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SCREENING OF PHYTOCHEMICAL AND IN VITRO ANTIOXIDANT PROPERTY OF N-MIRACLE (POLYHERBAL FORMULATION).

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ABSTRACT

Antioxidants are substances that inhibit oxidation, and are capable of counteracting the damaging effects of oxidation in body tissue. They prevent damage caused by free radicals. They create a barrier from free radial damage that results in decaying process of oxidation. Under most pathological conditions there is generation of reactive oxygen species and other free radicals. Polyherbal compounds are concentrated pharmaceutical preparations of plants obtained by removing active constituents with a suitable solvent, which is evaporated away, and adjusting the residue to a prescribed standard. The investigation of phytochemical compounds of the material extracted in ethanol, methanol and water revealed the presence of saponins, flavonoids, steroids, terpenoids, triterpenoids, alkaloids, carbohydrates, anthraquinone, phenolic compounds, and glycosides. Antioxidant activity of the N-Miracle (Polyherbal formulation) was also assessed by DPPH assay, total antioxidant assay, superoxide radical scavenging assay, and reducing power assay. They showed commendable antioxidant effect as that of the ascorbic acid. The antioxidant properties were due to the presence of promising phytoconstituents in N-Miracle (polyherbal formulation).

KEYWORDS: Antioxidants, Reactive Oxygen Species, N-Miracle, Phytoconstituents, Polyherbal formulation.

INTRODUCTION

Reactive oxygen species such as superoxide anions, hydroxyl radicals, hydrogen peroxide and singlet oxygen are formed as a result of normal metabolic activity and due to exogenous sources. The oxidative stress created by these radicals, leads to a range of biological and physiological lesions culminating in metabolic impairment, cell death, and degenerative diseases such as cancer, diabetes, obesity and neural disorder.[1]

Antioxidants are compounds that detoxify ROS and prevent their damage through multi mechanisms. Synthetic antioxidants have been in use as food additives for a long time, but reports on their involvement in chronic diseases have restricted their use in foods. Therefore, international attention has been focused on natural antioxidants mainly from plant sources. During certain diseased state, as well as during aging, there is a need to boost the antioxidant abilities, thereby potentiating the immune mechanism. The antioxidants preserve and stimulate the function of immune cells against homeostatic disturbances.[2]

The medicinal plants, which contain the high amount of polyphenols, are considered to be good source of natural antioxidant compounds and more often possess higher antioxidant potential than that of dietary fruits and vegetables. Consumption of these plant products certainly prevents the free radical mediated damage in the cell and therefore protects the body from several health problems. These antioxidant compounds can be used as natural antioxidant additives or nutritional supplements in the food products. As of natural origin, these antioxidants are much safe to use. Thus, much attention has been focused on the investigation of natural antioxidant compounds from plants, which can effectively scavenge ROS.[3] Polyphenols constitute a large group of naturally occurring substances in the plant kingdom, which include the flavonoids. These substances have considerable interest in the field of food chemistry, pharmacy and medicine due to a wide range of favorable biological effects including antioxidant properties. The antioxidant property of phenolics is mainly due to their redox properties. They act as reducing agents (free radical terminators), hydrogen donors, singlet oxygen quenchers and metal chelator.[4] Herbal medicines are not a simple task since many factors influence the biological efficacy and reproducible therapeutic effect. Standardized herbal products of consistent quality and containing well-defined constituents are required for reliable clinical trials and to provide consistent beneficial therapeutic effects.[5] However, there was a limited study on screening of phytochemical and in vitro antioxidant of activity of polyherbal formulation. Hence, the current study was intended to

evaluate the antioxidant potential of N-Miracle (polyherbal formulation). Therefore, the present study was carried out to demonstrate the screening of phytochemicals and in vitro antioxidant property of N-Miracle, a polyherbal formulation. In this study, we aim to investigate the methanolic extract of N-Miracle for its phytochemical composition, DPPH radical scavenging activity, total antioxidant activity, superoxide radical scavenging activity, iron chelating activity, and reducing power activity by various in vitro methods.

MATERIALS AND METHODS

Phytochemical Screening of N-Miracle

Preparation of N-Miracle: N-Miracle, a polyherbal formulation, was prepared by the combination of the following medicinal plants.

A. **Conium Maculatum**: The common name for Conium Maculatum plant is Poison Hemlock or the Spotted Hemlock plant. Conium is used for treating azoospermia and low sperm count after a careful examination of the symptoms related to male genitalia.

B. **Lycopodium Clavatum**: Lycopodium clavatum, commonly known as Club moss, Clubfoot Moss, Foxtail, Ground Pine, Sulfer, Wolf's Claw is one of the most widespread species belonging to family Lycopodiaceae. It is a pteridophyte, which is abundantly found in tropical, subtropical and in many European countries. This spore bearing vascular plant is used in various traditional system of medicines viz. stomach pain, against rheumatic disease, muscle pain, Alzheimers disease etc.[6]

C. **Selenium:** Selenium is an essential trace element for humans and animals.[7] Se plays an important role in the regulation of various metabolic processes in the body, being an integral part of selenoproteins.[8] Selenium (Se) is an important element for human and animal nutrition, due to its roles on a series of biochemical reactions enhancing antioxidant activity.[9]

D. **Vitex Agnus-castus**: Vitex is a deciduous shrub native to European, Mediterranean and Central Asian countries. It has slender, finger-like leaves, purple-black berries, and belongs to the Verbenaceae family.[10] The berries are used medicinally, with use dating back to the ancient Greeks and Romans. The berries were used by monks during the Middle Ages to sup-press sexual desire; hence its common names – monk's pepper and chaste tree.[11] Vitex agnus castus, rich in phenolics, is a new naturally potential antioxidant source.[12]

E. **Pausinystalia Yohimbe**: It is a member of the family Rubiaceae. It is a valuable medicinal tree, distributed in evergreen closed- canopy forests in West Africa. It is traditionally used for treatment of erectile dysfunction and diabetes.[13]

F. **Caladium Seguinum:** Caladium or Caladium Seguinum is also called the American Arum. Caladium is reputed to have a pronounced effect on the genital organs of both males and females.

Preparation of extract: 5g of the powder of N-Miracle were transferred into three different 250 mL conical flask, which contains 100 mL of three different solvents ethanol, methanol and water. The conical flask containing N-Miracle and solvent was mixed well for 48 hours by free hand. After 3 days, the extracts were filtered using Whatmann filter paper No.1.and was transferred into a china dish. The supernatant was completely removed by keeping the china dish over a boiling water bath at about 45^{o}C. The resultant extract was stored at 4^{o}C in airtight bottle until further use.

Qualitative Phytochemicals Screening: Methanol, ethanol and water extract of N-Miracle were tested for different phytoconstituents using standard procedures.[14]

Quantitative Phytochemicals Screening

Determination of total phenol: The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark in the 50ml flask and left to react for 30 min for colour development. This was measured at 505 nm.[15]

Determination of total flavonoids: 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No.42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.[16]

In vitro antioxidant activity of N-Miracle

DPPH radical-scavenging activity: DPPH radical scavenging activity was assessed according to the method of Shimada et al (1992). 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 mL sample solution at different concentrations. The mixture was

shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity. The percentage inhibition was calculated according to the following equation.

DPPH scavenging effect (%) = Ao – A1 / Ao X 100.

Where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample.[17]

Total antioxidant capacity: The antioxidant activity of the extract was evaluated by the phosphomolybdate method according to the procedure describe by Prieto et al. (1999). The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95^{o}C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

(%) Inhibition = Ao – A1 / Ao X 100.

Where A0 was the absorbance of the control (blank, without extract) and A1 was the absorbance in the presence of the extract.[18]

Superoxide anion scavenging activity: Measurement of superoxide anion scavenging activity of resveratrol was based on the method described by Liu *et al*., (1997). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation.

(%) Inhibition = ((A0-A1) / A0 × 100)

Where A0 was the absorbance of the control (blank, without extract) and A1 was the absorbance in the presence of the extract.[19]

Fe^{2+} chelating activity assay: To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2mM) was added. After 30 s, 0.1 ml ferrozine (5mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as.

$$\text{Chelating rate (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.[20]

Reducing power assay: The reducing power activity of N-Miracle was determined according to the method previously described by Oyaizu (Oyaizu, 1986). The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate (K3Fe(CN)6) (1%, w/v), followed by incubating at 50^{o}C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 800 rpm for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride (FeCl3) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.[21]

STATISTICAL ANALYSIS: Values were expressed as Mean ± SD for triplicates.

RESULTS

Phytochemical screening of N-Miracle: The different phytochemicals present in N-Miracle was presented in Table.1. N-Miracle showed positive results for the presence of secondary metabolites like steroids, saponins, triterpenoids, alkaloids, carbohydrates, flavonoids, polyphenols and glycosides. Tannins and amino acids are absent in all extracts prepared. Bioactive active compounds like saponin, flavonoids, steroids, terpenoids, alkaloids, carbohydrates, anthraquinone, polyphenols, and glycosides were present in ethanolic and methanolic extract of N-Miracle.

Results for total phenol and total flavanoid contents: Total phenol content and total flavanoid content were given in Table.2. The total phenolic content of N-Miracle was found

to have 123.45 mg/g and the total flavonoid content of N-Miracle was found to have 63.54 mg/g respectively.

In vitro antioxidant activity

DPPH free radical scavenging activity: Table.3 and Fig.1 showed the DPPH scavenging effect increased with the increasing concentrations of methanolic extract of N-Miracle as compared to standard ascorbic acid and IC_{50} value of the N-Miracle was observed as 51.83 µg/mL and IC_{50} value of standard ascorbic acid was 35.03 µg/mL, which indicates the DPPH scavenging effective of N-Miracle as compared to ascorbic acid.

Total antioxidant activity: Total antioxidant activity of the methanolic extract of N-Miracle was determined by phosphomolybdate method. The IC_{50} value of the methanolic extract of N-Miracle and standard (ascorbate) was found to be 42.69µg/ml and 42.41 µg/mL respectively. This was shown in Table.4 and Fig.2.

Superoxide anion scavenging activity: The methanolic extract of N-Miracle had a strong superoxide radical scavenging activity (Table.5 and Fig.3). The IC_{50} value was found to be 64.13 µg/mL for N-Miracle and for standard ascorbic acid, it was found to be 31.62 µg/mL.

Fe^{2+} chelating activity: The Fe^{2+} chelating activity of N-Miracle was given in Table.6 and Fig.4. The methanolic extract of N-miracle had a strong iron chelating activity. The IC_{50} value was found to be 53.13 µg/mL for N-Miracle and for standard ascorbic acid, it was found to be 30.96 µg/mL.

Reducing power activity Methanolic extract of N-Miracle has shown good reducing powder activity that was comparable with ascorbic acid (Table.7 and Fig.5). Increased absorbance of the reaction mixture indicated the increased reducing powder and the highest reducing power activity of methanolic extract of N-miracle was found to be 86% inhibition at 80 µg/mL concentration as compared to ascorbic acid.

Table. 1: Phytochemical screening of different extracts of N-Miracle (polyherbal formulation)

S. No.	Secondary Metabolites	Extracts		
		Ethanol	Methanol	Water
1.	Tannin	--	--	--
2.	Phlobatannins	--	--	--
3.	Saponin	+	+	+
4.	Flavonoids	+	++	+
5.	Steroids	+	+	+
6.	Terpenoids	+	+	+
7.	Triterpenoids	+	+	+
8.	Alkaloids	+	+	+
9.	Carbohydrate	+	+	+++
10.	Amino acid	--	--	--
11.	Anthroquinone	+	+	+
12.	Polyphenol	++	++	--
13.	Glycoside	++	+	+

(+) Presence: (-) Absence (++) High concentrations

Table. 2-Quantitative analysis of total phenols and flavonoids in N-Miracle (polyherbal formulation)

Phytoconstituents	Results (mg/gm)
Total Phenolics	123.45
Flavonoids	63.54

Values were expressed as Mean ± SD for triplicates

Table. 3-Percentage of DPPH radical scavenging activity of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations

Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)	IC_{50} (µg/ml)
Test Sample	17.81±1.24	31.81±2.22	63.81±4.46	78.77±5.51	51.83
Standard (Ascorbic acid)	25.6±2.04	61.26±4.90	88.98±7.11	99.34±7.94	35.03

Values were expressed as Mean ± SD for triplicates

Table. 4-Percentage of total antioxidant activity of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations.

Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)	IC_{50} (µg/ml)
Test Sample	28.32 ± 2.20	47.59 ± 3.71	65.56 ± 5.11	87.04 ± 6.69	42.69
Standard (Ascorbic acid)	22.35± 1.80	51.23± 4.09	72.54± 5.80	86.35± 6.91	42.41

Values were expressed as Mean ± SD for triplicates

Table. 5- Percentage of superoxide radical scavenging activity of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations

Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)	IC_{50} (µg/ml)
Test Sample	11.95±0.83	26.71±1.86	48.4±3.38	63.5±4.44	64.13
Standard (Ascorbic acid)	31.25 ± 2.50	64.23 ± 5.13	89.54 ± 7.16	98.51 ± 7.88	31.62

Values were expressed as Mean ± SD for triplicates

Table. 6- Percentage of iron chelating activity of methanolic extract N-Miracle (polyherbal formulation) at different concentrations

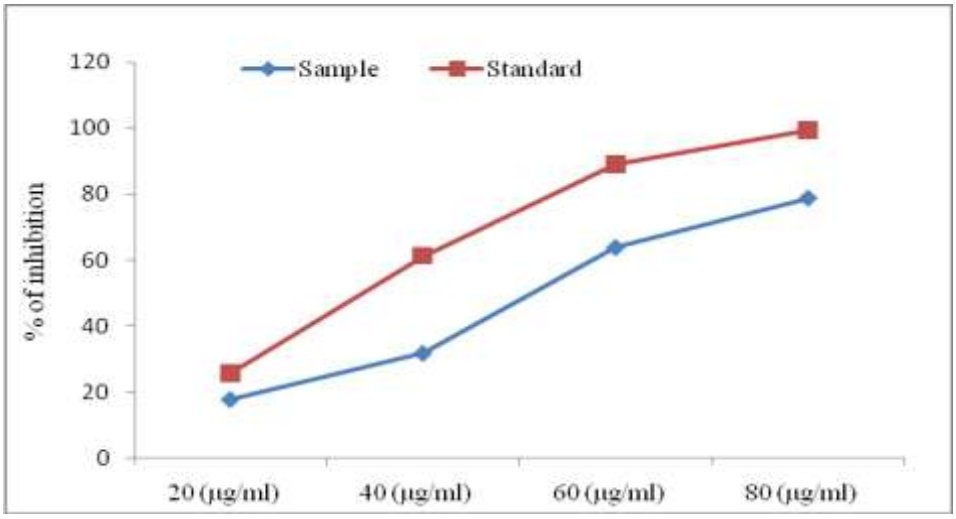
Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)	IC_{50} (µg/ml)
Test Sample	19.71±1.37	31.33±2.19	56.5±3.95	81.28±5.68	53.13
Standard (Ascorbic acid)	35.23 ± 2.81	65.21 ± 5.28	78.51± 6.28	98.65 ± 7.89	30.96

Values were expressed as Mean ± SD for triplicates

Table. 7- Reducing power assay of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations

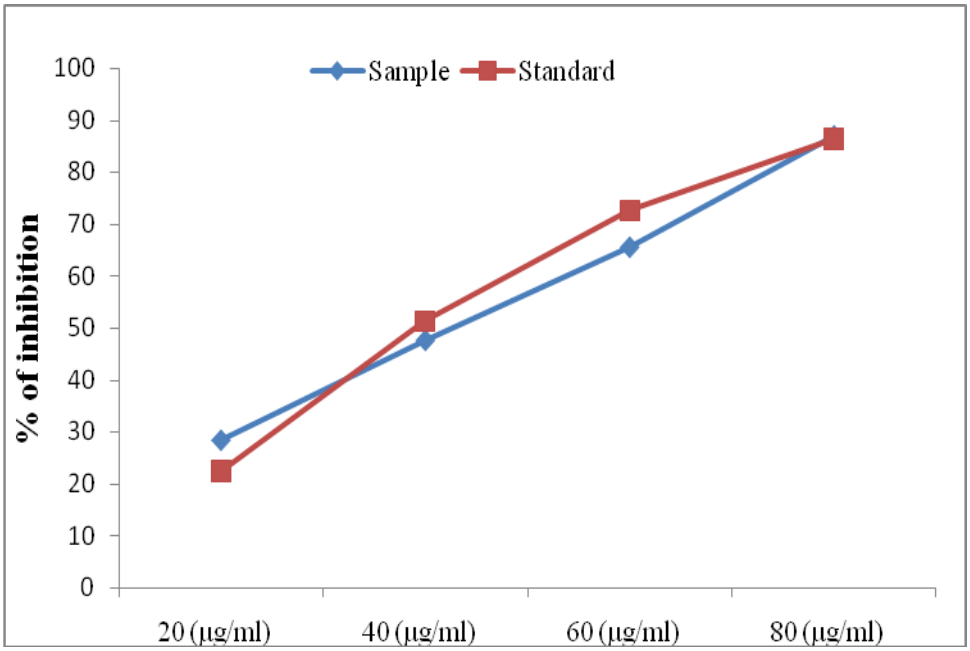
Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)
Test Sample	0.19±0.006	0.31±0.007	0.68±0.016	0.86±0.025
Standard (Ascorbic acid)	0.41± 0.03	0.71 ± 0.05	0.89± 0.07	0.98 ± 0.08

Values were expressed as Mean ± SD (Optical density) for triplicates



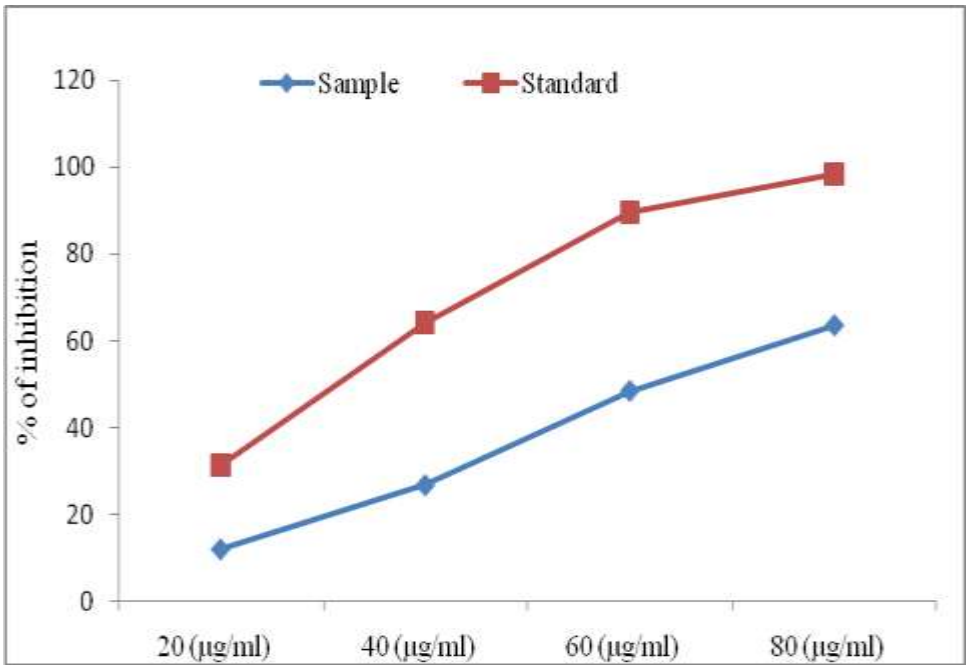
Values were expressed as Mean ± SD for triplicates

Fig. 1-Percntage of DPPH radical scavenging activity of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations



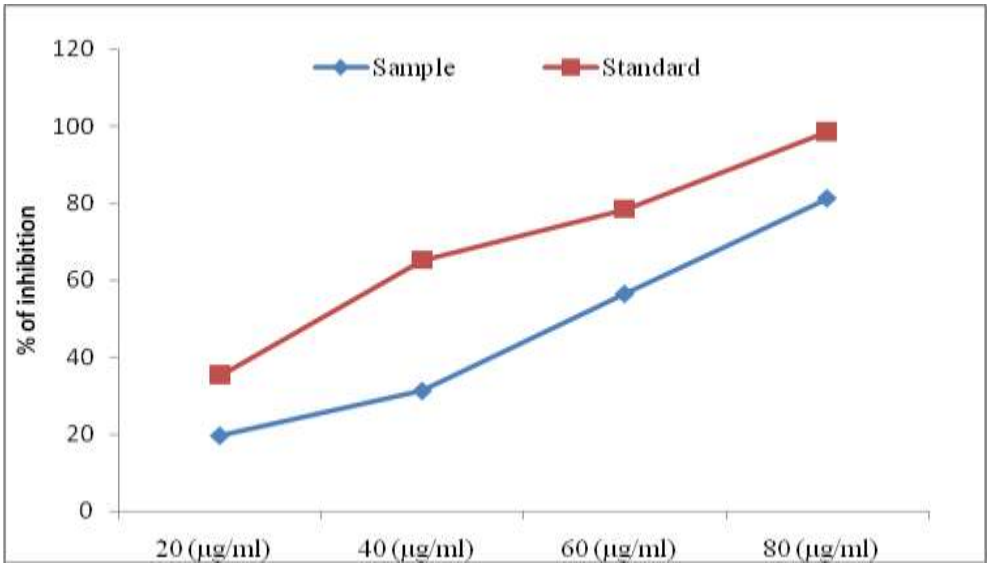
Values were expressed as Mean ± SD for triplicates

Fig. 2- Percentage of total antioxidant activity of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations



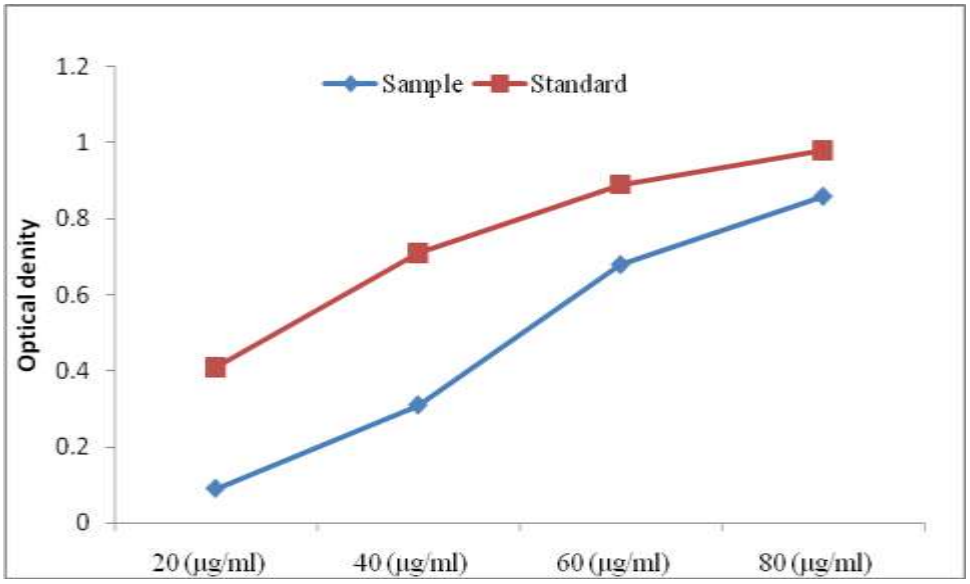
Values were expressed as Mean ± SD for triplicates

Fig. 3-Percentage of superoxide radical scavenging activity of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations



Values were expressed as Mean ± SD for triplicates

Fig. 4: Percentage of iron chelating activity of methanolic extract of N-Miracle (polyherbal formulation) at different concentrations



Values were expressed as Mean ± SD for triplicates

Fig. 5: Reducing power assay of methanolic extract N-Miracle (polyherbal formulation) at different concentrations.

DISCUSSION

The present study carried out on N-Miracle revealed the presence of medicinally active phytochemicals. These phytochemical compounds are known to support bioactive activities in N-Miracle and thus responsible for the antioxidant activity. The presence of flavonoids in the extract is likely to be responsible for the free radical scavenging effect. Flavonoids and

polyphenols are a major group of compounds that may act as primary antioxidants or free radical scavengers.[22]

Antioxidants block the action of free radicals, which have been implicated in the pathogenesis of many diseases and in the aging process.[23] Due to the presence of flavonoids and phenol suggests that the N-Miracle might have an antioxidant, anti-allergic, antiinflammatory, antimicrobial, and anticancer activity.[24] The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites. They possess biological properties such as antiapoptosis, antiaging, anticarcinogen, anti inflammation, anti atherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities.[25] Plants extract containing carbohydrates, glycosides are known to exert a beneficial action on immune system by increasing body strength and hence are valuable as dietary supplements.[26]

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation.[27] It was found that the radical-scavenging activity is increased with increasing concentration. This significant scavenging ability in the above extracts could be attributed to the presence of phenolic and flavonoids in methanolic extract of N-Miracle.

The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes.[28] The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.[29] The methanolic extract was found to be an effective scavenger of superoxide radical generated by photo reduction of riboflavin. Superoxide anions radical is one of the strongest ROS among the free radicals and get converted to other harmful reactive oxygen species such as hydrogen peroxide and hydroxyl radical, damaging biomolecules, which results in chronic diseases.[30] For the measurements of the reductive ability, it has been found that the $Fe3^{+}$ to $Fe2^{+}$ transformation occurred in the presence of extract samples.[31] The reducing properties are generally associated with the presence of reductones[32], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[33] Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation.

The reducing power of methanolic extract of N-Miracle was found remarkable and observed to rise as the concentration of the extract gradually increased.

CONCLUSION

The results obtained in the present study revealed the presence of important phytochemicals present in the methanolic extract of N-Miracle. The present study also demonstrated in vitro antioxidant efficacy of N-miracle. The finding of this study also suggests that N-Miracle could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases. The activity observed may be attributed to the presence of phenolic and flavanoid contents in the methanolic extract.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. The research received no specific grant from any funding agency in the public, community, or non-for profit sectors.

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