ORIGINAL ARTICLE

A comparative anti-inflammatory and antioxidative potent of *Nigella sativa* seeds extract and its oil

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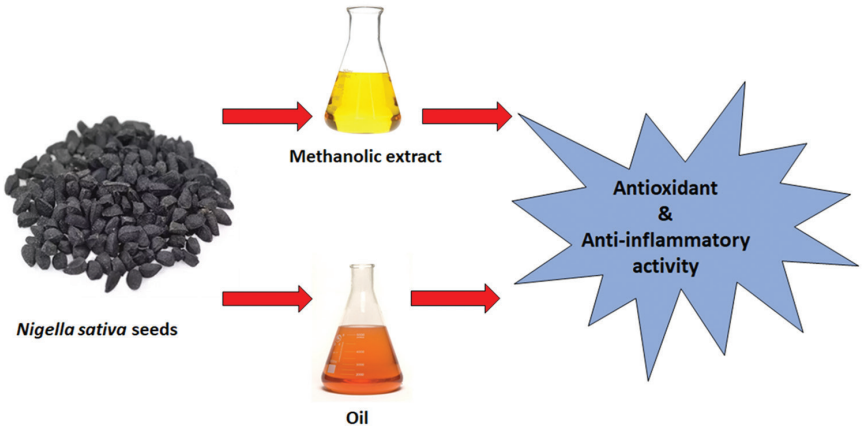
Graphical Abstract



Abstract

Objective: The purpose of the current work is to elucidate the therapeutic property of *Nigella sativa* seeds (methanolic extract and seeds oil). The extent of activity and correlation has been studied. **Materials and Methods:** *N. sativa* seeds used to prepare methanolic extract and its oil are used to investigate *in vitro* anti-inflammation and total antioxidative property. **Results:** The analysis revealed that protein (albumin) denaturation was inhibited maximum in seeds oil of *N. sativa* with 82.966 ± 3.704%. Proteinase inhibitory action of seeds oil showed significant action and was found to be maximum with 39.623 ± 5.820%. Heat-induced hemolysis of erythrocytes showed that it was found maximum inhibition with 65.866 ± 3.066% while hypotonicity-induced hemolysis activity was done that it was found maximum inhibition with 69.109 ± 3.054%. When anti-lipoxygenase and cyclooxygenase activities were estimated, it was found maximum in seeds oil with 83.383 ± 3.294% and 64.863 ± 4.029%, respectively. When antioxidant activity was observed, it was found maximum in oil with 85.201 ± 4.235%. When two-way ANOVA was applied between various parameters, it was found significant with $P < 0.05$. However, when correlation was established between various anti-inflammatory and antioxidant activities, a strong positive correlation of 0.9 was obtained. **Conclusion:** An overall strong positive correlation between anti-inflammatory and antioxidative property was found, signifying that the antioxidative property of *N. sativa* might be accountable for its anti-inflammatory action. Further, it was found that its seeds oil holds better quantity and types of phytochemicals which possess significant anti-inflammatory and antioxidative property. Thus, more efforts are desirable to effusively elucidate the antioxidants accountable for its anti-inflammation action, therefore, to develop improved herbal drug formulations.

Key words: Anti-inflammatory, antioxidant, cyclooxygenase, hemolysis, lipoxygenase, medicinal plants, *Nigella sativa*

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INTRODUCTION

Physiological process like inflammation is examined as a crucial progression that assists body to defend the situation contrary to innumerable harmful stimuli.[1] It is considered as a protection response, the crucial objective of it is to serve the organism to protect it from both primary source of damage, namely, toxins and microbes and secondary the outcome of such damage, namely, necrotic cells and tissues.[2] Although, several times inflammation goes uncontrolled leading to tissue damage and finally resulting in inflammatory disorder like rheumatoid arthritis.[3] An unrestrained and tenacious inflammation may perhaps can act as an etiologic reason for several chronic infections.[4] Inflammatory ailments are flattering common in people all over the world. At present, both nonsteroidal anti-inflammatory medicines and steroidal anti-inflammatory medicines are used to aid inflammation.[5,6] In inflammatory ailments, steroids have noticeable part in treatment, but their noxiousness, long-standing consumption of these medications is linked with severe adverse properties. Extended consumption of nonsteroidal anti-inflammatory treatments is also linked with other side effects as well.[7] Therefore, investigation for discovering improved and harmless medication for inflammation is incessant and never-ending progression. Herbal products, predominantly remedial floras, are supposed to be a significant source of phytochemicals that possess potential healing efficiency. Thus, in Unani, Ayurvedic, and Siddhi medication systems, *Nigella sativa* an herbal plant is extensively used for centuries in various ailments that include fever, pain, and inflammation.[8-12] At present, some studies put forward that *N. sativa* inhibits eicosanoid formation in lipid peroxidation and leukocytes.[13] Several investigations conducted previously also showed that *N. sativa* does possess substantial analgesic and anti-inflammatory property.[14,15] Although considerable efforts have been made to determine *N. sativa*'s antinociceptive property, very little exploration has been done to identify its anti-inflammatory property on chronic models as well as subacute models of inflammation. Henceforth, in the present study with considered to such a situation, *N. sativa*'s anti-inflammatory activity (pilot study) was established in various models of inflammation. Thus, the findings could be useful in various pharmaceutical formulations.

MATERIALS AND METHODS

National Research Centre on Seed Spices, Tabiji, Rajasthan, India, provided the standard seeds of *N. sativa* labeled as Ajmer Nigella 1 (AN1). The seed soil of *N. sativa* was purchased from local market (Vedaone natural kalonji oil/ black seed oil). To prepare extract, the seeds of AN1 were carefully washed with distilled water. The seed was then dried on blotting paper in shade. Shade dried out seeds was ground to fine powder by the help of tissue blender. The extracts were prepared following the protocol of Khoddami *et al*.[16] and Iqbal *et al*.[17] with minor amendments. To prepare extracts from the *N. sativa* seeds, methanol was used as solvent. For filtration, Whatman filter paper no. 1 was used and vaporized to obtain dried extract. The dried extract stock solution was prepared to 1 mg/mL concentration using methanol as solvent. The black seed oil was also diluted accordingly. The stock solutions of extract and oil were then diluted to different concentrations of 500 µg/mL, 400 µg/ mL, 300 µg/mL, 200 µg/mL, and 100 µg/mL for further analysis. Standard drugs of concentration 100 µg/mL were used as control.

DETERMINATION OF *IN VITRO* ANTI-INFLAMMATORY PROPERTY

Inhibition of Protein (Albumin) Denaturation

To inhibit the protein (albumin) denaturation**,** the investigation was performed, conferring to the procedure of Sakat *et al*.[18] and Rastogi *et al*.[19] with slight amendments. The experiment was conducted using equivalent volume of 1% bovine albumin (Fraction V) with test extracts of different concentrations as described previously, further to adjust the pH, 1 N HCl was used. The incubation was done at 37°C (20 min) followed by heating at 51°C (20 min) to complete the reaction. Thereafter, the mixture was cooled and absorbance was taken at 660 nm using ultraviolet (UV)-visible spectrophotometer (Shimadzu UV-1800). The percentage inhibitory activity of protein (albumin) denaturation was calculated. Aspirin was used as a reference drug.

$$\% \text{ inhibition} = [\{\text{Control}_{\text{Abs.}} - \text{Sample}_{\text{Abs}}\}/\text{Control}_{\text{Abs.}}] \times 100$$

Antiproteinase Activity

The activity for antiproteinase was executed following the procedure of Oyedepo and Femurewa[20] and Sakat *et al*.,[18] with some alterations. It was conducted by adding 0.001% trypsin (2 mL), 1 mM pH 7.4 Tris-HCl buffer (1 mL), and text extracts (1 mL) with incubation of 5 min at 37°C. After 5 min, 0.02% (w/v) casein (1 mL) was added followed by further 20 min of incubation at 37°C. To stop the reaction, 2% perchloric acid (2 mL) was added. The activity was measured at 210 nm and percentage antiproteinase was calculated. Aspirin was used as a reference drug.

$$\% \text{ inhibition} = [\{\text{Control}_{\text{Abs.}} - \text{Sample}_{\text{Abs}}\}/\text{Control}_{\text{Abs.}}] \times 100$$

Membrane Stabilization

Erythrocytes cells (red blood cells [RBCs]) suspension was prepared using the blood sample obtained from volunteers (healthy person) not taken nonsteroidal anti-inflammatory medicine for 2 weeks. An informed consent was obtained from the volunteers. To prepare suspension, the blood sample was centrifuged (3000 rpm for 10 min) and then washed with equal volume of normal saline and reconstituted as 10% v/v in normal saline suspension.[18,21]

Heat-induced Hemolysis

The activity was conducted by adding equal volume of test samples (1 mL) and 10% RBCs suspension (1 mL) followed by the incubation in water bath for 30 min, at 56°C. To stop the reaction, the reaction mixture was cooled and centrifuged (2500 rpm for 5 min) to obtain supernatant. The collected supernatant was taken for absorbance at 560 nm. The percent of heat-induced hemolysis was calculated.[18,22] Aspirin was used as a reference drug.

$$\% \text{ inhibition} = [\{\text{Control}_{\text{Abs.}} - \text{Sample}_{\text{Abs}}\}/\text{Control}_{\text{Abs.}}] \times 100$$

Hypotonicity-induced Hemolysis

The activity was performed by adding phosphate buffer pH 7.0 (1 mL), hyposaline (2 mL), and 10% RBCs suspension (0.5 mL) followed by the incubation of 30 min, at 37°C followed by centrifugation at 3000 rpm to obtain supernatant. The collected supernatant absorbance was taken at 560 nm. The percent hypotonicity-induced hemolysis was calculated by assuming control as 100%.[23] Diclofenac sodium was used as a reference drug.

$$\% \text{ protection} = 100 - (\text{OD sample}/\text{OD control}) \times 100$$

Antilipoxygenase (LOX) Activity

The anti-LOX activity was performed by following the protocol of Shinde *et al*.[22] with minor modifications, using lipoxidase as an enzyme and linoleic acid as a substrate. The test samples were dissolved in 2 M borate buffer of pH 9.0 and lipoxidase enzyme solution (0.25 mL) having concentration of 20,000 U/mL. Then, the incubation of 5 min at 25°C was given to the reaction mixture. After the completion of incubation, 1.0 mL of 0.6 mM linoleic acid was added and was vertexed for proper mixing. The absorption was taken at 234 nm and activity was calculated. Indomethacin was used as a reference drug.

$$\% \text{ inhibition} = [\{\text{Control}_{\text{Abs.}} - \text{Sample}_{\text{Abs}}\}/\text{Control}_{\text{Abs.}}] \times 100$$

Cyclooxygenase Activity

Activity was measured following the procedure of Viji and Helen[24] with some modifications. The assay combination consists of Tris-HCl buffer, hemoglobin, glutathione, and enzyme. The assay was instigated by adding arachidonic acid and was incubated at 37°C (20 min). The reaction was completed by adding 0.2 mL of 10% tricarboxylic acid in 1 N HCl and further added with 0.2 mL of *tert*-Butyl alcohol. The reaction mixture was kept for 20 min at boiling water bath and then was centrifuged (1000 rpm/5 min) to obtain supernatant and was measured at 632 nm. The activity was calculated. Ibuprofen was used as a reference drug.

$$\% \text{ inhibition} = [\{\text{Control}_{\text{Abs.}} - \text{Sample}_{\text{Abs}}\}/\text{Control}_{\text{Abs.}}] \times 100$$

Antioxidant Activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) Method

Using DPPH, the antioxidant activity was measured by free radical scavenging assay.[25] The procedure requires 3.0 mL absolute ethanol, with 0.5 mM DPPH (0.3 mL) and test samples (0.5 mL). The incubation of 60 min at room temperature was given to reaction mixture to complete the reaction. The change in the color of solution was measured at 517 nm and the percentage activity was calculated using the formula of Mensor *et al*.[26]

$$\% \text{ antioxidant activity} = 100 - \{[(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) \times 100]/\text{Abs}_{\text{control}}\}$$

Statistical Analysis

Mean ± standard deviation was used to express the results using Microsoft Excel. Correlation and two-way ANOVA were used for statistical analysis and to find the level of significance using GraphPad Prism software.

RESULTS

The present investigation was based on a comparative study of *in vitro* anti-inflammatory activity of *N. sativa* seeds methanolic extract and its seeds oil. It was found that for inhibition of albumin denaturation, maximum activity at 500 µg/mL of concentration was 82.966 ± 3.704% in oil while 52.996 ± 3.724% in methanolic extract, respectively. When antiproteinase activity was observed, maximum activity was observed at 500 µg/mL of concentration with 39.623 ± 5.820% for oil and 31.922 ± 3.411% for methanolic extract. The heat-induced hemolysis of erythrocytes showed significant results with maximum in oil with 65.866 ± 3.066% and in methanolic extract with 54.730 ± 2.366% at 500 µg/mL of concentration. The hypotonicity-induced hemolysis of erythrocytes showed maximum activity in oil with 69.109 ± 3.054% and in methanolic extract with 55.546 ± 3.687% at 500 µg/mL of concentration. When anti-LOX and cyclooxygenase activities were estimated, it was found maximum at 500 µg/mL of concentration in oil with 83.383 ± 3.294% and 64.863 ± 4.029%, respectively, and in methanolic extract 58.440 ± 3.963% and 56.216 ± 2.681%, respectively, the detailed are mentioned in Figure 1.

When antioxidant activity was observed, it was found maximum in oil with 85.201 ± 4.235% and 77.143 ± 2.280% in ethanolic extract at 500 µg/mL of concentration [Figure 2]. When two-way ANOVA was applied between methanolic extract of *N. sativa* seeds and its seeds oil, it was found significant with $P < 0.05$. However, when correlation was established between various anti-inflammatory activity and antioxidant activity, a strong positive correlation of 0.9 was obtained.

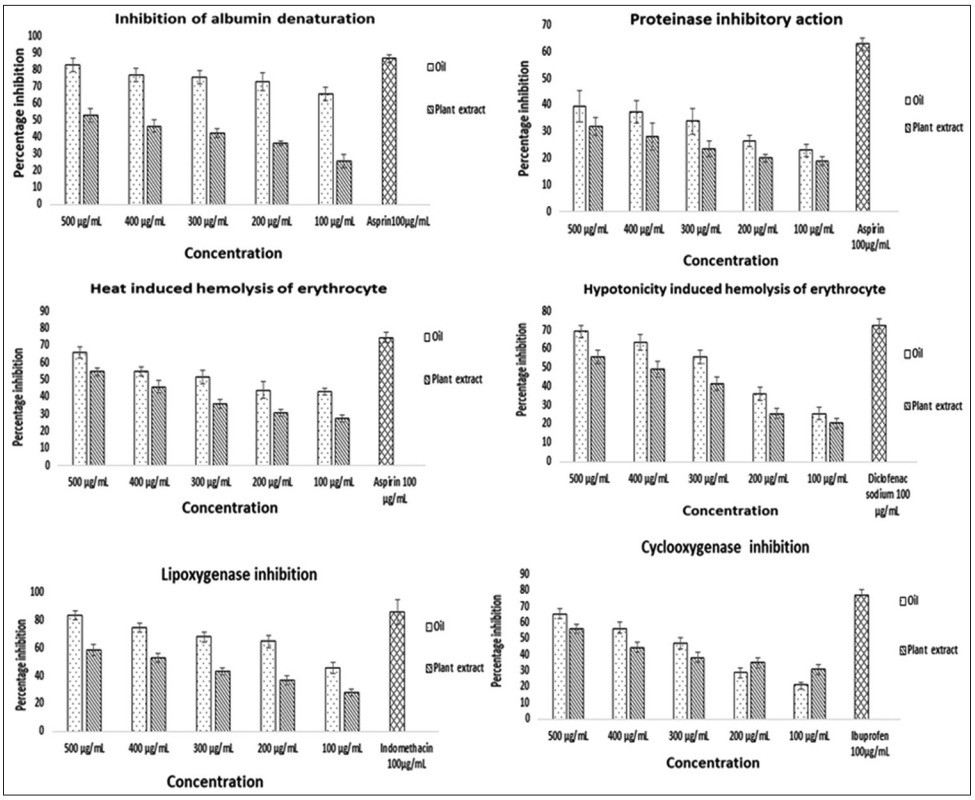

Figure 1: Comparison based on graphical representation of *in vitro* anti-inflammatory activity of methanolic extract of *N. sativa* seeds and its seeds oil showing significant variation. The results are expressed in ± standard deviation where *n*=3

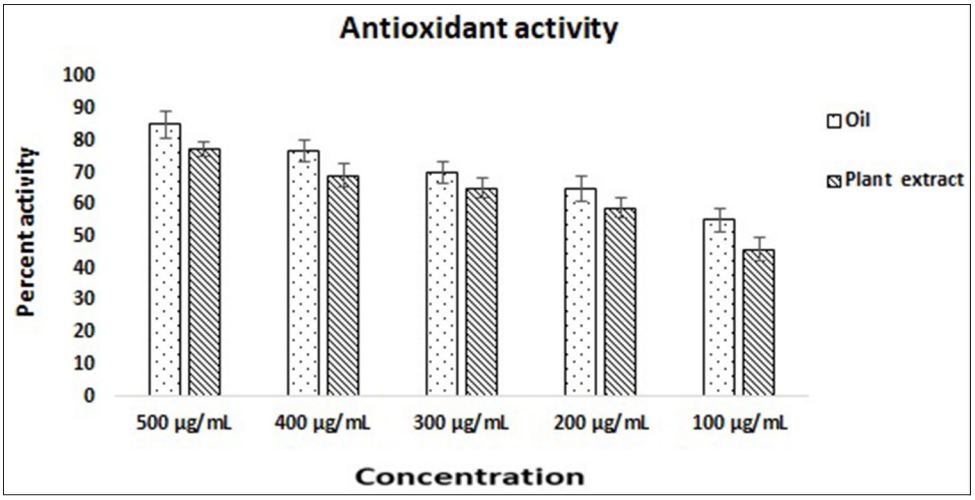

Figure 2: Comparison based on graphical representation of the antioxidant activity of methanolic extract of *N. sativa* seeds and its seeds oil showing significant variation. The results are expressed in ± standard deviation where *n* = 3

DISCUSSION

The *in vitro* anti-inflammatory and antioxidative activity of the methanolic extracts of *N. sativa* seeds and its seeds oil showed significant results. It was found that for inhibition of albumin denaturation, maximum activity was observed at 500 µg/mL of concentration in oil and in methanolic extract. When two-way ANOVA was applied, between methanolic extracts of *N. sativa* seeds and its seeds oil, it was found significant with $P < 0.05$ (0.00005). The results clearly indicate that the phytochemicals responsible for the inhibition of albumin denaturation activity are more in *N. sativa* seeds oil than *N. sativa* seeds methanolic extract. The results were found in accordance to the report of Belal *et al*.[27] where *in vitro* anti-inflammatory activity of the methanolic extract of *N. sativa* in egg albumin denaturation technique revealed mean inhibition of albumin denaturation of 55.55, 32.73, 50.15, and 58.18% at doses of 100, 200, 500, and 1000 µg/mL, respectively. In another study by Leelaprakash and Mohan[28] on methanol extract of *Enicostemma axillare*

showed maximum activity at 500 µg/mL of concentration, where maximum activity was observed. The antiproteinase activity was found maximum at 500 µg/mL of concentration with both the approaches. The two-way ANOVA showed significant results with $P < 0.05$ (0.002) between methanolic extract of *N. sativa* seeds and its seeds oil. This was the first report for antiproteinase activity of *N. sativa*. Investigations on other plants also revealed noteworthy antiproteinase inhibitory activity.[19] *Aegle marmelos* and *Ocimum sanctum* leaves aqueous extract revealed substantial antiproteinase activity. *A. marmelos* leaf extract showed maximum inhibition at 74.45 µg/mL while *O. sanctum* showed maximum inhibition at 49.70 µg/mL.[29] It was previously explored that leukocyte proteinase shows substantial part in augmentation of tissue injury during reactions of inflammatory activity and proteinase inhibitors play a defensive role.[30] The heat-induced hemolysis of erythrocytes was studied, it showed significant results with seeds oil than in methanolic extract of *N. sativa*. Two-way ANOVA revealed significant results with $P < 0.05$ (0.0006) between methanolic extract of *N. sativa* seeds and its seeds oil. *N. sativa* showed heat-induced hemolysis of erythrocytes which is the foremost information of its *in vitro* anti-inflammatory activity. However, other plants methanolic extract showed heat-induced hemolysis of human red blood cell membrane. It was reported that methanolic extract of *Ficus virens* is effective at 200 µg/mL with maximum inhibition of 64%.[31] In another research, inhibition of heat-induced hemolysis by ethanolic extract of *Wedelia trilobata* also showed maximum inhibitions of 78.11% in extract from leaf followed by stem with 74.17% and finally in flower with 58.74%.[32] The hypotonicity-induced hemolysis of erythrocytes showed maximum activity in oil than in methanolic extract at 500 µg/mL of concentration. The results were significant when two-way ANOVA was applied with $P < 0.05$ (0.002) between methanolic extract of *N. sativa* seeds and its seeds oil. In a report by Ranasinghe *et al*.,[33] hypotonicity-induced hemolysis showed significant results at a concentration of 37.5 µg/mL of the plant extract used. It can be supposed from the experiential results that the action of membrane stabilization and erythrocyte lysis inhibitory property of methanolic extract of *N. sativa* seeds and its seeds oil may probably be the mechanistic action of its anti-inflammatory activity. When anti-LOX and cyclooxygenase activities were estimated, it was found maximum at 500 µg/mL of concentration in methanolic extract of *N. sativa* seeds and its seeds oil. The results were in accordance to the report of Pise and Padwal[34] for anti-LOX and cyclooxygenase activities where *N. sativa* exhibited anti-inflammatory activity in acute inflammation, i.e., carrageenan-induced paw edema in rats, which was also observed statistically significant ($P < 0.001$). At present, some investigations recommend that *N. sativa* inhibits eicosanoid production in lipid peroxidation and leukocytes. It has been described that they obstruct the pathways of arachidonic acid metabolism for both cyclooxygenase and LOX.[35] Studies conducted previously reported that *N. sativa* does hold substantial analgesic and anti-inflammatory activity.[14,15] It was observed that as the anti-inflammatory activity increases the antioxidant activity increases with the upsurge in concentration of the various extracts. A noteworthy observation was observed when antioxidant activity was conducted. It was found that antioxidant activity showed maximum in oil than in methanolic extract at 500 µg/mL of concentration. The results were also found significant when two-way ANOVA was applied with $P < 0.05$ (0.0006) between methanolic extract of *N. sativa* seeds and its seeds oil. As we know various antioxidant present in plants, has the potency to scavenge free radicals,[36,37] which can reduce various ailments caused due to oxidative stress.[38,39] Phytochemicals such as flavonoid and phenolic compounds acquired from herbal plants are the chief source of antioxidative property,[36,40,41] and therefore, it acts as an anti-inflammatory moderator.[42] The formation of pro-inflammatory molecules such as TNF-α and nitric oxide can reduce inflammation. These anti-inflammatory molecules retort with free radicals that could be an effect of irretrievable defacement to cell membranes, leading to damage of tissue and even cell death.[43] Moreover, a strong positive correlation of 0.9 was observed between antioxidative property and various anti-inflammatory activities, which showed significant relationship among the two factors. The result was in accordance with the previous studies. It was observed in a study on culinary herbs by Chohan *et al*.[44] that the relationship between polyphenolic content with anti-inflammation and antioxidative property was significant. This noteworthy correlation indicated the relationship of antioxidant activity with anti-inflammatory activity.

CONCLUSION

The adverse health alignments and other side effects of presently used anti-inflammatory medications, naturally comprising anti-inflammatory supplements (generally obtained from plants) are nowadays being preferred. Various investigational practices are now engaged to validate antioxidant and anti-inflammatory medicines obtained from natural sources. The present investigation is an effort in the course of anti-inflammatory drug formulation from natural source (*N. sativa*) for numerous chronic inflammatory and its associated ailments. It can be elucidated that *N. sativa* possesses noteworthy anti-inflammatory and antioxidant activity. Further, it can be concluded that its seeds oil holds better quantity and types of phytochemicals which possess significant anti-inflammatory and antioxidant activity. The correlation established between the two activities showed significant results, thus elucidating that antioxidant present in *N. sativa* accounts for its anti-inflammatory activity. It is to be further needed to effusively explicate the antioxidants present in *N. sativa* accountable for this mechanism of action. Thus, it can be used in forthcoming drug formulations by herbal plants.

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