SYNOPSIS

Isolation, Characterization of Chemical Constituents and Validation of Herbal Potential of Folkloric Medicinal Plants

*Pisonia grandis* R.Br. and *Andrographis stenophylla* C.B Clarke

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Medicinal plants represent the eternal kindness of almighty for the perpetuation of life in the universe. In terms of life forms, medicinal plants are equally distributed across habits, viz, trees, shrubs and herbs. The history of the use of plants as a primary source of medicine can be traced back several millennia to the ancient written documents of early civilizations of India, China and the Middle East. The Charak Samhita written during 990BC has documented the production of around 340 herbal drugs and their indigenous uses for curing various ailments and diseases (Rahul et al., 2010).

India is one of the twelve biodiversity centres of the World. The Ministry of Environment and Forests, Government of India, has reported that there are over 8000 species of medicinal plants grown in the country. According to a study on export of Indian medicinal plants, 880 medicinal plant species find place in All India Trade but India’s share is only 0.5 % in global medicinal plant related export trade (Ramesh et al., 2011). Owing to the fact that the Indian subcontinent is a bountiful source of medicinal plants and has a rich heritage of knowledge on medicinal plant use for curing diseases, validation and standardisation of medicinal plants and their products will fetch huge returns in the global market which is big and expanding.

Based on this revelation, during the past decade, a large number of plant species have been taken up for validation studies and systematic documentation of data is being done. Therefore any herb based research must have an accompanying validation and standardization platform. Validation and standardization of medicinal plants has been mightily emphasized by the World Health Organization which has laid down recommendations for standardization (WHO 2007).

In the present research work two folkloric medicinal plants have been chosen for isolation and chemical characterisation of their phytoconstituents, which area of work is one major stage towards chemical standardisation of the plants. This being the prime focus of the study; herbal standardisation strategies have also been applied to make it a wholesome record of validation of the folkloric use of the chosen plants. The plants chosen are:

**Plant I: Pisonia grandis** R.Br. of plant family *Nyctaginaceae*

**Plant II: Andrographis stenophylla** C.B Clarke of plant family *Acanthaceae*

Both plants have been extensively used by local people and tribal folk as anti-diabetic and anti-inflammatory agents.

*Pisonia grandis* R.Br. is a flowering plant of the four O’clock family *Nyctaginaceae* and commonly called as *lettuce tree*. It is native to the Seychelles as well as other tropical areas of the world, especially the Indo-pacific islands. It is a small, evergreen foliage tree or a large shrub found in many gardens and is widely distributed throughout India. The history of the plant dates back to the 18th century when it was discovered by Robert Brown from islands in the Gulf of Carpentaria (Flinders 1814). An interesting observation by bird watchers in the Seychelles reveals that birds flying away from the plant shed the seeds and propagate its growth, however due to the bulkiness of a sticky substance in the seeds, they lose their flight later and fall to the ground and hence the plant earns the name “Bird Eating Tree.”

*Pisonia grandis* has been reported to have medicinal properties and is documented in NAPRALERTTM database. *Fresh leaves, moistened with Eau-de-Cologne, find use in reducing inflammation of a filarioid nature in the leg and other parts of the body. Chewing two leaves of the plant has been found to reduce sugar levels in the body and the roots are considered to be purgative* (Anonymous 1969). South Indians cook and eat the young leaves as a salad and also use it for the treatment of arthritis (The Hindu Daily Newspaper 2013).
The first scientific report on the medicinal potential of *Pisonia grandis* was published in 2002 which documented the analgesic, antipyretic and diuretic potential of the leaves (Anbalagan et al., 2002). Later studies confirmed the wound-healing (Prabu et al., 2008); anti-inflammatory (Radha et al., 2008); antidiabetic (Sunil et al., 2009a; 2009b); free radical-scavenging (Subhasree et al., 2009; Jagadeesan et al., 2011; Jayakumari et al., 2012); anti-fungal (Shubashini et al., 2010); anxiolytic (Habibur Rahman et al., 2011); antimicrobial (Nivedhitha et al., 2011); antipyretic (Elumalai et al., 2012); hepatoprotective (Majumdar et al., 2012; Thenmozhi et al., 2013); anti-arthritic (Elumalai et al., 2012) and *in-vitro* antiplasmodial (Sundaram et al., 2012) potential of the leaves of the plant.

There are only few reports on the chemical investigation of the leaves of the plant. As early as 1990, Natarajan et al. reported the presence of bio-active metabolites β-sitosterol, α-spinosterol, dulcitol, β-sitosterol glucoside, octacosanol and quercetin from the leaves of this plant. The first report of the isolation of highly valuable molecules pinitol and allantoin from the leaves of *Pisonia grandis* was published in 2011 a part of preliminary studies on this plant in our laboratory (Shubashini et al., 2011a; Shubashini et al., 2011b). Post this revelation, it was evident that publications on the medicinal potential of the leaves further mushroomed. There are not many reports on the chemical investigation of the stem and roots of the plant. Only one report on the hepatoprotective activity of root extract and one report on isolation of C-flavones from root extract exist. Hence the plant *Pisonia grandis* was chosen in the present study for a thorough chemical investigation and validation as a safe medicinal plant.

*Andrographis stenophylla* C.B Clarke is a medicinal plant of the *Acanthaceae* family. It is a rare and little known endemic species of India. Local tribes called this plant as “malai chiriyanagai”. *Andrographis stenophylla* is used in folk medicine as an anti-diabetic, anti-inflammatory agent, and as an antidote for snake bites. Local tribes claim that the leaves of this plant are potentially effective in the treatment of chronic fever, diabetes, wounds, ulcers, inflammations, cough, skin diseases and leprosy. There is only one report on the phytochemical investigation of leaves of the plant which describes the isolation of few flavones along with the terpene lactone andrographolide - a biomarker of the *Andrographis* genus (Neelaveni et al., 2010). There are no reports on scientific investigation of the stem, root, flower or pods of this plant. Hence the aerial part of this plant has been chosen for a thorough chemical investigation and validation of its herbal potential.

This research study will be presented in five chapters.

**Chapter I** comprises of a brief introduction to the study and a mention of its prime focus.

The main objectives of this research work are:

- To isolate and characterize the chemical constituents of two folkloric medicinal plants *Pisonia grandis* R.Br. and *Andrographis stenophylla* C.B Clarke
- To validate the herbal potential of the chosen plants by standardisation studies

A detailed review of literature pertaining to the research study will be presented in **Chapter II**.

The review will be presented in the following sections.

- Earlier work on the chosen plants *Pisonia grandis* and *Andrographis stenophylla*
- Earlier work on the bio-pharma potential of the bio-actives of the chosen plants

The methodology adopted for the research work comprising of two major stages will be presented in **Chapter III**

A brief outline of the strategy adopted for the research work is presented in the following sections:

- Isolation and characterization of chemical constituents from the chosen plants *Pisonia grandis* and *Andrographis stenophylla*
- Validation of the Herbal Potential of the chosen plants
ISOLATION AND CHARACTERIZATION OF CHEMICAL CONSTITUENTS FROM THE CHOSEN PLANTS

The following methodology was adopted for isolation and characterization of chemical constituents from chosen medicinal plants *Pisonia grandis* and *Andrographis stenophylla*

**Bulk Extraction of Plant Material**

Each of the chosen plant material (leaves, stem and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla*) was air-dried, pulverized and successively extracted with petroleum ether, ethanol and water. Two successive extractions of 3 hours duration each was carried out. Each extract was concentrated in vacuum and the residue weighed and stored.

**Column Chromatographic Isolation of Chemical Constituents**

Column chromatographic analysis of the non-polar and the high polar extract concentrates obtained for each plant material was carried out using silica gel as the adsorbent and employing a suitable solvent gradient as eluent. The following extract concentrates (residues) were analysed by column chromatography. Hitherto unexplored extracts were only taken up for the chromatographic analysis.

- Petroleum ether concentrate of the leaves of *Pisonia grandis*
- Dewaxed ethanol concentrate of stems of *Pisonia grandis*
- Methanol soluble concentrate of aqueous extract of stems of *Pisonia grandis*
- Petroleum ether concentrate of aerial parts of *Andrographis stenophylla*
- Dewaxed ethanol concentrate of aerial parts of *Andrographis stenophylla*

**Isolation of Chemical Constituents by Preparative HPLC**

The petroleum ether extracts of stems and roots of *Pisonia grandis* and dewaxed ethanol extract concentrate of roots of *Pisonia grandis* were analysed by preparative HPLC for isolating chemical constituents. Akta Prime Chromatographic system provided with inbuilt fraction collector was used for this analysis.

**Simple Method of Isolation of Constituents**

A portion of the dewaxed ethanol extract of leaves, stem and roots of *Pisonia grandis* was also analysed for isolation of one or more of their constituents by a simple protocol adopting solvent fractionation and recrystallization. Charts I and II represent the outline of the protocols adopted. Chart III represents yet another simple method of isolation of constituents from the dewaxed acetone extract of the leaves of *Pisonia grandis*. This work was taken up to analyse the feasibility of easy isolation of bio-active constituents from the extracts.

**Characterization of Isolated Compounds**

The isolated compounds were characterized by preliminary tests, physical data and spectral analysis. Spectral techniques used for structure elucidation include UV, IR, 1D-NMR, 2D-NMR and Mass spectrometry. Wherever necessary, thermanalytical and X-ray diffraction techniques were utilised to augment the structural analysis of the compounds.

**VALIDATION OF HERBAL POTENTIAL OF THE CHOSEN PLANTS**

In the present research work focus was on standardization of the chosen folkloric plants and their extracts with a greater impetus on the chemical constituent characterisation and quantification in the extracts. This part of the research work will validate the herbal potential of the plant for use as a Phytomedicine.

Herbal standardization was carried out by standard methods (AOAC 2012) as per protocol mentioned in *The Ayurvedic Pharmacopoeia of India (API) 2012*. The strategy adopted for the standardization is depicted in Chart IV.
**CHART I**

Dewaxed Ethanol Extract Concentrate of leaves of *Pisonia grandis* (600 mg)

- Hot Acetone
  - Acetone soluble fraction
    - Concentrated & Filtered
      - Compound A
  - Chloroform
    - Chloroform soluble fraction
      - Concentrated
        - Residue (140 mg)
          - Dissolved in hot alcohol
            - Alcohol fraction
              - Concentrated & Filtered
                - Compound B
          - Alcohol fraction
            - Concentrated & Filtered
              - Compound C
- Chloroform
  - Residue (460 mg)
    - Dissolved in hot alcohol

**CHART II**

Aqueous Extract of Stems of *Pisonia grandis* (1 g Concentrated Residue)

- Macerated with Methanol
  - Methanol Soluble Fraction
    - Concentrated & Cooled
      - Compound D
  - Methanol Insoluble Portion (Precipitate)
    - Washed & Vacuum Filtered
      - Compound E

**CHART III**

Dewaxed Acetone Extract Concentrate of Leaves of *Pisonia grandis* (Wt = 1 g)

- Dissolved in hot alcohol & Cooled
  - Compound F
The results pertaining to the present work titled "Isolation, Characterization of Chemical Constituents and Validation of herbal potential of two Folkloric Medicinal Plants - *Pisonia grandis* R.Br. and *Andrographis stenophylla* C.B Clarke" and the ensuing discussions will be presented in Chapter IV. Highlights of the results and their significance will be presented below.

**ISOLATION AND CHARACTERIZATION OF CHEMICAL CONSTITUENTS FROM THE CHOSEN MEDICINAL PLANTS**

**Bulk Extraction of Plant Material**

The yield of the extract concentrate of stems, roots and leaves of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* is presented in Table 1. The leaves of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* gave a good yield of the extracts in ethanol.

**Table 1. Yield of Crude Extracts**

<table>
<thead>
<tr>
<th>Plant Parts</th>
<th>Dry weight (Kg)</th>
<th>Yield of crude extracts (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Petroleum ether extract</td>
</tr>
<tr>
<td><em>Pisonia grandis</em> - Stems</td>
<td>0.75</td>
<td>2</td>
</tr>
<tr>
<td><em>Pisonia grandis</em> - Roots</td>
<td>0.75</td>
<td>4</td>
</tr>
<tr>
<td><em>Pisonia grandis</em> - Leaves</td>
<td>0.75</td>
<td>26</td>
</tr>
<tr>
<td><em>Andrographis stenophylla</em> - Aerial parts</td>
<td>0.75</td>
<td>14</td>
</tr>
</tbody>
</table>
Column Chromatographic Isolation of Chemical Constituents

Isolation of chemical constituents from the various extracts of chosen plants was mainly done by column chromatographic analysis. The separation has been achieved and the characterisation of the constituents is under progress. The chromatographic separation of polar extracts of both plants led to the isolation of valuable bioactives.

Column Chromatographic Analysis of Petroleum Ether Concentrate of Leaves of Pisonia grandis

Column chromatographic analysis of petroleum ether extract of leaves of Pisonia grandis led to the isolation three compounds. The characterisation of the compounds is under progress and the data will be presented in the thesis.

Column Chromatographic Analysis of Dewaxed Ethanol Concentrate of Stems of Pisonia grandis

Column chromatographic analysis of dewaxed ethanol extract concentrate of stems of Pisonia grandis led to the isolation of eight compounds. The isolation details, preliminary characterisation and spectral analysis of the isolated compounds will be dealt in the thesis.

Column Chromatographic Analysis of Methanol Soluble Concentrate of the Aqueous Extract of Stems of Pisonia grandis

Column chromatographic analysis of methanol soluble fraction of the aqueous extract of stems of Pisonia grandis led to the isolation of three compounds in appreciable yields which were taken up for characterisation. Other fractions eluted from the column gave meagre amount of compounds which were not taken up for the characterization. The valuable molecule allantoin was isolated in 0.4 % yield (200 mg)

Column Chromatographic Analysis of Petroleum Ether Concentrate of Aerial Parts of Andrographis stenophylla

Column chromatographic analysis of petroleum ether extract of aerial parts of Andrographis stenophylla is under progress and the results will be presented in the thesis.

Column Chromatographic Analysis of Dewaxed Ethanol Concentrate of Aerial Parts of Andrographis stenophylla

Column chromatographic analysis of dewaxed ethanol concentrate of aerial parts of Andrographis stenophylla led to the isolation of eight compounds characterized as flavonoidal moieties and their glycosides. One compound (ASAEC3) was characterized as the terpene lactone andrographolide. This bioactive molecule is considered as a biomarker of Andrographis genera.

Isolation of Chemical Constituents by Preparative HPLC

The petroleum ether concentrate of stems and roots of Pisonia grandis and dewaxed ethanol concentrate of roots of Pisonia grandis were analysed by preparative HPLC for isolating chemical constituents. The characterisation of the constituents will be presented in the thesis.

Isolation of Constituents by Simple Protocol

The column chromatographic analysis of the chosen plants led to isolation of medicinally valuable molecules and hence in order to analyse the feasibility of isolation of these bioactives directly from the extracts, a simple protocol comprising of fractionation and recrystallization was adopted. This led to the direct isolation of three bioactive molecules from extracts of Pisonia grandis in good yield. A similar protocol adopted for Andrographis stenophylla did not yield its bioactives directly.
HIGHLIGHTS OF CHEMICAL CHARACTERISATION STUDIES

Two medically valuable molecules pinitol and allantoin have been identified and characterized from the polar extracts of leaves, stem and roots of *Pisonia grandis* along with other metabolites. A notable aspect of the chemical characterisation study is the isolation of a new entity which comprises of pinitol and allantoin moieties held together by weak interactions probably by hydrogen bonding between the functionalities of both molecules. This compound herein named Pinallan. This is the first report of the identity of such a compound although pinitol exists in combined form with a host of small molecules like amino acids.

Extracts of *Andrographis stenophylla* were found to a rich source of flavonoidal moieties. The complete characterization of the isolated compounds will be dealt in the thesis.

VALIDATION OF HERBAL POTENTIAL OF THE CHOSEN PLANTS

**Authentication of Chosen Plants**

The chosen plants collected from local areas of Coimbatore were authenticated at the Institute of Forest Genetics & Tree Breeding (IFGTB). Voucher specimens have been deposited in the Herbarium of the Institute [F.No. 14932].

**Survey on Use of Plant by Local People**

A survey was conducted on the folkloric use of the chosen plants *Pisonia grandis* and *Andrographis stenophylla*. The survey led to the documentation of the folkloric use of the plants. It was found that the plants chosen for the present investigation have been extensively used by locals and tribals as anti-diabetic and anti-inflammatory agent. Mostly leaves of the chosen plants have been more prioritized among the people to treat various ailments. The survey also revealed that availability of the plant was reduced by commercial exploitation.

**Morphological Documentation**

Morphological data of the plant *Pisonia grandis* has been documented by Jayakumari et al. (2011). Morphological data of *Andrographis stenophylla* has been documented by Vijaya et al. (2007). This has mention in the present work since documentation of morphological data of the medicinal plants is an essential part of the validation and this will help in selection of the correct species of the plant for formulation.

**Physicochemical Analysis**

- **Organoleptic Study:** It is a sensory evaluation technique to analyse colour, odour and taste of the chosen plants to satisfy consumer’s expectation. Organoleptic analysis revealed that both the chosen plants (leaves, stems and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla*) are suitable for use as an Ayurvedic medicine.

- **Fluorescence Analysis:** The dried powdered parts of leaves, stems and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* depicted fluorescence with chemical reagents under day light and UV light indicating that the selected plant parts are source of natural metabolites

- **Elemental Analysis:** Elemental analysis of each of the chosen parts indicated that the chosen plants are good source of micronutrients. The content of carbon, hydrogen, nitrogen and sulphur was highest in leaves of *Pisonia grandis* which suggests that leaves are a good source of organic compounds compared to stem and roots.

- **Proximate Analysis:** Dried powdered parts of leaves, stems and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* were subjected to proximate analysis. The results indicated that the chosen plant parts are a good source of energy, minerals and fiber. The higher extractive value of aqueous extract of leaves of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* indicated higher assimilation efficacy on intake.
Toxic Metal Analysis: Medicinal herbs can be easily contaminated with heavy metals from the environment. Heavy metal analysis of leaves stem and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* revealed that the metal contaminations are within the WHO permissible limits and thus the chosen medicinal plant parts are non-toxic.

Extraction Mode: Reflux method gives higher yield of extract compared to soaking and ultrasonication.

Chemical Fingerprinting

Qualitative Phytochemical Tests: Phytochemical screening of the various extract concentrates of leaves stem and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* revealed the presence of phenols, flavonoids, alkaloids, terpenoids, sterols, tannins, carbohydrates, and proteins.

TLC Analysis: An optimized condition for TLC analysis was achieved for the various extracts with solvent mixtures.

HPLC Fingerprinting: HPLC finger printing of extracts of *Pisonia grandis* was carried out with various mobile phases as developing system. Optimized condition with respect to active constituent was achieved with CHCl₃: MeOH: H₂O as mobile phase and ammoniacal silver nitrate as a detecting agent. The HPLC finger printing of various extracts of *Andrographis stenophylla* is under progress and the results will be presented in thesis.

NMR Fingerprinting: NMR analysis of pet-ether, dewaxed-ethanol and overall ethanol extract of leaves, stem and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla* revealed that the spectra of overall extracts is the additive spectrum of pet-ether and dewaxed-ethanol extract. The chemical shift ranges exhibited by the extracts in 1H NMR and 13C NMR may represent the metabolite finger prints. The NMR finger print analysis will be part of the thesis.

GC-MS Fingerprinting: The GC-MS analysis of pet-ether concentrates of leaves, stems and roots of *Pisonia grandis* (PGSP, PGRP, PGLP) revealed that the most prevailing phytoconstituents is n-hexadecanoic acid (palmitic acid), 6-octadecenoic acid (petroselenic acid), 9-octadecenoic acid (oleic acid), 2,3-bis-[(9E)-9-octadecenoyloxy]propyl (9E)-9-octadecenoate (9-octadecenoicacid1,2,3-propanetriyl ester) and phytol. The GC-MS chromatograms represents the fingerprints.

The GC-MS analysis of pet-ether extract of *Andrographis stenophylla* (ASAP) revealed the presence of chondrillasterol, stigmasterol, n-hexadecanoic acid, 9-octadecenoic acid, dotriacontane, nonadecane and n-pentadecanol. The GC-MS chromatograms represent the fingerprints.

Chemical Standardization

The Chemical Characterisation studies led to the identification of the presence of medicinally valuable molecules from both the plant extracts. The bioactives isolated were quantified in the extracts by HPLC method. This analysis led to a revelation on the relative percentage of the bioactives in the various extracts. The relevance of this study lies in the validation of the extracts for its bioactive content which is of principal significance in any herbal formulation.
Quantitation of Bio-Actives in Various Extracts of *Pisonia grandis* by HPLC

HPLC quantitation of bioactive molecules pinitol and allantoin in the various extracts of *Pisonia grandis* was carried out by optimized conditions. **Table 2 below gives the percentage of pinitol and allantoin in various extracts of *Pisonia grandis***

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Pinitol (%)</th>
<th>Allantoin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPGLE</td>
<td>6.12</td>
<td>6.8</td>
</tr>
<tr>
<td>DPGSE</td>
<td>18.88</td>
<td>9.3</td>
</tr>
<tr>
<td>DPGRE</td>
<td>16.28</td>
<td>4.0</td>
</tr>
<tr>
<td>PGLAQ</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PGSAQ</td>
<td>5.24</td>
<td>4.6</td>
</tr>
<tr>
<td>PGRAQ</td>
<td>4.35</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Quantitation of Bio-Actives in Various Extracts of *Andrographis stenophylla* by HPLC

HPLC quantitation of bioactive molecule andrographolide in the various extracts of *Andrographis stenophylla* is under progress and the data will be presented in the thesis.

**Biological Standardization**

Leaves of *Pisonia grandis* have been explored extensively by many groups of workers for its bio-pharma potential. There is only one report on the hepato protective activity of root of *Pisonia grandis* exist. There are no reports on the activity of the stem extract. Hence, stems and roots along with leaves of this plant have been analysed for their herbal potential. In case of the plant *Andrographis stenophylla*, leaves have been taken up for the activity studies by earlier workers. The aerial parts have been chosen for assessing its herbal potential in the present work. For the various extracts of both plants, the anti-oxidant, anti-fungal, anti-cancer, *in-vitro* wound healing, *in-vivo* wound healing and *in-vitro* anti-arthritic activities have been assessed in the present work. The extract samples analysed are PGLP, PGSP, PGRP (Petroleum ether extracts of leaves, stems and roots of *Pisonia grandis*); DPGLE, DPGSE, DPGRE (Dewaxed ethanol extracts of leaves, stems and roots of *Pisonia grandis*); PGLAQ, PGSAQ, PGRQA (Aqueous extracts of leaves, stems and roots of *Pisonia grandis*); ASAP (Petroleum ether extract of aerial parts of *Andrographis stenophylla*); DASAE (Dewaxed ethanol extract of aerial parts of *Andrographis stenophylla*); and ASAAQ (Aqueous extract of aerial parts of *Andrographis stenophylla*). The efficacy of the isolated constituents also has been tested and compared with that of the extract concentrates of *Pisonia grandis* and *Andrographis stenophylla*. This study also forms part of the validation of the herbal potential of the chosen plants. The results of the biological activity studies will be presented in the thesis.

_In the present research work, two folkloric medicinal plants have been investigated thoroughly for their chemical constituents. Medically valuable molecules have been identified and characterized from the non-polar and polar extracts of leaves, stem and roots of *Pisonia grandis* and aerial parts of *Andrographis stenophylla*. All feasible herbal standardisation strategies have been applied to make it a wholesome record of validation of the folkloric use of both the plants. This work may lend a major lead to the development of herbal formulations particularly anti-diabetic, wound healing and anti-arthritic formulations._

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