IMPACT OF HERBAL SYNERGY ON ANTIOXIDANT EFFICACY OF A NOVEL FORMULATION

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ABSTRACT

Synergistic effect of herbs in a formulation leads to the development of combination therapy. Since, medicinal plants are a well known source of several natural antioxidants in the form of diet, therefore, two medicinally important edible plants viz. Murraya Koeingii and Sesamum indicum, known for their antioxidant efficacy were selected for the present study. Herbal formulation (HF) was prepared by the combination of standardized aqueous extracts of Murraya koeingii (MK) leaves and Sesamum indicum (SI) black seeds. The study was undertaken to explore the impact of synergy on antioxidant efficacy of individual herbs in a herbal formulation. Various assays used for evaluating antioxidant efficacy were total phenolics, total flavonoids, total antioxidant power (FRAP), Reducing power and Metal chelating activity. Free radical scavenging activity of herbal formulation as well as of its individual herbs was also assessed for various radicals viz. DPPH, ABTS, Nitric oxide, Hydroxyl and Superoxide radicals. The data reveals that the antioxidant efficacy of the herbal formulation significantly enhances due to the synergistic effect of both the herbs, Murraya Koeingii and Sesamum indicum. Thus, this formulation of MK leaves and SI seeds could be explored not only as an antioxidant therapeutic agent but also as an agent for managing diabetic complications viz. Oxidative Stress associate with hyperglycemia.

KEYWORDS: Herbal Formulation, Murraya Koeingii and Sesamum indicum.
INTRODUCTION
Synergy between two or more herbs plays a vital role in enhancing their bioefficacy. Oxidative stress, resulting from an imbalance between formation and neutralization of reactive oxygen species (ROS), is the root cause of today's diseases viz. cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases.\textsuperscript{[1,2]} Since, antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells\textsuperscript{[3]} therefore a large number of medicinal plants has been investigated for their antioxidant properties being cost effective and safe.\textsuperscript{[4]} Natural antioxidants either in the form of raw extracts or chemical constituents isolated from it are very effective to control the damages caused by oxidative stress.\textsuperscript{[5]} Though, human system has an inherent antioxidative mechanism but sometimes there may be an imbalance between formation of ROS and the inherent antioxidant capacity of the system.\textsuperscript{[6]} These free radicals cause various chronic disorders especially diabetes etc. The ingestion of natural antioxidants has been inversely associated with morbidity and mortality from such degenerative disorders as the antioxidant contents of medicinal plants contribute significantly to their therapeutic value. The use of medicinal plants rich in antioxidant constituents has been proposed by our research group as an effective therapeutic approach for controlling diabetes as well as diabetes induced oxidative stress.\textsuperscript{[7,8]}

There are several herbal formulations in the ayurvedic system of medicine which has been used to treat a wide variety of diseases. The formulations have the ability to act by various mechanisms and therefore it could be possible that different combinations of different plants will be more effective than the individual plant itself.\textsuperscript{[9]}

Hence, the present study explores the impact of synergy of two different medicinal plants viz. \textit{Murraya koenigii} L. Spreng (leaves) and \textit{Sesamum indicum} L. (black seeds) on their antioxidant capacity. The biherbal formulation was developed in the ratio 1:1 (50/50, w/w) and the synergy was assessed \textit{in vitro} for its antioxidant efficacy.

\textit{Murraya koenigii} L. Spreng is known as Curry leaf in hindi. It is a tropical tree and belongs to the family Rutaceae. It is cultivated in India not only for its characteristic flavour but also for its aroma. Its leaves are used in south Indian cuisine and ayurvedic medicine.\textsuperscript{[10]} It’s leaves has already been reported as anti-diabetic \textsuperscript{[11, 12]} and are also much valued as antioxidant.\textsuperscript{[13]} In addition to it, its antimicrobial \textsuperscript{[14]}, anti inflammatory \textsuperscript{[15]} and hepatoprotective \textsuperscript{[16]} attribute are also well established. Curry leaves also have a rich profile
of natural antioxidant viz. simple phenolic acids including tannic, gallic, cafffeic, cinnamic, chlorogenic, ferulic and vanillic acids.\cite{17} Thus, it could be explored for assessing its synergy with other herbs in a formulation.

Sesame (Sesamum indicum L.) belongs to the family Pedaliaceae.\cite{18,19} It grows in subtropical and tropical regions. It is believed that sesame originates from the Indian sub-continent.\cite{20,21} It is commonly known as ‘Til’ in Hindi in India. Sesame seeds are very nutritious and contain about 50% of oils and 20% of proteins. The traditionally obtained oil from the sesame seeds has an excellent quality and stability because it contains natural antioxidants such as sesamine, sesamol, sesaminol, phenols, flavonoids, γ-tocopherol, inositol hexaphosphate, lignans and their glucosides.\cite{18,22} Bioactive profile of sesame seeds is also very high, having antioxidant\cite{23}, antihyperlipidemic\cite{24}, anti-inflammatory\cite{25} and antihypertensive activities.\cite{26} Hence, it was selected as the counter herb for the formulation to be explored for evaluating the herbal synergy on antioxidant efficacy of the formulation.

*In vitro* antioxidant and free-radical scavenging activities of the herbal formulation was performed for total phenolics, total flavonoids, total antioxidant activity in terms of FRAP (Ferric Reducing Antioxidant Power), reducing power, metal chelating as well as DPPH (1, 1-diphenyl-2-picrylhydrazyl), ABTS, peroxide/hydroxyl, Superoxide, nitrosyl radical scavenging activities. Since, both the counter herbs of formulation had already been explored for their antioxidant efficacy individually. Therefore, the present study deals with the exploration of their synergistic effect on antioxidant efficacy of their formulation for treating diabetes induced oxidative stress and oxidative stress as well.

**MATERIALS AND METHODS**

*Plant collection and identification*

Fresh leaves of *M. koenigii*, and black seeds of *S. indicum* were purchased locally from Allahabad, U. P., India, and got identified by Prof. Satya Narayan, Taxonomist, Department of Botany, University of Allahabad, Allahabad, U. P., India. A voucher specimen has been submitted to the University herbarium. The leaves and seeds were first washed well, shed dried, and powdered. They were then extracted in the ratio 1:1 (50/50 w/w) with distilled water by hot extraction using soxhlet apparatus till colorless solvent was obtained. Extract obtained was allowed to dry till constant weight was obtained (10% w/w).
**Chemicals**

Folin-Ciocalteau reagent, Griess reagent, gallic acid, potassium ferricyanide, Tricarboxylic Acid (TCA), quercetin, BHT (Butylated Hydroxy Toluene), ascorbic acid, sodium carbonate, FeCl₃, DPPH, H₂O₂, KNO₂, MnO₂, AlCl₃, 2, 4, 6-tripyridyl-s-triazine (TPTZ), FeSO₄, HCl, Sodium Nitroprusside (SNP), ferrozine, sodium salicylate, acetic acid and sodium acetate were purchased from Merck Chemicals, New Delhi, India. All other chemicals and reagents were of analytical grade.

**Experimental Design**

Antioxidant potential of *Murraya koengii* (MK), *Sesamum indicum* (SI) and their herbal formulation (HF) was evaluated using different *in vitro* assays such as estimation of total phenolics, total flavonoids, reducing power, total antioxidant power in terms of FRAP and by measuring the scavenging activity of ABTS⁺, DPPH, NO⁻, OH⁻, and metal chelating activity. All the assays were carried out in triplicates and their average values were taken into consideration.

**Antioxidant assay - *in vitro***

- **Determination of Total Phenolics**

Total phenolic contents in MK, SI and their aqueous HF were determined by the modified Folin-Ciocalteau method by Wolfe et al. [27] An aliquot (100 µl) of the plant samples of MK, SI and their HF of different concentrations was mixed with 2.5 ml Folin-Ciocalteau reagent (diluted with water 1:10 v/v) and 2.0 ml (75 g/l) of sodium carbonate. The tube was vortexed for 15 s and allowed to stand for 30 min at 40°C for colour development. Absorbance was recorded against blank at 765 nm using Shimadzu UV-VIS spectrophotometer. The total phenolic contents were expressed as mg/g gallic acid equivalent. The calculation was based on the standard curve of gallic acid.

- **Determination of Total Flavonoids**

Total flavonoid contents were determined using the method of Ordon et al. [28] Solutions of different concentrations of MK, SI and their HF, as well as the standard, quercetin were prepared separately in methanol. A volume of 0.5 ml of 2% of AlCl₃-ethanol solution was added to 0.5 ml of varying concentrations. After 1 h at room temperature, the absorbance was measured at 420 nm. A yellow colour indicated the presence of flavonoids. The total flavonoid contents were expressed as mg/g quercetin equivalent.
Assessment of Ferric Reducing Antioxidant Power (FRAP)

A modified method of Benzie and Strain \cite{29} was adopted for the FRAP (Ferric Reducing Antioxidant Power) assay. The stock solutions included 300 mM acetate buffer (3.1 g CH3COONa and 16 ml CH3OOH), pH 3.6, 10 mM TPTZ solution in 40 mM HCl and 20 mM FeCl$_3$.H$_2$O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml FeCl$_3$.H$_2$O. The temperature of the solution was raised to 37°C before using. MK, SI & their HF as well as standard (150 µl) was allowed to react with 2850 µl of the FRAP solution for 30 min in dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm. Results were expressed in µM Fe$^{2+}$/g dry mass of extract.

Assessment of Reducing Power

The reducing power of MK, SI and their HF was determined by the method of Oyaizu et al.\cite{30} About 1 ml of the plant samples/standard in variable concentrations (100-500 µg/ml) were taken separately, mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% [K$_3$Fe(CN)$_6$]. The mixture was incubated at 50°C for 20 min followed by addition of 2.5 ml of 10% TCA and then centrifuged at 3000 rpm for 10 min. The upper layer was mixed with deionised water and FeCl$_3$, and then absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic Acid was used as standard.

Free radical scavenging assays

DPPH Radical Scavenging Assay

The effect of MK, SI and their HF on DPPH radical was determined using the method of Liyana-Pathiranan and Shahidi.\cite{31} The plant samples/standard of different concentrations (100-500 µg/ml) dissolved in de-ionised water, was mixed in 1.0 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.135 mM DPPH. The mixture was vortexed thoroughly and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a control. Ascorbic acid was used as standard. The decrease in absorbance of the mixtures indicates an increasing DPPH radical scavenging activity. The ability to scavenge DPPH radical was calculated by the following equation

DPPH radical scavenging activity (%) = [(A$_0$-A$_1$)/A$_0$ X 100],

where, A$_0$ is the absorbance of control and A$_1$ is the absorbance of MK, SI and their HF/standard.
**ABTS Radical Scavenging Assay**

The ability of MK, SI and their HF, to scavenge ABTS radical cation was determined by Re et al.\[32\]. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12–16 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS•+ solution with 60 mL methanol to obtain an absorbance of 0.706 units at 734 nm. ABTS•+ solution was freshly prepared for each assay. Plant extract with different concentrations (1 mL of each) were allowed to react with 1 mL of the ABTS•+ solution and the absorbance was taken at 734 nm after 7 min. The ABTS•+ scavenging capacity of the extracts were compared with different concentrations of Ascorbic Acid and percentage inhibition calculated as

\[
\text{ABTS radical scavenging activity (\%) } = \frac{(A_0 - A_1)}{A_0} \times 100,
\]

where, \(A_0\) is the absorbance of ABTS radical + methanol and \(A_1\) is the absorbance of ABTS radical + sample extract/standard.

**Metal Chelating Assay**

The chelating of ferrous ions by extract was determined by the method of Dinis et al.\[33\]. Briefly, the sample (MK, SI and their HF/standard, 100-500 µg/ml) was added to a solution of 2 mM FeCl\(_2\) (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was shaken vigorously and kept at room temperature for 10 min. The control contains FeCl\(_2\) and ferrozine only. The absorbance of the resulting solutions were measured at 562 nm against control. Ascorbic acid was used as standard. The decrease in absorbance of the mixtures indicates an increasing metal chelating activity. The ability to chelate ferrous ions was calculated by the following equation

\[
\text{Metal chelating activity (\%) } = \frac{(A_0 - A_1)}{A_0} \times 100,
\]

where, \(A_0\) is the absorbance of control and \(A_1\) is the absorbance of MK, SI and their HF/standard.

**NO Radical Scavenging Assay**

Scavenging of Nitrosyl radical was determined by incubating 5 mM SNP in PBS (Phosphate Buffered Saline), with different concentrations (50-400 µg/ml) of the plant samples/standard at 25°C. After 120 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent.\[34\] The absorbance was measured at 550 nm against a control. Ascorbic acid was used as standard and treated in the same way with Griess reagent and the absorbance
was measured. The decrease in absorbance of the mixtures indicates an increasing Nitrosyl radical scavenging activity. The amount of nitrite was calculated from standard curve constructed by sodium nitrite.

Nitrosyl radical scavenging activity (%) = [(A₀ - A₁)/A₀ × 100],
where, A₀ is the absorbance of control and A₁ is the absorbance of MK, SI and their HF /standard.

**Hydroxyl Radical Scavenging Assay**

Hydroxyl radical scavenging activity was measured according to a modified method of Smirnoff and Cumbes.\(^{[35]}\) Hydroxyl radicals were generated from the mixture of FeSO₄ and H₂O₂. The reaction mixture contained 1 ml FeSO₄ (1.5 mM), 0.7 ml H₂O₂ (6 mM), 0.3 ml sodium salicylate (20 mM) and sample (plant samples/standard, 100-500 µg/ml). After incubation, for 1 h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm against control. Ascorbic acid was used as standard. The decrease in absorbance of the mixture indicates an increasing hydroxyl/peroxide radical scavenging activity. The ability to scavenge hydroxyl radical was calculated by the following equation:

Hydroxyl radical scavenging activity (%) = [1 - (A₁ - A₂)/A₀ × 100],
where, A₀ is the absorbance of control and A₁ is the absorbance of MK, SI and their HF /standard, A₂ is the absorbance without sodium salicylate.

**Superoxide radical scavenging Assay**

Superoxide radicals were generated by the PMS/NADH system according to the method of Kakkar et al.\(^{[36]}\) The reaction mixture was composed of 1 ml of NBT (156 lM), 1 ml NADH (468 lM) and 100 µl either of plant sample (MK, SI & their HF) or standard compounds. The reaction was started by addition of 100 ll of PMS (60 lM) to the mixture. After 5 min incubation at 25°C, absorbance was measured at 560 nm against an appropriate blank to determine the quantity of formazan generated. Ascorbic Acid was used as a standard. The decrease in absorbance of the mixture indicates an increasing superoxide radical scavenging activity. The ability to scavenge superoxide radical was calculated by the following equation:

Superoxide radical scavenging activity (%) = [(A₀ - A₁)/A₀ × 100],
where, A₀ is the absorbance of control and A₁ is the absorbance of MK, SI and their HF /standard.
RESULTS AND DISCUSSION

Antioxidant assay - in vitro

Determination of total phenolic contents

Table 1, shows the results of total phenolic content of MK, SI & their aqueous HF. The results are expressed as equivalents of Gallic Acid. Standard curve equation of the standard taken, Gallic Acid was calculated with $y = 0.0042x + 0.1031$, $R^2 = 0.9932$. Among these three samples, aqueous HF was found to be the best source of phenolic contents in comparison to its individual counterparts i.e. MK and SI revealing thereby that the enhanced activity of the HF than the original plant extracts is due to the synergistic effect only.

Phenolics are the well-known group of secondary metabolites and comprise a large group of biologically active compounds.\textsuperscript{[37]} The antioxidant activities of phenols were credited to their redox properties, which permit them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, as well as their metal chelating abilities. The phenolic contents found in HF thus exhibited remarkable antioxidant efficiency which may be due to its phenolic hydroxyl groups that have the ability to scavenge free radicals.

Determination of total flavonoid contents

Table 1, also shows the results of total flavonoid contents of MK, SI & their aqueous HF. The results are expressed as equivalents of standard taken, Quercetin. Standard curve equation of Quercetin was calculated with $y = 0.0536x - 0.0038$, $R^2 = 0.968$. Among these three samples, aqueous HF was found to be a potential source of flavonoid contents as compared to its individual constituents i.e. MK and SI hence revealing once agains the herbal formulation’s synergistic effect.

Flavonoids are important secondary metabolite of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It is well established that pharmacological effect of flavonoids is associated with their antioxidant activities.\textsuperscript{[38]}

Determination of Ferric Reducing Antioxidant Power (FRAP) activity

Table 1, also reveals the results of the FRAP ability of MK, SI & their aqueous HF. The aqueous HF exhibited highest degree of FRAP ability, $149.7 \text{ mM Fe}^{2+}/g$, in terms of electron donation capacity. However, the two individual plant extracts of aqueous HF viz. MK & SI, recorded FRAP values of $99.25 \text{ mM Fe}^{2+}/g$ and $13 \text{ mM Fe}^{2+}/g$, showed much lesser values in comparison to their aqueous HF.
The FRAP assay provides a direct estimation of the level of antioxidants present in a sample based on its capability of reduction of ferric (Fe\(^{3+}\)) into ferrous (Fe\(^{2+}\)). Because an antioxidant is capable of donating a single electron to the ferric-TPTZ ((Fe\(^{3+}\) - TPTZ) complex causing thereby reduction of this complex into the blue ferrous-TPTZ (Fe\(^{2+}\) - TPTZ) complex which absorbs strongly at 593nm.

**Table 1: Total Phenolic & Total Flavonoid contents and FRAP activity of MK, SI & their HF.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Phenolic contents (mg/g of GAE)</th>
<th>Total Flavonoid contents (mg/g of QE)</th>
<th>FRAP activity (µM Fe(^{2+})/g of extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbal Formulation (HF)</td>
<td>82.75 ± 0.17</td>
<td>17.96 ± 0.13</td>
<td>147.9 ± 1.34</td>
</tr>
<tr>
<td><em>Murraya koenigii</em> (MK)</td>
<td>59.2 ± 0.12</td>
<td>3.73 ± 0.11</td>
<td>99.25 ± 1.67</td>
</tr>
<tr>
<td><em>Sesamum indicum</em> (SI)</td>
<td>12.87 ± 2.21</td>
<td>5.15 ± 0.18</td>
<td>13 ± 2.12</td>
</tr>
</tbody>
</table>

**Assessment of Reducing Power**

“Fig.1” indicates the results of Reducing Power of MK, SI & their aqueous HF in addition to the results of Standard, Ascorbic Acid (AA). The reducing power of the aqueous HF was found to be greater than the individual plant extracts. However, it was slightly lesser than the AA, the Standard. The reducing powers for all the four samples were found to be concentration-dependent i.e. absorbance recorded for the samples increased with increase in concentration with maximum absorbance recorded at the highest evaluated concentration of 500 µg/ml.

**Fig. 1: Reducing Power estimation of MK, SI, HF and Standard, Ascorbic Acid (AA)**
This reducing capacity of compounds is related to its electron-donating ability and may be due to phenolic substance mediated antioxidant activity.\cite{30,39,40} Thus, aqueous HF contained higher amounts of reducing compounds (electron-donating reductants), which serve as electron donors and reacts with free radicals, as a result, convert them to more stable products and terminate radical chain reaction.

**Free radical scavenging activity**

**DPPH radical scavenging activity**

“Fig. 2” exhibits the results of DPPH radical scavenging activity of MK, SI & their aqueous HF in addition to the results of standard, Ascorbic Acid (AA). It was observed that the aqueous HF showed higher DPPH activity than its individual counterparts viz. MK & SI. However, it was almost at par with the AA, the Standard. The scavenging activity of MK, SI & their aqueous HF was found to exhibit 78.54, 61.55 and 96.11\% inhibition, respectively at the highest evaluated concentration of 400μg/ml. While at the same concentration, inhibition by AA, the standard, was found to be 96.47\% which was almost comparable to that of HF. The IC\textsubscript{50} of HF, MK and SI were found to be 84.03, 150 and 260.83μg/ml, respectively (Table 2). On the other hand, the IC\textsubscript{50} value of AA, the standard, was found to be 11.08μg/ml.

![Fig. 2: DPPH Scavenging Activity of MK, SI, HF and Standard, Ascorbic Acid (AA)](image)

The approach of scavenging the stable DPPH radicals is a widely used method to evaluate the hydrogen donating capacity of antioxidants, since it involves a relatively short time compared to other methods. The decrease in absorbance may be due to the proton donating ability\cite{41} of phenolic constituents present in the plant samples. As the concentration of phenolic
compounds or degree of hydroxylation of the phenolic compounds increases their DPPH radical scavenging activity also increases and can be defined as antioxidant activity.\[42\]

**ABTS radical scavenging activity**

“Fig. 3” indicates the results of ABTS radical scavenging activity of *MK, SI* & their aqueous HF in addition to the results of Standard, Ascorbic Acid (AA). The total antioxidant activity of the *MK, SI* & their aqueous HF was based on the decolorization of ABTS\(^+\), which was measured spectrophotometrically at 734 nm. Interaction with the plant samples or standard, Ascorbic Acid suppressed the absorbance of the ABTS radical cation.

It was observed that the aqueous HF exhibited higher scavenging activity than its individual counterparts viz. *MK* & *SI*. However, it was slightly lesser than, AA, the Standard. At the highest evaluated concentration of 125\(\mu\)g/ml, the scavenging activity of the *MK, SI* & their aqueous HF showed, 78.48, 36.2 and 94.25% inhibition, respectively while at the same concentration, that of the AA recorded 99.12% inhibition. Also, the IC\(_{50}\) values of aqueous HF, *MK* and *SI* were 54.94, 72.90 and 177.90\(\mu\)g/ml, respectively (Table 2). While, the IC\(_{50}\) value of AA, the Standard was found to be 47.61\(\mu\)g/ml. Thus, the results depicted that the aqueous HF is a potentially better antioxidant than its individual plant extracts.

ABTS\(^+\) is a blue chromophore produced by the reaction between ABTS and potassium per sulfate. Addition of the plant samples to this pre-formed radical cation reduced it to ABTS in a concentration dependent manner.

![Fig. 3: ABTS Scavenging Activity of MK, SI, HF and Standard, Ascorbic Acid (AA)](image-url)
Metal chelating activity

“Fig. 4” illustrates the result of Metal chelating activity of MK, SI & their aqueous HF in addition to the results of Standard, Ascorbic Acid (AA). It was observed that the aqueous HF showed greater Metal chelating activity than its individual components viz. MK & SI. However, it was slightly lesser than AA, the Standard. At the highest evaluated concentration of 400μg/ml, the percentage inhibition of MK, SI and their aqueous HF were found to be 45.65, 55.46 and 76.42%. Whereas at the same concentration, that of standard, Ascorbic Acid was found to be 85.18%. Moreover, the IC₅₀ values of aqueous HF, MK and SI were found to be 209.83, 415.06 and 320.58μg/ml, respectively (Table 2). However, the IC₅₀ value of AA, the standard, was calculated to be 51.30μg/ml.

Metal chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation.[43] Ferrozine can quantitatively form complexes with Fe²⁺ producing a violet complex. In the presence of a chelating agent, complex formation is interrupted and as a result the violet color of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. According to the results, herbal formulation was found to be the most active than the two individual plant extracts thereby indicating its interference with the formation of ferrous and ferrozine complex, suggesting that it has enhanced chelating activity by capturing ferrous ion before ferrozine.

![Fig. 4: Metal Chelating Activity of MK, SI, HF and Standard, Ascorbic Acid (AA)](image-url)
**NO radical scavenging activity**

“Fig. 5” exhibits the results of NO scavenging ability of MK, SI & their aqueous HF in addition to the result of standard, Ascorbic Acid (AA). It was found that the aqueous HF showed higher NO scavenging ability than its individual components viz. MK & SI. However, it was slightly lesser to the AA, the Standard. At the highest evaluated concentration of 400μg/ml, the percentage inhibition of MK, SI & their HF was found to be 47.12, 42.92 and 65.6% whereas, that of Ascorbic Acid was found to be 83.43%. Moreover, the aqueous HF, MK and SI were found to cause a moderate dose-dependent inhibition of nitric oxide radical with an IC$_{50}$ value of 239.92, 366.20 and 417.20 μg/ml, respectively (Table 2). On the other hand, IC$_{50}$ value of AA, the standard, was calculated to be 98.19μg/ml. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. Results indicate that the HF studied has more potent nitric oxide radical scavenging activity than its individual counterparts viz. MK and SI due to its greater scavenging ability in terms of percent inhibition and lower IC$_{50}$ value.

![Fig. 5: Nitric oxide radical scavenging activity of MK, SI, HF and standard, Ascorbic Acid (AA)](image)

It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis.[44] The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion.
The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite.

**Hydroxyl radical scavenging activity**

“Fig. 6” depicts the result of Hydroxyl radical scavenging activity of *MK, SI* & their aqueous HF in addition to the result of standard, Ascorbic Acid (AA). It was observed that the aqueous HF showed higher Hydroxyl radical scavenging ability than its individual components viz. *MK & SI*. However, it was slightly lesser than the AA, the standard. The IC\(_{50}\) values of the aqueous HF, *MK, SI* and AA, the standard, were found to be 156.58, 303.27, 468.49 and 20.54μg/ml, respectively (Table 2). At the highest evaluated concentration of 400μg/ml, percentage inhibition shown by *MK, SI*, aqueous HF and AA, the standard, were found to be 44.15, 64.89, 85.71% and 92.22%, respectively. Thus, the aqueous HF was found to be a highly efficient scavenger of hydroxyl radicals with highest percent inhibition of 85.71% at the highest evaluated concentration of 400μg/ml. While, the standard, AA, exhibited maximum percent inhibition of 92.22 at the same evaluated concentration. Contrarily, the two plants, *MK* and *SI* showed maximum scavenging activity of 64.89 and 44.15% at the same concentration, respectively.

Hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage *in vivo*. Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage. The evaluation of radical scavenging activity was based on the generation of OH\(^{•}\) by Fenton reaction. Thus, by comparing the percent inhibitions of the herbal formulation to the two aqueous plant extracts separately, we can point out that the aqueous herbal formulation was a more efficient hydroxyl radical scavenger than its individual counterparts. Moreover, the higher scavenging activity in terms of percent inhibition and lower IC\(_{50}\) value of HF than the individual plant extracts indicates that the herbal formulation is a better hydroxyl radical scavenger than its individual constituents.
Superoxide radical scavenging activity

“Fig. 7” depicts the result of Superoxide radical scavenging activity of MK, SI & their aqueous HF in addition to the result of standard, Ascorbic Acid (AA). It was observed that the aqueous HF showed higher Superoxide radical scavenging ability than its individual components viz. MK & SI. However, it was slightly lesser to the Standard, Ascorbic Acid (AA). The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT. Aqueous HF, MK, SI as well as the standard, Ascorbic Acid indicates their abilities to quench superoxide radicals in the reaction mixture. The IC$_{50}$ values of HF, MK, SI (Table 2) and Ascorbic Acid were found to be 24.97, 72.5, 82.52 and 16.39 μg/ml, respectively. At the highest evaluated concentration of 100μg/ml, the percentage inhibition of aqueous HF, MK, SI was found to be 96.14, 57.86 and 54.3% whereas that of Ascorbic Acid was found to be 92.46%. Thus, the lower IC$_{50}$ value and higher percent inhibition exhibited by the HF than the individual plant samples indicated its higher superoxide radical scavenging efficacy.

Superoxide anion is very harmful to cellular components.[48] Robak and Glyglewski[49] reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions. The results suggest that the HF is a more potent scavenger of superoxide radical than its individual plants.
CONCLUSION

In the present study, we aimed to develop a biherbal formulation with minimum number of standardized ingredients. Both *Sesamum indicum* black seeds and *Murraya koenigii* leaves are well-known for their multiple therapeutic benefits and are clinically well-accepted. Though, several reports have been reported individually of these plants for their therapeutic efficacy, this is the first reporting of a biherbal formulation prepared from these plants to investigate their synergistic effects *in vitro* on their antioxidant potential. Combination of herbal ingredients into a formulation to achieve synergistic effect is a common practice in the herbal industry. The success of an herbal product is also governed by its quality, safety and efficacy.\textsuperscript{[50]} Modern medicine prefers to use single ingredients on the grounds that dosage can be more easily adjusted, drug interactions could be prevented and quality control measures could be achieved with ease. In case of herbal medicines, they synergistically work better than a single herb.
On the basis of the results obtained in the present study, it is concluded that the herbal formulation of two different plants viz. *M. koenigii* and *S. indicum*, which contains large amounts of flavonoids and phenolic compounds, exhibits high antioxidant and free radical scavenging activities. It also chelates iron and demonstrates reducing power significantly. These *in vitro* assays indicate that this herbal formulation is a significant source of natural antioxidant, which might be helpful in preventing the progress of various kinds of oxidative stress. Results suggest that a fixed combination of the plant extracts of *Sesamum indicum* and *Murraya koenigii* could be an ideal candidate for the antioxidant activity of their biherbal formulation. However, the components responsible for its antioxidant activity needs to be elucidated. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the herbal formulation. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed to support its therapeutic application during clinical conditions.

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