CHAPTER - 3

TO INVESTIGATE THE PHYTOCHEMICALS, ANTI-OXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF *Acacia ferruginea* EXTRACT

3.1 INTRODUCTION

Inflammation, a defensive reaction of tissues to injury or infection, is characterized by swelling, redness, heat, and pain and has vital functions in both host defense and pathophysiological events (Coussens and Werb, 2002). Sustained chronic inflammation can contribute to the pathogenesis of numerous ailments including diabetes as well as several neurodegenerative-, cardiovascular- and other life-debilitating diseases (Rus and Niculescu, 1997; Klegeris et al., 2007; Liu and Li, 2008; Chao et al., 2009). Presently, many non-steroidal anti-inflammatory drugs (NSAID) are routinely used to treat inflammatory disorders (Yesilada et al., 1997). However, long use of those medicines is related to adverse effects including gastro-intestinal irritation. Hence, there is need to develop alternative anti-inflammatory agents that possess minimal side effects. Development of drugs from natural sources that prevent or inhibit inflammation by down-regulating select inflammatory targets has become of keen interest in the field of drug development.

During inflammation, pro-inflammatory stimuli activate cellular responses to increase the production of various cytokines, inflammatory mediators (like nitric oxide [NO]) and prostaglandins (PG) secondary to the increased production/activity of inducible nitric oxide synthase (iNOS) and
cyclooxygenase-2 (COX-2) (Dinarello, 2010). It is known that inflammation-related proteins including COX-2, iNOS, matrix metalloproteinase (MMP)-9, and cytosolic phospholipase A2 (cPLA2) are associated with oxidative stress (excess production of reactive oxygen species [ROS]) induced by pro-inflammatory agents which include several cytokines, peptides and peroxidants (Chiurchiu and MacCarrone, 2011; Lee and Yang 2012). Apart from a role in inducing oxidative stress, ROS can cause deleterious effects due to reactivity with DNA, resulting in altered DNA structures and decreased DNA repair. In turn, these signaling cascades activate redox-regulated transcription factors such as activator protein-1 (AP-1) and nuclear transcription factor kappa- B (NF-κB) p53 protein that trigger adverse reactions (Valko et al., 2006; Franco et al., 2008; Christian and Kunal, 2009).

*A. ferruginea* has been used as traditional medicine for treating excessive mucous discharges, haemorrhages, stomatitis, irritable bowel syndrome, antileprotic drug (Rajanna et al, 2011) and also used to treat skin disease mainly scabies (Das, 1983). The current study was therefore designed to evaluate the anti-inflammatory effect of an *A. ferruginea* methanol extract against acute/chronic inflammation in a murine experimental model. *In vitro* experiments were also carried out to better understand the possible mechanisms of action underlying any protective impact of this extract against inducible oxidative stress. The phytochemical composition of *A. ferruginea* methanolic extract were determined using HPTLC, GC/MS and LC/MS analysis.

**3.2 MATERIALS AND METHODS**

**3.2.1 Plant material collection**

The fresh aerial parts of the plant were collected from Coimbatore, India. The plant was identified and authenticated at Botanical Survey of India,
Coimbatore (No: BSI/SRC/5/23/2011-12/Tech-687). A voucher specimen was retained in the Department of Biotechnology, Karunya University, Coimbatore.

3.2.2 Preliminary screening for phytochemicals

Qualitative phytochemical screening of the extract was carried out according to the methods of Harborne (1973). The extract was screened for alkaloids, flavonoids, phenolics, saponins, glycosides, steroids and tannins along with proteins, fats, and carbohydrates).

3.2.3 HPTLC fingerprint of the methanolic extract of A. ferruginea

An HPTLC system equipped with Linomat V applicator, TLC scanner 3, Reprostar 3 with 12 bit CCD camera for photo documentation, controlled by using WinCATS 4 software, was used. A total of 100 mg extract (semisolid paste) was dissolved in 5 ml methanol and the solution was centrifuged at 4000 rpm for 4 min for use in the analysis. The sample (2 μl) was spotted (in a band of 5-mm width) onto a pre-coated silica gel plate 60F-254 (20 cm × 10 cm × 250 μm; Merck, Darmstadt, Germany) using a Camag Linomat IV micro-syringe (Camag, Muttenz, Switzerland). The loaded plate was then maintained in a twin-trough chamber pre-saturated with a specified mobile phase. Different composition mobile phases were used to separate phenols and flavonoids. The plates were developed (in ascending manner) with respective solvents three times to obtain optimal phytochemical resolution. Optimized times for chamber saturation for the mobile phase containing toluene: ethyl acetate: formic acid and chloroform: methanol were 1 and 3 hr, respectively, at room temperature. The developed plates were dried in an oven to evaporate the solvents and then sprayed with ferric chloride reagent for phenols and diphenylborinic acid ethanolamine reagent for flavonoids. The plates were then re-dried at 100°C for 3
min and placed in a Camag Reprostar-3 chamber (Camag) for illumination and densitometric analyses at 254 and 366 nm, as well as in normal light.

3.2.4 GC/MS analysis

GC/MS analysis of the extract was performed using a Thermo GC-Trace Ultra VER: 5.0 (Bremen, Germany). For MS detection, the MS DSQ II electron ionization mode with ionization energy of 70 eV was used, with a mass range at m/z 50–650. A TR-5MS capillary column (30 m x 0.25 mm, film thickness = 0.25 μm) was used for the analyses. The column temperature was programmed from 80-250°C at a rate of 8°C/min, with the lower and upper temperature being held for 3 and 10 min, respectively. The GC injector and MS transfer line temperatures were set at 280 and 290°C, respectively. GC was performed in the split less mode. Helium (at flow rate = 1.0 ml/min) was used as the carrier gas. A 1 μl injection volume was used. Major and essential compounds were identified by retention times and mass fragmentation patterns (Vinod Prabhu and Guruvayoorappan, 2012).

3.2.5 LC/MS analysis

LC/MS analysis of the extract was performed using a 3200 Q-Trap system equipped with a degasser, binary pump, auto sampler, and column heater (Applied Biosystems, Foster City, CA). The system was coupled with Sciex turbo ion spray triple mass spectrometer (Darmstadt, Germany). Data acquisition and mass spectrometric evaluation were carried out in a personal computer with data analysis software (Analyst -1.4.1. & Light sight software1 (IDC, Framingham, MA). Chromatographic separation was carried out on Eclipse XDB - C8 column (5-μm, 150 x 4.6 mm; Agilent Technologies, Santa Clara, CA). Gradient elution was applied with a mobile phase comprised of 95% Solvent A (0.1%
acetic acid in water) and 5% Solvent B (0.1% acetic acid in acetonitrile) for 1 min, followed by an 11-min step gradient from 5% B to 100% B; thereafter, it was kept for 4 min with 100% B. Finally, elution was achieved with a linear gradient from 100% B to 5% B for 2 min. The flow-rate used throughout was 200 μl/min; sample injection volume was 10 μl. The following parameters were used throughout the MS experiment: for electrospray ionization with positive ion polarity, capillary voltage was set to 20 V, transfer capillary temperature to 300°C, nebulizer pressure to 40 psi, and the drying gas flow to 15 L/min. Data were acquired in the positive ion MS mode in the range of 50-1000 m/z (Vinod Prabhu and Guruvayoorappan, 2012).

3.2.6 Preparation of extract

The shade-dried aerial parts of the plant were subjected to mechanical size reduction. The powdered material (≈ 25 g) was then extracted with methanol in a Soxhlet apparatus. Traces of solvent were then removed by evaporation and the final extract concentrated in a vacuum rotary system; the percentage yield of extract was 12% [w/w]. The dose used in the present study was based on preliminary acute and chronic toxicity studies in mice (data not shown). Specifically, escalating concentrations (1-100 mg/kg B.wt.) A. ferruginea extract were administered and biochemical parameters, e.g., urea, creatinine (kidney function tests), SGOT and SGPT (liver function tests) were assessed. A dose of 10 mg/kg B.wt. was seen as non-toxic and so selected for use in the current experiments. For each exposure, the extract was re-suspended in 1% gum acacia for subsequent administration to the mice.
3.2.7 Animals

Male Balb/c mice (4-6-wk-old, 22-25 g) were obtained from the College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (Mannuthy, Kerala, India). All mice were maintained in a controlled sterile environment maintained at a constant temperature (24±2°C), 50% relative humidity, and a 12-hr light/dark cycle. All mice had ad libitum access to standard diet pellets (Sai Durga Feeds, Bangalore, India) and filtered water. After 2 weeks of acclimatization, the mice were randomly allocated into respective groups. All animal experiments were performed after obtaining approval from Institutional Animal Ethics Committee, Karunya University (Approval No: IAEC/KU/BT/2013/07).

3.2.8 Effect of extract against carrageenan (acute)-formaldehyde-induced (chronic) paw edema

Carrageenan regimen: Mice were divided into four groups (n = 6/group). Group I was kept as normal untreated controls, Group II was kept as irritant only group, Group III mice received a 100 µl dose of indomethacin (10 mg/kg B.wt.; Lucetti et al., 2010), and Group IV animals received A. ferruginea extract (10 mg/kg B.wt.) intraperitoneally (IP). Each treatment was daily for 5 consecutive days; the last dose was given 60 min before induction of inflammation. For the latter, mice in Groups II, III, and IV received a subcutaneous (SC) injection - into the plantar region of their right hind paw - of 0.1 ml of a solution of 1% (w/v) carrageenan in normal saline to induce edema; Group I mice received an injection of saline. The paw volume was measured using vernier caliper at 30-min intervals starting from 0 hr until 8 hr later, and then again at 12 and 24 hr.
**Formaldehyde regimen:** Mice was divided into four groups (n = 6/group) and received the same regimens as above. In this case, for induction of inflammation, the mice received an SC injection of 0.1 ml of 1% (v/v) formaldehyde solution into the plantar region of their right hind paw. The diameter of the hind paw was first measured before injection to obtain a baseline value; thereafter, measures of foot thickness (at metatarsal level) were performed at 24-hr intervals on five consecutive days using a vernier caliper.

### 3.2.9 Effect of extract on nitric oxide (NO) production during acute and chronic inflammation

Blood samples (~200 µl) were obtained from the tail-vein of the above-treated mice at different time intervals (i.e., 0, 4, 8, 12, and 24 hr post-injection for carrageenan groups; Day 0, 1, 2, 3, 4, and 5 post-injection for formalin groups). Serum was separated by centrifugation (3000 x g) and used for estimation of NO using a Griess reaction. The reaction mixture containing 50 µl sodium nitroprusside (10 mM) and 50 µl serum was incubated at 37°C for 3 hr. Thereafter, 1 ml of the mixture was combined with an equal volume of Griess reagent (0.1% α-napthyl-ethylene diamine dihydrochloride in water/1% sulphanilamide in 5% phosphoric acid) and incubated for 15 min at room temperature. The pink chromophore generated was measured at 540 nm in a Model:U-2190 UV-Vis spectrophotometer (Hitachi, Tokyo, Japan) and the sample values extrapolated from a standard curve generated in parallel with varying levels of sodium nitrite. All values were then expressed in µmoles (Green et al., 1982).
3.2.10 Effect of A. ferruginea extract on iNOS expression in serum

Blood samples obtained via the tail-vein (see above) at different time intervals were processed and the serum used for estimation of iNOS via ELISA (USCN Life science, Hubei, China), according to manufacturer instructions. Kit sensitivity was 0.1 ng iNOS/ml.

3.2.11 Histopathological analysis of paw

At the end of the experiment, i.e., 24 hr after carrageenan treatment (acute inflammation) and 5 d after formalin treatment (chronic inflammation), the skin tissues of the plantar region were collected for histopathological assessment. The hind paw tissues were fixed in 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin, dehydrated in alcohol, and then sections of 4-μm thickness were prepared and stained with haematoxylin and eosin (H&E). Thereafter, analyses were carried out using an EVOS-xl CORE light microscope (AMG, Bothell, WA). A certified histopathologist performed all analyses/interpreted the observed outcomes.

3.2.12 Immunohistochemical localization of COX-2

Skin tissues of the plantar region were collected from normal and inflammatory stimuli-injected paws of the mice, fixed in 4% neutral formalin, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5-μm thick) were mounted on slides, cleaned, hydrated. The sections were treated with buffered blocking solution (3% bovine serum albumin in PBS) for 15 min, then co-incubated with primary antibody against COX-2 (polyclonal murine anti-COX-2 antibody; dilution = 1:400 in PBS [v/v]) (ThermoScientific, Rockford, IL) at room temperature for 1 hr. The samples
were then gently washed with PBS and then co-incubated with secondary antibody (SS Polymer HRP/DAP; dilution = 1:500 in PBS [v/v]) (BioGenex, Fremont, CA), at room temperature for 1 hr. Thereafter, the sections were washed as before and with Tris-Hcl (0.05 M, pH 7.6), and then co-incubated with 3,3’-diamino-benzidine solution in the dark at room temperature for 10 min. The sections were then washed with Tris-Hcl, stained with hematoxylin, mounted with glycerin, and examined under a light microscope at 40X magnification.

3.2.13 In vitro determinations of extract anti-oxidant activity

3.2.13.1 DPPH scavenging assay

DPPH (1,1-diphenyl-2-picryl-hydrazyl) scavenging activity of *A. ferruginea* extract was determined according to Yen and Chen (1995). In brief, an aliquot of 0.5 ml extract [at escalating concentrations (20-100 µg/ml) in methanol] was mixed with 2.5 ml of a 0.5 mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 30 min in the dark at room temperature. The absorbance was then measured at 517 nm in the spectrophotometer against a blank sample that did not contain extract. Ascorbic acid was used as positive control. DPPH free radical scavenging ability (%) was calculated using: scavenging ability (%) = ([A \text{nm of control} - A \text{nm of sample}]/A \text{nm of control}) x 100. The concentration of extract necessary to reduce the initial concentration of DPPH by 50% (i.e., IC\text{50}) was then calculated.

3.2.13.2 Nitric oxide (NO) scavenging assay

NO production from sodium nitroprusside was measured according to Kang et al. (2006) An equal amount of 10 mM sodium nitroprusside solution in 0.5 M phosphate buffer (pH 7.4) was mixed with *A. ferruginea* extract [at
escalating concentrations (20-100 µg/ml) in a final volume of 3 ml, and incubated at 25°C for 3 hr. Thereafter, 1 ml reaction mixture was mixed with an equal amount of Griess reagent and the pink chromophore generated measured at 540 nm in the spectrophotometer. Sodium nitrite was used as standard. NO scavenging ability was calculated as: (\%) = ([A_{540nm of control} - A_{540nm of sample}]/A_{540nm of control}) \times 100. The concentration of extract necessary to reduce the initial NO concentration by 50% (IC\textsubscript{50}) was then calculated.

3.2.14 Statistical Analysis

All values are expressed as mean ± SD. For each endpoint, group means were compared using a one-way analysis of variance (ANOVA) followed by a Dunnett’s test, using Instat v3.0 software (Graphpad, San Diego, CA). A p-value <0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Preliminary phytochemical analysis

Preliminary phytochemical screening of the extract was carried out to determine the presence of constituents that could be responsible for any observed biological activities in these studies. The analyses indicated that there was an abundance of flavonoids, phenols, terpenoids, alkaloids, glycosides, quinone’s, saponins and tannins.

3.3.2 HPTLC profiling of \textit{A. ferruginea}

HPTLC qualitative analysis was done to confirm the presence of phenolics and flavonoids in the extract. Among the various peaks obtained, Peak
4 in densitogram 1 (Area = 43.92%, R_f = 0.41) represented flavonoids and Peak 6 in densitogram 2 (Area = 9.12%, R_f = 0.72) represented phenolics (Figures 3.1 and 3.2, respectively). Comparisons of R_f values were aided separately by using gallic acid and rutin, respectively, as phenolic and flavonoid standards.

3.3.3 GC/MS analysis of A. ferruginea

The GC/MS chromatogram of the extract is shown in Figure 3.3. GC/MS analysis resulted in identification of 18 different compounds as shown in Table 3.1. Derivatives of quinone (37.3%), quinoline (22.9%), imidazolidine (6.4%), pyrrolidine (4.5%), and cyclopentenone (3.5%) were identified as major components. Hexadecanoic acid, propanoic acid, pyridine, pyrazole and pyrimidine derivatives were also identified in the methanolic extract.

3.3.4 LC/MS analysis of A. ferruginea

The LC/MS chromatogram of the extract is shown in Figure 3.4. Table 3.2 presents the retention times, mode (+/-), λ max, and molecular weights of the respective components identified. In the LC/MS analysis, a positive molecular ion at [MS+H]^+ at an m/z of 146.5 corresponded to carboxamidine derivatives, 1H-pyrazole-1-carboxamidine monohydrochloride and 4-iodo-1-benzo thiophene-2-carboximidamide hydrochloride at an m/z of 338.6 was observed. Imidazole and thiazole derivatives such as 2-phenyl-4,4,5,5-tetramethylimidazoline-oxyl-1-oxyl-3-oxide, N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl) ethyl] phenyl] ben-zene sulphonamide, 2-(2’-methyl-n-propyl)-4,5-dimethyl-D3-thiazoline and thiazole,2,5-dihy-dro-4,5-dimethyl-2-(2-methylpropyl) were observed in a positive molecular ion at m/z values of 233.3, 481.3, and 171.3 respectively. At m/z = 633.3 and 381.1, 4-octadecanyl-amino-benzoyl-phenoxy-N-(2-
chlorophenyl)-acetamide and coumarin derivatives, 7-hydroxy-4-methyl-bis(2-chloroethyl) phosphate in a negative molecular ion at [MS-H]^+ and catechin derivatives at m/z=289 was also seen.

![HPTLC profile of methanolic extract of Acacia ferruginea for polyphenols](image)

**Figure 3.1** HPTLC profile of methanolic extract of *Acacia ferruginea* for polyphenols
Figure 3.2 HPTLC profile of methanolic extract of *Acacia ferruginea* for flavonoids
Figure 3.3 GC/MS chromatogram of methanolic extract of *A. ferruginea*.

Figure 3.4 LC/MS chromatogram of methanolic extract of *A. ferrugine*
Table 3.1 GC/MS analysis of the methanolic extract of A. ferruginea. Major component identification was aided by comparison against reference standards in NIST and Wiley 9.0 library.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Retention time</th>
<th>Name of the Compound</th>
<th>Molecular Formula</th>
<th>Molecular Wt.</th>
<th>% of relative Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.99</td>
<td>1,4,5,6-tetrahydropyrimidine</td>
<td>C₄H₈N₂</td>
<td>84</td>
<td>2.14</td>
</tr>
<tr>
<td>2</td>
<td>11.31</td>
<td>5-Fluoropentanenitrile</td>
<td>C₅H₃FN</td>
<td>101</td>
<td>2.29</td>
</tr>
<tr>
<td>3</td>
<td>12.05</td>
<td>1-Methyl-4-(1-imidazoyl)-1,2,3,6-tetrahydropyrimidine</td>
<td>C₆H₁₃N₃</td>
<td>163</td>
<td>1.58</td>
</tr>
<tr>
<td>4</td>
<td>12.56</td>
<td>Butyl ester, hydroxyl acetic acid</td>
<td>C₆H₁₂O₃</td>
<td>132</td>
<td>1.22</td>
</tr>
<tr>
<td>5</td>
<td>14.70</td>
<td>2-Hydroxy-3-tert-butyl-2-methylpropionitrile</td>
<td>C₈H₁₅NO</td>
<td>141</td>
<td>1.56</td>
</tr>
<tr>
<td>6</td>
<td>15.21</td>
<td>4-hydroxy-3-t-butyl-2-methyl-2-cyclopentenone</td>
<td>C₁₀H₁₆O₂</td>
<td>168</td>
<td>3.50</td>
</tr>
<tr>
<td>7</td>
<td>15.68</td>
<td>Propanoic acid, butyl ester (CAS)</td>
<td>C₇H₁₄O₂</td>
<td>130</td>
<td>0.84</td>
</tr>
<tr>
<td>8</td>
<td>16.59</td>
<td>1-(2,3-O-Anhydro-5-O-trityl-a-D-lyxofuranosyl)-2-pyrrolidino-4-pyrimidine</td>
<td>C₁₂H₃₁N₂O₄</td>
<td>521</td>
<td>1.03</td>
</tr>
<tr>
<td>9</td>
<td>18.92</td>
<td>7-Amino-3-carboxethoxy-1,8-naphthyridine-2(1H)-2-one</td>
<td>C₁₁H₁₁N₃O₃</td>
<td>233</td>
<td>0.91</td>
</tr>
<tr>
<td>10</td>
<td>20.07</td>
<td>1,8-Bis(1’-aza-4’,7,10’,13’-tetraoxycyclodecan-1’y)-9,10-</td>
<td>C₃H₆N₂O₁₀</td>
<td>642</td>
<td>37.28</td>
</tr>
<tr>
<td>11</td>
<td>21.89</td>
<td>Hexadecanoic acid, methyl ester (CAS)</td>
<td>C₁₇H₃₄O₂</td>
<td>270</td>
<td>2.45</td>
</tr>
<tr>
<td>12</td>
<td>22.76</td>
<td>3,4-dihyronathalene-1-oxo-2(H),3’-(2’,5’-diphenyl-4’-methyl-thio-5’-</td>
<td>C₂₀H₁₈N₂O₄</td>
<td>350</td>
<td>4.50</td>
</tr>
<tr>
<td>13</td>
<td>23.65</td>
<td>3-Chloro-2,2,5,8-tetrahydroxy-3-methyl-2,3-dihydro-1,4-naphthyridine</td>
<td>C₁₁H₇ClO₆</td>
<td>272</td>
<td>0.68</td>
</tr>
<tr>
<td>14</td>
<td>24.97</td>
<td>2-(Methylthio)-5-(furylmethylidene)-N(3)-(2’-chlorobenzyl-4-imidazolidine</td>
<td>C₁₆H₁₃ClN₂O₂</td>
<td>400</td>
<td>6.44</td>
</tr>
<tr>
<td>15</td>
<td>25.43</td>
<td>N,N-Dimethyl-2-Pyridine Methanamine</td>
<td>C₈H₁₂N₂</td>
<td>136</td>
<td>1.42</td>
</tr>
<tr>
<td>16</td>
<td>29.78</td>
<td>2,3,4,6-tetra (3,5-dimethyl pyrazol-1’y)-4-pyrazole-1’y) pyridine</td>
<td>C₂₈H₃₁N₁₁</td>
<td>521</td>
<td>0.95</td>
</tr>
<tr>
<td>17</td>
<td>30.86</td>
<td>5-(3,4-Dimethylphenyl)-4-phenylisoxazole</td>
<td>C₁₇H₁₅NO₃₄</td>
<td>281</td>
<td>0.78</td>
</tr>
<tr>
<td>18</td>
<td>39.19</td>
<td>Bis [2,2-(4-methylquinoline)]</td>
<td>C₂₀H₁₆N₂</td>
<td>284</td>
<td>22.94</td>
</tr>
</tbody>
</table>
Table 3.2 Identification of compounds in *A. ferruginea* methanolic extract using their Retention time and MS data derived in LC/MS analysis. Identification of compounds was aided by correlation with previous literature reports. *R*<sub>t</sub> refers to LC/MS chromatogram shown in Figure 3.4.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Rt* (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (min)</th>
<th>Molecular Weight</th>
<th>Mode (+/-)</th>
<th>Compounds (Tentative ID)</th>
<th>Molecular Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2.80</td>
<td>254</td>
<td>146.5</td>
<td>+</td>
<td>1H-pyrazole-1carboxamidine monohydrochloride</td>
<td>C&lt;sub&gt;4&lt;/sub&gt;H&lt;sub&gt;6&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;.Hcl</td>
</tr>
<tr>
<td>2.</td>
<td>3.12</td>
<td>254</td>
<td>130.4</td>
<td>+</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>9.52</td>
<td>254</td>
<td>233.3</td>
<td>+</td>
<td>2-phenyl-4,4,5,5-tetramethylimidazolineoxy-1-oxyl-3-oxide</td>
<td>C&lt;sub&gt;13&lt;/sub&gt;H&lt;sub&gt;17&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>4.</td>
<td>10.04</td>
<td>254</td>
<td>481.3</td>
<td>+</td>
<td>N-(2,2,2-Trifluoroethyl)-N-[4-2,2,2-Trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]benzenesulfonamide</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;F&lt;sub&gt;9&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt;S</td>
</tr>
<tr>
<td>5.</td>
<td>10.55</td>
<td>254</td>
<td>171.3</td>
<td>+</td>
<td>2-(2'-Methyl-n-propyl)-4,5-dimethyl-D3-thiazoline; Thiazole,2,5-dihydro-4,5-dimethyl-2-(2-methylpropyl)</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;17&lt;/sub&gt;NS</td>
</tr>
<tr>
<td>6.</td>
<td>10.92</td>
<td>254</td>
<td>338.6</td>
<td>+</td>
<td>4-ido-1-benzothiophene-2-carboximidamide hydrochloride</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;7&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;S.Hcl</td>
</tr>
<tr>
<td>7.</td>
<td>18.23</td>
<td>254</td>
<td>497.3</td>
<td>+</td>
<td>4-Epianhydrochlorotetracycline hydrochloride</td>
<td>C&lt;sub&gt;22&lt;/sub&gt;H&lt;sub&gt;21&lt;/sub&gt;CIN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;7&lt;/sub&gt;.Hcl</td>
</tr>
<tr>
<td>8.</td>
<td>19.11</td>
<td>254</td>
<td>633.3</td>
<td>+</td>
<td>4-octadecylamino-benzoyl-alpha-phenoxy-N-(2-chlorophenyl)-acetamide</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;51&lt;/sub&gt;CIN&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>9.</td>
<td>25.44</td>
<td>254</td>
<td>381.1</td>
<td>-</td>
<td>Coumarin, 7-hydroxy-4-methyl-.bis(2-chloroethyl)phosphate</td>
<td>C&lt;sub&gt;14&lt;/sub&gt;H&lt;sub&gt;15&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;P</td>
</tr>
<tr>
<td>10.</td>
<td>25.94</td>
<td>254</td>
<td>290.27</td>
<td>-</td>
<td>Catechin, 2-(3,4-dihydroxypheynyl)-3,4-dihydro-2H-chromene-3,5,7-triol</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
</tbody>
</table>
3.3.5 In vivo paw edema studies

3.3.5.1 Carrageenan model

The anti-inflammatory effect of *A. ferruginea* extract against subsequent carrageenan-induced paw edema is shown in Figure 3.5A. Pretreatment with extract resulted in reduced paw swelling size at 270 min post-carrageenan injection (0.46 $\pm$ 0.04 cm; 13.2%) relative to that seen in control counterparts (0.53 $\pm$ 0.02 cm). The inhibition of the inflammatory response exerted by the extract was comparable to that by indomethacin (0.47 $\pm$ 0.01 cm; 11.3%).

3.3.5.2 Formaldehyde model

The effect of *A. ferruginea* extract against subsequent formaldehyde-induced paw edema was also evaluated (Figure 3.5B). Pretreatment with extract resulted in reduced paw swelling size at Day 3 post-formaldehyde injection (0.51 $\pm$ 0.02 cm; 8.9%) relative to that seen in control mice (0.56 $\pm$ 0.02 cm). The inhibition of inflammatory response due to the extract was comparable to that by indomethacin (0.52 $\pm$ 0.01 cm; 7.1%).

3.3.6 Effect of extract on serum NO levels in mice with acute and chronic paw edema

The effect of *A. ferruginea* extract on serum NO levels during subsequent carrageenan- and formaldehyde-induced paw edema are shown in Figures 3.6 (A and B). Serum NO levels were significantly elevated in the carrageenan- and formaldehyde-induced control mice (relative to no treatment mice). NO levels in the carrageenan- and formaldehyde-induced control mice
were 16.48 [± 1.33] µM and 22.42 [± 2.54] µM compared to 10.52 [± 0.57] µM and 10.82 [± 0.92] µM for control hosts at, respectively, the 8th hour and on Day 3. Pre-treatment with extract significantly attenuated these increases in NO levels; in the carrageenan and formaldehyde mice, the levels were now found to be 13.42 [± 1.11] µM and 19.56 [± 1.62] µM, respectively (decreases of 18.5 and 12.75%) at the same time points. The effect of *A. ferruginea* extract pre-treatment were similar to those of indomethacin (vs. levels in carrageenan and formaldehyde mice at the same time points, levels were now found to be 13.94 [± 0.96] µM and 17.53 [± 1.23] µM respectively (decreases of 15.4 and 21.8 %) at the same time points.

### 3.3.7 Effect of extract on serum iNOS levels in mice with acute and chronic paw edema

The effect of *A. ferruginea* extract on serum iNOS levels was evaluated in mice with subsequent carrageenan- and formaldehyde-induced paw edema (Figures 3.7 [A and B]). The results showed that serum iNOS levels were elevated in the inflammatory controls (mice receiving only the irritants). iNOS levels in the carrageenan-induced control mice were 24.56 [± 2.21] ng/ml and 21.45 [± 2.12] ng/ml compared to 9.96 [± 0.54] ng/ml and 9.49 [± 0.72] ng/ml for the control hosts at 8 and 12 hr, whereas iNOS levels in the formaldehyde-induced control mice were 36.52 [± 2.56] ng/ml and 34.35 [± 1.94] ng/ml compared to 10.27 [± 0.76] ng/ml and 10.42 [± 0.74] ng/ml for the control hosts on Day 3 and 4. Pre-administration with *A. ferruginea* extract caused a significant reduction in these levels at 8 and 12 hr post-irritant injection in the carrageenan mice 19.56 [± 1.33] ng/ml and 17.88 [± 1.56] ng/ml [decreases of 20.3 and 16.6% vs. irritant only group values at same timepoints]. In the formaldehyde-treated mice, significant reductions in iNOS levels were noted from Day 2 through Day 5 post-irritant injection (23.18 [± 1.59] , 26.78 [± 2.24],
25.64 [± 2.01], and 23.45 [± 1.84] ng/ml) - decreases of 32.9, 26.6, 25.3, and 20.2% vs. irritant only group values at same time points. In both studies, administration of indomethacin significantly inhibited the iNOS production as well (average reductions of 21.2 and 23.4% vs. irritant-only counterparts over the indicated timeframes).

3.3.8 Histopathology of carrageenan-/formaldehyde-induced paw specimen

No inflammation, infiltration of inflammatory cells, or tissue destruction (erosion) was observed in the paw of mice in the control groups (Figure 3.8). Conversely, in each regimen, by the final time point post-irritant injection, the intra-plantar injection of carrageenan or formaldehyde into the hind paw had produced an intense edema, characterized by a presence of prominent epithelial infiltrates of inflammatory cells (neutrophils). Pre-treatment with the *A. ferruginea* extract significantly decreased this inducible inflammation as well as infiltration of neutrophils in both edema models. Indomethacin imparted the same beneficial effects against these pathologies.

3.3.9 Tissue COX-2 expression in induced edema models

The results show there were a great number of COX-2 immunoreactive cells (COX-2⁺; brown staining) in the paw tissues of mice injected with carrageenan or formaldehyde, as compared to in control (untreated) mice tissues (Figure 3.9). Mice injected with carrageenan and formaldehyde but pre-treated with *A. ferruginea* extract or indomethacin displayed a noticeable reduction in the presence of COX-2⁺ cells.
Figure 3.5 Effect of pre-treatment with methanolic extract of *A. ferruginea* on subsequent induction of (A) carrageenan- and (B) formaldehyde-induced paw edema.

Values shown are mean (± SD) of 6 mice/treatment group. \(^a_p < 0.01; \, ^b_p < 0.05\) for inflammation control (carrageenan/formaldehyde-alone) vs. extract-treated; \(^c_p < 0.01, \, ^d_p < 0.05\) for inflammation control vs. indomethacin treated.
Figure 3.6. Effect of pre-treatment with methanolic extract of *A. ferruginea* on nitric oxide (NO) production during subsequent induction of (A) acute and (B) chronic inflammation.

Values shown are mean (± SD) of 6 mice/treatment group. *a* $p < 0.01$; *b* $p < 0.05$ for inflammation control (carrageenan/formaldehyde-alone) vs. extract-treated; *c* $p < 0.01$, *d* $p < 0.05$ for inflammation control vs. indomethacin treated.
**Figure 3.7** Effect of pre-treatment with methanolic extract of *A. ferruginea* on serum iNOS level in mice during subsequent induction of (A) acute and (B) chronic inflammation.

Values shown are the mean (± SD) of 6 mice/treatment group. Values shown are mean ± SD. \(^a\)p < 0.01; \(^b\)p < 0.05 for inflammation control (carrageenan/formaldehyde-alone) vs. extract treated; \(^c\)p < 0.01, \(^d\)p < 0.05 for inflammation control vs. indomethacin-treated.
Figure 3.8 Representative histological changes in haematoxylin and eosin (H&E)-stained skin tissues of plantar region of mice paws.

At the end of the experiment, i.e., 24 hr. after carrageenan treatment and 5 d after formalin treatment, histologic examinations were carried out (magnification = 40X).
Figure 3.9 Immunohistochemical localization of COX-2.

Positively-stained [brown] granules for COX-2 were significantly increased in intensity in paw tissue in inflammatory controls and decreased in mice that had undergone pre-treatment with extract or indomethacin (magnification = 40X).
3.3.10 *In vitro* DPPH scavenging assay

The free radical scavenging activity of the *A. ferruginea* extract was assessed using DPPH assay. Figure 3.10 illustrates there was an increase in the percentage inhibition of DPPH radicals present due to scavenging and that the effect occurred in a concentration-related manner. The results allowed for calculation of an IC\(_{50}\) value for the *A. ferruginea* extract of 51.27 µg/ml.

3.3.11 *In vitro* NO radical scavenging assay

The *A. ferruginea* extract showed concentration-related inhibition in the presence of NO radicals in an *in vitro* assay (Figure 3.11). The results allowed for determination of an IC\(_{50}\) value for the extract of 61.42 µg/ml.
Figure 3.10 DPPH radical scavenging activity of *A. ferruginea* extract concentration (20-100 µg/ml) measured at 517nm.

Values are expressed as mean ± SD of triplicate experiments.
Figure 3.11 Nitric oxide radical scavenging activity of *A. ferruginea* extract concentration (20-100 µg/ml) measured at 540 nm.

Values are expressed as mean ± SD of triplicate experiments.
3.4 DISCUSSION

Murine models of carrageenan- and formaldehyde-induced inflammation are accepted experimental approach for evaluating anti-inflammatory effect of plants-/plant-derived drugs. In the study reported here, the effect of pre-treatment with an extract of Acacia ferruginea were evaluated using these model systems. Unlike earlier studies in which the anti-inflammatory effect of this extract was established in models of already extant inflammation (Lee at al., 2009; Mahmood et al., 2010), this study wanted to ascertain if the extract could also impart protective effects against subsequent induction of inflammation in situ. This approach would be in keeping with several other studies that examined effects of text agents (many of them, natural products) against inducible inflammation in various animal models (Kaur et al., 2004; Chao et al., 2009; Lucetti et al., 2010; Okokon et al., 2012; Golechha et al., 2014).

Intra-plantar injection of carrageenan causes leukocyte migration and is characterized as a bi-phasic event mediated by histamine and serotonin in the first phase (0-1 hr) and then by bradykinin and cyclooxygenase products in the second phase. The swelling accelerates in the second phase and is correlated with increased generation of prostaglandins, nitric oxide second-ary to induction/activation of COX-2 and iNOS. This leads to accumulation of fluids at the site, extravasation of plasma proteins, and ultimately, edema formation (Gamache et al., 1986; Silva et al., 2005; Perianayagam et al., 2006). In this present study, A. ferruginea extract imparted potent anti-inflammatory and anti-oxidant activities in acute and chronic murine models of inflammation. Pre-treatment with the extract significantly suppressed carrageenan- and formaldehyde-induced paw edema. Specifically, the study showed the extract significantly inhibited carrageenan-induced paw edema in the second phase of reaction (2-6 hr).
To assess the efficacy of the *A. ferruginea* extract against chronic inflammation, formaldehyde was employed. Administration of formaldehyde prompts chronic inflammation at the site of injection (in paw), leading to release of variety of inflammatory mediators. In the bi-phasic process, inflammatory stimuli and associated factors (certain hormones and growth factors) expand inflammation by enhancing the recruitment/activation of various immune cells; this, in turn, leads to increases in the release of select pro-inflammatory mediators, including interleukin (IL)-1 (Banerjee et al., 2000). Moreover, these exacerbated inflammatory pathways also accompanied by over-production of reactive oxygen species (ROS) and other free radicals by infiltrating inflammatory cells at the site of inflammation. This inappropriate generation of ROS (superoxide anion, singlet oxygen, hydroxyl radical, hydrogen peroxide) leads to a state of ‘oxidative stress’ that results in increased immune cell mortality and overall tissue damage.

It is known that large amounts of nitric oxide (NO; synthesized by various NOS iso-forms) are also produced at sites of inflammation (Fang et al., 2009). Activated macrophages increase their expression of iNOS and subsequently generate excess NO mainly after activation of NF-κB and MAPK signaling pathways (Kim et al., 2006). The excess NO helps to further the pathological complications associated with chronic inflammation (Bosca et al., 2005; Mekhora et al., 2012). In the study here, pre-treatment with extract elicited significant inhibition of formaldehyde-induced paw edema from Days 2 to 5 post-injection. The potent anti-edema effect was probably related to an ability to prevent NO production, in part, by causing an inhibition of neutrophil infiltration and iNOS synthesis in the region. Reductions in leukocyte migration and were induced by the extract pre-treatment, as evidenced histologically in both the acute and chronic models.
COX-2 is another enzyme involved in the inflammatory process. COX-2 mediates synthesis of prostaglandins (including PGE$_2$) and thromboxanes that promote cytokine secretion and suppresses immune function by inhibition of T$_H$1 cell activation and IL-2 secretion. Up-regulation of COX-2 has been reported to play a key role in regulating inflammatory responses that causes tissue injury in numerous cancers (Tu et al., 2014). Thus, the identification of a novel COX-2 inhibitor could be useful in helping to protect patients against chronic inflammatory disorders. Here, the presence of COX-2$^+$ cells (indicated by brown staining in paw tissues of both the acute and chronic inflammatory hosts) was ameliorated by treatment with the extract. Though the precise mechanism for this amelioration is still not known, it can be hypothesized that it occurred in part by means of an anti-oxidant effect as well as inhibition of synthesis, release, or action of pro-inflammatory mediators. Likewise, it is well known that indomethacin acts as potent anti-inflammatory agent by blocking prostaglandin synthesis through inhibition of the action of COX enzymes (Hull et al., 2003). The proposed effect of the test extract could also be related to a suppression of signaling pathways induced by oxidative stress, including NF-$\kappa$B, hypoxia-inducible factor (HIF)-$\alpha$, and signal transducer and activator of transcription (STAT)-3 (Marusawa and Jenkins, 2014). Because NF-$\kappa$B pathways are activated during inflammatory reactions, leading to induction of iNOS and NO production, extract-induced inhibition of NF-$\kappa$B could then also be contributing to previously observed reductions of iNOS and COX-2 (Ruiz et al., 2006).

It is likely a combinatorial effect from all the phytoconstituents present gave rise to these effects; future studies with individual constituents are necessary to better characterize the causative agent(s) for the observed outcomes. Earlier studies from our laboratory reports that the methanolic extract of A. ferruginea possessed numerous flavonoids, terpenoids, phenolics, steroids,
alkaloids, tannins, and saponins. Specifically, GC/MS and LC/MS studies of the extract revealed a presence of quinone, imidazolidine, pyrazole, pyrrolidine, thiazole, coumarin, and catechin derivatives. Previous literature has indicated that some of these agents impart potent anti-inflammatory effects. In particular, flavonoids were found to inhibit iNOS-mediated NO production and inhibit several enzymes like COX, lipoxy-genase (LOX), and xanthine oxidase that are often involved in inflammatory pathologies (Lim et al., 2013; Ravishankar et al., 2013). Polyphenols can work as modifiers of signal transduction pathways (NK-κB and MAPK) and also modulate expression of genes for COX, LOX, and NOS isoforms (Santangelo et al., 2007). Earlier studies with coumarin derivatives reported these were free radical scavengers as well as potent inhibitors of NF-κB activation via ERK/MAPK (extracellular signal regulated kinases) and PI3/AKT (phosphatidylinositol-3-kinases/protein kinase-B) pathways (Lin et al., 2008).

To ascertain the extent of any anti-oxidant capacity of the A. ferruginea extract, DPPH and NO radical scavenging assays were performed. In the study, the extract exhibited appreciable scavenging ability against DPPH (IC₅₀ = 51.27 µg/ml) and NO radicals (IC₅₀ = 61.42 µg/ml). As ROS play an important role in mediating chronic inflammation (Reuter et al., 2010), the free radical scavenging capability of the extract might be considered as one possible mechanism of action underlying the anti-inflammatory effect seen here and by others (Germano et al. 2008 and Dordevic et al. 2008). Among the known constituents in the extract, alkaloids and flavonoids are known to possess free radical scavenging activities. With less ROS available to impart effects on the local cells, it would be likely that there would also be less up-regulation of cell signaling cascades associated with mitogen-activated protein kinases (MAPK) and then reduced activation of redox-regulated transcription factors like AP-1 and NF-κB that trigger adverse reactions (Lee et al., 2006; Kim et al., 2009). Specifically, reductions in AP-1 and NF-κB expression/activity could in turn lead
to reduced induction/activation of iNOS and COX-2 as was observed here. This would be in keeping with previous reports showing that NF-κB and MAPK signaling pathway play a critical role in regulation of iNOS and COX-2 expression during development of inflammatory responses (Garcia et al., 2012; Lin et al., 2013).

In conclusion, based on the data in the present study, we conclude that *A. ferruginea* methanolic extract can impart a strong anti-inflammatory effect *in vivo*. The present study should lead to additional studies to explore targeted pathways underlying this anti-inflammatory action.