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PHYTOCHEMICAL STANDARDIZATION, TLC FINGERPRINTING AND ANTIOXIDANT EVALUATION OF AN OPTIMIZED POLYHERBAL FORMULATION

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ABSTRACT

In the present study, a polyherbal formulation with high antioxidant activity has been formulated and standardized with the help of medicinal plants used in traditional medicine. Twelve medicinal plants were used to prepare their hydroalcoholic extracts by cold percolation method in the ratio of 70% ethanol: 30% water. Three different polyherbal formulations (F1, F2 and F3) were prepared and analysed for phytochemical composition, TLC fingerprinting and in vitro antioxidant activity. The preliminary phytochemical screening of all the formulations showed presence of flavonoids, phenolics, tannins, glycosides, terpenoids and carbohydrates. TLC fingerprinting was done on silica gel 60 F254 plates using toluene:ethyl acetate:formic acid (5:4:1 v/v/v) as mobile phase, with well separated chromatographic profiles and typical Rf values that suggest the presence of several bioactive phytoconstituents. The antioxidant activity was conducted using DPPH radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay and Potassium Ferricyanide reducing power assay with the concentrations varying from 20 to 100 µg/ml. All formulations showed antioxidant activity in all experimental models with the highest activity observed for F3. The DPPH assay revealed that the formulation F3 exhibited 89.1 ± 1.5 % inhibition at 100 µg/mL while significant ferric reducing activity was obtained from formulation F3 in FRAP assay and significant reducing power activity was obtained from formulation F3 in potassium ferricyanide assay. The increased antioxidant activity of the formulation F3 can be explained by co-action of the phytoconstituents present in formulation and rich in these phenolics and flavonoids. Optimized formulation showed significant antioxidant activity and can be used as a potential natural source of antioxidant phytoconstituents.

KEYWORDS: Polyherbal formulation, TLC fingerprinting, antioxidant activity, phytochemical screening, hydroalcoholic extract, oxidative stress.

1. INTRODUCTION

During normal metabolism, free radicals and reactive oxygen species (ROS) are constantly generated in the body.[1]

These molecules are reactive and are neutralized with the natural antioxidant defense under physiological conditions. But, if the free radicals are produced in an excess amount or if there is a reduction in antioxidant defence mechanisms, oxidative stress occurs which may impair the lipids, proteins, and nucleic acids of the cells.[2] Several chronic and degenerative diseases such as inflammation, arthritis, cardiovascular diseases, diabetes mellitus, cancer, and neurodegenerative diseases have been linked to oxidative stress. Thus, there is a demand for the identification of effective and safe antioxidants, hence it has become an important field of research in pharmaceutical and biomedical sciences.[3]

Medicinal plants are well known as a rich source of natural products that have antioxidant properties. Flavonoids, phenolic compounds, tannins, terpenoids, alkaloids, and glycosides are phytoconstituents that are known for their free radical scavenging and reducing properties.[4] These compounds can transfer a hydrogen atom or an electron to an unstable free radical, reducing the amount of oxidative damage in biological systems. Herbal antioxidants have been a hot topic in recent years because synthetic antioxidants have been known to have side effects and toxicity, particularly when used over long periods.[5]

Polyherbalism is a part of traditional medicine and holds special significance in Ayurveda. Polyherbal formulations are made by mixing medicinal plants in proper ratio and amount in order to make the synergic enhancement of the therapeutic activity.[6] The formulations may be more pharmacologically active than the single plant extracts since their active components act at various levels. Furthermore, synergy or at least dose reduction effects may be achieved by using polyherbal combinations which may enhance therapeutic efficacy of individual herbs, so reducing the side effects of the dose administration.[7]

Standardization of herbal formulations is a necessary step to assure quality, safety, reproducibility and therapeutic uniformity. Thin Layer Chromatography (TLC) is one of the most popular analytical methods that is used for phytochemical profiling and preliminary standardization of herbal formulations and extracts. TLC fingerprinting is useful for the identification of characteristic phytoconstituents and a simple and reliable quality control system for the herbal preparations.[8]

Although some medicinal plants are reported individually for their antioxidant activity, there are very limited studies reported on scientifically standardized polyherbal formulations containing these medicinal plants for their antioxidant activity. Moreover, comparative phytochemical profiling of such formulations and chromatographic standardization is yet to be thoroughly explored.[9]

The present study was thus designed to formulate and assess the efficacy of the polyherbal product prepared from hydroalcoholic extract of 12 medicinal plants that are used traditionally for the management of inflammation and pain along with the oxidative stress related disorders.[10] The developed formulations were then subjected to preliminary phytochemical screening, TLC fingerprinting and antioxidant activity was evaluated by DPPH radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP)assay and Potassium Ferricyanide reducing power assay. The

purpose of the study was to find an optimized formulation with considerable antioxidant activity which could be applied further for pharmacological studies.

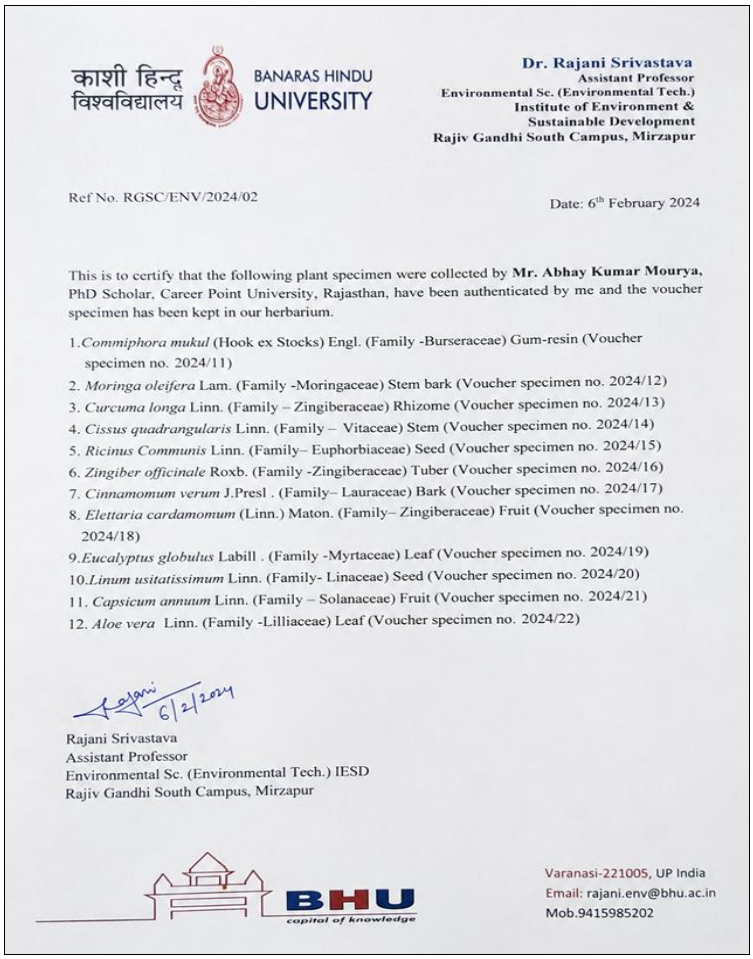
2. MATERIALS AND METHODS

2.1 Plant Materials and Authentication

The 12 medicinal plants traditionally used for inflammatory disorders and those related to oxidative stress were chosen for the present study. The plant materials were collected from authenticated herbal suppliers and natural habitats of Uttar Pradesh, India during February 2024. For experimental work only parts of healthy plants that were free from contamination were selected.[11]

Plants selected were Commiphora mukul (gum resin), Moringa oleifera (stem bark), Curcuma longa (rhizome), Cissus quadrangularis (stem), Ricinus communis (seed), Zingiber officinale (rhizome), Cinnamomum verum (bark), Elettaria cardamomum (fruit), Eucalyptus globulus (leaf), Linum usitatissimum (seed), Capsicum annuum (fruit) and Aloe vera (leaf).

All the plant materials were taxonomically authenticated by Dr. Rajani Srivastava, Assistant Professor, Institute of Environment and Sustainable Development, Banaras Hindu University, Mirzapur, Uttar Pradesh, India. Voucher specimens were finally lodged in the departmental Herbarium for future reference. The following is presented in Figure 1 the authentication certificate and the voucher specimen details.



काशी हिन्दू विश्वविद्यालय BANARAS HINDU UNIVERSITY

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Ref No. RGSC/ENV/2024/02

Date: 6th February 2024

This is to certify that the following plant specimen were collected by **Mr. Abhay Kumar Mourya,** PhD Scholar, Career Point University, Rajasthan, have been authenticated by me and the voucher specimen has been kept in our herbarium.

1. *Commiphora mukul* (Hook ex Stocks) Engl. (Family -Burseraceae) Gum-resin (Voucher specimen no. 2024/11)
2. *Moringa oleifera* Lam. (Family -Moringaceae) Stem bark (Voucher specimen no. 2024/12)
3. *Curcuma longa* Linn. (Family – Zingiberaceae) Rhizome (Voucher specimen no. 2024/13)
4. *Cissus quadrangularis* Linn. (Family – Vitaceae) Stem (Voucher specimen no. 2024/14)
5. *Ricinus Communis* Linn. (Family– Euphorbiaceae) Seed (Voucher specimen no. 2024/15)
6. *Zingiber officinale* Roxb. (Family -Zingiberaceae) Tuber (Voucher specimen no. 2024/16)
7. *Cinnamomum verum* J.Presl . (Family– Lauraceae) Bark (Voucher specimen no. 2024/17)
8. *Elettaria cardamomum* (Linn.) Maton. (Family– Zingiberaceae) Fruit (Voucher specimen no. 2024/18)
9. *Eucalyptus globulus* Labill . (Family -Myrtaceae) Leaf (Voucher specimen no. 2024/19)
10. *Linum usitatissimum* Linn. (Family- Linaceae) Seed (Voucher specimen no. 2024/20)
11. *Capsicum annuum* Linn. (Family – Solanaceae) Fruit (Voucher specimen no. 2024/21)
12. *Aloe vera* Linn. (Family -Lilliaceae) Leaf (Voucher specimen no. 2024/22)

6/2/2024

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Figure 1: Authentication of selected medicinal plants.

2.2 Chemicals and Reagents

Chemicals and reagents used in the study were all analytical grade. Ethanol and methanol were used for extraction procedures. Antioxidant studies were carried out using DPPH (2,2-diphenyl-1-picrylhydrazyl), ferric chloride, potassium ferricyanide and TPTZ (2,4,6-tripyridyl-s-triazine). The reference antioxidant standard was ascorbic acid. All chemicals and solutions were prepared in distilled water during the study.[12]

2.3 Preparation of Plant Extracts

The collected plant materials were washed with distilled water and were dried in the shade at room temperature for 10–15 days. The dried materials were coarsely powdered with mechanical grinding and sieved with the no. 40 sieve to get uniform particle size.[13]

A preliminary study of the solubility was carried out with water, methanol, ethanol and the hydroalcoholic solvent systems in order to select the most appropriate extraction solvent. The absorbance analysis results indicated hydroalcoholic solvent (ethanol:water 70:30 v/v) as the extraction solvent.[14]

Hydroalcoholic extracts were obtained individually by cold percolation method. In brief, 100 g of the powdered plant material was extracted in 1000 mL of hydroalcoholic solvent for seven days with intermittent shaking at room temperature. The extracts were filtered through muslin then Whatman filter paper No. 1 and concentrated under reduced pressure with a rotary vacuum evaporator. The dried extracts obtained were stored at 4°C until further use.[15]

2.4 Preparation of Polyherbal Formulations

Hydroalcoholic extract of selected medicinal plants was blended in different proportions (1:1, 1:3; and 3:1) to get three polyherbal formulations - F1, F2 and F3 that possessed different therapeutic importance and phytochemical compositions. Table 1 displays the formulations that were developed.[16]

The individual extracts required were accurately weighed and mixed homogeneously and uniformly according to the geometric dilution method to ensure that the formulations were prepared correctly and uniformly. The mixing process was continued until a uniform free flowing blend was achieved without any aggregation or phase separation was visible.[17]

The prepared formulations were graded visually in terms of color, texture, appearance and uniformity prior to being placed in airtight containers. The formulations were kept in a cool and dry conditions till further phytochemical and antioxidant evaluation.[18]

Table 1: Composition of polyherbal formulations (F1, F2, and F3).

Plant Extract	F1 (%)	F2 (%)	F3 (%)
Commiphora mukul	20	15	15
Moringa oleifera	15	15	14
Curcuma longa	15	10	13
Cissus quadrangularis	10	10	12
Ricinus communis	10	10	9
Zingiber officinale	5	10	8
Cinnamomum verum	5	8	7
Elettaria cardamomum	5	7	6
Eucalyptus globulus	5	6	5
Linum usitatissimum	5	4	4

Capsicum annuum	2.5	2.5	3.5
Aloe vera	2.5	2.5	3.5

2.5 Preliminary Phytochemical Screening

The hydroalcoholic extracts of the leaves and developed polyherbal formulations were subjected to preliminary phytochemical screening using the standard qualitative methods as described in pharmacognostic literature which was used for the identification of major classes of secondary metabolites. Alkaloids, flavonoids, tannins, saponins, glycosides, steroids, terpenoids and carbohydrates were tested for using chemical reagents and colour reactions. The presence of distinct changes of color or precipitate was taken as an indication of the presence of the corresponding phytoconstituent(s).[19]

2.6 TLC Fingerprinting

Qualitative phytochemical profiling and preliminary standardization of the hydroalcoholic extracts and prepared polyherbal formulations (F1, F2 and F3) was carried out by thin layer chromatography (TLC). TLC analysis was carried out to check for different phytoconstituents in the formulations and to obtain representative chromatographic fingerprint of the formulations.[20]

Silica gel 60 F254 aluminium TLC plates (Merck, Germany) were used as stationary phase. The plates were dried in a hot air oven at 110°C for 30 min before chromatographic analysis, to attain better adsorption efficiency.

The dried extract, polyherbal formulation was dissolved separately in 1 mL of methanol and made into sample extracts. The solution was filtered to exclude the particles and the clear solution was analysed chromatographically. The gel filter paper TLC plates were spotted with about 5.0 µL of each of the sample solutions at a distance of ~ 1 cm from the bottom of the plate. The distance between the spots were reasonable to ensure that the spots do not overlap in the chromatographic development.

After the preliminary optimization trials, the mobile phase used was toluene:ethyl acetate:formic acid (5:4:1 v/v/v) for better separation of the phytoconstituents. The mobile phase was introduced to a chromatographic chamber and the chamber was saturated for about 20–30 minutes prior to development. The spotted TLC plates were then inserted into the chamber and the level of the solvent was kept below the sample application line. Chromatographic development was continued until the solvent front has travelled about 8cm of the plate length.[21]

Once developed, TLC plates were taken out of the chamber and air dried at room temperature. Developed chromatograms were checked in the UV chamber in both U.V. lights (254 nm and 366 nm). Under UV light, different coloured and fluorescent bands were identified and recorded. The separation of spots was performed by measuring the retention factor (Rf) by the following equation:

$$Rf\ value = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent front}}$$

where,

- **Distance travelled by the solute** = distance moved by the phytoconstituent spot from the point of application
- **Distance travelled by the solvent front** = distance moved by the mobile phase from the point of application

The obtained TLC fingerprint profiles and Rf values were used for comparative phytochemical evaluation and preliminary standardization of the developed polyherbal formulations.

2.7 In Vitro Antioxidant Activity

2.7.1 DPPH Radical Scavenging Assay

The free radical scavenging activity of the prepared polyherbal formulations was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay as described by Blois with a little modification. The various concentrations (20, 40, 60, 80 and 100 µg/mL) of the formulations were prepared in methanol and added to freshly prepared DPPH solution. The reaction mixtures were kept in dark conditions at room temperature for 45 min to prevent the degradation of DPPH radicals by light.[22]

The absorbance was then measured at 517 nm after the incubation on a UV–Visible spectrophotometer. The reference standard used was ascorbic acid. The percentage inhibition of DPPH radicals was obtained by the following equation:

$$\% \, Inhibition = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c = absorbance of control; A_s = absorbance of sample

2.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric Reducing Antioxidant Power (FRAP) assay was used to assess the FRCA of the formulations as described by Benzie and Strain.[4] Various concentrations of formulation samples were added to freshly prepared FRAP reagent and were incubated at 37°C for 30 min.

This led to the formation of a blue coloured complex and absorbance was measured at 593 nm with a UV–Visible spectrophotometer. The more absorption was observed, the higher ferric reducing antioxidant activity was observed.[23]

2.7.3 Potassium Ferricyanide Reducing Power Assay

The reducing power activity of the formulations was carried out by potassium ferricyanide method as reported by Oyaizu.[5] Each formulation sample was prepared in various concentrations and mixed with phosphate buffer and potassium ferricyanide solution and incubated at 50°C for 20 min. Trichloroacetic acid was then added to stop the reaction and ferric chloride solution was added.

The absorbance of the reaction mixture obtained was measured with a UV–visible spectrophotometer at 700 nm. The higher absorbance of the reaction mixture was an evidence of greater reducing power activity of the formulations.[24]

2.8 Statistical Analysis

All experiments were performed in triplicate (n = 3), and the results were expressed as Mean ± SEM. One way analysis of variance (ANOVA) was used to analyze the data, which were tabulated and presented graphically.[25]

3. RESULT AND DISCUSSION

3.1 Preliminary Phytochemical Screening

Table 2: Preliminary Phytochemical Screening of Polyherbal Formulations

Phytoconstituent	F1	F2	F3
Alkaloids	+	+	++
Flavonoids	++	++	+++
Tannins	++	++	+++
Glycosides	+	++	++
Saponins	+	+	++
Steroids	+	+	++
Terpenoids	++	++	+++
Carbohydrates	+	+	++

Note: (+) Present; (++) Moderately present; (+++) Strongly present.

Phytochemical screening of the developed polyherbal formulations (F1, F2 and F3) was done initially for the presence of major classes of secondary metabolites. The findings of the qualitative phytochemical analysis are shown in Table 2.

The phytochemical analysis identified several key bioactive ingredients like alkaloids, flavonoids, tannins, glycosides, saponins, steroids, terpenoids, and carbohydrates in different concentrations in the formulated products. Alkaloids, tannins, and terpenoids were highly abundantly detected in all formulations while steroid and carbohydrates were moderately detected and carbohydrates were not detected in the formulations.All the formulations showed presence of flavonoids, tannins and terpenoids in high quantity, alkaloids in moderate quantity, steroids in moderate quantity and carbohydrates were not detected.

F3 showed comparatively high phytochemical responses for flavonoids, tannins and terpenoids than other formulations which suggested that F3 contained comparatively high phytochemical content than F1 and F2. This increased phytochemical profile may be due to an optimized ratio of hydroalcoholic extracts of plants in formulation F3.

Because of their ability to donate hydrogen atoms or electrons to neutralize free radicals, flavonoids and phenolic compounds have long been known for their antioxidant properties. Likewise, terpenoids and glycosides have been shown to have various biological properties such as antioxidant and anti-inflammatory. Hence, the developed formulations' antioxidant activity may be further increased with these phytoconstituents because of their high concentration.

The results obtained indicated the successful incorporation of various bioactive phytoconstituents into the poly-herbal formulations and supported the further antioxidant evaluation.

3.2.1 TLC Fingerprinting Profile of Individual Plant Extracts and Polyherbal Formulations

Phytochemical profiling and preliminary standardization of individual hydroalcoholic plant extracts and developed polyherbal formulations (F1, F2, and F3) were carried out by using thin layer chromatography (TLC). Under UV light at 254 and 366 nm, multiple chromatographic bands were recorded which represents the presence of several phytoconstituents present in the extracts and formulations. The results of the TLC fingerprinting are shown in Table 3 and Figure 2.

Table 3: TLC Fingerprinting Profile of Selected Plant Extracts and Polyherbal Formulations.

Sample	No. of Major Spots	R_f Values (254 nm)	Rf Values (366 nm)	Probable Phytochemical Class
Commiphora mukul	4	0.18, 0.36, 0.62, 0.78	0.22, 0.40, 0.65, 0.80	Terpenoids, steroids, resins
Moringa oleifera	5	0.12, 0.29, 0.47, 0.59, 0.73	0.15, 0.33, 0.49, 0.62, 0.75	Flavonoids, phenolics
Curcuma longa	4	0.28, 0.41, 0.55, 0.69	0.30, 0.44, 0.58, 0.71	Curcuminoids, phenolics
Cissus quadrangularis	4	0.16, 0.31, 0.52, 0.67	0.19, 0.35, 0.54, 0.69	Flavonoids, glycosides
Ricinus communis	3	0.21, 0.48, 0.70	0.24, 0.51, 0.72	Steroids, fatty constituents
Zingiber officinale	4	0.25, 0.39, 0.60, 0.76	0.27, 0.42, 0.63, 0.79	Gingerols/shogaols, terpenoids
Cinnamomum verum	3	0.20, 0.46, 0.68	0.23, 0.49, 0.70	Phenolics, cinnamaldehyde-type compounds
Elettaria cardamomum	3	0.22, 0.44, 0.66	0.25, 0.47, 0.68	Volatile terpenoids
Eucalyptus globulus	4	0.17, 0.34, 0.57, 0.74	0.20, 0.37, 0.60, 0.77	Terpenoids, essential oils
Linum usitatissimum	3	0.14, 0.38, 0.63	0.16, 0.41, 0.66	Lignans, glycosides
Capsicum annuum	3	0.26, 0.50, 0.72	0.29, 0.53, 0.75	Capsaicinoids, terpenoids
Aloe vera	4	0.10, 0.32, 0.54, 0.70	0.12, 0.35, 0.57, 0.73	Anthraquinones, polysaccharide-linked compounds
Polyherbal Formulation F1	5	0.14, 0.26, 0.41, 0.58, 0.72	0.17, 0.29, 0.44, 0.61, 0.75	Flavonoids, terpenoids, phenolics
Polyherbal Formulation F2	6	0.12, 0.22, 0.36, 0.49, 0.63, 0.76	0.15, 0.25, 0.39, 0.52, 0.66, 0.79	Flavonoids, glycosides, terpenoids
Polyherbal Formulation F3	7	0.12, 0.22, 0.34, 0.47, 0.59, 0.68, 0.78	0.15, 0.25, 0.37, 0.49, 0.62, 0.71, 0.80	Composite profile (flavonoids, terpenoids, phenolics, glycosides)

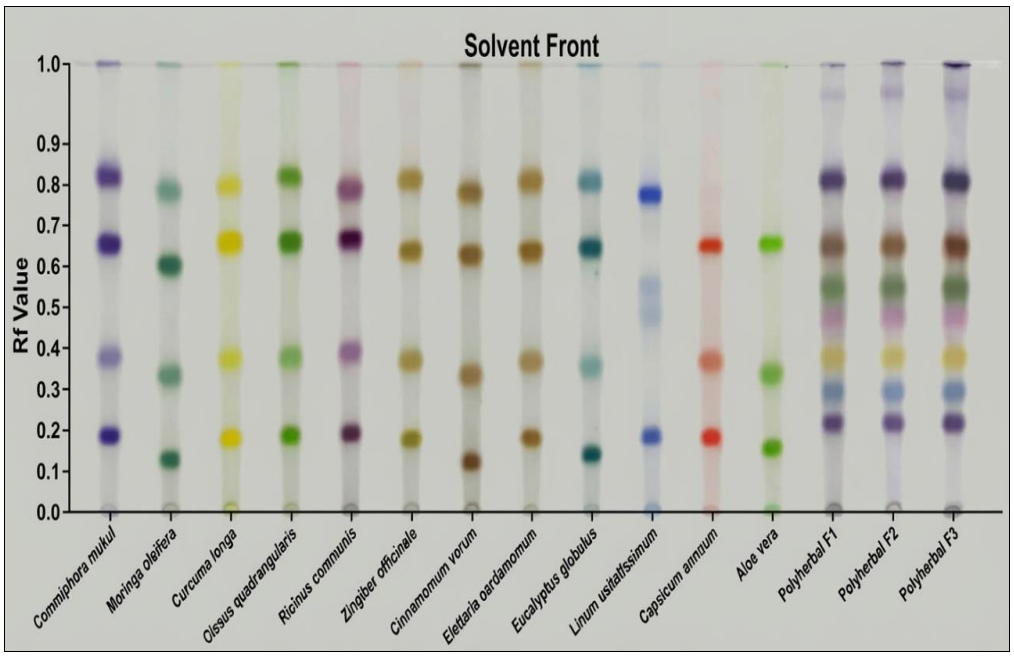

Figure 2: TLC chromatographic fingerprints of hydroalcoholic plant extracts and polyherbal formulations (F1, F2, and F3) visualized under UV light at 254 nm and 366 nm.

The results of the chromatographic analysis showed the presence of flavonoids, phenols (phenolic compounds), volatile oils, curcuminoid type compounds, glycosides, steroids, terpenoids and lignans in extracts of various plants. The level of spots and their variation were noted between the formulations.

In the developed formulations, F3 showed the highest number of well-resolved chromatographic bands under both the UV wavelengths which suggests that it has comparatively richer phytochemical composition and better phytochemical integration. Formulation F1 exhibited lesser number of chromatographic bands while formulation F2 exhibited moderate chromatographic separation.

R_f values of the individual plant extracts and developed formulations were similar, indicating successful incorporation and retention of phytoconstituents during formulation development. The developed formulations may contain flavonoids, phenolics, terpenoids and glycosides as identified through TLC fingerprinting which could have a significant role in their antioxidant activities.

In general, the TLC fingerprint profiles established in the present study can be used as reference profiles for initial phytochemical characterization, quality control and standardization of the formulated polyherbal products.

3.3 *In Vitro* Antioxidant Activity

The antioxidant activities of the developed polyherbal formulations (F1, F2 and F3) were tested with three established in vitro antioxidant assays which include DPPH radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, and potassium ferricyanide reducing power assay. The assays were used to evaluate various modes of antioxidant activity such as free radical scavenging, electron donating and reducing potential. The reference antioxidant standard used was ascorbic acid. Each experiment was repeated 3 times (n = 3) and the results reported as Mean ± SEM.

In the range of concentration tested (20-100 µg/mL), all the developed formulations showed a concentration-dependent antioxidant activity. In all evaluated experimental models, F3 formula exhibited the highest antioxidant activity followed by F2 and F1.

3.3.1 DPPH Radical Scavenging Assay

The free radical scavenging potential of the developed polyherbal formulations was assessed by DPPH radical scavenging assay. The antioxidant activity of all the formulations was concentration dependent within the range of concentration (20 - 100 µg/mL). The results obtained are reported in Table 4 and plotted in Figure 3.

Table 4: DPPH Radical Scavenging Activity of Polyherbal Formulations.

Concentration (µg/ml)	F1 (% inhibition)	F2 (% inhibition)	F3 (% inhibition)	Ascorbic acid
20	31.4± 1.9	38.7± 2.2	44.1± 1.6	50.8± 1.5
40	42.6± 2.4	43.4± 1.7	53.8± 2.3	66.1± 1.8
60	49.9± 1.8	63.5± 2.5	72.4± 1.9	74.5± 2.1
80	64.8± 2.7	70.1± 2.0	83.6± 1.7	89.3± 1.6
100	79.2± 2.1	82.4± 1.8	89.1± 1.5	94.2± 1.4

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

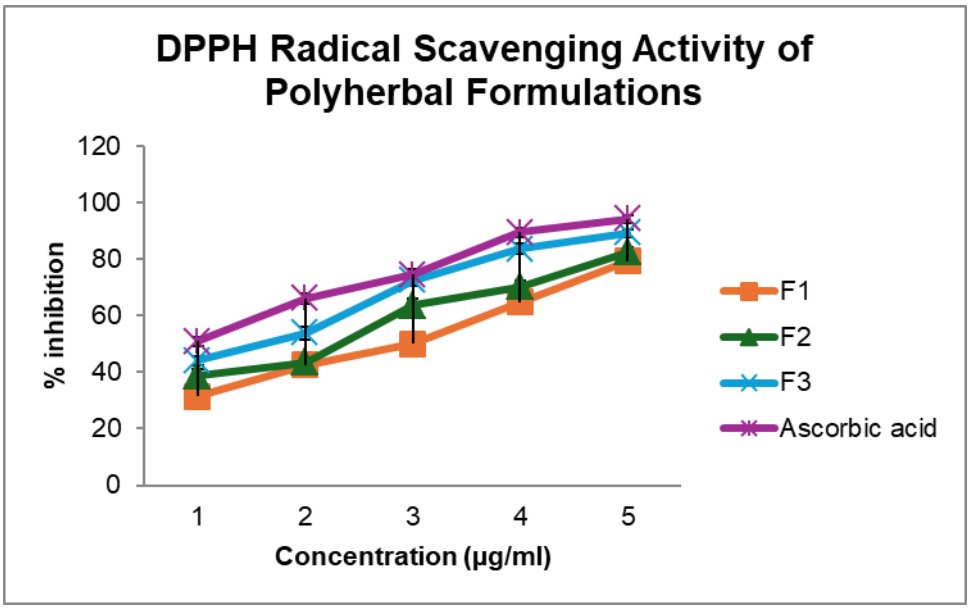

Figure 3: DPPH radical scavenging activity of polyherbal formulations at different concentrations.

The DPPH radical scavenging assay demonstrated that all the polyherbal formulations exhibited concentration-dependent antioxidant activity. The percentage inhibition increased progressively with increasing concentration from 20 to 100 µg/ml. Among the tested formulations, F3 showed the highest radical scavenging activity at all concentrations.

At 100 µg/ml, formulation F3 exhibited 89.1 ± 1.5% inhibition, followed by F2 (82.4 ± 1.8%) and F1 (79.2 ± 2.1%), whereas the standard ascorbic acid showed 94.2 ± 1.4% inhibition. The enhanced antioxidant activity of F3 may be attributed to the higher content of phenolic compounds, flavonoids and terpenoids present in the formulation. The results indicate that the antioxidant potential of the formulations followed the order: Ascorbic acid > F3 > F2 > F1.

These findings suggest that formulation F3 possesses strong free radical scavenging activity and may serve as a promising natural antioxidant formulation for combating oxidative stress-induced cellular damage.

Similar findings have been reported in previous studies, where polyherbal formulations rich in phenolic and flavonoid constituents demonstrated significant DPPH radical scavenging activity due to their hydrogen-donating and free radical neutralizing properties. The enhanced antioxidant activity observed in formulation F3 may therefore be attributed to the synergistic interaction of its bioactive phytoconstituents.

3.3.2 Ferric Reducing Antioxidant Power (FRAP) Assay

The developed polyherbal formulations were assessed for their electron donating ability and ferric ion reducing capacity using ferric reducing antioxidant power (FRAP) assay. The activity of Ferric reducing was concentration-dependent in all the formulations within the range of concentration used (20–100 µg/mL). The results obtained are presented in Table 5 and Figure 4.

Table 5: Ferric Reducing Antioxidant Power (FRAP) of Polyherbal Formulations.

Concentration (µg/ml)	F1 (% inhibition)	F2 (% inhibition)	F3 (% inhibition)	Ascorbic acid
20	0.28 ± 0.05	0.36 ± 0.06	0.41 ± 0.05	0.48 ± 0.04
40	0.38 ± 0.07	0.49 ± 0.05	0.51 ± 0.05	0.59 ± 0.05
60	0.44 ± 0.06	0.52 ± 0.06	0.72 ± 0.06	0.79 ± 0.06
80	0.65 ± 0.07	0.75 ± 0.06	0.79 ± 0.06	0.83 ± 0.05
100	0.72 ± 0.06	0.81 ± 0.06	0.92 ± 0.05	1.08 ± 0.06

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

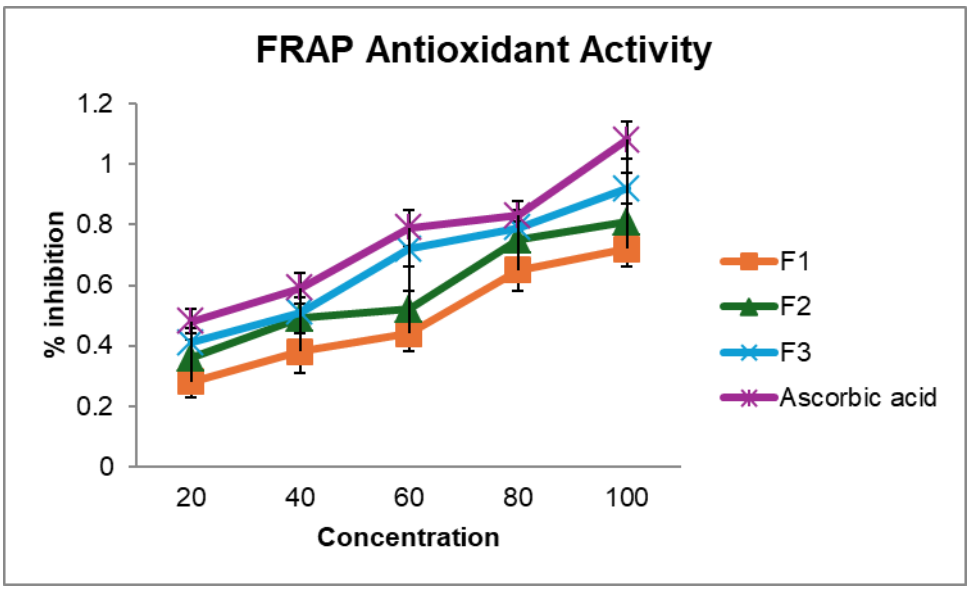

Figure 4: Ferric reducing antioxidant power of polyherbal formulations at different concentrations.

The highest antioxidant activity as ferric reducing activity (FRA) was observed for F3 formulation at all concentrations tested. The absorbance value of formulation F3 was 0.92 ± 0.05 which is comparatively close to the standard ascorbic acid value (1.08 ± 0.06) at 100 µg/mL concentration. The ferric reducing activity of formulations F2 and F1 was also found to be appreciable, but less than that of F3.

The linear rise in absorbance with rise in concentration showed that electron donating and reducing property of the phytoconstituents of formulations were effective. The higher concentration of the flavonoids, phenolic compounds and terpenoids found during the phytochemical screening and TLC fingerprinting studies might explain the relatively higher reducing activity of formulation F3.

The results obtained indicate that formulation F3 has potent ferric reducing antioxidant activity, thus it could help in the prevention of cellular damages caused by oxidative stress.

3.3.3 Potassium Ferricyanide Reducing Power Assay

Polyherbal formulations were developed and the reducing power activity was assessed by Potassium ferricyanide reducing power assay. The reducing activity of all the formulations was concentration dependent in the range tested (20–100 µg/mL). The results obtained are listed in Table 6 and plotted in Figure 5.

Table 6: Reducing Power Activity of Polyherbal Formulations.

Concentration (µg/ml)	F1	F2	F3	Ascorbic acid
20	0.27 ± 0.05	0.34 ± 0.06	0.41 ± 0.05	0.46 ± 0.04
40	0.37 ± 0.07	0.47 ± 0.05	0.52 ± 0.06	0.63 ± 0.05
60	0.48 ± 0.06	0.52 ± 0.06	0.71 ± 0.05	0.74 ± 0.06
80	0.52 ± 0.07	0.74 ± 0.06	0.81 ± 0.06	0.93 ± 0.05
100	0.77 ± 0.06	0.82 ± 0.07	0.97 ± 0.05	1.09 ± 0.06

Values are expressed as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

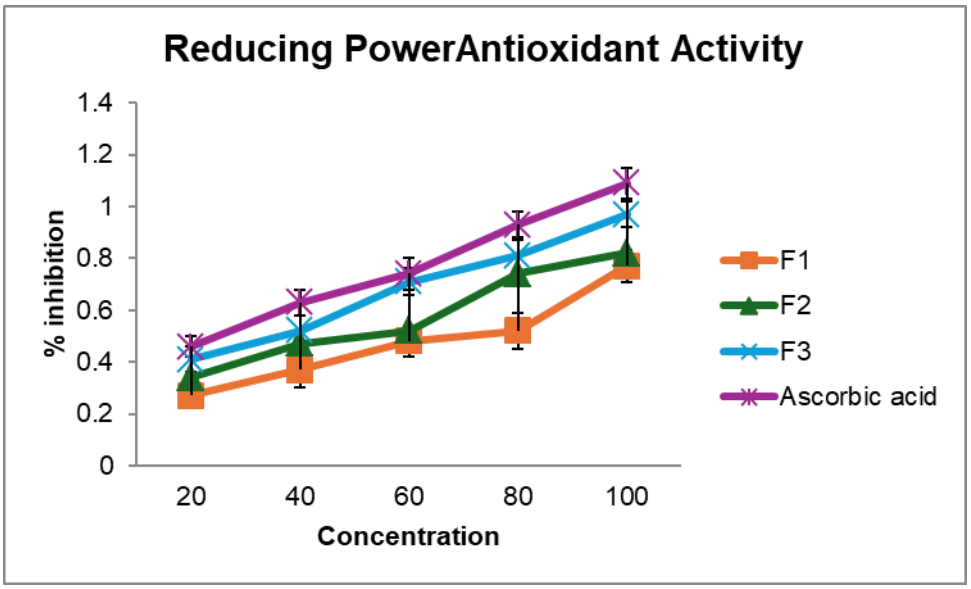

Figure 5: Reducing power activity of polyherbal formulations at different concentrations.

The reducing power activity was greatest for F3 in all the tested concentrations. The absorbance value for formulation F3 at 100µg/mL was 0.97 ± 0.05 which was comparatively close to the absorbance value of the standard ascorbic acid (1.09 ± 0.06). The reducing power activity of formulations F2 and F1 were also significant but not as high as F3.

As the concentration of the formulations increased, the absorbance also increased, showing good electron donating and reducing properties of the phytoconstituents present in the formulations. The more prominent reducing activity recorded in formulation F3 might be due to higher concentration of flavonoids, tannins and phenolic compounds observed from the phytochemical screening and TLC fingerprinting studies.

The results obtained indicate that formulation F3 has high reducing power activity and may be potential to protect against oxidative stress induced cell damage.

3.3.4 Comparative Interpretation and Selection of Optimized Formulation

Comparative antioxidant potential of the developed polyherbal formulations was carried out using DPPH radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, potassium ferricyanide reducing power assay, which revealed that all the developed polyherbal formulations showed appreciable antioxidant potential. But in all the tested experimental models formulation F3 always had the highest antioxidant activity.

The DPPH assay results indicated that the formulation F3 exhibited the highest free radical scavenging activity with a result of 89.1 ± 1.5% at a concentration of 100 µg/mL which was comparatively close to the standard ascorbic acid. Likewise, FRAP assay and reducing power assay showed that formulation F3 had higher ferric reducing power and electron donating ability than the formulations F1 and F2.

The increased antioxidant activity of formulation F3 could be due to the synergic effect of the flavonoids, phenolic compounds, tannins, terpenoids and glycosides, which were also found in the preliminary phytochemical screening and TLC fingerprint analysis. These phytoconstituents are well characterized for their hydrogen and electron donating capabilities, and their potent antioxidant effects in protecting cells from oxidative stress damage.

The increased number of chromatographic bands and higher antioxidant profile found in formulation F3 confirms its better antioxidant activity. Optimized proportion of plant extracts in formulation F3 might have played a role in better integration of phytochemicals and synergistic antioxidant effects of the bioactive ingredients.

It has also been reported earlier that polyphenol containing herbal formulation has better antioxidant and reducing property due to synergistic effect of flavonoids, phenolics, etc. with each other. Thus, the results obtained are consistent with the previously reported antioxidant activity of formulations from medicinal plants.

The overall results of the present investigation show that formulation F3 has comparatively higher antioxidant activity among all the developed formulations. Formulation F3 was chosen as the optimized polyherbal formulation for further pharmacological study based on the highest phytochemical content, TLC fingerprint and antioxidant activity.

4. CONCLUSION

In the present study, polyherbal formulations were successfully developed and evaluated with hydroalcoholic extracts of the selected medicinal plants traditionally used for oxidative stress related disorders. The phytochemical screening of all developed formulations was carried out to confirm the presence of some important bioactive phytoconstituents such as flavonoids, phenolic compounds, tannins, glycosides, terpenoids and carbohydrates.

The TLC fingerprinting studies showed characteristic chromatographic patterns with characteristic Rf values, proving the successful incorporation and retention of phytoconstituents in the formulations. The developed formulations showed comparatively high phytochemical composition and good chromatographic fingerprint profile for F3, having more number of well-resolved bands.

Both antioxidant evaluation conducted using DPPH radical scavenging assay and Ferric Reducing Antioxidant Power (FRAP) assay and Potassium ferricyanide reducing power assay showed significant antioxidant effect for all the formulations with concentration dependent manner. All formulations exhibited antioxidant activity, with formulation F3 exhibiting the highest antioxidant activity, and comparable activity to standard ascorbic acid.

The higher antioxidant activity of formulation F3 could be due to the synergistic effect of antioxidants such as phenolic compounds, flavonoids, tannins and terpenoids present in the formulation. The results obtained indicate that the optimized polyherbal formulation has significant natural antioxidant properties and could be a useful herbal system for future drug development studies on oxidative stress related disorders.

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