA. The cells were grown in a 6-well plate at a density of 7 x 10^4 cells per well per milliliter of medium. Following the treatment with peroxide, the cells were subjected to the treatment with methanolic extract at the IC_{50} concentration. The H_2O_2 treatment on its own was employed to serve as a positive control for the experiment, and all of the groups were allowed to incubate for twenty-four hours. At the conclusion of the incubation period, the cells were subjected to 10 μM DCFH-DA for a period of 30 min. After that, the cells were collected, and the fluorescence intensity was determined using a BD FACS calibur (Ex/Em – 488 nm/530 nm). The analysis was carried out with the help of the BD Cell Quest Pro program.

3. Results

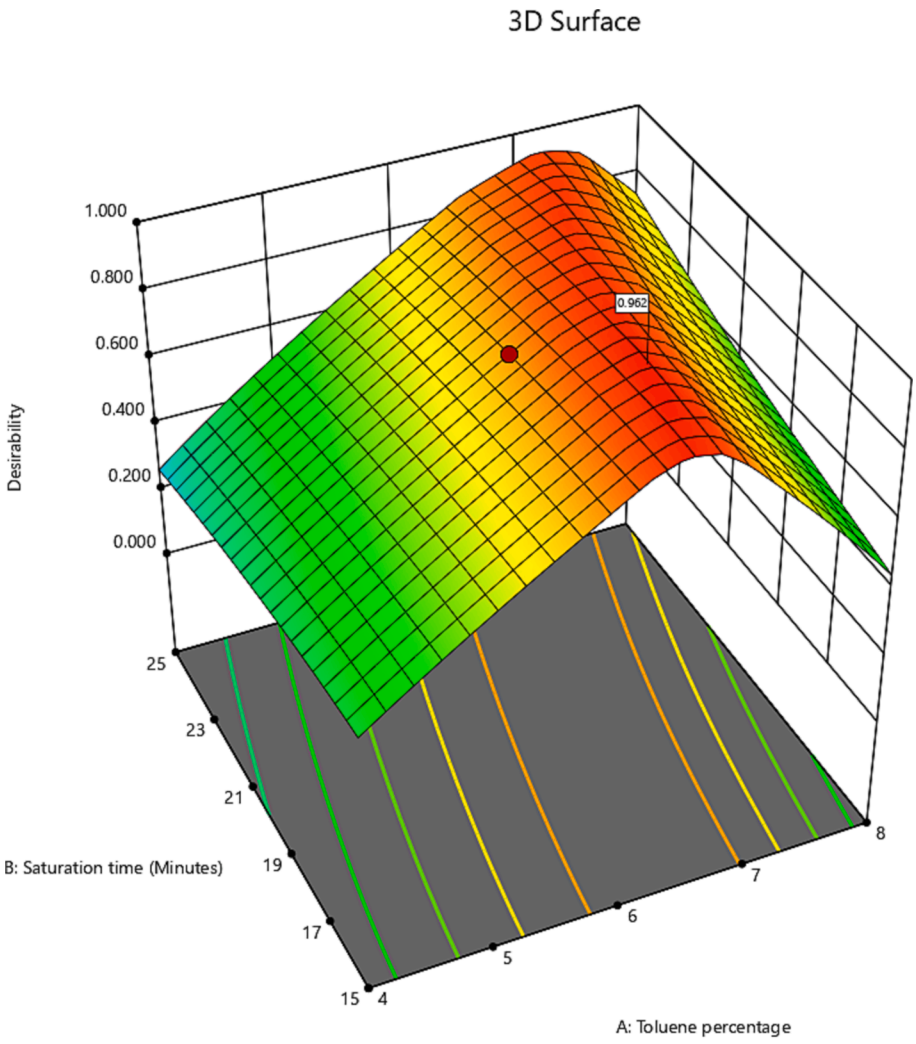
Using ultrasonicator bath method we obtained the percentage yield of 59 % w/w which was used for further analysis.

3.1. FTIR analysis

The FTIR spectrum was used to identify the functional groups present in the methanolic extract, which shows the hydroxyl, double bond, aromatic, carbonyl, nitrogen-containing compounds. The FTIR spectrum of 10 individual plant extracts along with mixture of extract were shown in Fig. 1.

3.1.1. Interpretation of IR

GC- Garcinia Cambogia



(i)

Fig. 5. (*continued*).

FT-IR (cm^{-1}): 3452(–OH), 3003 (Alkene C–H (Aliphatic)), 2850 (Alkane C–H), 1737 (Carbonyl group bound to ester, O-C = O), 1627 (Olefin group, C = C), 1213 (Carbonyl single bond stretch, C-O).

VV- Vitis vinifera

FT-IR (cm^{-1}): 3402 (–NH), 2933(Alkane C–H), 1730 (Aldehyde, C = O), 1639(Amide, N–H), 1537 (Nitro, N-O), 1350 Amine(C-N).

TA- Terminalia arjuna

FT-IR (cm^{-1}): 3439(–OH), 2939(C–H), 1716 (carboxylic acid), 1625 and 1595 (amines), 1051(Glycosidic linkage).

HA- Helianthus annus

FT-IR (cm^{-1}): 3338(–OH), 3008 (Alkene C–H), 2924 (Alkane C–H), 1746(Aldehyde, C = O), 1163(C–H, C-N).

CM- Commiphora mukul

FT-IR (cm^{-1}): 3404(–OH), 2968(–CH2, –CH3), 2843 (Lipid), 1452 (ester groups), 1112 (C-O-C & C–C-O vibration).

LU- Linum usitatissimum

FT-IR (cm^{-1}): 3309(–OH), 2926(Alkane C–H), 1741(-C = O), 1618 (C = C), 1417 ester (C–H), 1149(C–H, C-N).

RC- Rubia cordifolia

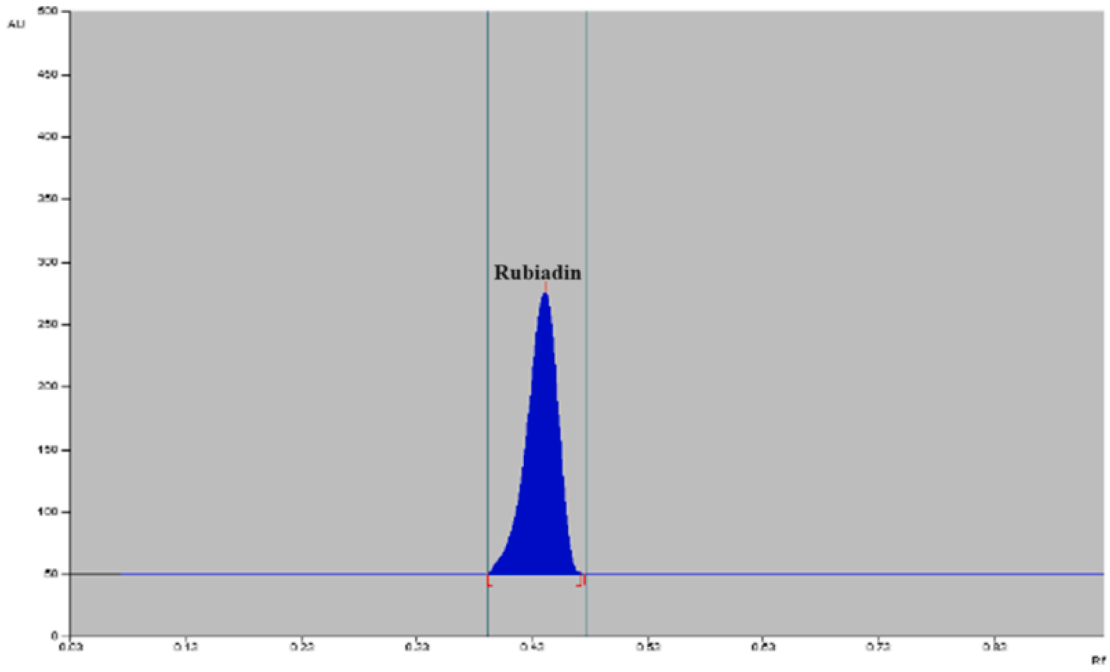
FT-IR (cm^{-1}): 3377(–OH), 2877(carboxylic acid (–OH)), 1743(-C = O), 1508, 1465 (Aromatic C–C).

AS- Allium sativum

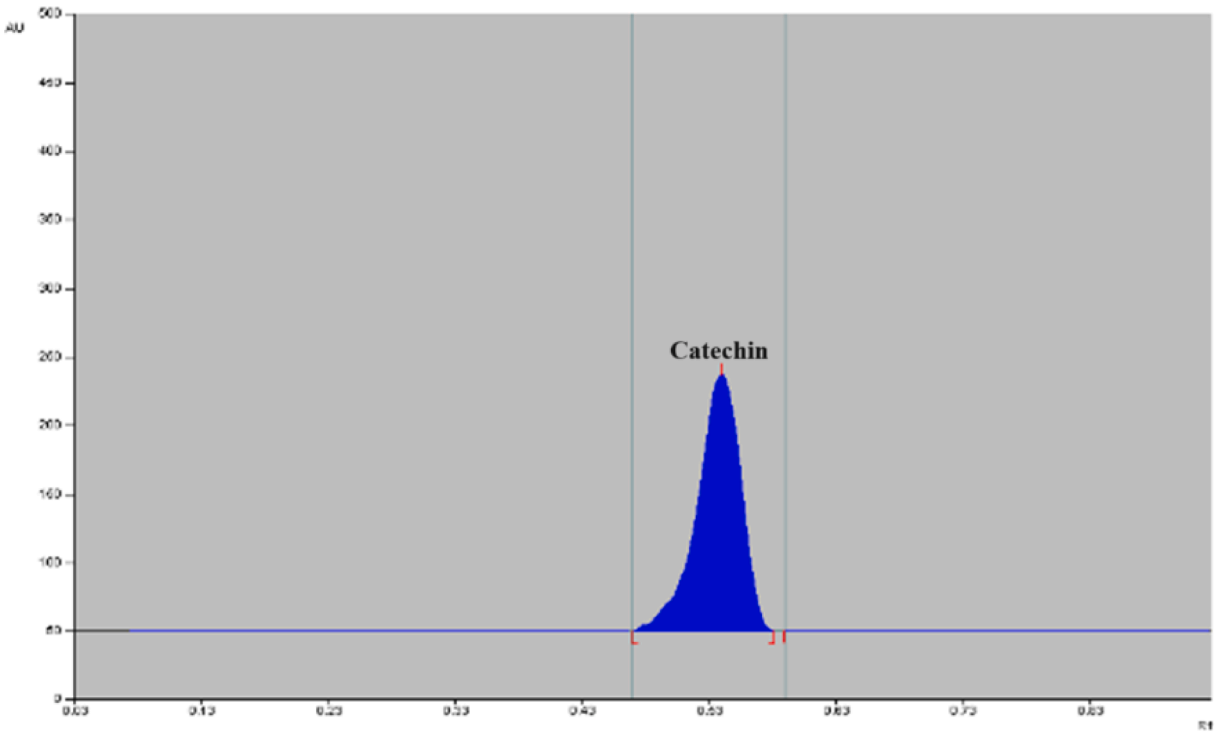
FT-IR (cm^{-1}): 3419(–OH), 2929 (Alkane C–H), 1643 (N–H), 1124 (C–H, C-N), 1053 (S = O).

AC- Acacia catechu

FT-IR (cm^{-1}): 3404(–OH), 2922(Alkane C–H), 1618(C = C alkenes),



(a)



(b)

Fig. 6. HPTLC chromatograms of standards and sample under UV 366 nm (a) Rubiadin (b) Catechin (c) Ellagic acid (d) PHF Sample.

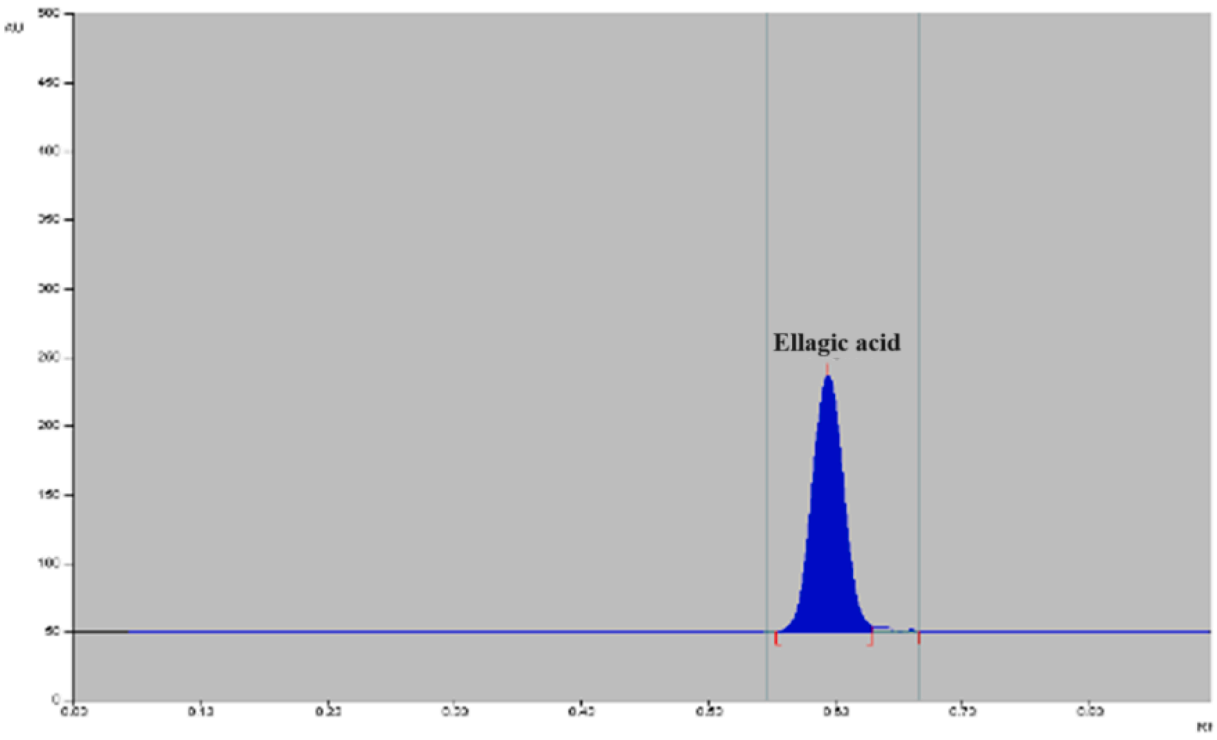
1523 (C = C aromatic ring), 1369 (Aromatic –OH), 1244 (-C-O alcohols).

PN- Piper nigrum

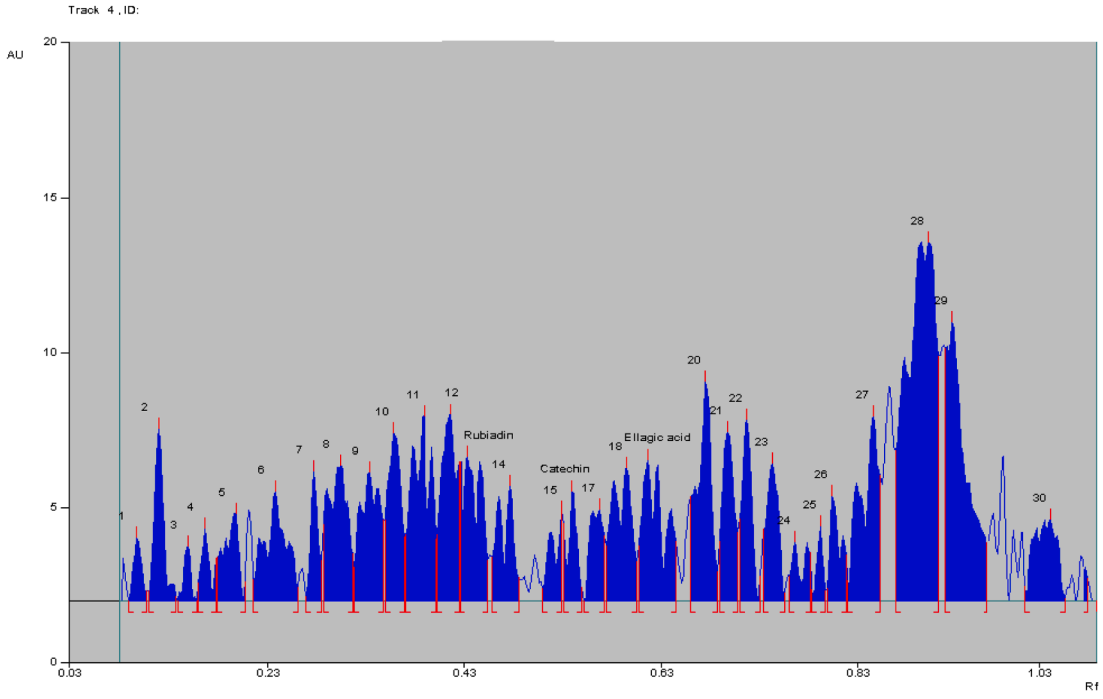
FT-IR (cm^{-1}): 3350(–OH), 2920, 2852(Alkane C–H), 1635, 1616 (amides and amines), 1253 (Aromatic –OH), 1195(C–H, C-N).

PHF- Poly herbal formulation

FT-IR (cm^{-1}):3380 (–OH), 3258(–NH), 3165(-Ar H), 1700(-C = O), 1619(-C = C), 1400(C–C Aromatic), 1193(C-O), 1126 (C–H, C-N), 869 (CH Aromatic), 601(C = C–H Alkyne).



(c)



(d)

Fig. 6. (*continued*).

3.2. LC-MS analysis

The HPLC chromatograms at four different wavelengths (215, 254, 280, and 366 nm) were scanned, recorded, and shown in the Fig. 2a, 2b, 2c, 2d. Table 3 lists the compounds detected their actual mass- to-charge ratios, chemical formulas, and retention time (Rt) in positive and negative ion mode in ESI. Ten compounds have been recognized in the extract of PHF such as Gallic acid ($m/z = 170.12$) (Fig. 3a), Alliin ($m/z = 177.90$) (Fig. 3b), Citric acid ($m/z = 192.20$) (Fig. 3c), Hydroxycitric acid ($m/z = 208.50$)(Fig. 3d), Resveratrol ($m/z = 228.40$) (Fig. 3e), Rubiadin ($m/z = 254.75$) (Fig. 3f), Kaempferol ($m/z = 286.05$) (Fig. 3g), Catechin ($m/z = 290.45$) (Fig. 3h), Quercetin ($m/z = 301.05$) (Fig. 3i),

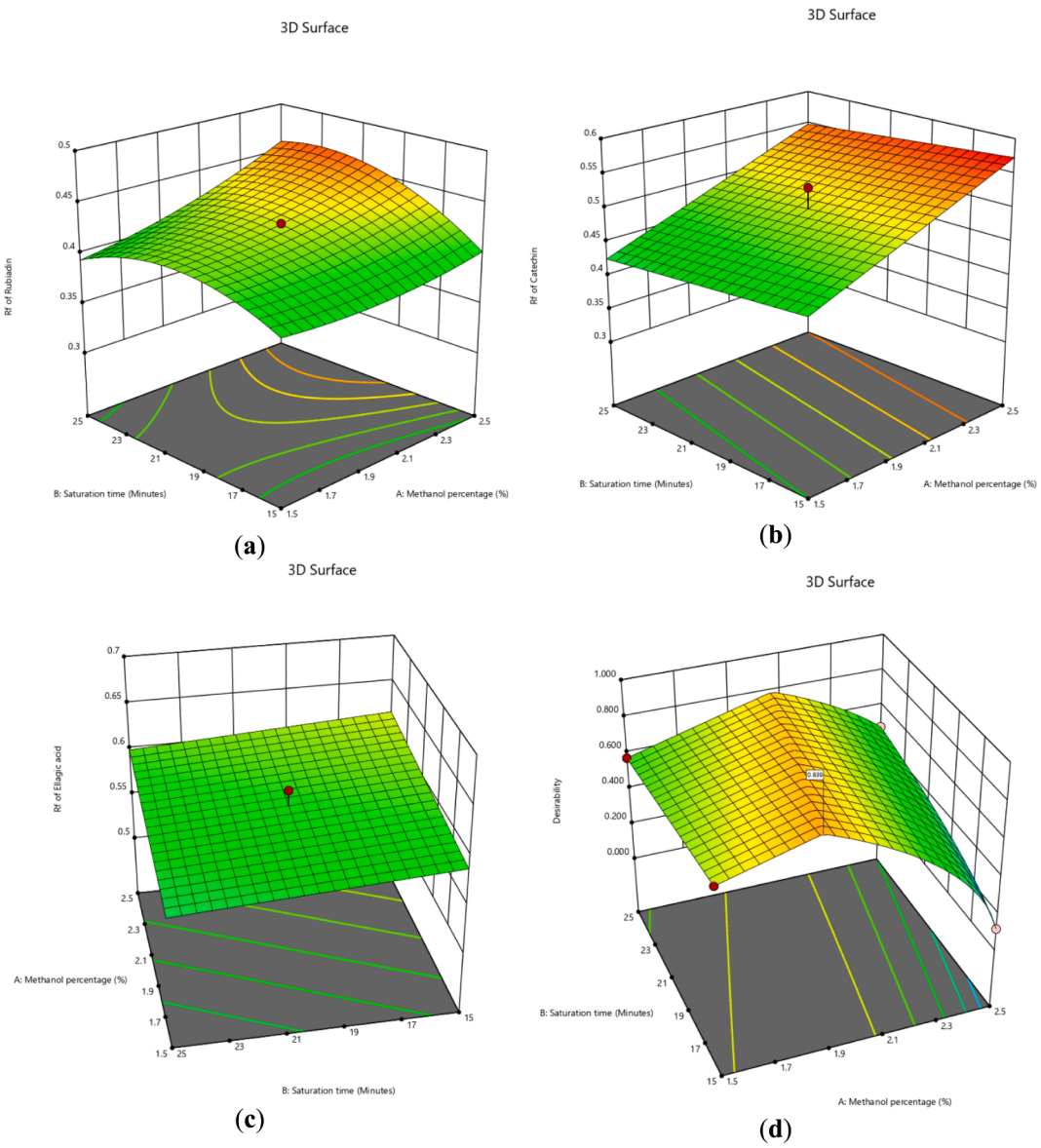


Fig. 7. 3D response surface plots of (a) Rubiadin (b) Catechin (c) Ellagic acid (d)Desirability.

Ellagic acid ($m/z = 302.75$) (Fig. 3j).

3.3. Quantification of eight biomarkers by HPTLC studies

Various combinations of toluene, ethyl acetate, and formic acid were used as the mobile phase on silica gel plates using high-performance thin-layer chromatography (HPTLC) technique based on AQbd-CCD approach. After experimentation, it was determined that a ratio of (6:3:0.8:0.2 v/v/v/v) resulted in satisfactory resolution. The diagram shown in Fig. 4 illustrates the visual representation of the data.The R_f values of quercetin, kaempferol, *trans*-ferulic acid, gallic acid, resveratrol, piperine, vanillin, and caffeine were determined to be 0.47, 0.63, 0.50, 0.21, 0.53, 0.58, 0.62, and 0.67, respectively. The peak areas of the standards (quercetin, kaempferol, *trans*-ferulic acid, gallic acid, resveratrol, piperine, vanillin, and caffeine) were compared to the peak area of the extract. Based on this comparison, the estimated content of quercetin, kaempferol, *trans*-ferulic acid, gallic acid, resveratrol, piperine, vanillin, and caffeine in the extract was found to be 0.1658 %, 0.7166 %, 0.2831 %, 0.4379 %, 0.3746 %, 0.3301 %, 0.1879 %, and 0.1191 % w/w, respectively. The correlation coefficient was found to be between 0.9946 and 0.09976 for standard concentration range of 40–120 mcg per ml.

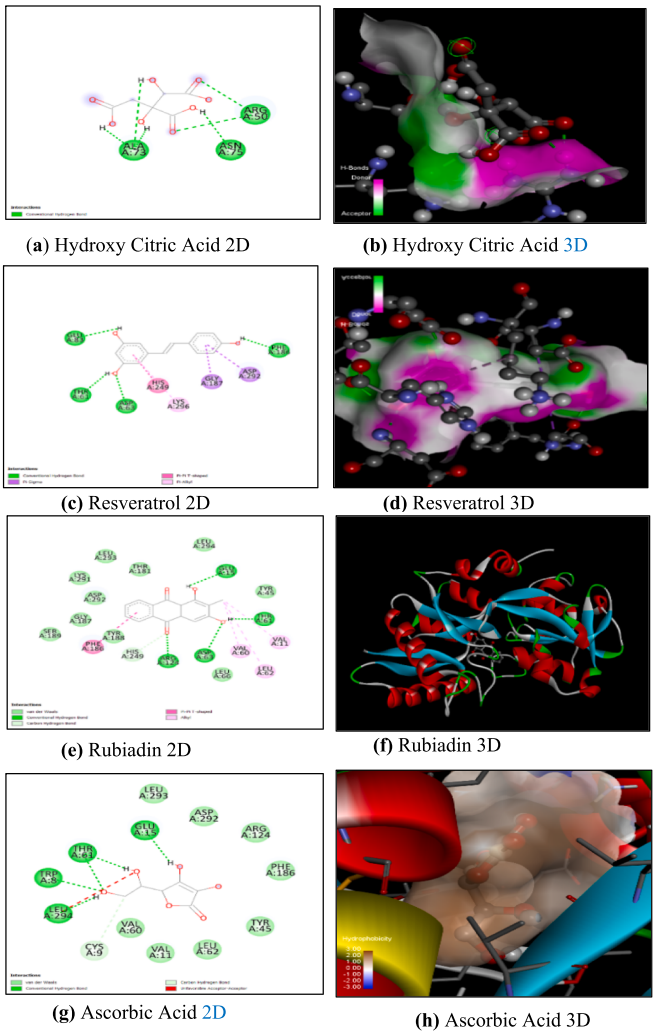
The desirabilty for the method optimised for eight markers quercetin, kaempferol, *trans*-ferulic acid, gallic acid, resveratrol, piperine, vanillin, and caffeine was found to be 0.962. (Fig. 5).

The Rf values of rubiadin, catechin and ellagic acid were determined to be 0.44, 0.53, and 0.61 respectively. Comparing to the peak area of the standards the content of rubiadin, catechin and ellagic acid in the extract was found to be 0.0399 %, 0.0532 %, and 0.100 % w/w, respectively. The correlation coefficient was found to be between 0.9974, 0.09993, and 0.9982 respectively for standards rubiadin, catechin and ellagic acid over the concentration range of 40–120 mcg per ml. (Fig. 6).

The desirabilty for the method optimised for three markers rubiadin, catechin and ellagic acid was found to be 0.839. (Fig. 7).

3.4. Bioactivity prediction and molecular docking

In order to find a possible candidate for antioxidant activity, molecular docking was done on active components derived by LC-MS fingerprinting analysis on the binding pocket of transferrin (PDB ID: 1D3K, 1D4N and 1 N84). Rubiadin was determined to have the highest



(a) Hydroxy Citric Acid 2D **(b)** Hydroxy Citric Acid 3D

(c) Resveratrol 2D **(d)** Resveratrol 3D

(e) Rubiadin 2D **(f)** Rubiadin 3D

(g) Ascorbic Acid 2D **(h)** Ascorbic Acid 3D

Fig. 8. 2D and 3D images of docked compounds against 1D3K.

Table 4
Binding energies and binding interactions of the active compounds with transferrin.

S. No	Compound code	Binding energies			Binding interactions with 1D3K
		1D3K	1D4N	1 N84	
1	Gallic Acid	−4.66	−5.19	−5.94	PRO A:308, LYS A:78, LYS A: 304, TRP A:264, ASP A:261
2	Alliin	−4.26	−5.45	−4.28	LYS A:307,PRO A:306
3	Citric Acid	−6.96	−6.5	−5.65	ARG A;308, PRO A:79
4	Hydroxy Citric Acid	−7.02	−5.85	−6.81	VAL A:305, VAL A: 80
5	Resveratrol	−7.25	−7.89	−6.99	GLU A:260
6	Rubiadin	−8.05	−7.96	−7.48	LEU A:293, LYS A:291,THR A:181, ASP A: 292, GLY A: 187, PHE A: 186, VAL A: 11, VAL A:60, LEU A:62
7	Kaempferol	−6.55	−6.87	−4.87	VAL A:81
8	Catechin	−6.48	−5.95	−5.64	PRO A:79
9	Quercetin	−6.4	−5.44	−7.77	LYS A: 304
10	Ellagic Acid	−6.78	−6.82	−7.52	ASP A: 292
11	Standard-Ascorbic Acid	−8.95	−7.52	−8.45	LEU A:293, LYS A:291,THR A:181, LEU A:62

docking score after the aforementioned optimization, followed by Resveratrol and hydroxycitric acid. These compounds were docked against the target enzyme transferrin and assessed based on their docking energies (Fig. 8). It is believed that substances with a dock score of 7.0 or nearer are a better reflection of antioxidant activity. Table 4 contains a list of the active compounds together with their corresponding binding energies.

3.5. Cell viability activity by MTT assay

The methanolic extract showed significant viability of 98.325 %, 93.121 %, 86.432 %, 79.264 %, 74.320 %, and 71.489 % respectively in THP-1 cells (Fig. 9). The IC_{50} concentration of the extract was 18.55 ± 0.51 µg/ml. Findings were reported as minimal cell death, which indicate that formulation was found to be safe.

3.6. Evaluation of cytotoxicity using AO/EtBr staining

The AO/EtBr staining technique was used to differentiate between viable and non-viable cells. The IC_{50} value of the PHF compound was evaluated on THP-1 human monocytic cell lines, and cell death was quantified using the Carl Zeiss Confocal Laser Scanning Microscope (CLSM 700). Following the completion of the treatment, the cells undergo staining with acridine orange (AO) and ethidium bromide (EtBr), and then undergo observation under confocal laser scanning microscopy (CLSM) to determine the proportion of viable cells (green) and non-viable cells (red) at wavelengths of 526 and 650 nm. Both the untreated THP-1 cells (referred to as the Positive Control) and the THP-1 cells treated with PHF exclusively absorbed AO, indicating the presence of living cells with intact membranes, as expected. The results presented in this study provide more evidence in favor of the MTT experiment, indicating that the presence of PHF does not have detrimental effects on the host cells, as seen in Fig. 10.

3.7. Intracellular ROS assay

THP-1 cells that had been labelled with DCFDA were employed in the FACS analysis in order to have a better understanding of the impact of the extract on the intracellular oxidative stress. The non-fluorescent DCFH-DA dye that readily penetrates into the cells becomes DCFH after being digested by intracellular esterase to form DCFH. This chemical that is confined within the cells further gets oxidized by peroxide. According to the results of the FACS study, the extract drastically cut down on the amount of free radical formation when it was present in their IC_{50} concentrations. When compared to cells that had just been treated with peroxide, THP-1 cells that had also been treated with an extract and a concentration of 50 µm of peroxide exhibited a substantial decrease ($p < 0.001$) in the fluorescence intensity. (Fig. 11).

4. Discussion

Ayurvedic and herbal medical preparations consist of a blend of botanical ingredients, each of which includes various chemical components that together contribute to the desired therapeutic effects. The simultaneous action of many herbal combinations might potentially target different physiological pathways, so offering comprehensive treatment. The burgeoning demand for Ayurvedic products is being driven by the increased interest in plant-based formulations(Bhope et al., 2011).Polyherbal formulations (PHFs) enhance patient comfort by reducing the need of consuming many individual herbal formulations simultaneously. This subsequently promotes increased compliance and therapeutic efficacy(Dubey & Dixit, 2023). The selected formulation comprises a compilation of ten plants (*Garcinia cambogia, Vitis vinifera, Terminalia arjuna, Helianthus annus, Commiphora mukul, Linum usitatissimum, Rubia cordifolia, Allium sativum, Acacia catechu, Piper nigrum*). *Garcinia cambogia* is a well-known plant that is used to cure obesity due to its major component hydroxy citric acid (HCA)(Sripradha et al., 2016). Flavonoids, phenolic acids, and resveratrol are among the polyphenol chemicals found in grapes (*Vitis vinifera*) which reduces the risk

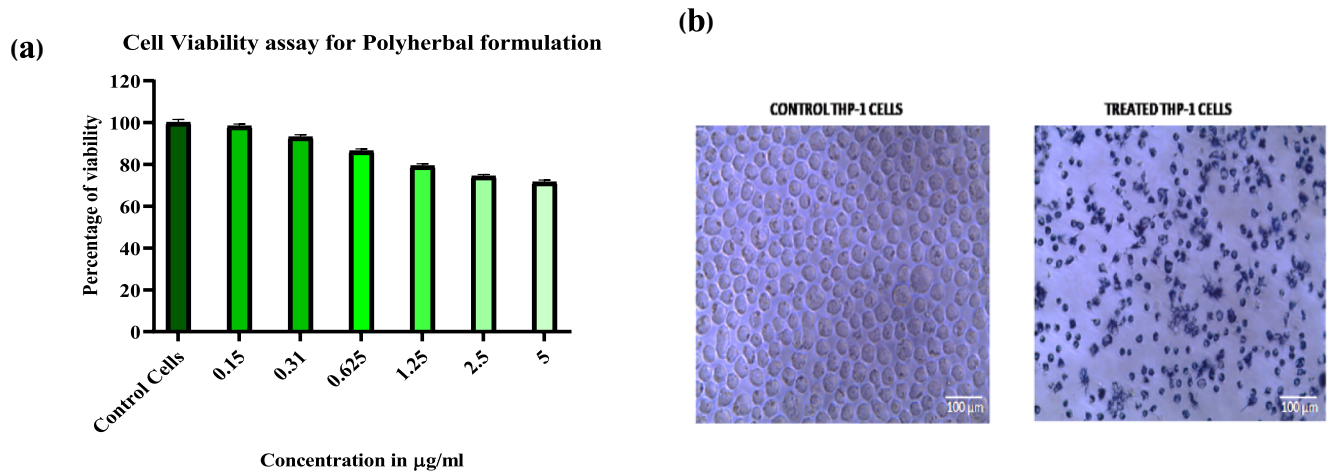


Fig. 9. Cell viability activity by MTT assay for polyherbal formulation (**a**) Graph showing viability of cells based on concentrations (**b**) Control and treated THP-1 cells under microscope.

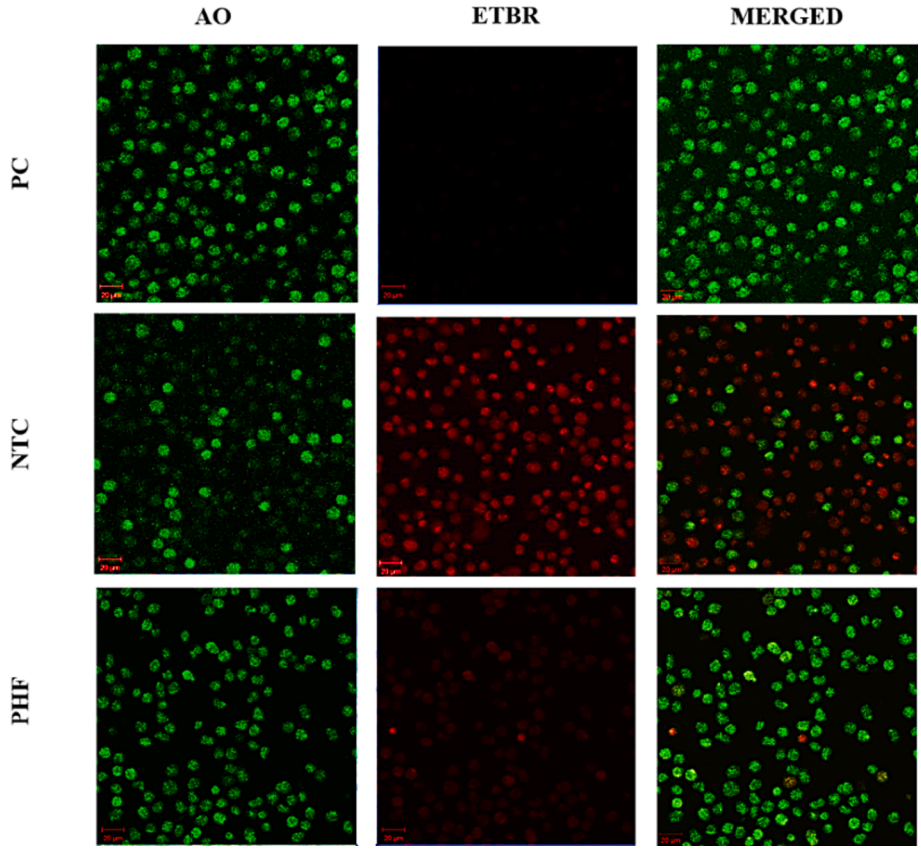


Fig. 10. CLSM images of live and dead cells by AO/ EtBr staining technique.

of stroke when added to our diet(Dohadwala & Vita, 2009). The bark of *Terminalia arjuna*, has been used to lower blood pressure and heart rate mainly because of presence of arjunic acid, arjunone, arjunolone, luteolin and derivatives(Mandal et al., 2013). *Helianthus annuus* L. has well known for its antioxidant, anti- inflammatory and cardiovascular benefits mainly because of gallic acid, coumaric, ferulic acid, and sinapic acids, flavonoids like heliannone, quercetin, luteolin, apigenin, kaempferol etc.,(Guo et al., 2017).

Commiphora mukul is effective in treating obesity, dyslipidemia, and inflammation. Guggulsterone is the main active compound present in this plant to show its biological activity(Kumar et al., 2019). *Linum usitatissimum*, generally known as flaxseed, is a plant that helps the body maintain healthy cholesterol and blood sugar levels due to alpha-linolenic acid(Dupasquier et al., 2007; Bassett et al., 2011). *Rubia cordifolia*, often known as Common Madder, has antioxidant and anti-inflammatory properties mainly because of rubiadin(Wen et al., 2022; Verma et al., 2016).Garlic (*Allium Sativum* L.), and its components such as alliin and allicin have been discovered to have significant antioxidant capabilities and are also utilized to treat atherosclerosis(Batiha et al., 2020). Chemical constituents include catechin, epicatechin, gallocatechin, catechin, catechutannic acid, kaempferol etc., are responsible for 7antioxidant activity in *Acacia catechu*(Baranitharan et al., 2021). *Piper*

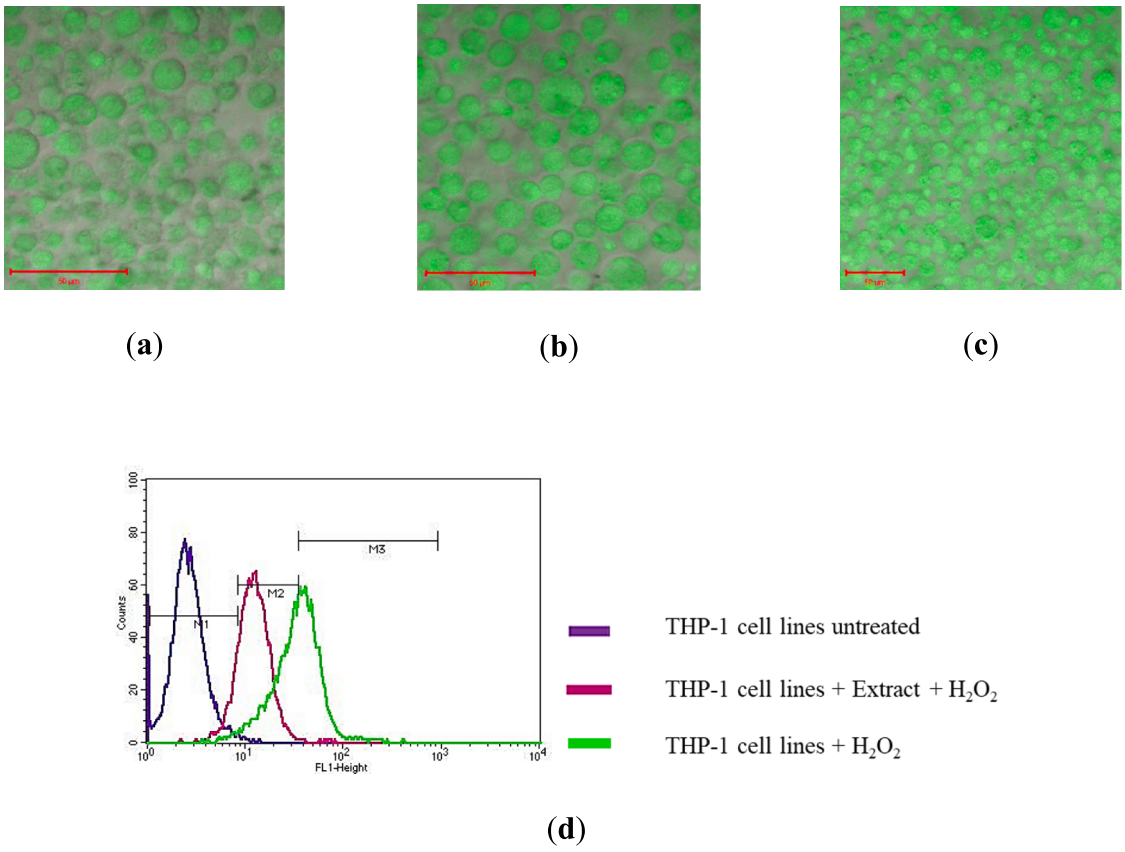


Fig. 11. Intracellular ROS activity of PHF in THP-1 cell lines CLSM images of (**a**) THP- 1 cells untreated (**b**) THP- 1 cells treated with PHF + H_2O_2 (**c**) THP- 1 cells treated with H_2O_2 (Positive control) (**d**) Overlay spectrum of all groups.

nigrum L., often known as Indian Long Pepper, was mainly used as antioxidant, antibacterial, anticonvulsant, and anti-inflammatory activity due to the presence of phytoconstituents such as piperine, 4-Carvacrol, Terpeneol, Piperonal(Ashokkumar et al., 2021).

Rashid claims that comparable species or variants are often intentionally or unintentionally replaced for or added to herbal medications. Standardization is used to validate the identity, quality, and purity of herbs and herbal products (Rashid et al., 2018). Using ultra wave assisted ultrasonic bath extraction procedure within less time and using less solvent we achieved best yield (59 %w/w). Then methanolic extract of formulated medicinal plant powder has undergone characterisation techniques like FTIR and LC-MS studies. The FTIR results states the functional groups present in the formulation in which mostly are aromatic compounds and some are hydroxyl, carbonyl, nitrogen-containing compounds. The LC-MS technique identifies ten phytocompounds such as Gallic acid, Alliin, Citric acid, Hydroxycitric acid, Resveratrol, Rubiadin, Kaempferol, Catechin, Quercetin, Ellagic acid based on *m/z* values. M. T. Celestino et al. suggested that establishing a correlation between antioxidant activity on tests in solution and computer simulations in solid oral formulations would be a useful way to predict how well a class of antioxidants would work and to make their use more efficient in the technology of pharmaceutical formulations (Celestino et al., 2012). So the identified compounds went for bioactivity prediction and molecular docking using transferrin enzyme. Most of the compounds has good binding energies with transferrin confirming that this formulation has significant antioxidant activity. Clarifying the composition of components is crucial since the effects of multibotanical formulations depend on the synergistic interactions between many parts of each herb rather than on the inclusion of each herb alone. HPTLC analysis is the main tool for confirming the presence of phytomarkers by comparing the R_f values with standard markers according to Attimarad M (Attimarad et al., 2011). Potential phytocompounds has been confirmed and quantified using novel methods. The application of the AQbD idea in the HPTLC process helps to increase the system's suitability. If a developed method is designed without applying the AQbD approach on either side, it may result in unsatisfactory method accomplishment and require revalidation. According to Barbara Steinhoff, it is crucial to evaluate the presence of toxic contaminants in the herbal components and preparations used in the development of herbal therapeutic products (Steinhoff, 2019). So, toxicity assessment in THP-1 cell lines through MTT assay results shows viability upto 24 hrs which scientifically proves that the formulation is safer for oral use. Further the live and dead cells were also studied using AO/EtBr staining technique in which higher percentage of green fluorescence concludes the safety aspect of the formulation. In addition intracellular ROS studies explains that the formulation has excellent antioxidant properties.

5. Conclusions

Multiherbal Churna was assessed for standardisation parameters using spectroscopic, and chromatographic parameters. FT-IR analysis of all extracts of the formulation confirmed the presence of – OH functional groups among phytochemical compounds, indicating the possibility of an antioxidant nature. An LC-MS fingerprinting investigation of ten phytocompounds revealed that they had docked with the human transferrin protein. Antioxidant activity, which can be utilised to treat atherosclerosis, is controlled by this protein. The ten phytocompounds for interactions with protein ligands were determined using docking scores nearer or above 7.0. The analytical standards for the product were developed with reference to quality-based raw ingredients. According to the HPTLC findings eight markers namely quercetin, kaempferol, Transferulic acid, Gallic acid, Resveratrol, Piperine, Vanillin, Caffeine was

quantified simultaneously and reported for the first time in the novel formulation. Additionally, the MTT test was performed in THP-1 cell lines in consideration of the toxicity profile, and it indicated cell viability of up to 98 % for 24 h, proving the formulation's non-toxicity. Intracellular ROS assay proves the antioxidant action of the formulation. Since the bioactive chemicals present in the extracts may have synergistic effects with other substances and demonstrate a greater therapeutic potential, further preclinical research is currently being conducted to support the notion that the observed results would be significant for upcoming clinical aspects. As a result, this study offers standardised and potentially therapeutic information about effective polyherbal formulations for atherosclerosis.

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CRediT authorship contribution statement

Rayadurgam Sireesha: Conceptualization, Methodology, Software, Data curation, Supervision. **Manikandan Krishnan:** Conceptualization, Validation, Formal analysis. **Muhammad Sadiq:** Investigation. **Munuswamy Ramanujam Ganesh:** Validation, Visualization. **Chinnaperumal Kamaraj:** Validation, Writing – original draft. **Naiyf S. Alharbi:** Writing – review & editing, Project administration. **Muthu Thiruvengadam:** Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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