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PHYTOCHEMICAL SCREENING AND *IN-VITRO* ANTIOXIDANT PROPERTY OF POLYHERBAL MIXTURE OF *RUBIA CORDIFOLIA, VITEX NEGUNDO, PIPER NIGRUM, MYRISTICA FRAGRANS*

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ABSTRACT

The antioxidant activity of a hydroalcoholic extract of polyherbal mixture (RVMP) using four different herbs, namely *Rubia cordifolia, Vitex negundo, Piper nigrum,* and *Myristica fragrans*, was determined in this study using 1,1-diphenyl-2-picrylhydrazyl (DPPH), reducing power assay, hydroxyl radical scavenging assay, and nitric oxide scavenging assay (NO).The findings show that polyherbal mixture (RVMP) has significant antioxidant activity. The total phenolic content and flavanoids were also ascertained. The polyherbal mixture contained a significant amount of phenolic content. The current study's antioxidant activity data suggest that polyherbal mixture (RVMP) is a superior source of natural antioxidants, which may aid in the prevention of oxidative stress progression.

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INTRODUCTION

Plants contain natural antioxidants that scavenge damaging free radicals from our bodies.[1] If not removed immediately, these hazardous by-products can cause oxidative damage to functioning macromolecules like DNA, proteins, and lipids.[2]. Superoxide anions (O2. -), hydroxyl radical (.OH), singlet oxygen, hydrogen peroxide (H_2O_2), ferric ion, nitric oxide (NO), and perhaps other reactive oxygen species (ROS) are examples. [3] When all these free radicals are produced in excess, they can be detrimental, causing inflammation, cancer, ischemia, lung damage, rheumatoid arthritis, ageing, cardiovascular disease, and other degenerative diseases. [4, 5] Reactive oxygen species aggravate cell membrane disruption by oxidising polyunsaturated fatty acids within cell membranes and lipoproteins via metal ion-dependent hydroxyl radical formation. Proteins that are attacked by free radicals may fragment or aggregate, interfering with ion channels, cell receptors, and oxidative phosphorylation.[6] Plants are rich in antioxidant compounds such as phenolic acids, polyphenols, and flavonoids, which have been linked to a broad spectrum of biological effects including antioxidant activity.[7] The phytochemicals have been discovered to act as antioxidants by scavenging free radicals, and they may have therapeutic potential for free radical-related disorders. [8]

Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are restricted in foods, and they have been suspected of being carcinogenic. As a matter of fact, the search for natural antioxidants, particularly ones derived from plants, has soared in recent years.[9] Because of safety concerns, plant-based antioxidants are now preferred over synthetic antioxidants. Natural antioxidants, such as polyphenols found in medicinal and dietary plants, are gaining popularity as a means of preventing oxidative damage. Natural antioxidants boost antioxidant capacity and lower disease risk. Different parts of the plant, such as the seeds, leaves, and bark of the stem and root, are known to contain significant amounts of phyto-constituents such as phenolics, flavonoids, and tannins, which have the ability to inhibit the excessive production of free radicals and thus can act as antioxidants. The enduring interest in natural antioxidants among plant secondary metabolites has received growing attention as people become more aware of herbal remedies as potential sources of phenolic antioxidants. The focus of this research was to take a gander into the antioxidant properties, total phenolic and flavonoid contents of a polyherbal mixture that included roots of *Rubia cordifolia*, fruit of *Piper nigrum*, leaves of *Vitex Negundo* and seed of *Myristica fragrans*

MATERIALS AND METHODS

Collection of plant materials

Rubia cordifolia and *Vitex negundo* were collected from our college's herbal garden, and *Piper nigrum* and *Myristica fragrans* were collected from a local market in Vadodara, Gujarat. Botanists identified and authenticated the various parts of plants.

Preparation of Polyherbal Mixture

The roots, fruits, leaves, and seeds were all shade dried for 3-4 weeks before being finely powdered in a mixture and sieved twice to obtain a fine powder.

Step I: 100gm of powdered crude plant materials (*Rubia Cordifolia* roots, *Piper nigrum* fruit, *Vitex Negundo* leaves, and *Myristica fragrans* seed) were weighed and transferred into conical flasks. N-Hexane was measured and filled in the flask approximately three times the volume of powder for defatting. For 24 hours, the conical flasks were kept at room temperature. After 24 hours, the n-Hexane-containing powders were filtered and air dried.

Step II: Following the defatting process, the dried powders were transferred to conical flasks. Each flask was filled with a 3:7 mixture of ethanol and water, so that the volume of liquid was approximately three times that of the dry powder. For 24 hours, the conical flasks were kept at room temperature. The contents of the flasks were filtered after two days. The filtrates were placed in a porcelain dish and dried by heating them in a water bath. Scraping the powders from the porcelain dish was used to collect them. The extract was dried until it reached a constant weight. *Rubia Cordifolia* yielded 7.13 %, *Piper nigrum* 14.18 %, *Vitex Negundo* 19.72 %, and *Myristica fragrans* 11.32 %. 30 mg of *Rubia Cordifolia*, *Vitex Negundo*, *Myristica fragrans*, and 10mg of *Piper nigrum* extract were combined and labelled as Polyherbal mixture (RVMP).This extract mixture was dissolved in 10ml of ethanol, boiled on a water bath for 5 minutes, cooled, and centrifuged at 4000 rpm for 10 minutes. The antioxidant property was assessed using clear supernatant.

General chemical tests for preliminary phytochemical screening[10]

According to a methodology, dried hydroalcoholic extracts of *Piper nigrum, Myristica fragrans*, *Vitex Negundo*, and *Rubia Cordifolia* were tested for the presence of alkaloids, tannins, glycosides, carbohydrates, saponins, flavonoids, phenols, terpenoids, and phytosterols. The results of phytochemical analysis were expressed as the presence (+) or absence (-) of phytochemicals.

Phytochemical analysis

To determine the presence of phytochemical constituents, the Polyherbal mixture (RVMP) was subjected to preliminary phytochemical studies using standard procedures. The hydroalcoholic extract of polyherbal mixture (RVMP) was found to contain important phytochemicals such as flavonoids, phenols, and others, which enhanced the anti-oxidant potential.

Evaluation of *in vitro* antioxidant activity

All chemicals and solvents used in the study were of analytical grade and the chemicals were purchased from Merck.

Determination of Total Phenolics [11]

The total phenolic content of a polyherbal mixture (RVMP) was determined using the Folin-reagent Ciocalteu's (FCR) and gallic acid as a standard (10-100 µg/ml). Various concentrations of polyherbal mixture (RVMP) 1mg/mL were mixed with 0.25mL of FCR and incubated at room temperature for 15 minutes before adding 1.25 mL of 20% w/v sodium carbonate solution and adjusting the volume with double distilled water. The mixture was kept for 30 minutes until the blue colour developed. The absorbance was measured in a UV spectrophotometer at 725 nm. The total phenolic content was calculated as a gallic acid equivalent from the calibration curve using the following formula:

Formula:

$$T=C*V/M$$

Where,
T = Total content of phenolic compound (mg/gm of plant extract)
C = the concentration of gallic acid established from the calibration curve.
V = the volume of extract (ml)
M = the gram of weight of plant extract.

Determination of Total Flavonoid Content [12]

The flavonoid content of the polyherbal mixture (RVMP) was determined by creating a calibration curve with standard Quercetin (10-100µg/mL in methanol).

A 0.5mL standard solution was combined with 1.5mL of 95% ethanol, 0.1mL of 10% aqueous aluminium chloride, 2.8mL of distilled water, and 0.1mL of 1M potassium acetate. After 30 minutes at room temperature, the absorbance of the reaction mixture was measured with a UV spectrophotometer at 415nm. The blank solution was made by combining 10% aluminium chloride and the same amount of distilled water.

The absorbance of the polyherbal mixture (RVMP) was measured at 510 nm versus water as a blank. The analysis was carried out in triplicate, and the results were expressed as quercetin equivalents.

DPPH radical scavenging activity [13]

The antioxidant activity of the polyherbal mixture (RVMP) was determined in terms of radical scavenging ability or hydrogen donating property, using the stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33). The polyherbal mixture (RVMP) at various concentrations (10 - 100µg/mL) was added to 1 ml of a DPPH solution (0.2 mM in ethanol) as the source of free radical and was kept for 30 minutes at room temperature. The decrease in the solution absorbance, due to proton donating activity of polyherbal mixture, was measured at 517 nm. Ascorbic acid was used as positive control. The percentage DPPH radical scavenging activity was calculated as follows:
DPPH radical scavenging activity (%) = $\{(A_0 - A_1)/ A_0 \text{ X } 100\}$

Where A_0 is the absorbance of the control and A_1 is the absorbance of Polyherbal mixture (RVMP) or of the standard sample (Ascorbic acid).

Nitric oxide radical scavenging activity [5, 14, 15]

The nitric oxide scavenging activity of the polyherbal mixture (RVMP) was determined by mixing 3ml of 10mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) with various concentrations (10 - 100µg/mL) of polyherbal mixture (RVMP) and incubating at room temperature for 150 minutes. Following the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 0.1 % naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. A chromophore was formed during the diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylethylenediamine, and the absorbance of that chromophore was measured at 546 nm. The sample's percentage radical scavenging activity was calculated as follows:
% NO radical scavenging activity = $\{(A_0 - A_1)/ A_0 \text{ X } 100\}$
Where A_0 is the absorbance of the control and A_1 is the absorbance of Polyherbal mixture (RVMP) or of the standard sample.

Hydroxyl radical scavenging activity [16]

The polyherbal mixture RVMP (10-100µg/mL) was mixed with 1ml of iron-EDTA solution (0.13 % ferrous sulphate and 0.26 percent EDTA), 0.5 ml of EDTA solution (0.018 %), and 1ml of dimethyl sulfoxide (DMSO) (0.85 % v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 0.5ml of ascorbic acid (0.22 %), and the reaction mixture was incubated in a water bath at 80-90°C for 15 minutes. After 15 minutes of incubation, the reaction was stopped by adding 1ml of ice-cold TCA (17.5% w/v). Three millilitres of Nash reagent (75.0g ammonium acetate, 3 ml glacial acetic acid, and 2 ml acetyl acetone were mixed with distilled water to make a final volume of 1 L) was added and left at room temperature for 15 minutes. The intensity of the formed colour was measured spectroscopically at 412nm against a blank. By following formula, the percentage inhibition was calculated.

$$\% \text{ Inhibition} = \{(A_0 - A_1) / A_0 \text{ X } 100\}$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of Polyherbal mixture (RVMP) or of the standard sample.

Reducing power assay [17]

In this method, aliquots of the standard and Polyherbal mixture (RVMP) (10 to 100 µg/ml) in 1.0 ml of distilled water were mixed with 2.5 ml of (pH 6.6) phosphate buffer and 1% potassium ferricyanide (2.5ml). In a water bath, the mixture was incubated for 20 minutes at 50°C. After cooling, 2.5 ml of 10% tri-chloro-acetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of solution (5 mL) was mixed with 5 mL of distilled water and 1 mL of freshly prepared (0.1%) ferric chloride solution. The absorbance was measured in a UV spectrophotometer at 700 nm (UV-2201, Systronic double beam). A blank was created by omitting the Polyherbal mixture. Ascorbic acid was used as a standard at various concentrations (10 to 100 µg/ml). According to these findings, an increase in the absorbance of the reaction mixture indicates an increase in reducing power.

RESULTS AND DISCUSSION

Medicinal plants with high polyphenol content are thought to be a good source of antioxidant compounds. Ingestion of these plant products can shield from a variety of health problems by halting free radical-mediated cell damage. The antioxidant activity was assayed using the DPPH, Reducing power assay, Nitric oxide scavenging, and Hydroxyl radical scavenging assays. According to the findings of this study, a polyherbal mixture of *Piper Nigrum, Myristica Fragrans, Vitex Negundo,* and *Rubia Cordifolia* contained a significant portion of flavonoids and phenolic compounds with high antioxidant activity. This recommends that plants are a notable source of natural antioxidants, which may aid in the prevention of the progression of various oxidative stresses. However, further isolation of bioactive compounds would aid in determining its potency and safety as one of the leading candidates for antioxidant activity for pharmaceutical applications. Table 1 shows the results of phytochemical screening, which confirmed the presence of various phytoconstituents such as alkaloids, phenols, carbohydrates, glycosides, flavonoids, tannins, saponins, essential oils.

Table: 1 Qualitative Analysis *Piper nigrum, Myristica fragrans , Vitex Negundo, Rubia Cordifolia.*

Sr. No	Chemical constituent	Piper Nigrum	Myristica Fragrans	Vitex Negundo	Rubia Cordifolia
1	Carbohydrates	+	+	+	+
2	Alkaloids	+	+	+	+
3	Flavonoids	+	+	+	+
4	Steroids	-	+	-	-
5	Glycosides	-	-	-	-
6	Tannins	-	+	-	+
7	Phenolic compound	+	+	+	+
8	Terpenoids	+	-	-	-
9	Essential oil	+	+	+	+
10	Saponins	+	+	-	+

Total phenolic content

The total phenolics content of Polyherbal mixture (RVMP) was found to be 325.5 $\pm$ 2.3 GAE/g dry weight of extract. Owing to specific environmental conditions and ongoing physiological cellular functions in the body, generation of free radical is prominent. This radical lack an electron thus they have an electric charge making them highly unstable. In order to stabilize it-self they either remove an electron from or donate it to a neighbouring molecule, ultimately leading to a chain reaction wherein the newly formed radical now tries to withdraw or donate the electron causing damage to other millions of molecules. Phenolic compounds by virtue of their hydroxyl group bestow the scavenging ability. The standard and sample extracts show comparable linear relation of total phenol.

Total Flavonoid Content

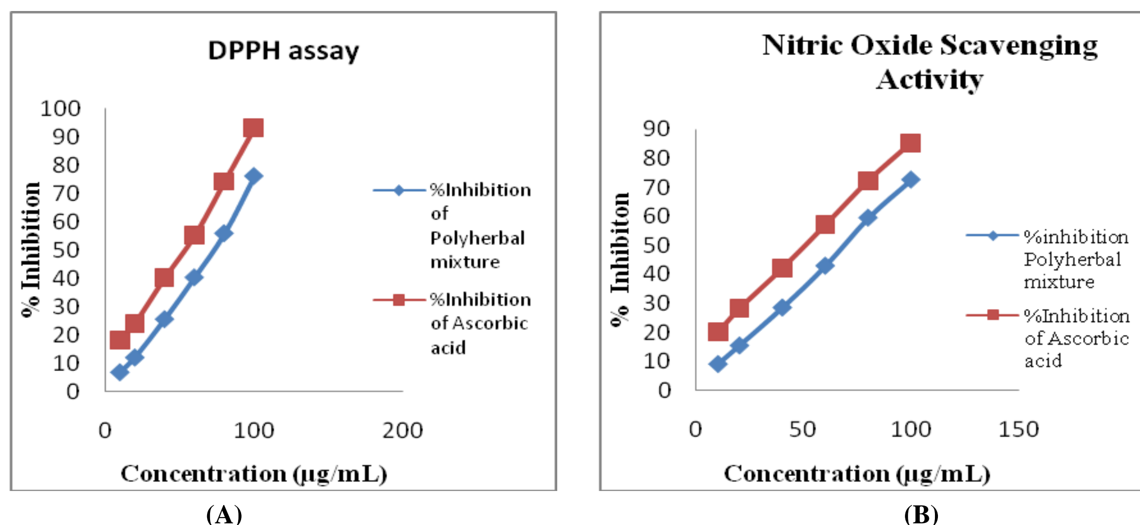
The total flavonoid content Polyherbal mixture (RVMP) was found to be 211.3 $\pm$ 3.4 mg quercetin / g of plant extracts. Flavonoids are diphenyl propane compounds that are commonly found in leaves, flowering tissues, stems, and barks. Flavanoids have inherent ability to inhibit plethora of enzymes involved in oxidation system, thereby conferring protection against free radical owing to their potent antioxidant property.

DPPH free radical scavenging activity

The DPPH radical is reasonably stable. The assay is based on measuring antioxidants' ability to scavenge the stable DPPH radical, when it interacts with a suitable reductant. The electrons become paired off, and the solution ends up losing hue stoichiometrically as the number of electrons taken up 20 increases. Fig. 1A showed the DPPH scavenging effect of the polyherbal mixture (RVMP) increased with increasing concentrations as compared to standard ascorbic acid, and the IC_{50} value of the polyherbal mixture (RVMP) was observed as 51.08 µg/mL and the IC_{50} value of standard ascorbic acid was 39.45 µg/mL, inferring DPPH scavenging as compared to ascorbic acid.

The Polyherbal mixture (RVMP) was reported to have higher radical scavenging. At a concentration of 100 µg/mL, the scavenging activity of hydroalcoholic extracts of polyherbal mixture (RVMP) is 76.07 %, while the standard's scavenging activity is 93.08 %. In Fig. 1B, the scavenging ability of Hydroalcoholic extracts of polyherbal mixture (RVMP) at 100 µg/mL was found to be responsible for 72.65 % of nitric oxide free radical scavenging activity, whereas standard ascorbic acid inhibited activity by 85.43 % at the same concentration.

At a concentration of 100μg/mL, the hydroalcoholic extracts of polyherbal mixture (RVMP) showed significant hydroxyl radical scavenging activity of up to 79.63 %, while ascorbic acid showed 89.61 % inhibition at the same concentration shown in Fig. 1C.



(A) (B)

Fig. 1: A) DPPH radical scavenging activity B) Nitric oxide scavenging activity of polyherbal mixture and Standard Ascorbic Acid.

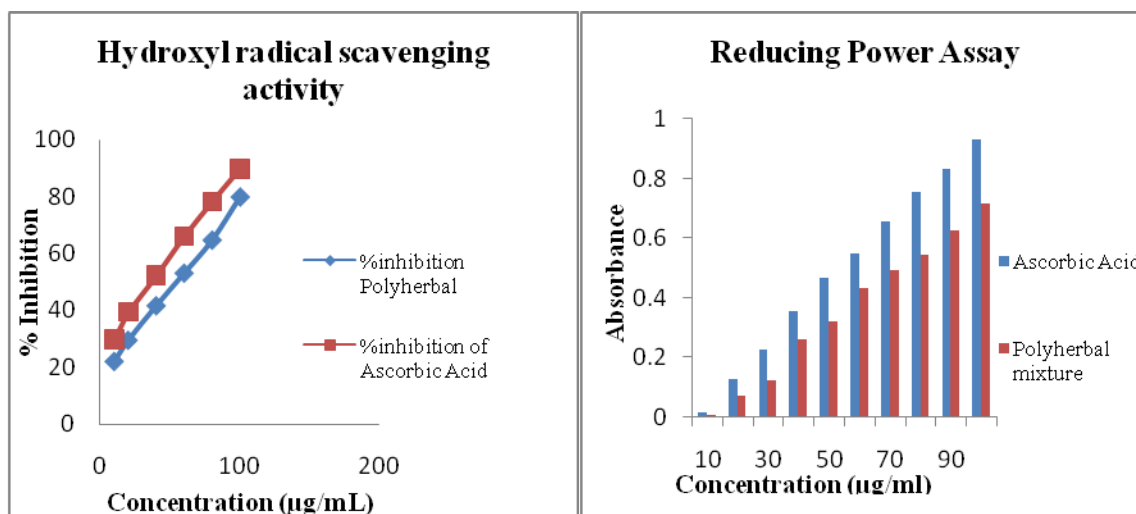


Fig1: C) Hydroxyl radical scavenging activity D) Reducing Power Assay of Polyherbal mixture and

CONCLUSION

The polyherbal mixture (RVMP) of *Rubia cordifolia, Vitex negundo, Myristica fragrans* and *Piper nigrum* possess high free radical scavenging activity and phytochemical constituents which may prove useful for future studies to discover novel treatment strategies.

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