Antioxidant Availability of Beheda (Terminalia bellerica (Roxb.)) in Relation to its Medicinal Uses

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INTRODUCTION

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body’s normal use of oxygen such as respiration and some cell mediated immune functions. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects.[1] The ROS inducing superoxide anionic radical (O$_2^-$), hydrogen peroxide (O$_2$H) and hydroxyl radicals (·OH) are implemented in oxidative damage to various cellular macromolecules. Increasing number of evidence suggested that oxidative stress induced biochemical changes are crucial etiological factors in several chronic human disease such as diabetes mellitus, cancer, atherosclerosis, arthritis, inflammation and neurodegenerative disease.[2] There have been many studies undertaken on how to delay or prevent the onset of these diseases. The most likely and practical way to fight against degenerative diseases is to improve body antioxidant status, which could be achieved by higher consumption of vegetables and fruits. Foods from plant origin usually contain natural antioxidants that can scavenge free radicals. The antioxidants may mediate their effect by directly reacting with ROS, quenching them and / or chelating the catalytic metal ions. Several synthetic antioxidants, e.g. BHA and BHT are commercially available but they are suspected to cause or prompt negative health effects, and also show low solubility and moderate antioxidant activity. Natural antioxidants, especially

Keywords: Antioxidant, FRAP assay, DPPH assay, Terminalia bellerica, ABTS assay, Total phenolic content.

ABSTRACT

Oxidative stress arises from an imbalance in the generation and metabolisms of ROS, with more of ROS being produced, than that is metabolized. Oxidative stress plays a significant pathological role in human diseases. However, the antioxidant supplements may be used to help the human body to reduce oxidative damage. Therefore the present study evaluated the antioxidant activity of Terminalia bellerica (Gertn.) Roxb. fruits. The study was designed to evaluate and compare the antioxidant activity, total phenolics, flavonoids content of aqueous (AETB) and ethanolic (EETB) extracts of fruits. The antioxidant activity was assessed by DPPH (1,1-diphenyl-1,2-picryl hydrazyl), ABTS (2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt), nitric oxide, superoxide and hydroxyl radical scavenging assay, FRAP (Ferric Reducing Antioxidant Power), reducing power and TAC (Total antioxidant capacity). AETB has shown higher antioxidant activity (% inhibition) as compared to EETB in nitric oxide, superoxide, ABTS(2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt) radical scavenging assay with IC$_{50}$ values 41.42±1.23, 892.85±2.73, 23.74±2.0 in AETB and 93.16±1.93, 3496.50±5.21, 55.89±2.21 in EETB respectively. Like antioxidant activity the reducing power increases in a dose dependent manner showing higher absorbance at 700 nm for AETB i.e. 0.95±0.03 as compared to EETB i.e. 0.43±0.01 at 500μg/ml. The FRAP values were found for AETB 1.68±0.07, EETB 1.06±0.03 and TAC values were found for AETB 2.53±0.07 and EETB 2.90±0.01. The amounts of total phenolic (TPC) and flavonoid content (TFC) were also determined. The results suggest that TPC and TFC contribute significantly to the antioxidant activity of the Terminalia bellerica (Gertn.) Roxb. Fruits.

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phenolic and flavonoids are safe and also bioactive. Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for human consumption.[3]

*Terminalia bellerica* Roxb. (family: Combretaceae), commonly known as belleric myrobalan and locally known as beheda, is an edible plant found throughout Central Asia.[4] Its fruit has been used in traditional medicinal system for anemia, asthma, cancer, colic, constipation, diarrrhea, dysuria, headache, hypertension, inflammations, and rheumatism.[5,6] It contains termilignan, thannilignan, 7-hydroxy-3,4′-(methylenedioxy) flavone, anolignan B,[7] gallic acid, ellagic acid, β-sitosterol,[8] arjungenin, belleric acid, bellericoside[9] and cannogenol 3-O-β-D-galactopyranosyl-(1→4)-(O-α-Lrhamnopyranoside).[10]

*Terminalia bellerica* is known to lower the lipid levels in hypercholesterolemic animals.[11] The ethanolic extract of *Terminalia bellerica* was found effective against several pathogens including *Bacillus subtilis*, *Proteus vulgaris*, *Salmonella typhimurium*, *Salmonella typhimurium*, *Escherichia coli*, and *Staphylococcus aureus*.[12] On the other hand, methanolic extract (75%) of *Terminalia bellerica* reduced the serum glucose level both in normal and alloxan-induced diabetic rats,[13] showing preventive effect against the myocardial necrosis in rats[14] water soluble fraction obtained from the defatted fruits of *Terminalia bellerica* caused hepatoprotection against CCl₄-induced hepatotoxicity.[15,16,17] reported that *Terminalia bellerica* lowers blood pressure (BP). In this study, we explored the *in vitro* antioxidant activity of the fruits of this plant.

**MATERIAL AND METHODS**

**Chemicals**

Trolox (6-hydroxy-2,5,7,3-tetramethylchroman-2-carboxylic acid), ABTS(2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diaminium salt) was purchased from Sigma Chemical Co. Ltd USA. DPPH (1,1 - diphenyl – 1,2 – picryl hydrayl), TPTZ(2,4,6-tripryidy-s-triazine), potassium ferricyanide, trichloroacetic acid (TCA), FeCl₃, sodium nitroprusside, sulphanilamide, naphthylethenediamine dihydrochloride, TPTZ(2,4,6-tripryidy-s-triazine), ascorbic acid, NBT (nitroblue tetrazolium), reduced NADH (nicotinamide adenine dinucleotide), PMS (phenazine methosulfate), sulphuric acid (H₂SO₄), ammonium molybdate, ammonium persulphate, ascorbic acid/ standard Vitamin C (Vit. C), quercetin and pyrocatechol was purchased from HiMedia, Mumbai. All other unlabelled chemicals and reagents were of analytical grade and used without further purification.

**Plant material**

The *Terminalia bellerica* (Roxb.) fruits were collected from Govali village, Kalyan, Thane district, Maharashtra, India. The plant material was taxonomically identified by Blatter Herbarium St.Xavier’s College, Mumbai. A voucher specimen (No. T-1114 of S.C. Tavakari) has been preserved in a laboratory for further reference. The collected fruits were dried under shade and powdered with a mechanical grinder and stored in an air tight container. The dried powder material of the fruits was soaked in distilled water and ethanol for 10hrs, to get an aqueous (AETB) and ethanolic extract (EETB) after filtration through Whatman paper No. 42.

**Preliminary phytochemical screening**

Qualitative phytochemical analysis of AETB and EETB was carried out as follows: Phenolics: 2ml of filtrate + 2ml FeCl₃, blue precipitate indicated presence of phenolics. Saponins (frothing test): 0.5 ml filtrate +5ml distilled water; frothing persistence indicated presence of saponins. Alkaloids: 2ml of filtrate +1%HCl+Dragendorff reagent, orange precipitate indicate the presence of alkaloids. Flavonoids: 5ml dilute ammonia was added to a portion of filtrate +concentrated sulphuric acid; yellow colour indicates presence of flavonoids. Steroids (Liebermann-Burchard reaction: 2ml filtrate +2ml acetic anhydride +concentrated sulphuric acid; green color indicates the presence of steroids. Terpenoids: 4ml of filtrate +concentrated sulphuric acid 3ml was added to form a layer; reddish brown colouration interface indicates the presence of terpenoids. Cardiac glycosides (Keller-Kinlini test): 2ml filtrate + 1ml of glacial acetic acid + FeCl₃ +concentrated H₂SO₄; brown colour indicates the presence of cardiac glycosides.[18]

**Total phenolic content**

The total phenolic content of different extracts was measured using colorimetric Folin –Cio-calteu method. The reaction mixture consisted 5ml of diluted sample to which 3 ml of distilled water and 0.5 ml Folin –Cio-calteu reagent was added. After 3minutes, add 2ml of 20% Na₂CO₃ solution and place the tubes in boiling water bath for one min, cooled and the absorbance was measured at 760 nm. Standard graph was prepared by using different concentration of pyrocatechol.[19]

**Total flavonoid content**

The flavonoid content of different extracts was measured using a modified colorimetric method. 0.5ml of sample was
mixed with 0.5 ml of 2% AlCl₃ and incubated for 10 mins. and the absorbance was measured at 415 nm. The measurement was compared to a standard graph for quercetin.²⁰

**Antioxidant Activity**

**Determination of reducing power (Fe³⁺ - Fe²⁺ transformation ability)**

The reducing power of a compound serves as a significant indicator of its potential antioxidant activity. Increased absorbance of the reaction mixture indicates increased reducing power.

Various conc. of the extracts in 1 ml of water were mixed with phosphate buffer (2.5 ml, 0.2 M pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged at 3000g for 10 min. Upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared FeCl₃ solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm.²¹

**Super oxide anion scavenging activity**

1 ml of NBT solution (144μM in 100mM phosphate buffer, pH 7.4), 1 ml of reduced NADH (677μM in 100mM phosphate buffer, pH 7.4) and 0.5 ml of sample extract was mixed and the reaction was started with adding 100 μl of PMS solution (60 μM PMS in 100mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 25°C for 5 min, and the absorbance 560 was measured against blank.²²

Insert using Microsoft formulae

\[
\% \text{ scavenging} = \left( \frac{A_{\text{con}} - A_{\text{test}}}{A_{\text{con}}} \right) \times 100
\]

**Nitric oxide radical scavenging activity**

Sodium nitroprusside (5 mM, 1 ml) in phosphate buffer saline (PBS) (0.1 M, 7.4 pH) was mixed with 3 ml of different conc. of the extract and incubated at 25°C for 150 min. 0.5 ml of the samples was mixed with 0.5 ml of Griess reagent (1% sulphanilamide, 2% H₃PO₄ and 0.1% naphthylethylenediamine dihydrochloride). Measure the absorbance at 546 nm.²³

Insert using Microsoft formulae

\[
\% \text{ scavenging} = \left( \frac{A_{\text{con}} - A_{\text{test}}}{A_{\text{con}}} \right) \times 100
\]

**DPPH radical scavenging activity**

The assay is based on the measurement of the scavenging ability of antioxidant towards the stable radical DPPH. DPPH radical react with suitable reagent, the electrons become paired off and the solution looses color stoichiometrically depending on number of electrons taken up. A volume of 2 ml of sample was added to 2 ml of phosphate buffer (0.02 M, pH 6) and 2 ml of 0.2 mM DPPH in 95% ethanol. The mixture was shaken and left for 30 min. at R.T. and the absorbance was measured at 517 nm.²⁴

The capability to scavenge the DPPH radical was calculated using following equation:

Insert using Microsoft formulae

\[
\% \text{ scavenging} = \left( \frac{A_{\text{con}} - A_{\text{test}}}{A_{\text{con}}} \right) \times 100
\]

**ABTS radical scavenging assay**

For ABTS assay, the method of Re²⁵ was adopted. The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate/ammonium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 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 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS+ scavenging capacity of the extract was calculated as ABTS radical scavenging activity (%) = [(Abscontrol - Abssample)/(Abscontrol)] × 100 where Abscontrol is the absorbance of ABTS radical + methanol; Abssample is the absorbance of ABTS radical + sample extract/standard.

**FRAP assay**

The ferric reducing ability was measured by Ferric Reducing Antioxidant Power (FRAP) assay. The stock solutions of 10mM TPTZ in 40 mM HCl, 20 mM FeCl₃·6H₂O and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml of acetate buffer. It was prepared freshly and warmed at 37°C. 900 μl of FRAP reagent was mixed with 90 μl of distilled water and 30 μl of sample solution. The reaction mixture was then incubated at 37°C for 30 min and absorbance was recorded at 595 nm. The concentration of FeSO₄ was in turn plotted against concentrations of the standard antioxidants (L-ascorbic acid and Trolox).²⁶

**Total antioxidant capacity**

0.1 ml of extract was combined in eppendorf tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM
Antioxidant Availability of Beheda (*Terminalia bellerica* (Roxb.))

sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in thermal block at 95°C for 90 minutes. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695 nm against blank. (27)

**Statistical analysis**

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance i.e. one way ANOVA and student’t’ test using GraphPad QuickCalcs.. The two-tailed P <0.05 the difference is considered to be statistically significant and if P <0.0001 the difference is considered to be extremely statistically significant The IC₅₀ values were calculated from linear regression analysis.

**RESULT AND DISCUSSION**

**Preliminary phytochemical screening**

Preliminary phytochemical screening of AETB and EETB revealed the presence of various bioactive components like flavonoids, sterols, terpenoids and phenolics while negative for the rest of classes of compounds. The presence of flavonoids and tannins in *Terminalia bellerica* might be contributing in its cardiovascular effects. (28)

**Total phenolic and flavonoid content**

Total phenolic contents are reported as pyrocatechol equivalents. The total phenolic contents of AETB and EETB were 10.92±0.05 and 10.32±0.09 pyrocatechol equivalent/g of sample, respectively. The P value < 0.0001, this difference is considered to be extremely statistically significant. The total flavonoid contents of AETB and EETB were 10.27±0.11 and 2.9±0.01 mg quercetin equivalent/g of sample. The P value < 0.0001, this difference is considered to be extremely statistically significant. AETB had higher total phenol and flavonoids contents than EETB. It has been reported that soft fruits and medicinal plants exhibited higher levels of flavonoids. (29) Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities. (30)

**Antioxidant activity**

Antioxidant activity of the extracts of varying concentrations ranging form 10- 10000 μg/ml was evaluated by various in vitro models. It was observed that the test compounds scavenged free radicals in concentration dependent manner in all the models. The result of FRAP and TAC assay were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) and Ascorbic acid Equivalent Antioxidant Capacity as described. TEAC is the concentration of Trolox (μmol/L) required to give the same antioxidant capacity as 1% (w/v) test substance. AEAC is the same for Ascorbic acid. (31)

**Determination of reducing power (Fe³⁺ – Fe²⁺ transformation ability)**

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl’s Prussian blue at 700nm. (32) Increasing absorbance at 700 nm indicates an increase in reductive ability. Table 1 shows that the reducing powers of all the extracts and standards also increased with the increase of their concentrations. There was an extremely significant difference (p< 0.001) among the AETB and EETB in reducing power. The activity of AETB and EETB was not comparable with Vit. C and trolox (p< 0.05) there were significant differences between them.

**Super oxide anion scavenging activity**

Superoxides are produced from molecular oxygen due to oxidative enzymes (33) of body as well as via nonenzymatic

<table>
<thead>
<tr>
<th>Table 1 Reducing power ((Fe³⁺ - Fe²⁺ transformation ability) of AETB, EETB, Vit C and Trolox</th>
<th>conc.(μg/ml)</th>
<th>Absorbance at 700nm</th>
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<tbody>
<tr>
<td></td>
<td>AEMP</td>
<td>EEMP</td>
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<td>50</td>
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<tr>
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<td>0.32±0.01</td>
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<td>500</td>
<td>0.95±0.01</td>
<td>0.43±0.01</td>
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</table>

Values are means ± S.D. (n=4)
reaction such as autoxidation by catecholamines.\textsuperscript{34} In the present study, superoxide radical reduces NBT to a blue colored formazan that is measured at 560 nm.\textsuperscript{35} Table 2a shows the superoxide scavenging effect of AETB in comparison to EETB on the PMS/NADH-NBT system. The increase of percentage scavenging activity thus indicates the consumption of superoxide anion in the reaction mixture by the plant extracts. AETB had strong superoxide radical scavenging activity as compared to EETB as IC\textsubscript{50} value of AETB (892.85±2.73 μg/ml) is less than EETB (4166.66±5.21 μg/ml). There were significant differences (p < 0.05) between AETB and EETB. The IC\textsubscript{50} value of Vit C is 66.31± 3.14, whereas Trolox shows at 2857.14± 1.01 μg/ml. Lower the IC\textsubscript{50} value of better is the scavenging ability of the sample.

**Nitric oxide radical scavenging activity**

The extract effectively reduced the generation of nitric oxide from sodium nitroprusside (Table 2a). In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent.\textsuperscript{36} Both AETB and EETB decreased the amount of nitrite generated from the decomposition of sodium nitroprusside in vitro which may be due to the presence of antioxidant principles in the extract. The percentage scavenging activity increased with increasing concentration of the extract. Lower the IC\textsubscript{50} value of better is the scavenging activity of the sample. There were significant differences (p < 0.05) between AETB and EETB. The IC\textsubscript{50} value of AETB and EETB was found to be 41.42±1.23, 89.28±2.17. However, Std Vit. C activity of was very more pronounced than that of our extracts (162±1.32μg/ml.)

**DPPH radical scavenging activity**

DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts.\textsuperscript{37} DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow coloured product, diphenyl picryl hydrazine, with the addition of all fractions in a concentration-dependent manner. All the concentration of AETB and EETB demonstrated H-donor activity. Lower the IC\textsubscript{50} value of better is the scavenging ability of the sample. The IC\textsubscript{50} values of AETB and EETB were 38.4±2.28μg/ml and 28.24±1.18μg/ml respectively. These activities are less than that of the Std Vit C and Trolox i.e. 10.40±1.43μg/ml and 17.42±1.21μg/ml respectively (Table 2b). There were significant difference (p < 0.05) between AETB and EETB.

**ABTS radical scavenging activity**

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals.\textsuperscript{38} The AETB and EETB were fast and effective scavengers of the ABTS radical and this activity was comparable to that of Vit. C. Higher concentrations of the extracts were more effective in quenching free radicals in the system. AETB has shown higher antioxidant activity (% inhibition) as compared to EETB in ABTS (2,2-Azino-bis 3-ethyl benothiazoline-6-sulfonic acid diammonium salt) radical scavenging assay with IC\textsubscript{50} values 24.71±2.21 in AETB and 50.12±0.87 in EETB respectively (Table 2a). There was significant difference (p< 0.05) between AETB and EETB. These activities are less than that of the Std Vit C and Trolox i.e. 33.94±1.23μg/ml and 5.42±0.27μg/ml respectively.

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**Table -2a % Radical scavenging activity of AETB and EETB in in vitro Assays at various concentrations**

<table>
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<tr>
<th>conc.(μg/ml)</th>
<th>% scavenging activity</th>
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<tr>
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<td>Superoxide anion radical</td>
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Values are mean ± S.D (n=4)
Table 2b % Radical scavenging activity of AETB and EETB in in vitro Assays at various concentrations

<table>
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<th>conc. (μg/ml)</th>
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<td>DPPH radical</td>
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Values are mean ± S.D (n=4)

FRAP assay

In FRAP (Ferric reducing antioxidant power) assay the ability of plant extract to reduce ferric ions was determined. FRAP assay measures the changes in absorbance at 593 nm owing to the formation of blue colored Fe^{2+} tripyridyltriazine compound from the colourless oxidized Fe^{3+} form by the action of electron donating antioxidants.[39] The FRAP values of AETB is higher as compared to EETB (Table 3). The P value < 0.0001, this difference is considered to be extremely statistically significant. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present it can be reported that AETB and EETB may act as free radical scavenger, capable of transforming reactive free radical species into stable nonradical products.

Total antioxidant capacity

Total Antioxidant capacity of AETB and EETB is shown in Table 3. The phosphomolybdenum method was based on reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH.[40] In this assay EETB was found to have higher activity, AETB showed lower activity. The P value < 0.0001, this difference is considered to be extremely statistically significant. The extracts demonstrated electron donating capacity and thus they may act as radical chain terminators, transforming reactive free radical species into stable non reactive products.[41]

CONCLUSION

The results of present study demonstrate that the radical scavenging of AETB, indicate that the use of Terminalia bellerica (Roxb.) as a medicinal plant for the treatment of hypertension and possessing properties like antispasmodic and bronchodilatory seems quite justified.

The percentage scavenging activity (with reference to IC_{50} value) in super oxide anion, nitric oxide, ABTS radical scavenging assays shows that AETB has better percentage scavenging activity as compared to EETB. AETB thus also give effective reducing power and FRAP values, But the TAC values AETB are lower as compared to EETB. The overall better antioxidant and free radical scavenging activities of AETB might be due to the presence higher amounts of phenolic and flavonoid compounds in aqueous extract.

Further studies are in progress in our laboratory to evaluate the in vivo antioxidant potential of this extract in various animal models and phytochemical studies are required to establish the types of compounds responsible for the bioactivity of this medicinal plant.

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