8. Herb drug interaction of *Andrographis paniculata* Nees on Pharmacokinetic and pharmacodynamic of Naproxen

8.1. Experimental

8.1.1. *In vivo* pharmacokinetic study in rats

8.1.1.1. Chemicals and reagents: 
Naproxen was obtained as a gift sample from Cadila Healthcare Ltd. Ahmedabad and Carbamazepine (IS) was obtained as a generous gift from Emcure Pharmaceuticals Pvt. Ltd. Pune. HPLC grade Acetonitrile was purchased from Merck Chemicals, Mumbai, Maharashtra, India. Andrographolide (AN) was purchased from Research Organic Pvt. Ltd, Chennai. *Andrographis paniculata* Nees extract (APE) was procured from Natural Remedies Pvt. Ltd, Bangalore (Batch No. FAPEX/2013110012). Phytochemical analysis performed by HPLC with stating ≈ 30 % w/w andrographolide in the extract. High purity deionized water was obtained from Millipore, Milli-Q (Bedford, MA, USA) water purification system.

8.1.1.2. Preparation of stock solutions, calibration samples, and quality controls: 
Stock solutions were prepared by dissolving accurate amounts of reference standards in methanol at a concentration of 1.0 mg/ mL for AN, NP and internal standard carbamazepine (IS). A series of working standard solutions were obtained by further diluting the stock solutions in methanol. The IS working solution (200 µg/mL) was obtained by diluting the stock solution in methanol. Calibration standards were prepared by spiking the appropriate amounts of the standard solutions into 320 µL of blank plasma to yield final concentrations of 0.5, 1, 2.5, 5, 10, 15, 20, 25, 50 and 60 µg/ mL. The quality control (QC) samples were similarly prepared at concentrations of 1, 20 and 60 µg/mL for AN and NP for the low, medium and high concentration QC samples, respectively. All solutions were kept refrigerated (-80°C) and brought to room temperature before use.
8.1.1.3. Sample preparation:

10 µl of each solution of AN and NP were added into 320 µl drug free plasma separately. For each calibration standard, 10 µl of internal standard carbamazepine (200 µg/ml) solution was added and vortexed for 2 min. 1.1 ml of ethyl acetate was added to each calibration standard and vortexed for 2 min. After centrifugation at 15000 rpm for 30 min, the supernatant was transferred to another clean tarson microtube and the solvent was evaporated to dryness under a gentle stream of nitrogen gas at 35°C. The residue was reconstituted with 200 µL of methanol, and a 20 µL aliquot was injected into the HPLC system for analysis. The quality control (QC) samples were also prepared in the same manner as a bulk based on an independent weighing of standard drugs, at concentrations of 1, 20 and 60 µg/mL as a single batch at each concentration.

8.1.1.4. Method development using HPLC:

The chromatographic separation was performed using a Jasco PU-1580 gradient liquid chromatography instrument, equipped with an autosampler system and a UV detector UV-1575 (Jasco, Japan) with a Thermo Hypersil ODS column (250 x 4 mm, 5 μm). The mobile phase system was optimized to give a good resolution of AN, NP and carbamazepine (internal standard) from other endogenous substances in plasma sample. Mobile phase consisted of the mixture of solvent A (Acetonitrile), solvent B (MeOH) and solvent C (0.01 M Acetic acid) in the ratio of 20:30:50 (v/v/v) for 25 minutes at a flow rate of 0.5 mL/min. The concentration of AN was determined using the peak area ratio of pure AN to I.S. The concentration of AN in APE was determined using the peak area ratio of AN in APE and I.S. Similarly, the concentration of NP was determined using the peak area ratio of NP and I.S. Each calibration curve was analyzed individually using least squares weighted linear regression. Further validation of the method was done according to the US Food and Drug Administration (FDA) guidelines for selectivity, matrix effects, recovery and stability.

8.1.1.5. Method Validation:

8.1.1.5.1. Selectivity:

Selectivity was performed by the comparison of blank plasma from six individual rats to the corresponding spiked plasma samples. All plasma lots were found to be free of interferences with the compounds of interest.
8.1.1.5.2. Sensitivity and linearity:
The lower limit of quantitation (LLOQ) of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 20% of nominal and precision not exceeding 20%. The repeatability of LLOQ was determined by examining five LLOQ samples independent from the standard curve. The curves were fitted by a weighted (1/x) least-squares linear regression method through the measurement of the peak area ratio of the analyte to IS versus analyte concentration.

8.1.1.5.3. Accuracy and precision:
The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (RE). Intra- and inter-day precision was assessed from the results of QCs. The mean values and RSD for QCs at three concentration levels were calculated over three validation days by using a one-way analysis of variance (ANOVA).

8.1.1.5.4. Extraction recovery:
Recovery presents the extraction efficiency of a method, which was determined at three QC levels (five samples each). The recoveries were evaluated by comparing peak areas of analytes in spiked plasma samples with those of samples to which the analytes had been added after extraction. Each of the samples was also spiked with IS at the working concentration of 200 µg/mL. The samples were subsequently processed using the procedure described previously. A second set of plasma samples was processed and spiked post-extraction with the same concentrations of the analytes and IS that actually existed in pre-extraction spiked samples (i.e. 80% of the concentration of the analytes and IS in the pre-extraction spiked samples). Extraction recovery values for each analyte and IS were determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to that of the samples spiked after extraction.

8.1.1.5.5. Stability:
Bench-top stability was investigated to ensure that analytes were not degraded in plasma samples at room temperature for a time period to cover the sample preparation and was assessed by
exposing the QC samples to ambient laboratory conditions for 2 h. Freeze–thaw stability was assessed over three cycles. QC samples were thawed at room temperature and refrozen at −80°C over three cycles and assayed. Due to the need for occasional delayed injection or reinjection of extraction samples, the stability of reconstituted samples in auto-sampler vials was assessed at ambient temperature for 12 h. The freezer storage stability of the analytes in rat plasma at −80°C was evaluated by assaying QC samples at beginning and 45 days later. All stability QC samples were analyzed in three replicates.

8.1.1.6. Animals:

Female wistar rats weighing 180–220 g were purchased from National Institute of Biosciences. Six rats were placed in one cage, and maintained under controlled room temperature (25 ± 2°C) and humidity (60–70%) with day/night cycle (12 h/12 h). All animals had free access to food and water. After acclimatization for 7 days animals were fasted overnight (12 h) prior to each experiment. All experiments were performed as per the guidelines of CPCSEA after obtaining approval (1703/PO1C/13/CPCSEA) from the Institutional Animal Ethics Committee.

8.1.1.7. Drug administration and blood sampling:

Experimental animals were randomly divided into three groups of 18 animals of each as follows. Oral administration of Group 1- NP alone (7.5 mg/kg, p.o.), Group 2-co-administration of AN with NP (60 mg/kg+7.5 mg/kg, p.o.) and Group 3-co-administration of APE with NP (200 mg/kg+7.5 mg/kg, p.o.) for 7 days. After drug administration, 18 animals were further subdivided into three groups with six animals each. Blood samples (1 ml each) were collected at 0, 1.5, 4 and 10 h in first subgroup, 0.5, 2, 6, and 12 h in second subgroup and 1, 3, 8 and 24 h in third subgroup. Only four blood samples were collected from individual animal within 24 h from the retro- orbital plexus under light ether anesthesia. The samples were transferred to EDTA tubes and centrifuged at 15000 rpm for 20 min. Plasma was separated from the blood and stored at −80°C until further analysis.
8.1.8. Data analysis:
The plasma concentrations versus time profiles from individual animals were estimated by non-compartmental model using Win Nonlin software (Pharsight Corporation, Mountain View, CA, USA). The peak plasma concentration ($C_{\text{max}}$) and time reaching $C_{\text{max}}$ ($t_{\text{max}}$) was read directly from the observed individual plasma concentration-time data. All data were expressed as mean ± SEM. Differences between groups were evaluated by one-way ANOVA (Dunnett test). The differences were considered to be significant at *$P<0.05$, **$P<0.01$, ***$P<0.001$.

8.1.2. Pharmacodynamic study

8.1.2.1. Arthritis assessments:
The animals were divided into seven groups of six animals each as follows Group A-vehicle control group, Group B-arthritic control group, Group C-standard group (10 mg/kg NP, p.o). Group D-arthritic animals treated with APE 200 mg/kg p.o.; Group E-arthritic animals treated with APE 200 mg/kg co-administered with 10 mg/kg NP, p.o., Group F-arthritic animals treated with AN 60 mg/kg p.o., Group G-arthritic animals treated with AN 60 mg/kg co-administered with 10 mg/kg NP, p.o. Arthritis was induced to all the groups of animals except vehicle control group by a single injection of 0.1mL FCA in to sub plantar region of left hind paw on day 1 under light ether anesthesia (Mali et al., 2011). The dosing of all the groups started from day 12 and continued till day 28 once daily orally. Anti-arthritic activity was evaluated on paw volume, pain threshold and mechanical withdrawal threshold on day 0, 1, 4, 10, 14, 17, 21, 24 and day 28. On day 28, blood was withdrawn under light ether anesthesia for hematological analysis. The animals were sacrificed on day 28 to study the joint histopathology.

8.1.2.2. Paw volume:
The left hind paw volumes of all animals were measured just before FCA injection on day 0 and thereafter at different time intervals till day 28 using a plethysmometer (UGO Basile, Italy). The change in paw volume was measured as the difference between the final and initial paw volumes.

8.1.2.3. Mechanical hyperalgesia:
Mechanical hyperalgesia of left hind paw was evaluated by Randall and Selitto test using analgesiometer (UGO Basile, Italy). The left hind paw was placed between flat surface and blunt
pointer applying steadily increasing pressure. The threshold was determined when rat exhibited a stereotype flinch response and attempted to remove the foot from the apparatus. The cut-off pressure was 450 g.

8.1.2.4. Mechanical nociceptive threshold
Nociceptive threshold to mechanical stimulation was determined using Von Frey hairs (ALMEMO, Germany) of increasing gauge. The animals were allowed to acclimatize for 10 min in the Perspex box and Von Frey hairs (0.6 to 12.6 g) were applied to plantar surface of left hind paw. A series of three stimuli were applied to each paw for each hair within a period 2–3 s. The lowest weight of Von Frey hair to evoke a withdrawal from the three consecutive applications was considered to indicate the threshold.

8.1.2.5. Histological analysis:
The animals were sacrificed on day 28 by cervical dislocation. Ankle joints were separated from the hind paw, weighed and immersed in 10% buffered formalin for 24 h followed by decalcification in 5% formic acid, processed for paraffin embedding sectioned at 5µ thickness. The sections were stained with haematoxylin and eosin and evaluated under light microscope for the presence of hyperplasia of synovium, pannus formation and destruction of joint space.

8.1.2.6. Hematology analysis:
The haematological parameters like haemoglobin (Hb), red blood cell (RBC), white blood cell (WBC) and platelets were determined by standardized laboratory method.

8.1.2.7. Statistical analysis
The data was analyzed by two way ANOVA followed by Bonferroni test. The values of P < 0.05 were considered statistically significant.
8.2. Results

8.2.1. Method optimization:
The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for each analyte and the IS as well as a short run time. After comparison of few columns, a Thermo Hypersil ODS column (250 x 4 mm, 5 μm) was finally selected with a flow rate of 0.5 mL/min to achieve an efficient chromatographic separation of the analytes and the endogenous plasma components for eliminating the matrix effects. Mobile phase consisted of the mixture of solvent A (Acetonitrile), solvent B (MeOH) and solvent C (0.01 M Acetic acid) in the ratio of 20:30:50 (v/v/v) with run time 25 minutes.

8.2.2. Method Validation:

8.2.2.1. Selectivity:
The retention time of NP, AN and I.S. were detected at 7.05 ± 1, 11.44 ± 1 and 16.95 ± 1 min respectively (figure 22).

Figure 22: Chromatogram of NP, AN and I.S. in rat plasma

(A) Chromatogram of blank plasma sample
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(B) Chromatogram of blank plasma sample with carbamazepine (I.S.)

(C) Chromatogram of plasma sample spiked with NP (10 µg/ml), AN (10 µg/ml) and I. S.
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(D) Chromatogram of a plasma sample obtained from rat at 1.5 hr after oral administration of 60 mg of AN and 7.5 mg of NP.

8.2.2.2. **Sensitivity and linearity:**

The lower limit of quantitation (LLOQ) for andrographolide and naproxen was 1 µg/mL. Calibration standards were prepared by spiking the appropriate amounts of the standard solutions into 320 µL of blank plasma to yield final concentrations of 1, 2.5, 5, 10, 15, 20, 25, 50, and 60 µg/mL for AN and NP. Typical equations of the calibration curves of AN and NP were $y = 0.0287x + 0.0146$ and $y = 0.0747x - 0.0646$ respectively with good correlation coefficient ($r^2$ = 0.9972 and 0.9987 respectively).
8.2.2.3. Accuracy and precision:
Table 12 shows a summary of intra- and inter-day accuracy and precision for analytes from QC samples, respectively. Intra and inter batch precisions were within limits (R.S.D. < 15 %) and accuracy was in between the range 85 to 115 %. The method showed good accuracy and
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precision. In this assay, the intra-day precision was less than 6.6 % for each QC level of AN and 6.9 % for NP. The inter-day precision was less than 2.78 % for each QC level of AN and 6.57 % for NP.

**Table 12:** Intra and inter-batch precision and accuracy for determination of AN and NP in rat plasma.

<table>
<thead>
<tr>
<th>Nominal concentration (µg/mL)</th>
<th>Intra day</th>
<th>Inter day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured concentration (µg/mL) (mean±S.D.)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>AN 1</td>
<td>1.05 ± 0.06</td>
<td>6.60</td>
</tr>
<tr>
<td>20</td>
<td>20.00 ± 0.14</td>
<td>0.73</td>
</tr>
<tr>
<td>60</td>
<td>59.38 ± 0.68</td>
<td>1.15</td>
</tr>
<tr>
<td>NP 1</td>
<td>1.02 ± 0.07</td>
<td>6.90</td>
</tr>
<tr>
<td>20</td>
<td>19.95 ± 0.13</td>
<td>0.67</td>
</tr>
<tr>
<td>60</td>
<td>60.03 ± 0.09</td>
<td>1.51</td>
</tr>
</tbody>
</table>

**8.2.2.4. Extraction recovery:**

Extraction recovery values for each analyte and IS were determined by calculating the ratios of the peak areas of the pre-extraction spiked samples to that of the samples spiked after extraction. The results are indicated in Table 13.
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### Table 13: Extraction recovery of AN and NP in rat plasma (n = 5).

<table>
<thead>
<tr>
<th>Nominal concentration (µg/mL)</th>
<th>Peak area&lt;sup&gt;a&lt;/sup&gt; (n=5) (Mean ± S.D.)</th>
<th>Peak area&lt;sup&gt;b&lt;/sup&gt; (n=5) (Mean ± S.D.)</th>
<th>Extraction Recovery&lt;sup&gt;c&lt;/sup&gt; (%) (A/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN 1</td>
<td>119167.4 ± 4393.94</td>
<td>113751.2 ± 9573.49</td>
<td>105.29</td>
</tr>
<tr>
<td>20</td>
<td>1663755 ± 75134.9</td>
<td>1621447 ± 76643.72</td>
<td>102.71</td>
</tr>
<tr>
<td>60</td>
<td>4621360 ± 114848.4</td>
<td>4674194 ± 63626.03</td>
<td>98.85</td>
</tr>
<tr>
<td>NP 1</td>
<td>147569.8 ± 355.85</td>
<td>147156.6 ± 438.77</td>
<td>100.28</td>
</tr>
<tr>
<td>20</td>
<td>3995474 ± 88677.96</td>
<td>4004881 ± 126855.9</td>
<td>99.85</td>
</tr>
<tr>
<td>60</td>
<td>11362066 ± 843378.2</td>
<td>11113752 ± 769537.2</td>
<td>102.72</td>
</tr>
<tr>
<td>I.S.</td>
<td>2805129 ± 96299.29</td>
<td>2710935 ± 95000.33</td>
<td>103.53</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standards spiked before extraction.

<sup>b</sup> Standards spiked after extraction.

<sup>c</sup> Extract recovery (%) expressed as the ratio of the mean peak area of the analytes spiked into plasma pre-extraction (A) to the mean peak area of the analytes spiked into plasma post-extraction (B).

#### 8.2.2.5. Stability:

All stability QC samples were analyzed in three replicates. The results indicated that each analyte had an acceptable stability, as shown in Table 14.
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**Table 14:** Stability of AN and NP in rat plasma (n = 3).

<table>
<thead>
<tr>
<th>Nominal concentration (µg/mL)</th>
<th>Sample conditions</th>
<th>AN</th>
<th>NP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bench top stability&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9977 ± 0.02</td>
<td>1.0661 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Auto-sampler stability&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0259 ± 0.07</td>
<td>1.0126 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Freeze-thaw stability&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0786 ± 0.12</td>
<td>1.0375 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>45 days storage stability&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.0091 ± 0.06</td>
<td>1.0177 ± 0.07</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>19.8908 ± 0.10</td>
<td>19.8617 ± 0.11</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>59.6332 ± 0.75</td>
<td>59.7693 ± 0.67</td>
</tr>
<tr>
<td>NP 1</td>
<td></td>
<td>1.0661 ± 0.05</td>
<td>59.7693 ± 0.67</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>19.8617 ± 0.11</td>
<td>59.7693 ± 0.67</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>59.6332 ± 0.75</td>
<td>59.6332 ± 0.75</td>
</tr>
</tbody>
</table>

<sup>a</sup> Exposed at ambient temperature (25 °C) for 2 h.

<sup>b</sup> Kept at ambient temperature (25 °C) for 12 h.

<sup>c</sup> After three freeze–thaw cycles.

<sup>d</sup> Stored at −80 °C for 45 days.

**8.2.3. Pharmacokinetic study:**

The developed HPLC method was applied successfully to the pharmacokinetic study in the rat plasma for the respective groups. The mean plasma concentration time curves are shown in Figure 24 and the pharmacokinetic parameters are shown in Table 15. NP was absorbed into the circulatory system and reached its peak concentration approximately 2 h after administered individually. $T_{\text{max}}$ of groups co-administered AN+NP and APE +NP has been changed to 1.5 hr and 1 hr respectively. The $C_{\text{max}}$ of NP was decreased significantly with the groups administered with AN + NP and APE + NP than the group administered NP individually. Co-administration of AN with NP significantly ($P < 0.05$) decreased the $C_{\text{max}}$, $T_{\text{max}}$, AUC<sub>0-5</sub>, $T_{1/2}$ MRT<sub>0-4</sub>, CL and increased the AUC<sub>0-∞</sub>, MRT<sub>0-∞</sub> and Vd of NP when compared to NP alone group. Co-administration of APE with NP significantly ($P < 0.001$) decreased the $C_{\text{max}}$, $T_{\text{max}}$, AUC<sub>0-4</sub>,
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AUC$_{0-\infty}$ and increased the MRT$_{0-t}$, MRT$_{0-\infty}$, CL and Vd of NP when compared to NP alone group.

**Table 15:** Results of pharmacokinetic parameters of all the experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NP alone group (Group 1)</th>
<th>Co-administration of NP with AN (Group 2)</th>
<th>Co-administration of NP with APE (Group 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>15.88 ± 0.084</td>
<td>9.733 ± 0.057***</td>
<td>12.27 ± 0.022***</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>2.01 ± 0.04</td>
<td>1.51 ± 0.026***</td>
<td>1.01 ± 0.052***</td>
</tr>
<tr>
<td>AUC$_{0-t}$ (h/µg/ml)</td>
<td>89.79 ± 0.0457</td>
<td>67.19 ± 0.026***</td>
<td>72.19 ± 0.020***</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (h/µg/ml)</td>
<td>131.5 ± 0.138</td>
<td>241.0 ± 0.020***</td>
<td>100.6 ± 0.026***</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>10.95 ± 0.426</td>
<td>7.30 ± 0.036***</td>
<td>10.92 ± 0.034</td>
</tr>
<tr>
<td>MRT$_{0-t}$ (h)</td>
<td>15.47 ± 0.032</td>
<td>9.56 ± 0.032***</td>
<td>17.47 ± 0.031***</td>
</tr>
<tr>
<td>MRT$_{0-\infty}$ (h)</td>
<td>7.64 ± 0.028</td>
<td>8.65 ± 0.032***</td>
<td>8.71 ± 0.042***</td>
</tr>
<tr>
<td>Vd (l/kg)</td>
<td>0.90 ± 0.000</td>
<td>32.77 ± 0.014***</td>
<td>11.74 ± 0.000***</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>0.05 ± 0.002</td>
<td>0.03 ± 0.000***</td>
<td>0.07 ± 0.000**</td>
</tr>
</tbody>
</table>

*Differences between groups were evaluated by one-way ANOVA (Dunnet test). Data are expressed as mean ± S.E.M.; n=6 rats per group. ***P<0.001 when compared to group 1.*

Where oral administration of,

Group 1- NP alone (7.5 mg/kg, p.o.),
Group 2-co-administration of AN (60 mg/kg, p.o.) with NP (7.5 mg/kg, p.o.) and
Group 3-co-administration of APE (200 mg/kg, p.o) with NP (7.5 mg/kg, p.o.).
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Figure 24: Mean concentration-time curves of NP pharmacokinetic study

Mean concentration-time curves of NP (7.5 mg/kg, p.o.) alone, after co-administration of AN (7.5 mg/kg+60 mg/kg, p.o.) and after co-administration with APE (7.5 mg/kg +200 mg/kg, p.o.)

8.2.4. Pharmacodynamic studies (Anti arthritic studies):

8.2.4.1. Effect on change in paw volume:

A significant increase in paw volume in all FCA induced groups was observed compared to vehicle control group A. The paw volume was maximum on day 12 in all FCA administered groups. On administration of APE (200 mg/kg) + NP (10 mg/kg) and AN (60 mg/kg) + NP (10 mg/kg) from day 12 onwards significantly (P < 0.05) decreased the paw volume, which was observed till end of the study. Co-administered and alone groups with significant change in paw volume were further compared with each other and the results are given in Figure 25.
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Figure 25: Effect of NP, APE, NP + APE, AN and NP + AN on paw volume.

Effect of NP (10 mg/kg), APE (200mg/kg), NP (10 mg/kg) + APE (200 mg/kg), AN (60 mg/kg) and NP (10 mg/kg) +AN (60 mg/kg) on paw volume. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

8.2.4.2. Effect on mechanical hyperalgesia:

There was significant decrease in pain threshold on administration of FCA which was continued till day 12. On administration of APE (200 mg/kg) + NP (10 mg/kg) and AN (60 mg/kg) + NP (10 mg/kg) from day 12 onwards there was significant increase in pain threshold from day 16 onwards which was observed till end of the study. On further comparison of co-administered and alone groups with each other, significant (P < 0.05) change on mechanical withdrawal threshold observed and the results obtained are given in Figure 26.
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Figure 26: Effect of NP, APE, NP + APE, AN and NP +AN on mechanical withdrawal threshold.

Effect of NP (10 mg/kg), APE (200mg/kg), NP (10 mg/kg) + APE (200 mg/kg), AN (60 mg/kg) and NP (10 mg/kg) +AN (60 mg/kg) on mechanical withdrawal threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

8.2.4.3. Effect on nociceptive threshold:

There was a significant decrease in mechanical withdrawal threshold observed in all the animals treated with FCA. On administration of APE (200 mg/kg) + NP (10 mg/kg) and AN (60 mg/kg) + NP (10 mg/kg) from day 12 onwards there was significant increase in the mechanical withdrawal threshold from day 16 when compared to arthritic control. Co-administered and alone groups with significant (0.05) change on nociceptive threshold were further compared with each other and the results are given in Figure 27.
Figure 27: Effect of NP, APE, NP + APE, AN and NP + AN on nociceptive threshold.

Effect of NP (10 mg/kg), APE (200mg/kg), NP (10 mg/kg) + APE (200 mg/kg), AN (60 mg/kg) and NP (10 mg/kg) +AN (60 mg/kg) on nociceptive threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

8.2.4.4. Effect on histopathology of inflamed joints:

The histopathological evaluation of the tibiotarsal joint showed prominent inflamed degenerative connective tissue associated with cellular inflammation edema, granuloma formation in the vehicle treated animal. No infiltration of inflammatory cells and fibrous tissue was observed with NP (10 mg/kg) treated group. Moderate infiltration of inflammatory cells and fibrous tissue proliferation were observed with group treated with APE (200 mg/kg). Similarly, minimal infiltration of the inflammatory cells and prominent blood vessels in AN (60 mg/kg) treated group was observed. Infiltration of inflammatory cells and minimum fibrous tissue proliferation were observed for NP+APE (10+200 mg/kg) and NP+AN (10+60 mg/kg) treated groups (Figure 28).
Figure 28: Histopathological representation of tibiotarsal joints of animals of NP antiarthritic study.

A Healthy control (Joint bone with no infiltration of inflammatory cells exudates in joint tissue). B. Arthritic control (Joint bone surrounded by fibrous tissues with normal bone marrow cavity). C. NP 10 mg/kg (Joint bone with no infiltration of inflammatory cells and fibrous tissue). D. APE 200 mg/kg. (Moderate infiltration of inflammatory cells and fibrous tissue proliferation). E. NP+ APE (10+200 mg/kg) (Infiltration of inflammatory cells and minimal fibrous tissue proliferation). F. AN 60 mg/kg. (Moderate infiltration of inflammatory cells and fibrous tissue proliferation.). G.
NP + AN (10+60 mg/kg) (Minimal infiltration of inflammatory cells and fibrous tissue proliferation). Stain: H & E 10 x

8.2.4.5. Effects on hematological parameters FCA-induced in rats:
Levels of Hb, and RBC were decreased in arthritic rats with concomitant increases in ESR, WBC and platelet count. These changes were significantly (P<0.05) reverted to near normal levels in NP (10 mg/kg), NP (10 mg/kg) + AN (60 mg/kg), and NP (10 mg/kg) + APE (200 mg/kg) treated animals as compared to treated with AN and AE alone groups (Table 16).

Table 16: Effect of APE 200mg/kg, NP+APE (7.5+200 mg/kg), AN (60 mg/kg) and NP+AN (7.5+60 mg/kg) on hematological parameters in arthritic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Arthritic Control</th>
<th>Healthy Control</th>
<th>NP (60 mg/kg)</th>
<th>AN (200 mg/kg)</th>
<th>NP +AN (10+60 mg/kg)</th>
<th>NP+APE (10+200 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (gm/100ml)</td>
<td>8.87 ± 0.14</td>
<td>14.37 ± 0.21***</td>
<td>13.59 ± 0.32***</td>
<td>10.04 ± 0.06***</td>
<td>10.99 ± 0.03***</td>
<td>12.37 ± 0.12***</td>
</tr>
<tr>
<td>WBCs (thousands/µl)</td>
<td>15.29 ± 0.18</td>
<td>7.75 ± 0.15 ***</td>
<td>8.460 ± 0.16***</td>
<td>12.07 ± 0.16***</td>
<td>13.39 ± 0.13***</td>
<td>9.332 ± 0.1602***</td>
</tr>
<tr>
<td>RBCs (million/µl)</td>
<td>3.23 ± 0.05</td>
<td>7.04 ± 0.16 ***</td>
<td>6.57 ± 0.18</td>
<td>4.10 ± 0.06***</td>
<td>5.31 ± 0.06***</td>
<td>6.35 ± 0.21**</td>
</tr>
<tr>
<td>Platelets (lacks/ µl)</td>
<td>17.94 ± 0.09</td>
<td>9.147 ± 0.10***</td>
<td>9.39 ± 0.17</td>
<td>16.77 ± 0.25***</td>
<td>13.50 ± 0.09***</td>
<td>10.94 ± 0.06***</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>16.34 ± 0.74</td>
<td>8.417 ± 0.19 ***</td>
<td>9.46 ± 0.08</td>
<td>13.68 ± 0.32***</td>
<td>12.31 ± 0.07***</td>
<td>10.50 ± 0.09***</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett’s test when compared with arthritic control group *P<0.05, **P<0.01, ***P<0.001
8.3: Discussion

Use of herbal products has increased steadily among adults over the past few years. In fact, many people think that all herbs are safe owing to their natural origin. However, herbs may interact with conventional medications either taken as over the counter or prescribed by physicians resulting in various side effects. Such interactions may result in a new side effect that is not seen with the use of the herb or drug alone. Being natural in source the herbal products have many side effects like direct toxic effects, allergic reactions, effects from contaminants, and interactions with drugs and other herbs.

Co-administration of herbal medicines and drugs was prevalent for the treatment of some chronic and refractory diseases in clinical practice. As a result, the potential risk from herb–drug interaction increased significantly. Herb–drug interactions are classified as pharmacodynamic (PD) and pharmacokinetic (PK) in nature (Xia et al, 2009). When constituents of herbal products have either synergistic or antagonist activity in relation to a conventional drug it’s called as PD interaction. At the end, concentration-dependent activity of a therapeutic molecule is altered at the site of action at the drug-receptor level. Alteration of absorption, distribution, metabolism, or elimination of a conventional drug by an herbal product or other dietary supplements is called as PK interaction. Most of the herb-drug interactions are negative in nature, but sometimes interactions may have a beneficial effect on drug therapy. To date, there have been many reports on pharmacokinetic interactions between herbs and drugs. Some of the recent investigations have suggested the modulation of cytochrome P-450 enzymes (CYP)-mediated drug elimination as a major mechanism responsible for such types of interactions (Pandit et al., 2012). Most of the population do not reveal to their physician or pharmacist about the herbal products they are using. Because of which herb–drug interactions are not identified and resolved immediately.

When the body’s immune system gets confused about its own healthy tissue as foreign bodies and attacks them, it is known as autoimmune disease. Women are more prone to autoimmune disease than men (Anaya, 2012). Rheumatoid arthritis is an autoimmune disease and can attack many other organs so it is called systemic disease and sometimes rheumatoid disease. Symptoms of rheumatoid arthritis are persistent joint synovial tissue inflammation. Being a chronic condition with multiple causes, it affects the people in their most active period of life. An autoimmune or infectious triggering incident cause joint damage in RA which starts with the proliferation of synovial macrophages and fibroblasts. It further goes up to bone erosion
and irreversible joint damage leading to permanent disability. Due to its articular manifestations, multiple organ systems may be affected and may result in shortened life expectancy, with increased deaths due to cardiovascular disease, infection, and cancer.

There are three general classes of drugs commonly used in the treatment of rheumatoid arthritis: non-steroidal anti-inflammatory agents (NSAIDs), corticosteroids, and disease modifying anti-rheumatic drugs (DMARDs). Commonly used NSAIDs in RA treatment are aceclofenac, ibuprofen, etoricoxib, nabumetone and naproxen. Near about 80% of the population in various developing countries use herbal medicines in the treatment of diseases such as cold, inflammation, heart disease, diabetes and central nervous system diseases and about 60–90% of persons with arthritis use CAM (traditional or complementary and alternative medicine). Most of the population, particularly those living in villages depend largely on herbal remedies and use herbals for curing most of the diseases. However, no scientific data regarding their identity and effectiveness of these herbs was available except that in the treatise of Ayurveda and Unani medicine. Depending on the geographical, cultivation and cure conditions number of plants are reported to have anti rheumatic properties.

Metabolism is the most common etiology of pharmacokinetic drug interactions, and phase I metabolism by cytochrome P450 (CYP) enzymes is often involved. CYPs are found primarily in the liver and at lower levels in organs such as the kidneys, skin, gut and lung. In humans there are a group of at least fifty-seven different CYP proteins. CYP 450 enzymes increase the solubility of ‘foreign’ compounds which are sparingly soluble in water, including carcinogens, mutagens, and other toxic xenobiotics as well as drugs, allowing these compounds to be excreted in the urine with the help of oxidative metabolism. The CYP enzymes comprise a superfamily of heme-containing mono-oxygenases. In the presence of carbon monoxide, they have an absorption maximum at wavelength 450 nm and are therefore called P-450. The major CYPs in human liver are in families 1, 2 and 3, which are responsible for xenobiotic and drug metabolism. Isoforms include CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. CYPs can be inhibited and some isoforms can be induced by exogenous compounds or host molecules. Inhibition and/or induction of CYPs that metabolize current drugs are important causes of drug interactions (Martignoni et al, 2006; Morris and Zhang, 2006). Animal studies may give important information on herb-CYP
interactions, but inter-species variations in the substrate specificity, catalytic features and amino acid sequences of CYPs may cause difficulty in extrapolating animal data to humans. Due to differences in the species the selection of appropriate animal model becomes crucial. Due to high cost, for initial studies rat or mouse models can be used instead of pigs, minipigs and monkeys which have more similar CYPs to humans.

Andrographis paniculata Nees (AP) is a traditionally used medicinal plant in Korea, Thailand, China, Japan, South Africa, India, Pakistan and Sri Lanka for the treatment of fever, cold, inflammation, diarrhea and other infectious diseases. The extracts of AP and its isolated compounds are also reported to have various pharmacological activities, including hepatoprotective, antidiabetic, inhibition of replication of the HIV virus, antimalarial and principally anti-inflammatory properties. Near about 26 Ayurvedic formulations in Indian pharmacopoeia mentioned AP as a predominant constituent (Jarukamjorn and Nemoto, 2008; Ooi et al., 2011; Qiu et al., 2012; Dhiman et al., 2012; Shen et al., 2013, Carretta et al., 2009). Traditional use of AP as anti-inflammatory herb has been studied by various scientists and proved the mechanism (Hidalgo et al., 2005b, Abu-Ghefreh et al., 2009; Shen et al., 2013; Low et al., 2015). Andrographolide (AN), one of the active constituent of AP has been reported to have anti arthritic effect. Production of pro-inflammatory mediators such as COX-2, iNOS and cytokines has been reduced by AN (Carretta et al., 2009). In clinical trials, Andrographis paniculata extract (30% AN) showed effectiveness for symptom relief and reduce serological parameters in patients with Rheumatoid Arthritis (Hidalgo et al., 2005a). It is a common practice in South India that the arthritic patients take herbal remedies that contains APE as their main ingredient, along with NSAIDs such as ETO, NAB and NP with or without the knowledge of their health care provider.

CYP2C9 and CYP1A2 together account for the majority of R- and S-naproxen 0-demethylation in human liver in vitro and acts as a substrate in the metabolism of naproxen. As per the previous studies, AN induces CYP1A2 (Jaruchotikamol et al., 2007; Pekthong et al, 2009; Koe et al, 2014) and also inhibits CYP1A2 (Ooi et al., 2011; Chen et al, 2013). APE has CYP1A2 inhibitory activity in vitro (Pekthong et al., 2008). Thus the hypothesis was any substance influencing the CYP1A2 enzyme is likely to affect the metabolism of NP in rats, which should be studied.
Pharmacokinetic interactions of AN and APE were found with NP which was observed by a significant decrease in $C_{\text{max}}$, $T_{\text{max}}$, AUC$_{0-t}$ of NP by APE and AN significantly ($P<0.05$). Fast elimination of NP was observed with decreased $T_{1/2}$ when co-administered with AN. But decrease in CL and MRT$_{0-t}$ and increase in Vd and MRT$_{0-\infty}$ indicated changes in elimination pattern of NP on co-administration with AN. Whereas $T_{1/2}$ of NP remained same when co-administered with APE and an increase in CL, Vd, MRT$_{0-t}$ and MRT$_{0-\infty}$ has been observed. Co-administration of NP with AN and APE decreased the systemic exposure level of NP in vivo with decreasing $C_{\text{max}}$ and AUC$_{0-t}$ but this decrease is significantly more with AN+NP treated group compared to APE+NP treated group. It indicates that AN or some other ingredients of APE might have decreased the bioavailability of NP in rats. AN is well known for its CYP1A inducing properties. This decrease in $C_{\text{max}}$ and AUC of NP by AN might be due to CYP1A2 inducing property of AN.

To evaluate further possible herb-drug interaction pharmacodynamic anti-arthritic study has been performed in the groups with co-administration of APE, AN with NP followed by histopathological and hematological evaluation. In anti-arthritic study, increase in change in paw volume, decrease in mechanical nociceptive threshold and mechanical hyperalgesia, it was observed that NP treated group showed better activity than NP+AN and NP+APE treated groups. Significant decrease in activity of AN+NP compared to NP may be due to the CYP1A inducing property of AN. Further, even though the dose of AN is equal in both groups treated with APE and AN there was better activity observed with AN treated group. This can be attributed to the interference of other components present in the extract. The minimum infiltration of inflammatory cells and fibrous tissue in histopathology and increased levels of Hb, and RBC with concomitant decrease in ESR, WBC and platelet count in the hematological study of NP+AN (10+60 mg/kg) supports the findings.

Andrographolide has anti-inflammatory activity by suppressing inducible nitric oxide synthase expression in RAW 264.7 cells (Chiou et al., 2000), prevents oxygen radical production by human neutrophils (Shen et al., 2002), inhibits NF-kappaB activation(Xia et al., 2004), reduced COX-2 expression induced by platelet activating factor and N-formyl-methionyl-leucyl-phenylalanine in HL60/neutrophils (Hidalgo et al., 2005), it also showed interaction with arginine and histidine in the cyclooxygenase site of COX-2 and reduced PGE 2 production in human fibroblast cells induced by lipopolysaccharide (Levita et al., 2010; Akbar,
2011). Naproxen has reported to have COX-2 inhibitor activity (Warner et al., 1999). As per the previous literature AN, APE and NP have COX-2 inhibition activities and on co-administration the activity should increase synergistically. In this study, the antiarthritic activity of co-administered groups has been decreased as compared to NP alone administered group. The exact mechanism for decrease in the activity is not clear and should be further studied. The study observed that co-administration of AN and APE changes the action of metabolic enzymes of NP.