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ORIGINAL ARTICLE

In-Vitro Antioxidant Evaluation of Polyherbal Formulation by DPPH and NO^- Methods

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ABSTRACT
The objective of present study was to develop a polyherbal formulation (PHF) using ethanolic extracts of five different herbs and to evaluate their phytochemicals and determination of their antioxidant activity by DPPH (2, 2-Diphenyl-1-picrylhydrazyl) and (Nitric oxide) NO method. The PHF authenticated plants were defined by investigating their pharmacognostic and phytochemical properties. Phytochemical screening showed the presence of alkaloids, glycosides, carbohydrates, amino acid, tannin, steroids, and flavonoids in the combination extracts. The ethanolic extracts of selected plants are good source of compounds with antioxidant properties and these extracts exhibited significant Free Radical Scavenging activity. The antioxidant activity of the PHF was tested using DPPH and NO free radical scavenging techniques. From current research it was concluded that the polyherbal formulation prepared from combination of ethanolic extracts of selected plants have shown significant antioxidant when it was compared with ascorbic acid as the reference standard.
Keywords: Polyherbal formulation (PHF), Phytochemical, DPPH, NO, Antioxidant Activity, Ascorbic acid.

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INTRODUCTION

In recent years the field of herbal medicine has seen tremendous expansion because of its natural origin and fewer adverse effects; it is being promoted in emerging nations [1].It falls under the category of traditional Indian medicine. A molecule is said to be an antioxidant if it can prevent other molecules from oxidizing. The chemical process of oxidation involves the transfer of electrons from a material to an oxidizing agent. Free radicals may be produced via oxidation processes. These radicals have the ability to initiate cell-damaging chain reactions. By eliminating free radicals, antioxidants stop these chain reactions and prevent further oxidation reactions. Since they undergo oxidation themselves, antioxidants frequently function as reducing agents like polyphenols, ascorbic acid, and thiols. The antioxidant properties of plants have been linked to their phenolic content, according to numerous studies. Phenolics and polyphenols chemicals found in plants have antioxidant properties [2]. Free radicals are most frequently produced by oxidative processes in food, medications and even biological systems [3].Free radicals that harm biological systems are mostly oxygen radicals. Additionally, antioxidants have the ability to scavenge radicals, donate electrons and hydrogen, breakdown peroxide, quench singlet oxygen, inhibit enzymes, and chelate metals [4]. In contrast to synthetic antioxidants, which are hazardous to humans; natural antioxidants are harmless and nontoxic and are necessary because of their impact on the immune system.

As we know that from previous studies individual medicinal plants contains various active phytoconstituents in minute amount which are insufficient to achieve the desirable therapeutic effects. When these Plant medicines are more often used in combination rather than in a single form in order to acquire maximum benefit from their combined potential to reduce side effects of one another. Keeping the above information in view, an indigenous polyherbal preparation was developed. Polyherbal formulations, which combine multiple herbal ingredients, have been used in traditional medicine systems like Ayurveda and Traditional Chinese Medicine for centuries. These formulations leverage the

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synergistic effects of various bioactive compounds found in plants, enhancing their therapeutic efficacy, including antioxidant activity [5].To support this antioxidant potential of combined mixture of ethanolic extracts i.e. polyherbal formulation of *Ricinus communis* L., *Moringa oleifera* Lam, *Azadirachta indica* A.Juss, *and Tinospora cordifolia*(Thumb.) Miers, *Cymbopogon citratus*(DC.) Stapf of Indian origin was examined by *in vitro* studies using DPPH and NO assay method.

MATERIAL AND METHODS:

MATERIALS:

Chemicals and Reagents:

Analytical-grade chemicals and solvents like petroleum ether, ethanol, chloroform, and ethyl acetate. DPPH (2,2-diphenyl-1-picrylhydrazyl), sodium nitroprusside, Griess reagent, and ascorbic acid for antioxidant assays were employed in this investigation.

METHODS:

Plant materials Collection and Authentication:

Fresh parts of specific plant materials—such as *Ricinus communis* L. (leaves), *Moringa oleifera* Lam. (leaves), *Azadirachta indica* A. Juss. (leaves), *Tinospora cordifolia* (Thumb.) Miers (leaves and stem), and *Cymbopogon citratus* (DC.) Stapf (leaves)- that were needed for the current investigation were gathered from the environs of the districts of Latur and Osmanabad in Maharashtra, India and authenticated by renowned botanist Prof. D.L. Shirodkar of Botanical Survey of India (BSI) having reference voucher number Ref.No. BSI/WRC/Iden. Cer./2021/3108210000452**.** These fresh plant materials were washed, air-dried at room temperature, and coarsely powdered using an electric grinder. Morphological characteristics (color, odor, taste, and texture) were recorded. For potential use in future studies; this dried plant material was stored in an airtight container.

Extraction:

Using a Soxhlet extraction system, plant materials that had been air-dried and coarsely powdered were first defatted with petroleum ether. Following that, they were extracted using a sequence of solvents, including ethanol, chloroform, and ethyl acetate, in ascending order of polarity. The residue was then dried over sodium sulfite in a desiccator after the extracts were concentrated using a rotary flash evaporator operating at low pressure. Following drying, the relevant extracts were weighed, their yield percentage was calculated, and they were subsequently placed in an airtight container [6-8].

Polyherbal Formulation:

A polyherbal formulation (PHF) suspension (100 ml) was prepared by triturating ethanolic extracts of the selected plants.

Phytochemical Screening:[9,10]

Phytochemical tests were performed to detect compounds like alkaloids, glycosides, flavonoids, phenolics, terpenoids, steroids, carbohydrates, and proteins.

Determination of Antioxidant Activity:

DPPH method:[11]

The free radical scavenging activity of test sample was determined by DPPH scavenging method (Table-3). 0.1mM DPPH solution was prepared in ethanol by adding 39.4 mg of DPPH in 1000 ml of ethanol and to 0.5 ml of this solution, 1.5 ml of test sample was added of various concentrations. The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes in dark conditions. Then the absorbance was measured at 517 nm using spectrophotometer (UV-VIS Shimadzu). Ascorbic acid was used as standard compound.

The scavenging activity of sample by the DPPH radical was calculated using the formula;

$$\text{DPPH scavenging effect (\% inhibition)} = \{(A0 - A1)/A0) \times 100\}$$

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance of test sample or Ascorbic acid.

Nitric Oxide Method:[12]

The free radical scavenging activity of test sample was determined by Nitric oxide (NO) scavenging method (Table-4). 10 mM sodium nitroprusside solution was prepared in phosphate buffered saline. Test sample of various concentrations was prepared in ethanol or water. 0.5 ml of 10 mM sodium nitroprusside, 1 ml of test samples of various concentrations and equal volume of freshly prepared Griess reagent was added, solution was then incubated at 25°C for 3 hours. Then the absorbance was measured at 546 nm.Ascorbic acid was used as standard compound. The percentage of nitrite radical scavenging activity of test compounds was calculated by;

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$$\text{NO scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test compounds}}{\text{Absorbance of control}} \times 100$$

Data Analysis:

Results were expressed as mean ± SD, with statistical significance set at <0.05, $p<0.05$.

RESULTS

The study aimed to develop and evaluate the antioxidant potential of a polyherbal formulation (PHF-C) using ethanolic extracts of five selected herbs evaluated morphologically (Table 1) and phytochemically.

Table 1: Morphological characters

Sr. No.	FEATURES	OBSERVATIONS					
		RC	MO	AI	TC	CC	PHF-C
1.	**Color**	Green	Green	Dark Green	Dark Bright Green	Dark Green	Greenish Black
2.	**Odour**	Odourless	Characteristic	Characteristic	Indistinct	Lemon Like	Characteristic
3.	**Taste**	Astringent	Slight	Bitter	Bitter	Bitter	Slight Bitter
4.	**Texture**	Soft	Soft	Papery	Soft	Soft	Sticky

Phytochemical Screening:

The polyherbal formulation (PHF-C) contained significant levels of bioactive compounds, including phenolics, flavonoids, alkaloids, glycosides, terpenoids, steroids, tannins, carbohydrates, and proteins (Table 2).

Flavonoids and phenolic compounds were particularly abundant in the formulation, contributing to its antioxidant properties.

Table 2: Phytochemical Screening

PHYTO - CONSTITUENTS	OBSERVATIONS					
	RCL	MOL	AIL	TCLS	CCL	PHF-C
Carbohydrate	+	-	+	+	+	+
Glycoside	+	+	+	+	+	+
Protein	-	+	-	+	+	+
Steroids	-	-	-	-	-	++
Terpenoids	+	-	+	+	+	+
Tannins	+	-	+	+	+	+
Saponins	+	+	-	+	-	-
Phenols	+++	+++	+++	+++	+++	+++
Alkaloids	+	+	-	+	-	+
Flavonoids	+++	+++	+++	+++	+++	+++

Key: + = Present, -= Absent

DPPH Scavenging Activity:

The PHF-C exhibited a concentration-dependent increase in free radical scavenging activity, with percentage inhibition values ranging from 18.13% at 20 µg/ml to 64.24% at 100 µg/ml (Table 3).

The antioxidant activity of PHF-C was comparable to the standard ascorbic acid, especially at higher concentrations.

Table 3: DPPH Activity of Polyherbal Formulation with Reference To Ascorbic Acid

Sr. No.	CONCENTRATION (µg/ml)	ASCORBIC ACID (% inhibition)	POLYHERBAL FORMULATION (PHF-C) (% inhibition)
1	20	15.02	18.13
2	40	21.24	32.12
3	60	44.04	48.7
4	80	62.17	53.37
5	100	73.05	64.24

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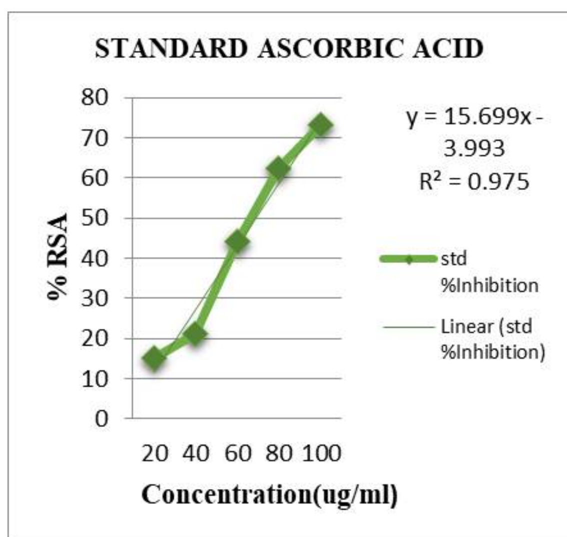


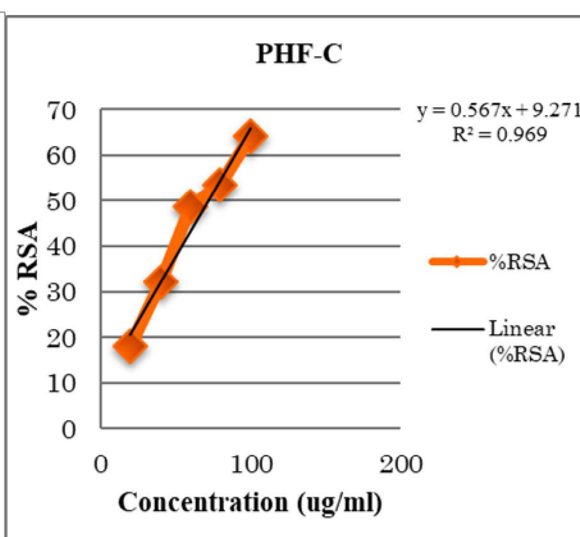


Fig No-1 Antioxidant Activity of Ascorbic Acid

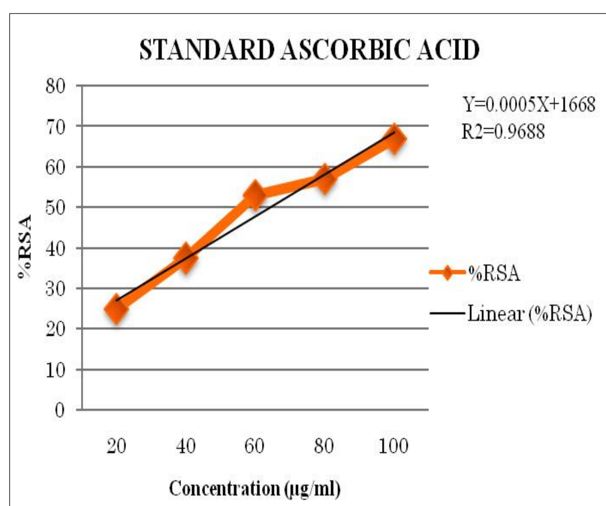
Fig No-2 Antioxidant Activity of PHF-C

Nitric Oxide (NO) Scavenging Activity:

The PHF-C demonstrated concentration-dependent NO scavenging activity, with inhibition values from 16.58% at 20 µg/ml to 54.92% at 100 µg/ml (Table 4). While slightly lower than ascorbic acid, the PHF-C showed significant activity, indicating its ability to neutralize NO radicals.

Table 4 : Nitric Oxide Scavenging Activity of Polyherbal Formulation With Reference To Ascorbic Acid

SR. NO	CONCENTRATION (µg/ml)	ASCORBIC ACID (% inhibition)	POLYHERBAL FORMULATION (PHF-C) (% inhibition)
1	20	24.87	16.58
2	40	37.30	30.05
3	60	52.84	43
4	80	56.99	48.7
5	100	66.83	54.92



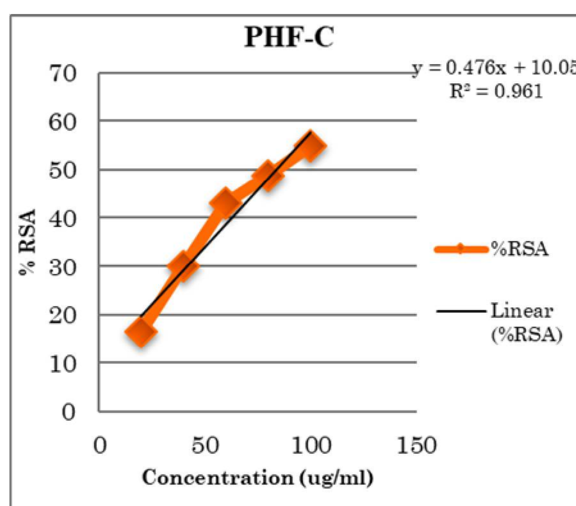


Fig No-3 Antioxidant Activity of Ascorbic Acid

Fig No-4 Antioxidant Activity of PHF-C

DISCUSSION

The presence of flavonoids, phenolics, and alkaloids in PHF-C provides a strong basis for its antioxidant potential. These compounds are well-documented for their roles in neutralizing free radicals through electron donation and radical scavenging mechanisms. The PHF-C showed robust scavenging of DPPH radicals, with effectiveness comparable to ascorbic acid at higher concentrations. This underscores the synergistic action of its phytochemical constituents in mitigating oxidative damage. The moderate yet significant NO scavenging activity further validates the PHF-C's ability to counteract reactive nitrogen species. This activity may be enhanced by the terpenoids and glycosides present in the formulation. The combined antioxidant activities demonstrated by DPPH and NO methods affirm the therapeutic relevance of PHF-C. The polyherbal approach effectively leverages the individual strengths of its components, enhancing overall efficacy while reducing potential side effects. The findings highlight PHF-C as a promising candidate for managing oxidative stress-related conditions. Future studies could focus on *in vivo* evaluations, clinical trials, and further exploration of its molecular mechanisms to establish therapeutic applications.

CONCLUSION

The present study highlights the presence of a diverse range of bioactive compounds, including proteins, carbohydrates, alkaloids, glycosides, steroids, flavonoids, terpenoids, and phenolic compounds, which are known to contribute the significant antioxidant potential of a polyherbal formulation (PHF-C), prepared using ethanolic extracts of five selected medicinal plants. The antioxidant evaluation using DPPH and NO free radical scavenging methods demonstrated that the PHF-C exhibits notable free radical scavenging activity in a concentration-dependent manner. The percentage inhibition values indicate the PHF-C's efficacy in neutralizing oxidative stress, comparable to the standard antioxidant, ascorbic acid. These findings suggest that the PHF-C is a promising natural source of antioxidants, potentially useful in mitigating oxidative stress-related damage. The combination of phytochemicals likely acts synergistically to enhance the antioxidant activity of the formulation. This provides a strong foundation for its application in developing therapeutic agents. This study sets the stage for further pharmacological evaluations, including in vivo investigations and clinical trials, to confirm the efficacy and safety of the PHF-C. The formulation holds the potential for addressing oxidative stress-related disorders and contributing to the development of novel, plant-based therapeutic solutions.

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