Extraction And Evaluation of Herbs Having Antimicrobial and Antioxidant Activity

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Abstract
The present study investigates the pharmacological potential of a polyherbal extraction (PHE) designed for the treatment and protection of normal cells through antioxidant mechanisms. Extracts from ten medicinal plants were individually prepared using double-distilled water, with Terminalia chebula (17.5%) and Emblica officinalis (13.5%) incorporated in higher proportions to enhance therapeutic efficacy. In phytochemical screening observed constituents like flavanoids, tannins, glycoside, alkaloids, all of which are known to contribute to antimicrobial and antioxidant activities. Antibacterial efficacy was assessed using agar disc diffusion method, with Ofloxacin as the reference standard. Extraction demonstrated pronounced inhibitory activity against gram-positive bacteria (16.7 mm), while moderate inhibition with gram-ve strains, Escherichia coli and Proteus (15.3 mm). Quantitative estimation of phytochemicals revealed that TPC and TFC measured by Folin–Ciocalteu, and aluminium chloride colorimetry, were strongly correlated (r = 0.97), suggesting that flavonoids constitute a major fraction of phenolic compounds. Antioxidant potential was comprehensively evaluated using multiple assays, including ABTS, DPPH, FRAP, and β-carotene bleaching. Free radical scavenging, thereby accounting for differences in radical species and reaction mechanisms. The results highlight that antioxidant activity is a multifactorial process influenced by the synergistic interplay of diverse phytoconstituents. Collectively, these findings support the therapeutic promise of the PHE as both an antibacterial and antioxidant agent, providing a scientific basis for its potential application in managing skin infections and protecting cellular integrity.

Keywords: Poly-herbal extraction. Antibacterial activity. Antioxidant assays. Phenolic content. Flavonoid correlation.

Introduction

Antibiotic resistance has become one of the most critical global health concerns, with pathogens such as Staphylococcus, Streptococcus, Pseudomonas, and members of the Enterobacteriaceae family rendering many conventional drugs ineffective[1]. Continuous usage of antibiotics contributing to high mortality rates, particularly among immunocompromised patients in hospital environments[2]. As a result, increasing attention has been directed toward herbs, which are rich in biometabolite demonstrated the antibacterial and antioxidant properties[3]. Traditional remedies, long embedded in community health practices, now serve as valuable resources for drug discovery and development, with nearly 70% of modern medicines in India derived from natural products[4].Polyherbal extractions (PHEs) are gaining attention for their low toxicity, affordability[5]. However, the assumption that traditional PHEs are inherently safe and effective is misleading, and rigorous scientific validation is essential[6]. Recent studies highlight the need for conservation of medicinal plants and scientific validation of therapeutic claims to ensure sustainability and safety[7]. In this study evaluated a polyherbal extraction composed of ten medicinal plants for its antibacterial and antioxidant potential. We hypothesize that synergistic interactions among phytochemicals in this formulation enhance its therapeutic efficacy. By combining phytochemical screening, antibacterial assays, and antioxidant evaluations, this work aims to provide scientific evidence supporting the role of PHEs as promising alternatives in combating resistant infections. Importantly, this study contributes novel quantitative data on the correlation between phytochemical composition and biological activity, thereby advancing the understanding of how polyherbal extractions can be optimized for modern therapeutic use[8].

Materials And Methods

Plant Extract[9]

On the basis of ethnomedicinal knowledge, tribal practices, pharmaceutical applications and literature survey, ten medicinal plants[10] were selected for the present study and these plants involved in identification of antimicrobials and antioxidants in the form of bioconstituents[11]. Specific plant parts used were as follows:

- Sapindus trifoliatus — pericarp of dried ripe fruits
- Emblica officinalis — whole plant
- Andrographis paniculata — whole plant
- Achyranthus aspera — root
- Eclipta prostrata — leaf
- Curcuma longa — rhizome
- Azadirachta indica — leaf
- Terminalia chebula — whole plant

- Allium sativum — clove
- Pongamia pinnata — leaf

The selected parts were thoroughly cleaned, shade-dried, and powdered prior to extraction. Each plant material was extracted separately using double-distilled water by heat reflux extraction (HRE) for 2 hours. This technique increases solvent temperature, enhances diffusion of active compounds, and improves extraction efficiency. However, prolonged heating may degrade heat-sensitive constituents, so process optimization is essential[12].

Preparation of Formulation

Based on literature reports, Andrographis paniculata exhibits strong antimicrobial activity[13], while Allium sativum has demonstrated potent antimicrobial properties in recent studies[14]. Similarly, Terminalia chebula shows significant antioxidant potential in animal models[15], and Emblica officinalis possesses high flavonoid and phenolic content represented with antioxidant activity[16]. Considering these findings, extracts were combined in specific proportions to develop an extraction effective against skin infections.

The composition was as follows:

The formulation prepared as per the following:

- Extract of Sapindus trifoliatus- 10%
- Extract of Emblica officinalis- 13.5%
- Extract of Andrographis paniculate- 12.5%
- Extract of Achyranthus aspera- 3%
- Extract of Eclipta prostrata- 5%
- Extract of Curcuma longa- 12.5%
- Extract of Azadirachta indica- 10.5%
- Extract of Terminalia chebula- 17.5%
- Extract of Allium sativum- 12.5%
- Extract of Pongamia pinnata- 3%

Qualitative Chemical Tests for Phytoconstituents in Polyherbal Extraction

Initial phytochemical screening is an essential step in assessing polyherbal extractions, as it highlights key secondary metabolites. These constituents are often linked to therapeutic properties, particularly antimicrobial and antioxidant activities. For
instance, investigations on Goniothalamus velutinus confirmed notable levels of phenolics and strong antioxidant potential in bark and leaf extracts[17]. Likewise, Canarium patentinervium demonstrated diverse phytochemicals associated with antimicrobial and antioxidant effects[18]. Screening of herbs traditionally treat malaria revealed rich phytochemical profiles that correlated with significant antioxidant activity[19]. Collectively, scores importance of qualitative chemical tests in validating efficacy of polyherbal extractions and guiding their therapeutic application.

Antibacterial Screening

Nutrient agar medium was used for antibacterial screening. Test organisms were obtained from Pharmaceutical Biotechnology Lab, CMR College of Pharmacy. Staphylococcus aureus NCIM 2079, Bacillus subtilis NCIM 2718 were selected as grampositive bacteria and Escherichia coli, Proteus vulgaris NCIM 2027 as gramnegative bacteria.

Working conditions All procedures were performed in a horizontal laminar air-flow hood under aseptic conditions. Air sampling was conducted by exposing sterile nutrient agar plates inside the hood; absence of microbial growth after incubation confirmed sterility. Inocula were prepared and incubated overnight at 37 °C.

Test materials

Test: polyherbal extract
Standard drug: ofloxacin (INN)
Solvent: sodium hydroxide

Nutrient agar poured into petridishes aseptically to a thickness of 4–5 mm. After solidification, dishes were inverted to prevent condensation and dried at 37 °C before inoculation. Standardized inocula were applied using sterile swabs streaked on the agar surface in three directions, by 60° each time. Inoculated plates were allowed to dry at room temperature. Sterilized discs dipped in the PHE, ofloxacin and sodium hydroxide were kept on the agar surface, and refrigerated for 60min to ensure uniform diffusion. Triplicates of PHE prepared and incubated at 37 °C for 18–24 hours, ZOI compared with the standard, following established protocols for the Kirby–Bauer disc diffusion method[20].

Determination of Total Phenolic and Flavonoid Content

The method used to study TPC of extract was Folin–Ciocalteu (FC), a widely accepted assay for quantifying dietary polyphenols due to their antioxidant relevance[21]. Standard prep of gallic acid (50–250 µg/mL) was used to construct a calibration curve. For the assay, 1 mL of gallic acid solution was mixed with 5 mL of FC reagent

and allowed to stand for 5 minutes, and absorbance was measured by UV–Visible spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Japan) at 765 nm. In the same way 1 mL of polyherbal extraction (PHE) was used in place of gallic acid. Results were expressed GAE, consistent with established protocols in herbal extraction studies[22].

Total Flavonoid Content

Aluminium chloride colorimetric method with a digital colorimeter was used to measure TFC of individual herbal extracts and PHE (Labomed Inc., USA). Catechin solutions (6.25–200 µg/mL) were prepared for calibration. Flavonoid–aluminium complex absorbance was measured at 510 nm. 1 mL of herbal extract or PHE at 1 mg/mL used as test samples.

Antioxidant Assays

Free radicals, which play a critical role in biological damage and disease progression are neutralised by antioxidant activity of herbal extracts[23]. Since no single assay can fully represent the antioxidant profile, multiple in vitro methods were employed to provide a comprehensive evaluation[24]. Radical neutralizing capacity of the polyherbal extraction (PHE) was assessed using ABTS, DPPH, FRAP, and free radical scavenging assays.

ABTS assay Test samples at varying concentrations were dispensed in triplicate into a 96-well microtiter plate. Trolox and ascorbic acid served as positive controls. The $ABTS^{\bullet+}$ solution was prepared, kept in the dark for 12–16 hours, diluted with ethanol to get an absorbance of 0.65 ± 0.01 at 734 nm at 37 °C. For the assay, 100 µL of $ABTS^{\bullet+}$ solution was added to 100 µL of extract or PHF and kept at room temperature for 7 minutes. Antioxidant activity was observed spectrophotometrically (Shimadzu UV-1800, Shimadzu Corporation, Japan) at 734 nm.

DPPH radical scavenging assay The DPPH assay was conducted. Aliquots of extracts and PHE were dispensed in triplicate into a 96-well plate. DPPH solution of 0.1Mm mixed with methanol serving as the control. Plates were shaken for 120 s, kept in dark for 30 minutes. Absorbance was measured at 550 nm, EC_{50} values were determined by plotting scavenging activity against concentration.

FRAP assay: FRAP reagent 180µL was added to 6.25 µg/ml of extract and incubated for 30 minutes. Absorbance was recorded at 593 nm. Results were expressed as µM $FeSO_4$ equivalents per µg of sample.

Free radical scavenging assays Hydroxyl and nitric oxide radical scavenging activities were evaluated. For hydroxyl radical scavenging, extracts and PHE at concentrations of 15.625–250 µg/mL were mixed with $FeCl_3$ (1 mM), H_2O_2 (0.17 M), phosphate buffer (0.2 M, pH 7.8) and 1,10-phenanthroline (1 mM) . Incubated for 5 minutes, absorbance was measured at 560 nm. Scavenging activity was calculated as:

Hydroxyl scavenging (%) $=(A_0-A_1/A_0) \times 100$

Phosphate-buffered saline (pH 7.4) with sodium nitroprusside (10 mM), used for nitric oxide scavenging was incubated with extracts, ascorbic acid, or butylated hydroxytoluene (BHT) for 150 minutes at 25 °C. Followed by sulfanilic acid and naphthyl ethylenediamine dihydrochloride (0.1% w/v) again incubated for 30 minutes at room temperature, and absorbance was measured at 540 nm.

Pearson correlation coefficient analysis was used to evaluate relationships between phytochemical contents (TPC, TFC) and antioxidant activities.

$$r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\bar{x})^2}\sqrt{\sum(y-\bar{y})^2}}$$

Student's t-test, and p-values < 0.05 were considered statistically significant.

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

Result

Phytochemical screening of PHE emphasized the presence of secondary metabolites as summarized in **table 1** below.

Table 1 Qualitative chemical tests for phytoconstituents in the polyherbal formulation

S.No	Chemical constituents	Name of the Test	Methodology	Result
1	Test for Steroids, Triterpenes	1.Salkowski	Few drops of concentrated sulfuric acid added to the Polyherbal formulation. Shaken.	positive
		2.Liebermann Burchard	few drops of acetic anhydride are added and mixed well. One ml of concentrated sulphuric acid is added from sides of test tube.	positive
2	Test for Saponins	1.Foam	small amount of extract shaken with little quantity of water.	positive

		2.Haemolysis	To the 2ml of 1.8% of sodium chloride solution, 2ml of distilled water is added to 2 ml of 1% extract to the 5 drops of blood is added to each tube and gently mixed with the contents.	
3	Test for steroidal saponins		The extract is hydrolyzed with sulphuric acid and extracted with chloroform. The chloroform layer is tested for steroids.	negative
4	Test for triterpenoid saponins		The extract is hydrolyzed with sulphuric acid and extracted with chloroform. The chloroform layer is tested for steroids.	negative
5	Test for alkaloids	1.Mayers	The acid layer was mixed with Mayer's reagent(potassium mercuric iodide solution)	positive
		2.Dragon dorf	the acid layer with few drops of Dragon Dorf's reagent (potassium bismuth iodide)	
		3.Wagner	the extract was heating with Wagner's reagent	
		4.Hager	the acid layer was mixed with few drops of Hager's reagent (saturated solution of picric acid)	
6	Test for carbohydrates	1.Fehling	the extract was heated with Fehling's A and B solutions	negative
		2.Molisch	the extract was treated with Molisch's reagent and concentrated sulphuric acid added along the sides of the test tube.	
		3.Bendict	the extract was heated with Benedict's reagent	
		4.Barford	reagent was added and boiled on a water bath for few minutes	
7	Test for flavanoids	1.Shinoda	the alcoholic solution of few fragments of magnesium ribbon and concentrated hydrochloric acid added to the extract	positive
		2.Ferric chloride	few drops of neutral ferric chloride solution are added to little quantity of alcoholic extract	
		3.Lead acetate	few drops of lead acetate solution are added to the alcoholic solution	
		4.Zinc hydrochloric acid reduction	the alcoholic solution is treated with pinch of zinc dust and few drops of concentrated hydrochloric acid	
8	Test for tannins	1.Ferric chloride	1% ferric chloride solution added to the extract	positive
		2.Gelatin	the extracts are treated with 1% solution of gelatin containing 10% sodium chloride	
9	Test for glycosides	1.Baljit's	to the extract, sodium picrate solution is added	positive
		2.Legal	the extract is dissolved in pyridine, sodium nitroprusside solution is added	
		3.Keller- Kilian	the extract was dissolved in glacial acetic acid and after cooling,2 drops of ferric chloride solution are added to it. These contents are transferred to a test tube containing 2ml of conc. sulphuric acid.	

Antimicrobial activity of polyherbal formulation of selected medicinal plants:
The polyherbal formulation exhibited significant ZOI ranging from 15 mm to 35 mm as illustrated in Figure 1.

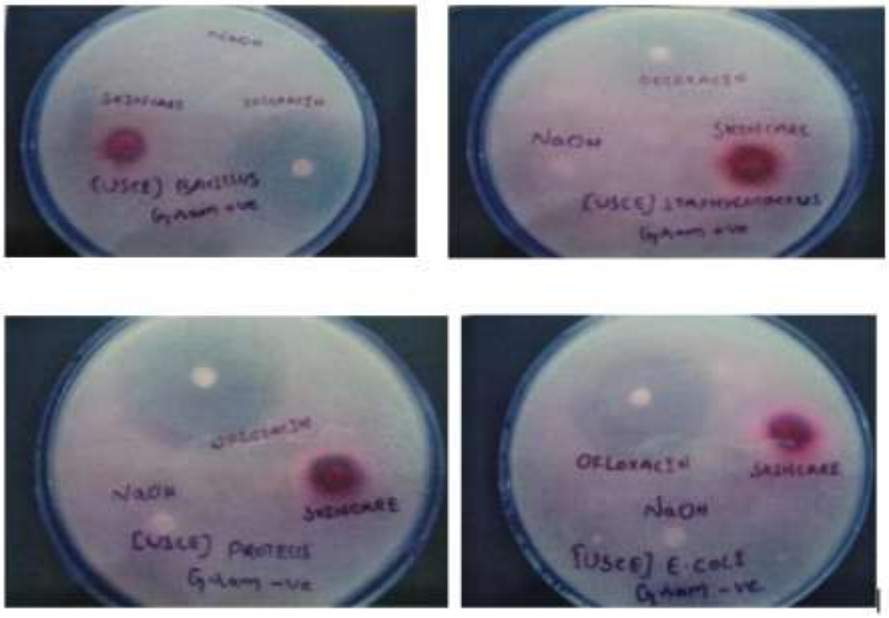

Figure 1 zone of inhibition of Bacillus, Proteus, Staphylococcus and Ecoli

As shown in **Table 2** and **Figure 2**, Ofloxacin(INN) was used as the standard antibiotic in this study. It exhibited the highest zone of inhibition (3.23 cm) against Staphylococcus. In comparison, the polyherbal extraction (PHE) demonstrated greater activity against Gram+ve bacteria (1.67 cm) compared to Gram-ve bacteria (1.33 cm).

Table 2 Diameter of zone of inhibtion of polyherbal extract

Test organism	Diameter of zone of inhibition(cm)											
	Ofloxacin (0.5ml)			Avg	Extract(0.5ml)			Avg	Sodium (0.5ml) hydroxide			Avg
	1	2	3		1	2	3		1	2	3	
Gram positive bacteria: 1. Bacillus	2.7	2.7	2.8	2.73	1.5	1.6	1.7	1.60	-	-	-	-
2. Staphylococcus	3.2	3.3	3.2	3.23	1.6	1.7	1.7	1.67	-	-	-	-
Gram negative bacteria: 1. E. coli	2.8	2.8	2.7	2.73	1.3	1.2	1.5	1.33	-	-	-	-
2. Proteus	2.7	2.8	2.8	2.77	1.6	1.4	1.6	1.53	-	-	-	-

TPC and TFC of individual plant extracts and the polyherbal extract (PHE) were quantified GAE and CE, respectively, as presented in **figure 2**. TPC values ranged from 9.84 ± 0.37 mg GAE/g in Achyranthus aspera to 69.28 ± 0.84 mg GAE/g in Terminalia chebula. Similarly, TFC values varied from 42.32 ± 1.29 mg CE/g in Achyranthus aspera to 210.36 ± 1.48 mg CE/g in Terminalia chebula. Both TPC and TFC analyses revealed that PHF contained the highest levels of phenolic and flavonoid compounds (65.43 ± 0.74 and 200.43 ± 1.45), followed by Terminalia chebula and Emblica officinalis.

In recent decades, numerous assays have been developed to assess antioxidant potential. However, a single method is often inadequate to fully characterize antioxidant capacity, as variations in reaction mechanisms, radical species, and assay conditions can influence outcomes. Therefore, in vitro assays including ABTS, DPPH, BCB, FRAP, hydroxyl radical, and nitric oxide radical scavenging tests were conducted to study the antioxidant potential of PHE. Antioxidant assays are classified as single electron transfer (SET) and hydrogen atom transfer (HAT) methods. To measure the capacity of antioxidants to donate electrons and reduces target compounds SET assay was employed, whereas HAT assays assess their capacity to quench reactive oxygen species (ROS) by donating hydrogen atoms. The FRAP assay operates via the SET mechanism, the BCB assay is HAT-based, while DPPH and ABTS assays incorporate both SET and HAT principles.

ABTS, DPPH, and BCB were performed for individual herbal extracts and the polyherbal extraction (PHE), as presented in **figure 2**. Ascorbic acid(INN) and Trolox served as standards.

ABTS assay: The capacity of antioxidants to quench the blue-green $ABTS^{\bullet+}$ cation radical at 734 nm was measured by ABTS assay, resulting in its conversion to the colorless ABTS form. IC_{50} values ranged from 3.05 ± 0.34 µg/mL for ascorbic acid to 89.54 ± 0.65 µg/mL for Pongamia pinnata. Among the tested herbs, Terminalia chebula (IC_{50}: 14.74 ± 0.27 µg/mL) and Emblica officinalis (IC_{50}: 27.49 ± 0.73) exhibited the strongest radical scavenging activity. The PHE demonstrated moderate activity 35.63 ± 0.85 µg/mL.

β-carotene bleaching assay (BCB) β-carotene bleaching was done by hydroperoxide radicals produced by oxidation of linoleic acid. Antioxidants slow this decolorization by competing with β-carotene for radical scavenging. The PHE primarily acted via a hydrogen atom transfer (HAT) mechanism, with an IC_{50} of 98.07 ± 4.43 µg/mL, second only to Trolox (IC_{50}: 2.63 ± 0.22). Ascorbic acid, Achyranthus aspera, and Pongamia pinnata did not inhibit β-carotene bleaching, indicating limited activity against linoleate-derived radicals.

DPPH assay The yellow α, α-diphenyl-β-picrylhydrazine was observed through reduction of purple DPPH. The PHE showed moderate act with an IC_{50} of 175.83 ± 9.64. Lower IC_{50} values observed with ABTS assay compared to the DPPH assay.

FRAP assay (**Figure 2**) revealed that Terminalia chebula exhibited the strongest reducing capacity (24.25 ± 1.53 µM $FeSO_4$/µg), followed by Curcuma longa (20.23 ± 2.78 µM $FeSO_4$/µg), Eclipta prostrata (18.96 ± 1.92 µM $FeSO_4$/µg), and the polyherbal extraction (PHE) (19.55 ± 1.65 µM $FeSO_4$/µg). Comparison across assays demonstrated that the PHE did not show a marked improvement in antioxidant activity relative to the individual extracts of Terminalia chebula, Curcuma longa, and Emblica officinalis.

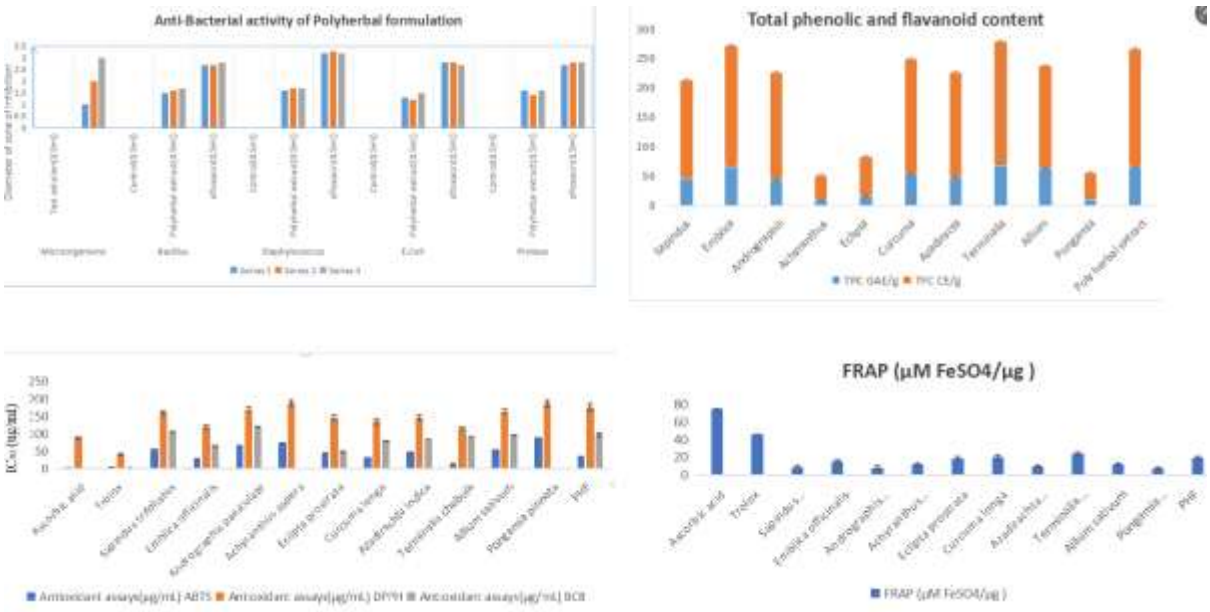

Fig:2 a) zone of inhibition b) total phenolic and flavanoid content c) ABTS,DPPH, BCB d) FRAP

Hydroxyl and Nitric Oxide Radical Scavenging Activity

The results presented in **Figure 3** showed that OH and NO_2 radical scavenging activities increased proportionally with concentration. Among the tested samples, Terminalia chebula demonstrated the prominent hydroxyl RSA (75.24 ± 0.23 µg/mL), next one was the polyherbal extraction (PHE) (73.45 ± 0.36 µg/mL) and Emblica officinalis (72.23 ± 0.34 µg/mL). In contrast, the PHE exhibited the highest nitric oxide radical scavenging activity (66.45 ± 0.38 µg/mL), surpassing the individual extracts.

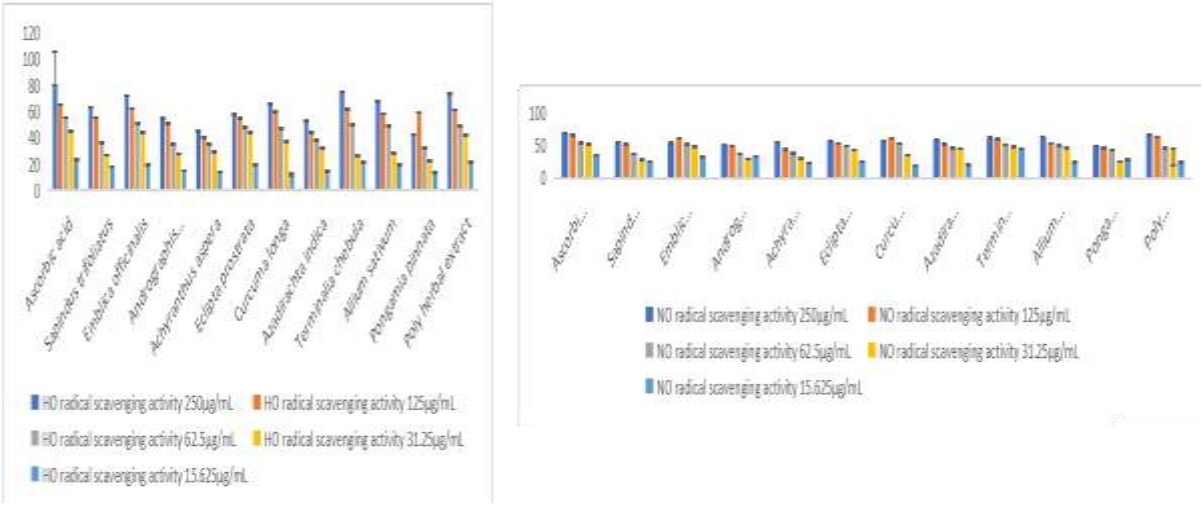

Fig: 3 Bar graph representing NO radical scavenging activity

Correlation results

Pearson Correlation Analysis

The correlation between antioxidant activities and phytochemical contents of the selected herbal extracts

Table: 3 Correlation table with significance

Parameter	TPC	TFC	ABTS	DPPH	BCB	FRAP	HO	NO
TPC	1	0.97***	-0.78**	-0.58	0.78**	0.41	0.89***	0.6
TFC	0.97***	1	-0.73**	-0.59	0.85***	0.33	0.81***	0.47
ABTS	-0.78**	-0.73**	1	0.86***	-0.52	-0.81***	-0.88***	-0.6
DPPH	-0.58	-0.59	0.86***	1	-0.36	-0.6	-0.65*	-0.2
BCB	0.78**	0.85***	-0.52	-0.36	1	0.13	0.65*	0.42
FRAP	0.41	0.33	-0.81***	-0.6	0.13	1	0.68*	0.59
HO	0.89***	0.81***	-0.88***	-0.65*	-0.65*	0.68*	1	0.66*
NO	0.60	0.47	-0.60	-0.2	0.42	0.59	0.66*	1

(** p < 0.05, **** p < 0.01, **** p < 0.001)

Parameter	TPC	TFC	ABTS	DPPH	BCB	FRAP	HO	NO
TPC	**1**	**0.97**	**-0.78**	**-0.58**	**0.78**	**0.41**	**0.89**	**0.6**
TFC	**0.97**	**1**	**-0.73**	**-0.59**	**0.85**	**0.33**	**0.81**	**0.47**
ABTS	**-0.78**	**-0.73**	**1**	**0.86**	**-0.52**	**-0.81**	**-0.88**	**-0.6**
DPPH	**-0.58**	**-0.59**	**0.86**	**1**	**-0.36**	**-0.6**	**-0.65**	**-0.2**
BCB	**0.78**	**0.85**	**-0.52**	**-0.36**	**1**	**0.13**	**0.65**	**0.42**
FRAP	**0.41**	**0.33**	**-0.81**	**-0.6**	**0.13**	**1**	**0.68**	**0.59**
HO	**0.89**	**0.81**	**-0.88**	**-0.65**	**-0.65**	**0.68**	**1**	**0.66**
NO	**0.60**	**0.47**	**-0.60**	**-0.2**	**0.42**	**0.59**	**0.66**	**1**

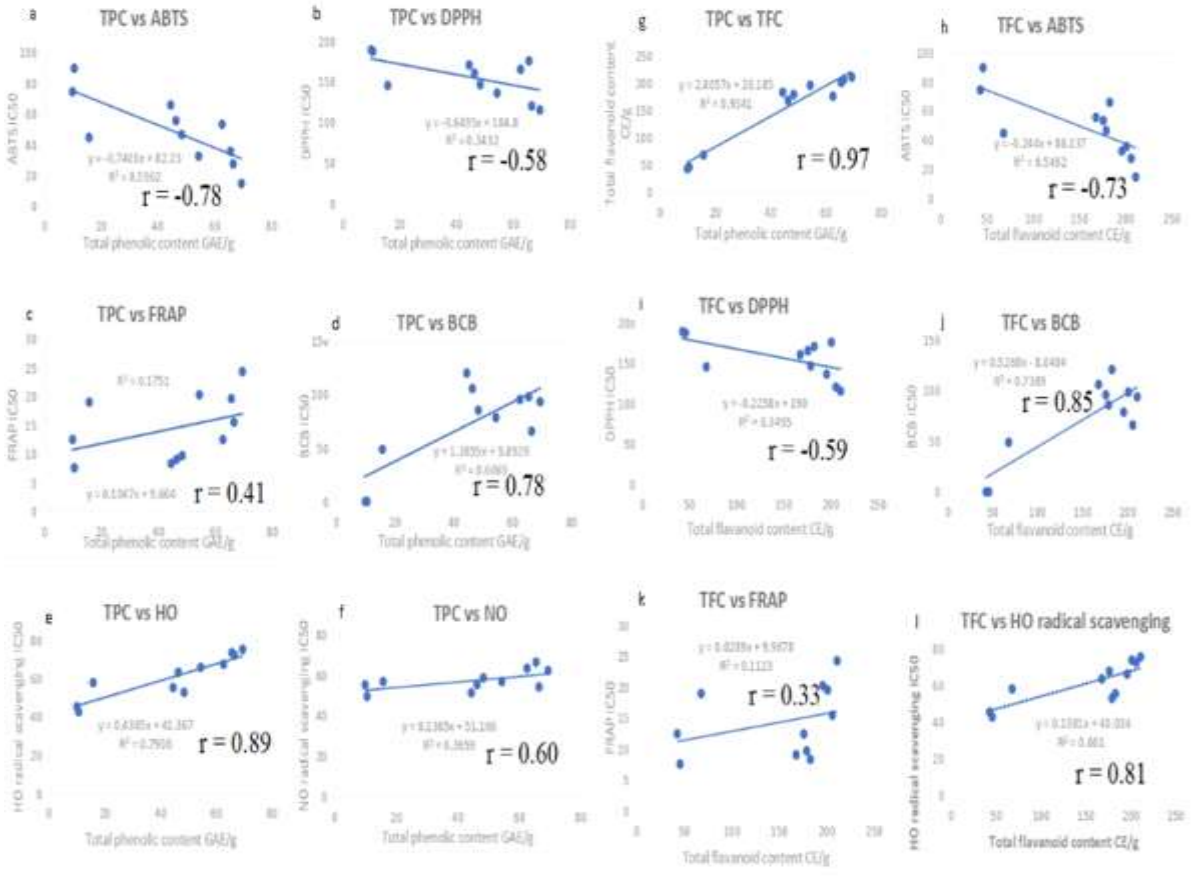

Fig:4 Correlation heat map

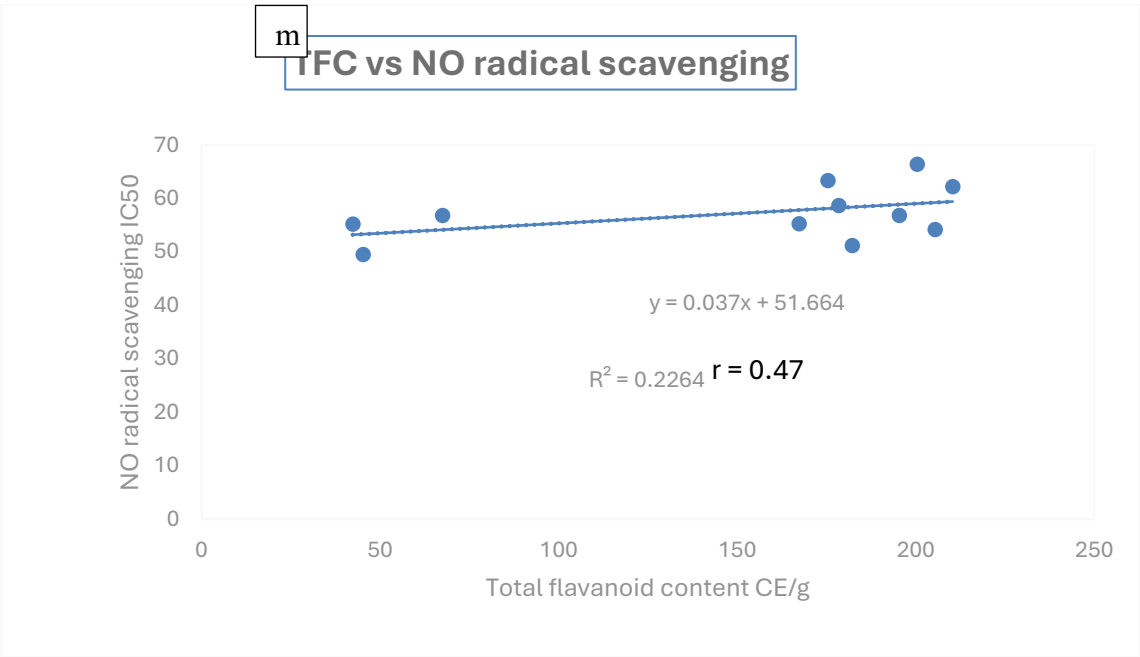

Figure: 5(a-m) Scatter plots representing correlation coefficients

A very strong positive correlation was observed between TPC and TFC ($r = 0.97$, $p < 0.001$), confirming that flavonoids represent a major fraction of phenolic compounds. TPC showed strong negative association with ABTS ($r = -0.78$, $p < 0.01$) and moderate -ve association with DPPH ($r = -0.58$), indicating that lower IC_{50} values associated with higher phenolic levels and enhanced antioxidant potential. In contrast, TPC exhibited strong +ve association with the β-carotene bleaching assay (BCB) ($r = 0.78$) and hydroxyl radical scavenging activity (HO) ($r = 0.89$, $p < 0.001$), moderate +ve association with FRAP ($r = 0.41$) and nitric oxide scavenging activity (NO) ($r = 0.60$).

TFC displayed similar trends, showing strong +ve association with BCB ($r = 0.85$) and HO ($r = 0.81$), and moderate -ve correlations with ABTS ($r = -0.73$, $p < 0.01$) and DPPH ($r = -0.59$). Among antioxidant assays, ABTS and DPPH were strongly positively correlated ($r = 0.86$, $p < 0.001$), while ABTS showed strong -ve association with FRAP ($r = -0.81$) and HO ($r = -0.88$, $p < 0.001$). Moderate correlations such as HO–FRAP ($r = 0.68$, $p < 0.05$) and HO–NO ($r = 0.66$, $p < 0.05$) were statistically significant, whereas weak correlations ($r < 0.60$) were not significant ($p > 0.05$).

The results indicate major contributors to antioxidant potential were phenolic and flavanoid compounds, particularly in hydroxyl radical scavenging and lipid peroxidation inhibition. The moderate correlations observed with DPPH and FRAP suggest that other bioactive constituents, including terpenoids, alkaloids, and vitamins, also play a role. The strong inter-correlation between ABTS and DPPH highlights their shared mechanisms, whereas their negative correlation with FRAP reflects differences in underlying reaction pathways.

CONFLICTS OF INTEREST

The authors declares that they have no conflicts of interest related to this study or its publication.

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