1 **Anti-Inflammatory activity and *Insilco* Approaches on root extract of *Decalepis hamiltonii***
2 **Wight & Arn**

3 **ABSTRACT**

4 **Aims**: The present study was aimed to evaluate anti-inflammatory and antioxidant
5 potential of methanol root extract of *Decalepis hamiltonii*. **Results** The methanolic extract
6 showed *in vitro* anti-inflammatory activity inhibition cells 96.1%μg/ml. MTT (Thiazolyl Blue
7 Tetrazolium Bromide) assay was significant inhibited by the methanol (96.2) and inhibition
8 addition the Nitric oxide production 25μg/ml 95% inhibition. The methanol extract of *Decalepis*
9 *hamiltonii* shows a Thus, a very strong approach to In-silico docking and in vitro anti-
10 inflammatory study for whole with these compounds/extract. **Conclusion.** From the study it is
11 concluded that the root extract of *Decalepis hamiltonii* showing a strong approach to *In-silico*
12 docking and anti inflammatory activity.

13 **Keywords:** *Decalepis hamiltonii;* .MTT (Thiazolyl Blue Tetrazolium Bromide); Nitric oxide;
14 Ferric reducing antioxidant power

15 **1. Introduction**

16 Medicinal plants have been used as age long remedies for human diseases because they
17 contain components of therapeutic value. The plants are naturally large source of antioxidants,
18 anticancer, antidiabetic agents they might be serve as leads for the development of novel drugs.
19 The research and analysis on plants employed as pain-relievers and anti-inflammatory agents in
20 traditional ethno medicine is one of the productive and logical strategies in the search for new
21 drugs [1-2]. Free radicals, which have one or more un-paired electrons (superoxide, hydroxyl,
22 peroxyl) are produced in normal or pathological cell metabolism and the compounds that can
23 scavenge free radicals have great potential in ameliorating the diseases and pathological cells [3-

24 6]. The increase in the prevalence of multiple drug resistance has shown down the development
25 of new synthetic anti-inflammatory drugs and the new drug is necessary to search for new anti-
26 inflammatory from alterative sources. Phytochemicals from medicinal plants showing anti-
27 inflammatory activities have the potential of filling this need because of the structures of
28 phytochemical different from those of the more studied and they're those of the more action may
29 too very likely differ [7]. In this growing interest, many of the phytochemical bioactive
30 compounds from medicinal plants have shown many pharmacological activities [8-11]
31 Inflammation are pathological condition in which highly reactive species are produced. Nitric
32 oxide (NO) is a small diffusible molecule responsible for vasodilatation, neurotransmission and
33 inflammation. This molecule is produced by the organism at a basal concentration. Nevertheless,
34 under stimulation by pathogens, NO is generated in higher amounts by the inducible nitric oxide
35 synthase (iNOS) in activated macrophages. The over production of NO is involved in the
36 pathogenesis of septic shock, tissue damage, multiple organ dysfunctions and carcinogenesis
37 processes [12]. Natural products have contributed significantly towards the development of
38 modern medicine. Recently traditional medicine worldwide has been re-evaluated by extensive
39 research on different plant species and their active therapeutic principles. The rich wealth of
40 plant kingdom can represent a novel source of newer compounds with significant anti-
41 inflammatory activities. The major merits of herbal medicine seem to be their perceived efficacy,
42 low incidence of serious adverse effects, and low cost. This *Decalepis hamiltonii* (Wight& Arn),
43 popularly known as swallow root in English, belongs to the family *Asclepiadaceae.* It is a
44 climber with stout, smooth branches shrub and a native of the forests of Deccan peninsula and
45 Western Ghats of India. The leaves are curvaceous, orbicular or elliptical, with rounded tip. Its
46 tubers are consumed as the pickles and the juice for its alleged health promoting properties The

47 Root has a sweet sarsaparilla-like taste; contains 9.2% fleshy matter and 8% woody core [13]. In
48 this context the present research work is on antioxidant and anti-inflammatory activity of
49 Decalepis hamiltonii (Wight & Arn).
50

51 ## 2. Material and Methods

52 ### 2.1 Collection of material

53 The Root of *Decalepis hamiltonii* has been collected from kolli hills, Namakkal district of
54 Tamilnadu, India. The taxonomic identification of plant was done with comparing the Flora of
55 Presidency of Madras, by Gamble J.S., 1921.

56 ### 2.2 Preparation of solvent extraction

57 50gm of *Decalepis hamiltonii* root was packed in Soxhlet apparatus for extraction and
58 500 ml of methanol was used as solvent. Soxhlet was kept running for 72 hours, until the solvent
59 color appears in the collection tube. Methanol was removed by evaporation using rotary vapour
60 at not more than 40°C. The residue was then placed in an oven at 40°C for about 48hours to
61 remove the moisture. The resulting dried mass was then powdered and used for further studies.

62 ### 2.3 Ferric Reducing Antioxidant Power

63 The Ferric reducing antioxidant power of different parts of the various crude extracts of
64 *Decalepis hamiltonii* was evaluated [14]. The samples were mixed with 2.5 ml of 0.2 M Phosphate
65 buffer (pH 6.6) and 2.5 ml of 1 per cent potassium ferric cyanide. After the mixture was incubated at 50
66 ºC for 20 min, 2.5 ml of 10 per cent Trichloroacetic acid, 2.5 ml distilled water and 0.5 ml of 0.1 per
67 cent ferric chloride was added and then the absorbance was measured at 700 nm against a blank. The
68 blank consist of all the reagents without the test sample. The reducing power of Gallic acid was also

69 determined for a comparison. High absorbance of the reaction mixture indicates strong ferric reducing
70 antioxidant power.

71 **2.4 MTT assay**

72 The Peripheral blood mononuclear layer cell culture was trypsinized and the cell count
73 was adjusted to 1.0×10^5 cells/ml using medium containing 10% new born calf serum. To each
74 well of the 96 well micro titreplate, 0.1 mL of the diluted cell suspension (approximately 10,000
75 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was
76 flicked off, washed the monolayer once and 100 µL of different drug concentrations was added
77 to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO2
78 atmosphere and microscopic examination was carried out and observations recorded every 24
79 hours. After 72 hours, the drug solutions in the wells were discarded and 50 µL of MTT
80 (2mg/mL) prepared in Hank's balanced salt solution (HBSS) without phenol red was added to
81 each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO2
82 atmosphere. The supernatant was removed and 50 µL of propanol was added and the plates were
83 gently shaken to solubilize the formed formazan[15]. The absorbance was measured using a
84 microplate reader at a wavelength of 540 nm.

85 **2.5 Nitric Oxide Production**

86 The Nitric oxide (NO) production in cell culture were determined by measuring the
87 nitrite (NO_2) formation by using Griess assay [16]. Briefly, 100µL of griess reagent (Sigma-
88 Aldrich, USA) containing 1% sulfanilamide and 0.1% naphtylethyene diamine dihydrochloride
89 in 2.5% H_3PO_4 was added to 100 µl of cell culture supernatant and incubate for 15 minutes at
90 room temperature in the dark. The color density was measured at 540 nm using a microplate

91 reader (Molecular Devices, USA). A standard curve was generated in the same method using
92 $NaNO_2$.

93 The compounds namely 2-Hydroxy-4-methoxybenzaldehyde was identified by GC-MS
94 analysis were screened against the anti diabetic, anti - inflammatory and cancer protein. The
95 compound details were retrieved from the Pubchem database and the chemical structures were
96 generated from SMILES notation (Simplified Molecular Input Line Entry Specification) by
97 using the Chemsketch Software (www.acdlabs.com).

98 **2.7 Protein Data Bank**

99 The Protein Data Bank (PDB) archive is the single worldwide repository of information
100 about the 3D structures of large biological molecules, including proteins and nucleic acids. The
101 Protein Data Bank (PDB) is a repository for the three-dimensional structural data of large
102 biological molecules, such as proteins and nucleic acids. The data, typically obtained by X-ray
103 crystallography or NMR spectroscopy and submitted by biologists and biochemists from around
104 the world, are freely accessible on the Internet via the websites of its member organizations
105 (PDBe, PDBj, and RCSB). The PDB is overseen by an organization called the Worldwide
106 Protein Data Bank, WWPDB.

107 **2.8 Preparation of protein structure**

108 The structural information of the macromolecules determined by x-ray crystallographic
109 and NMR methods are available in the PDB. The 3D structure protein Receptor (PDB I.D: 4IFI)
110 was downloaded from the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/) the water
111 molecules were removed from protein file 4IFI before docking. Energy minimization by
112 applying for CHARMM (Chemistry at Harvard Macromolecular Mechanics) force fields, It's a

113 program for macromolecular dynamics; it can be used for energy minimization, normal modes
114 and crystal optimizations and also incorporates free energy methods for chemical and
115 conformational free energy calculations.

116 **2.9 PUBCHEM**

117 The Pubchem bioassay data (http://pubchem.ncbi.nlm.nih.gov) is a public repository for
118 biological Activities of small molecules and small interfering RNAs (siRNAs) hosted by the US
119 National Institutes of Health (NIH). It archives experimental descriptions of assays and
120 biological test results and makes the information freely accessible to the public. A Pubchem
121 Bioassay data entry includes an assay description, a summary and detailed test results. Each
122 assay record is linked to the molecular target, whenever possible, and is cross-referenced to other
123 National Center for Biotechnology Information (NCBI) database records. 'Related Bioassays'
124 are identified by examining the assay target relationship and activity profile of commonly tested
125 compounds. A key goal of PubChem Bioassay is to make the biological activity information
126 easily accessible through the NCBI information retrieval system- Entrez, and various web-based
127 PubChem services. An integrated suite of data analysis tools is available to optimize the utility of
128 the chemical structure and biological activity information within PubChem, enabling researchers
129 to aggregate, compare and analyze biological test results contributed by multiple organizations.
130 Describe the PubChem Bioassay database, including data model, bioassay deposition and
131 utilities that PubChem provides for searching, downloading and analyzing the biological activity
132 information.

133 **2.10 Preparation of Ligand structures**

134 The identified Chemical compound namely 2-Hydroxy-4-methoxybenzaldehyde was
135 derived from *Decalepis hamiltonii* Wight & arn and this compound structure were retrieved
136 from Pubchem online server both of these compounds were under investigation of Chemsketch
137 (Chemically intelligent drawing interface free ware developed by Advance Chemistry
138 Development, Inc., (http://www.acdlabs.com) was used to construct the structure of the ligands.
139 The ligand molecules were generated and the three dimensional optimizations were done and
140 then saved MOL file (a file format for holding information about the atoms, bonds, connectivity
141 and coordinates of a molecule).

142 **2.11 Drug likeliness prediction**

143 Ligand property was predicted by using "Lipinski drug Filters" (http://www.scfbio-
144 iitd.res.in/utility/LipinskiFilters.jsp). Lipinski rule of five helps in distinguishing drug-like and non-
145 drug-like properties and predicts high probability of success or failure due to drug likeliness for
146 molecules. The Lipinski filter helps in early preclinical assessment and thereby avoiding costly
147 late stage preclinical and clinical failures.

148 **2.12 Docking analysis:**

149 The docking analysis is performed by Argus lab 4.0.1 for the antidiabectic and cancer
150 protein interacts with Gas-Chromatography of *Decalepis hamiltonii* 2-Hydroxy-4-
151 methoxybenzaldehyde compound. The compound or ligand selected for based on Lipinski's rule of
152 five. Fitting points are added to hydrogen bonding groups on the protein. The interaction between
153 the binding pockets of target protein, antidiabetic protein and 3v6r inflammation protein and
154 investigation compound to find out the accurate binding model for the active site of protein. The
155 mechanism of ligand placement is based on binding site position. The protein ligand docking

156 energy values performance of this compound was based on the Scoring functions which is
157 implemented in docking program to make various assumptions and implications to fit best
158 complexes, which includes terms of hydrogen bonds employed by Argus lab 4.0.1 to rank the
159 docked bases and to assess the binding site and the number of rotatable bonds present.

160 **2.13 Ligand binding sites prediction**

161 After docking the docked structure was saved as ".Pdb" file and further explored to
162 predict the binding sites using "ligand explorer" software. The predicted binding sites, based on
163 the binding energy, and amino acids make up the binding cavity. Here ligand binding site
164 represents the site where the ligands most efficiently bind with the protein, among all the active
165 site.

166 **2.13 Discovery Studio Visualizer**

167 The docking results were visualized using Accelyrs Discovery Studio 4.1 Visualizer. The
168 discovery studio visualizer is also a free viewer that is designed to offer an interactive
169 environment for viewing and editing molecular structures, sequences, X-ray reflection data,
170 script and other data. DS Visualizer is handier for analyzing the docking results.

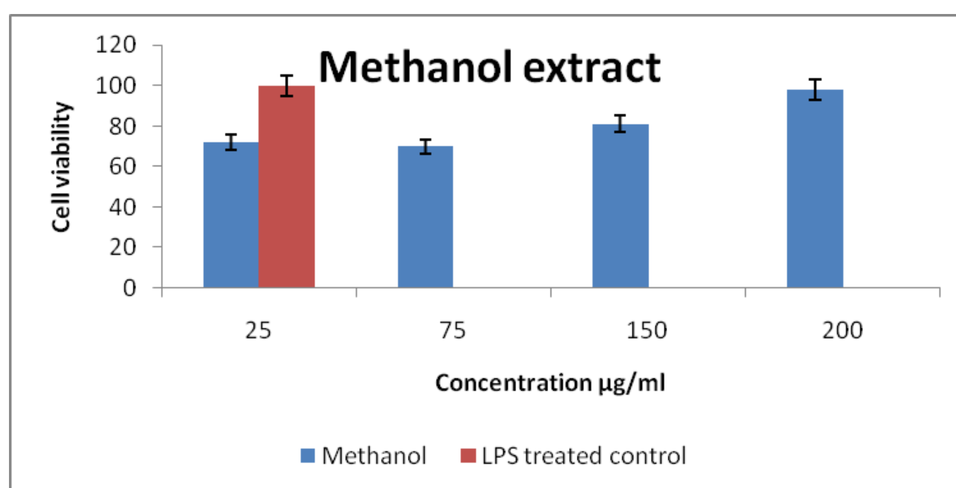
171 **3.0 Results and Discussion**

172 **3.1 In-vitro Cytotoxicity activity**

173 **Cytotoxicity effect of the root extract of *Decalepis hamiltonii* in Peripheral Blood**
174 **Mononuclear cells (PBMC)**

175 MTT (3-(4,5 dimethylthiazole- 2yl) - 2, 5-Diphenyl Tetrazolium Bromide) assay is
176 widely used in the *in vitro* evaluation of the biosafety of plant extracts. Determination of

177 cytotoxic effect of plant extracts is needed before studying the mechanism of action in various
178 cells / cell lines. The cytotoxicity activity of methanolic root extract of *Decalepis hamiltonii* on
179 cell viability was determined using cell line culture at various concentrations is shown in Fig 1.
180 The values of cell viability were found to be in the range of 68% to 98% at various concentrations
181 tested. The highest concentration of 200µg/ml showed moderate Cytoxicity. Cell viability was
182 evaluated by MTT assay, hence the concentrations of sample (cell viability ≥90) were selected for
183 subsequent iNOS inhibition experiment.



184

185 Results are expressed as mean ± Standard deviation

186 **Figure. 1 Cytotoxicity effect of the root extract of *Decalepis hamiltonii***

187 **3.2 Inhibition of Lipopolysaccharide – induced iNOS production by root extracts**

188 To investigate the inhibitory effects of the methanolic extracts from the LPS–induced
189 iNOS production in peripheral blood mononuclear cell (PBMC) the accumulation of iNOS in
190 the culture medium is measured. As shown in Figure 2, root extracts significantly suppressed
191 LPS-induced iNOS product in a dose-dependent manner. The root extract at 75µg/ml
192 significantly inhibited the production of iNOS up to 90.2±0.86.

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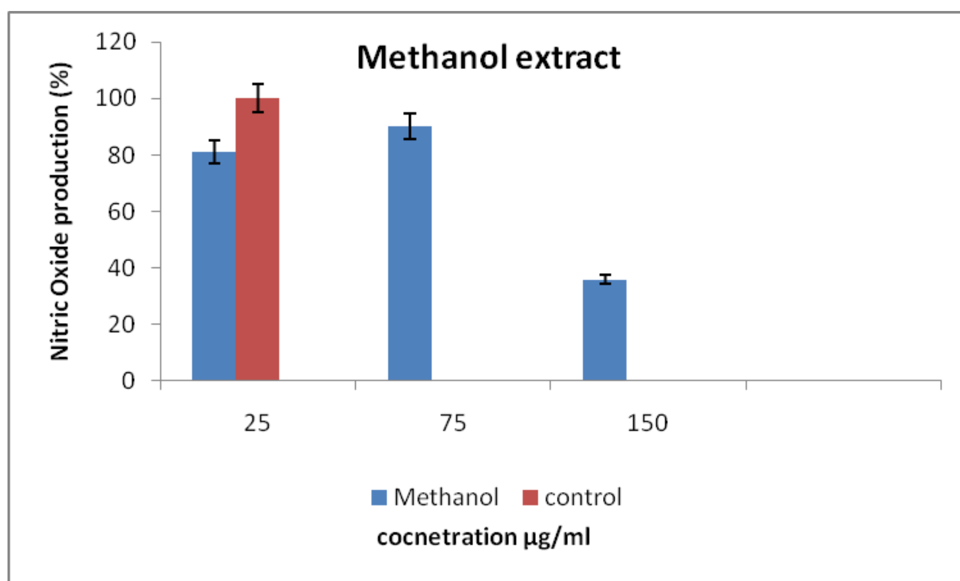

194 Results are expressed as means ± standard deviation

195 **Fig. 2 Nitric oxide effect of the root extract of *Decalepis hamiltonii***

196 3.3 **Ferric reducing antioxidant power**

197 Ferric reducing antioxidant power of Petroleum ether, Chloroform, Ethyl acetate and
198 Methanol extracts of root plant parts of *Decalepis hamiltonii* is shown in Fig. 3. IC_{50} values were
199 calculated as the amount of antioxidant present in the sample necessary to decrease the initial
200 ferric reducing antioxidant concentration by 50%. Petroleum ether, Chloroform, Ethyl acetate
201 and Methanol root extracts of *Decalepis hamiltonii* exhibited the highest activity. The IC_{50}
202 values of Methanol extracts of *Decalepis* root and Gallic acid values were 392.7, 442.5, 311.4
203 and 171.02 µg/ml respectively.

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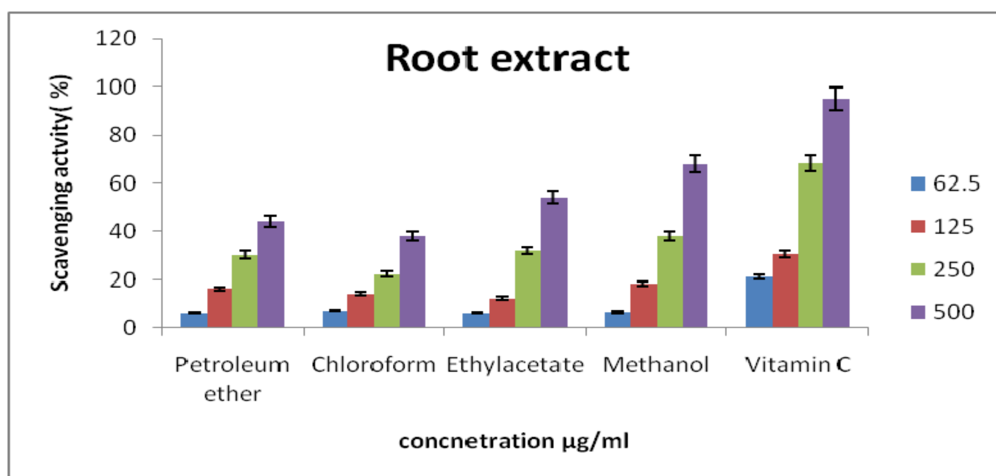


205 ***Fig. 3 In silico*** **analysis of anti- inflammatory activity of 2-hydroxy-4-methoxybenzaldehyde**

206 **from root extract of *Decalepis hamiltonii***

207 The molecular interaction between the ligand and the protein (receptor) was investigated

208 using the Ligand fit protocol. The 2-Hydroxy- 4 -Methoxy Benzaldehyde was docked with active

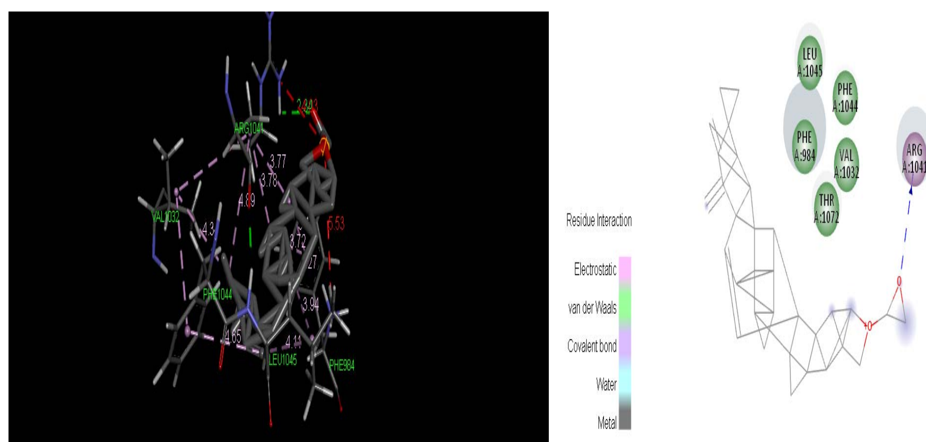
209 site inflammation receptor protein 3V6R-docking energy-17.45 kcal/mol. 3D structure and 2D

210 structure interaction to interact with Targeted protein; amino acid region and distance. The

211 docking studies further confirmed the anti-diabetic property of the bioactive compounds via GC-

212 MS analysis and suggested that first time demonstrated a strong 3v6r (Inflammation) inhibitory

213 property of this extract, maybe due to the identified bioactive compounds (Figure.4).



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215 **Figure.4 3D and 2D structures for amino acid regions for 2-Hydroxy-4- Methoxy**
216 **Benzaldehyde interact with Targeted protein**

217 In the present study methanolic root extract of *Decalepis hamiltonii* was found to have maximum
218 inhibition concentration (99.5% at 200 µg/ml. Similar results were observed [17], who had
219 shown moderate cytotoxicity of *Polygonum chinensis* extracts (400- 125 µg/ml). [18] also
220 compared root, stem and leaf extracts of *Jatropha curcas* in anti-inflammatory activity study
221 towards carrageen an induced *rat paw edema*. Groups of albino rats were injected by
222 inflammation inducer (Carrageenan) and the levels of inflammation mediators (histamine,
223 serotonin and kinin) were monitored. Their results showed that root extract inhibited histamine,
224 serotonin and kinin released up to 70% - 80% compared to leaf (50% - 55%) and stem (40% -
225 45%). However, these studies did not indicate the nature of compounds involved in the anti-
226 inflammatory activity.

227 Ferric reducing properties are generally depend on the presence of reductions which have
228 been shown to exert antioxidant activity by breaking the free radical chain by donating a
229 hydrogen atom [19].The present study of the ferric radical activity of the all the extracts of the
230 *Decalepis hamiltonii* leaves, stem and root were analyzed. The root extract was found to increase
231 in a concentration depended manner. Among all the four extracts, methanol root extract was
232 recorded the highest scavenging activity of 78.0% at a concentration of 500µg/ml. The results
233 suggest that methanol extracts have more reducing power than ethyl acetate, petroleum ether and
234 chloroform but not as efficient as standard gallic acid. Similar type of work has also been carried
235 out in *Azima tetracantha* [20]. It is believed that antioxidant activity and reducing power are
236 related [21]. Reuctones donate a hydrogen atom and inhibit LPO by donating hydrogen. Atom
237 and there by terminating the free radical chain reaction [22].

238 **3.5 Conclusion**

239 Thus, a very strong approach to *In-silico* docking and in vitro anti-inflammatory study for
240 whole with these compounds/extracts. Further studies regarding isolation and characterization of
241 bioactive principles responsible for this action may be further carried out in future.

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