In-vitro, in-vivo and in-silico studies on the phytoconstituents of the plants Rubiacordifolia and Pimentadioica

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Abstract: The present work aims at providing scientific validation for ethnomedicinal claims of Rubiacordifolia and Pimentadioica. The ethnomedicinal claims for the above mentioned plants reveals that, these plants process potent anti-inflammatory, rejuvenators and anti-cancer property. In the presence study crude drug is prepared using soxhlet process, the anti-inflammatory studies were done through albumin denaturation and protein inhibition studies and cytotoxicity through MTT assay using MCF-7 cell line study. The study reviled that the plant P. dioica and R. cordifolia process potent albumin denaturation ability (0.5 mg/ml and 1 mg/ml) and protein inhibitory activity (0.5 mg/ml and 1.5 mg/ml). MTT assay on MCF-7 cell lines reveals that both the plants possess anti-proliferative property at the range of 100 µg/ml with % IC_{50} value 34.13191 of R. cordifolia showed highest anti-proliferative activity against MCF-7 cell line. In order to screen affinity characteristics of anti-inflammatory, wound healing, antibacterial activity (gram positive and negative) of R. cordifolia and P. dioica natural product. To understand the molecular mechanism, Insilico studies were done and the study reviled that they have good affinity for the target. Out of 8 ligands, 3 had good affinity score towards gram +ve (of S. aureus), garam –ve (E. coli) bacteria and GSK 3 β wound healing target (Homo sapiens). The ligands such as Betacaryophyllene, Purpurin, Quercetinhaave shown good affinity scores as well as better ADMET properties. by using ADMET SAR software.

Key words: Rubia cordifolia, Pimenta dioica, anti-inflammatory properties, anti-cancer, ADMET SAR software.

I. INTRODUCTION

Rubia cordifolia, also known as, Manjishtha, Indian madder, distributed throughout India [7]. It is found throughout the hilly districts of India from northwest Himalayas eastwards, ascending to 8000 ft and southwards to Ceylon. The roots of this plant are of high medicinal value and are recognized as official [8]. Rubia cordifolia is an important medicinal plant which is used for treatment of various ailments in the Ayurvedic system of medicine. The biological investigations have shown that many of the medicinal properties claimed for the plant in the historical texts do, indeed, have sound scientific basis. It has a variety of uses such as blood purifier, immune-modulator[11], anti-inflammatory [12] and antioxidant [13]. It is helpful in treating skin diseases.

Pimenta dioica Allspice is derived from the plant Pimento dioica, and is called allspice because of its unique flavor, which is a combination of cinnamon, cloves, ginger, and nutmeg [2]. Allspice is also obtained from another plant closely related to P. dioica called Pimenta racemose, mostly found in Central America [3]. Allspice is predominantly found in Jamaica, Mediterranean area and Asia. Some of the secondary metabolites include terpenoids, alkaloids, polyphenols, and glycosides [4]. They are also inhibitors of enzymatic activities in living organisms such as inflammatory enzymes like cyclooxygenase-2 [5]. Eugenol is a phenyl propene predominantly found in clove oil, cinnamon, ginger, nutmeg and has anti-bacterial, and anti-inflammatory effect [6]. Review of the literature reveals that, these plants are less studied for the claimed activity. In this context, in the present study an attempt is made to provide scientific validation for the ethnomedicinal claims of the mentioned plant genetic resources.

Methods and materials

1. Collection and processing of the plant samples: The leaves of P. dioica, and bark of R. cordifoliae were collected near the western ghats of Karnataka near Sringeri region. These plants leaves and tree bark was washed, shade dried and powdered. The powder was preserved in air sealed polythene bag for further evaluation.

2. Preparation of plant extract

The plant parts were shade dried, powdered mechanically and material was defatted with petroleum ether (30-50°C) by hot extraction method in a soxhlet apparatus. The defatted powder materials were further extracted with methanol and concentrated methanol extracts were used for the analysis. The extracts were concentrated using Rotavapor at 40°C under reduced pressure and stored in dessiccator for further use.
3. Biological Screening:

3.1 Anti-inflammatory assay: Anti-inflammatory assay for crude drug, fraction and isolated compounds were studied using

- **Inhibition of albumin denaturation:** The reaction mixture (1ml) was consisting of test extract at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using small amount of 1N HCl. The samples were incubated at 37°C for 20 minutes and then heated for 57°C for 20 minutes. After cooling the samples, the turbidity was measured spectrophotometrically at 660nm [1]. The experiment was performed in triplicate. Percentage of protein denaturation was calculated as follows:

$$\text{Percentage inhibition} = 1 - \frac{\text{Asample}}{\text{Acontrol}} \times 100$$

- **Proteinase inhibitory action:** The test was performed according to the modified method of Oyedepo. The reaction mixture (2ml) was containing 0.06mg trypsin, 1ml 20 mMTrisHCl buffer (pH 7.4) and 1ml test sample of different concentrations. The mixture was incubated at 37°C for 5 minutes and then 1ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 minutes. 2ml of 70% per-chloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210nm against buffer as blank[1]. The experiment was performed in triplicate. The percentage inhibition of Proteinase inhibitory activity was calculated.

$$\text{Percentage inhibition(%) = } 1 - \frac{\text{OD of sample}}{\text{OD of control}} \times 100$$

3.2 Anti-cancer activity: Cytotoxicity study using MTT assay on MCF7 cell line

**MTT assay is an well established method to study the cytotoxicity,** the reduction of tetrazolium salts is widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The assay was done following the method mentioned in Benencia, et al., 2009 with modification, in brief.

1. The cells were trypsinized and aspirated into a 50ml centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM Low Glucose medium containing 10% FBS, such that 200 µl of suspension contained approximately 15,000 cells.
2. To each well of the 96 well microtitre plate, 200 µl of the cell suspension was added and the plate was incubated at 37°C and 5% CO₂ atmosphere for 24 h.
3. After 24 h, the spent medium was aspirated. 200 µl of different test concentrations of test drugs were added to the respective wells. The plate was then incubated at 37°C and 5% CO₂ atmosphere for 24 h.
4. The plate was removed from the incubator and the drug containing medium was aspirated. 200µl of medium containing 10% MTT reagent was then added to each well to get a final concentration of 0.5mg/ml and the plate was incubated at 37°C and 5% CO₂ atmosphere for 3 hr.
5. The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilisation solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilize the formed formazan.
6. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) was generated from the dose-response curve for the cell line.

4. IN SILICO MOLECULAR DOCKING:

The receptor molecule of *S.aureus*, *E.coli*and *Homo sapiens* origin were retrieved from PDB (proline database) the ligand molecule for each plant was retrieved from the PubCem. The ligands selected were Beta-caryophyllene, Purpurin and Quercetin For the plant *Rubiacordifolia* and *Pimentadioica* respectively. The receptor and ligand molecule were selected and their fee availability and feasibility to convert the fail format of the ligand to one that is compatible with the docking software .the ligand and the receptor were uploaded in the autodoc software and result were viewed and photographed using PyMOL viewer.

Results and discussion

1. Anti-inflammatory assay

**Inhibition of albumin denaturation:** Protein denaturation has been employed as an in-vitro screening method for anti-phlogistic agents. Drug binding to plasma albumin may inhibit thermal denaturation of albumin which perhaps block ø-NH2 groups in case
of histidine decarboxylase or may displace urate from albumin. Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability of extracts, protein denaturation was studied. The methanolic extracts showed the higher percentage inhibition at(0.5mg/ml and 1mg/ml) for both P.dioica and R.cordifolia. Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability of extracts, protein denaturation was studied. The methanolic extracts showed the higher percentage inhibition (%). The study revealed that P.dioica had the maximum inhibition at 0.5mg/ml and R.cordifolia at 1mg/ml of concentration.

**a) Proteinase inhibitory action:** Proteinases have been implicated in the arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocyte proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitory. The study was conducted for the methanolic extracts. The methanolic extracts showed higher inhibition activity at(1.5mg/ml and 0.5mg/ml) of extract Rubiacordifolia and Pimentadioica. It was previously reported that leukocyte proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitory. The study reveals that, P.dioea has highest activity at 0.5mg/ml and R.cordifolia at 1.5mg/ml.

![Figure 4: Protein inhibitory action of Pimenta dioica](image)

**2) MTT Assay:**

**MCF-7 cell line vs Rubiacordifolia**

The study reveals that, the standard drug Camptothecin at 40µM showed viability percentage of 44.067% however the test drug Rubiacordifoliulate at 200µg/ml concentration showed the closest viability percentage compared to the standard drug i.e., 45.48%, with IC\(_{50}\) value IC\(_{50}\) 144.085µg/ml as shown in the table………………

<table>
<thead>
<tr>
<th>MCF-7 cell line vs RC</th>
<th>BLAN K</th>
<th>UNTREATED Camptothecin (40µM)</th>
<th>Concentration Unit: µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 200 300 400 500</td>
<td></td>
</tr>
<tr>
<td>Reading 1</td>
<td>0.006</td>
<td>0.534 0.242 0.305 0.247 0.18</td>
<td></td>
</tr>
<tr>
<td>Reading 2</td>
<td>0.003</td>
<td>0.537 0.235 0.314 0.245 0.161</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.0045</td>
<td>0.5355 0.2385 0.3097 0.246 0.1705</td>
<td></td>
</tr>
<tr>
<td>Mean OD-Mean B</td>
<td>NA</td>
<td>0.531 0.234 0.3052 0.2415 0.166</td>
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</tr>
<tr>
<td>STANDARD DEVIATION</td>
<td>0.00212132</td>
<td>0.004949747</td>
<td>0.0060 0.0014 0.0134</td>
</tr>
<tr>
<td>STANDARD ERROR</td>
<td>0.001500227</td>
<td>0.003500529</td>
<td>0.0042 0.001 0.0095</td>
</tr>
<tr>
<td></td>
<td>0.282528541</td>
<td>0.659233262</td>
<td>0.8047 0.1883 1.7893</td>
</tr>
<tr>
<td>Viability %</td>
<td>NA</td>
<td>100 44.06779661 57.4816 45.480</td>
<td></td>
</tr>
</tbody>
</table>

|                      | 23 31.26177 30.03766 27.77778 |

Liver damage by arthritic reactions.

![Liver damage](image)
3. **Insilico studieds:** The in silico work was carried out for two of the most pathogenic organisms such as E.coli and S. aureus, wound healing target from Homo sapiens and breast cancer target. LT enterotoxin and ISDH linker domain were the receptors for E.coli and S. aureus respectively. These receptors and ligand were docked using Autodock vina 4.0 software and following results were obtained. It was found that the ligand β-caryophylline from the plant *Pimentadiocia* and Quercetin from the plant *Rubiacordifolia* have shown good affinity score towards the active binding site.

**TABLE 1:** In silico molecular docking result for receptor of *S.aureus*:

<table>
<thead>
<tr>
<th>PLANT</th>
<th>LIGAND</th>
<th>CID</th>
<th>MOLECULAR WEIGHT (g/wt)</th>
<th>MOLECULAR FORMULA</th>
<th>logp</th>
<th>HBD</th>
<th>HBA</th>
<th>SCORE (1T2P) <em>S.aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pimentadiocia</em></td>
<td>β-caryophylline</td>
<td>5281515</td>
<td>204.19</td>
<td>C_{15}H_{24}</td>
<td>6.044</td>
<td>0</td>
<td>0</td>
<td>-7.5</td>
</tr>
<tr>
<td><em>Rubiacordifolia</em></td>
<td>Quercetin</td>
<td>5280343</td>
<td>302.04</td>
<td>C_{13}H_{10}O_{7}</td>
<td>1.834</td>
<td>5</td>
<td>7</td>
<td>-7.5</td>
</tr>
<tr>
<td></td>
<td>Purpurin</td>
<td>6683</td>
<td>256.04</td>
<td>C_{16}H_{10}O_{5}</td>
<td>0.976</td>
<td>3</td>
<td>5</td>
<td>-7.1</td>
</tr>
</tbody>
</table>

**Fig:** G+VEβ-caryophylline Fig: G+VE purpurin Fig: G+VE Quercetin
TABLE 2: In silico molecular docking result for receptor of E. coli

<table>
<thead>
<tr>
<th>PLANT</th>
<th>LIGAND</th>
<th>CID</th>
<th>MOLECULAR WEIGHT (g/wt)</th>
<th>MOLECULAR FORMULA</th>
<th>logp</th>
<th>HBD</th>
<th>HBA</th>
<th>SCORE</th>
</tr>
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<tr>
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<td>5281515</td>
<td>204.19</td>
<td>C_{15}H_{24}</td>
<td>6.044</td>
<td>0</td>
<td>0</td>
<td>-5.5</td>
</tr>
<tr>
<td><em>Rubiacordifolia</em></td>
<td>Quercetin</td>
<td>5280343</td>
<td>302.04</td>
<td>C_{15}H_{10}O_{7}</td>
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<td>5</td>
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<tr>
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<td>Purpurin</td>
<td>6683</td>
<td>256.04</td>
<td>C_{14}H_{8}O_{5}</td>
<td>0.976</td>
<td>3</td>
<td>5</td>
<td>-7.3</td>
</tr>
</tbody>
</table>

Fig 5: G-veβ-caryophyllene  Fig 6: G-vePurpurin  Fig 7: G-veQuercetin

TABLE 3: In silico molecular docking result for receptor of wound healing:

<table>
<thead>
<tr>
<th>PLANT</th>
<th>LIGAND</th>
<th>CID</th>
<th>MOLECULAR WEIGHT (g/wt)</th>
<th>MOLECULAR FORMULA</th>
<th>logp</th>
<th>HBD</th>
<th>HBA</th>
<th>SCORE Wound healing</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pimentadiocia</em></td>
<td>β-caryophylline</td>
<td>5281515</td>
<td>204.19</td>
<td>C_{15}H_{24}</td>
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<td>-5.6</td>
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<tr>
<td><em>Rubiacordifolia</em></td>
<td>Quercetin</td>
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<td>302.04</td>
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<td>0.976</td>
<td>3</td>
<td>5</td>
<td>-8.5</td>
</tr>
</tbody>
</table>

Fig 7: Wound healing β-caryophyllene  Fig 8: Wound healing Purpurin  Fig 9: Wound healing Quercetin
CONCLUSION:

- From the present study it can be concluded that the medicinal plants tested viz., *Pimenta dioica* and *Rubia cordifolia* possess anti-inflammatory activity found to be higher in the methanol extracts. In vitro cell line based MTT assay using MCF-7 cell lines revealed that, the extract possess potent anticancer activity with IC₅₀ values of the test compounds found to be *Rubia cordifolia* (144.085 µg/ml), in order to reduce the expenses of drug In silico studies was conducted to 10 ligands from each plant *Pimenta dioica* and *Rubia cordifolia* out of 8 different plant 3 molecule showed good affinity value towards the target binding pocket.

REFERENCES