“STUDIES ON HERB-DRUG INTERACTIONS ON SELECTED HERBS AND DRUGS”

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This is to certify that the work incorporated in the thesis entitled **"Studies on herb-drug interactions on selected herbs and drugs"** for the degree of **'Doctor of Philosophy'** in the subject of **Pharmaceutical Chemistry** under the faculty of **Pharmaceutical Sciences** has been carried out by **Ms. Aishwarya Ramchandra Balap** in the Department of Pharmaceutical Chemistry at **Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune** during the period from 03/07/12 to 31/12/16 under the guidance of **Dr K. R. Mahadik**.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Studies on herb-drug interactions on selected herbs and drugs"** submitted by me to the Bharati Vidyapeeth University, Pune for the degree of **Doctor of Philosophy (Ph.D.)** in **Pharmaceutical Chemistry** under the faculty of **Pharmaceutical Sciences** is original piece of work carried out by me under the supervision of **Dr. K. R. Mahadik** and **Dr. L. Sathiyanarayanan.** I further declare that it has not been submitted to this or any other university or Institution for the award of any degree or Diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place :

Date :

Ms. Aishwarya R. Balap

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INDEX

CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

6-MNA	6-methoxy-2-naphthylacetic acid
AN	Andrographolide
ANOVA	Analysis of variance
AP	*Andrographis Paniculata*
APE	*Andrographis Paniculata Nees* Extract
$AUC_{0-\infty}$	Area under plasma concentration-time curve extrapolated to infinity
AUC_{last}	Area under plasma concentration-time curve from zero to last time point
$AUMC_{0-\infty}$	Area under the concentration time (c×t) versus time (t) curve for extrapolated to infinity
$AUMC_{last}$	Area under the concentration time (c×t) versus time (t) curve for last time point
CAM	Complementary and alternative medicine
CL	Plasma clearance
C_{max}	Peak plasma concentration
CV	Coefficient of variation
CYP	Cytochrome P450
DMARD	Disease-modifying antirheumatic drug
DME	Drug metabolising enzyme
EDTA	Ethylenediaminetetra-acetic acid
ETO	Etoricoxib
HPLC	High performance liquid chromatography
I.S.	Internal Standard
Ke	Elimination rate constant
$MRT_{0-\infty}$	Mean Residence Time extrapolated to infinity
MRT_{0-t}	Mean Residence Time from zero to t hours
NAB	Nabumetone
NP	Naproxen
NSAID	Nonsteroidal anti-inflammatory drug

r	Correlation coefficient
SD	Standard deviation
SEM	Standard error of the mean
$t_{½}$	Elimination half-life
t_{max}	Time when Cmax occurs
UGT	UDP-glucuronosyltransferase
Vd	Apparent volume of distribution

1. Introduction:

1.1 Herb-drug interaction

One third of adults in developed nations and 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. Herbs are often co-administered with therapeutic drugs which may lead to herb-drug interactions or may cause unexpected interactions. Severity of this area can be proved with increased number of clinical reports of such interactions (Zhou et al, 2007). Herbal medicinal products include dietary supplements containing medicinal herbs or the medicines traditionally used in phytotherapy for treating or preventing diseases. Increased sales of herbal medicinal products over recent years have inspired many researchers to turn their focus to the pharmacological mechanisms underlying herb–drug interactions. The efficacy of drug therapy depends on many factors related to a drug's pharmacokinetic and pharmacodynamic properties (Colalto, 2010).

In United States (US) self-directed herbal use increased by 10% between 1997 and 2002 and less than 40% of users of complementary and alternative medicine (CAM) disclose use to their physician (Chavez, 2005). Pharmacokinetics and/or pharmacodynamics of the drug may be altered due to co-administration with herbal remedies, which is the major cause for the life-threatening adverse reactions. Because of the clinical significance of drug interactions with herbs, it is important to identify drugs and compounds in development that may interact with herbal medicines. Timely identification of such drugs using proper *in vitro* and *in vivo* approaches may have important implication for drug development. Herb drug interactions can be caused by either pharmacodynamic or pharmacokinetic mechanisms. Hence, herb–drug interactions are classified as pharmacodynamic (PD) and pharmacokinetic (PK) in nature.

1.2 Pharmacodynamic (PD) herb-drug interactions

These interactions may occur when a herbal product produces additive, synergistic or antagonist activity in relation to the conventional drug. Pharmacodynamic interactions are related to the pharmacological activity of the interacting agents and can affect organ systems, receptor sites or enzymes.

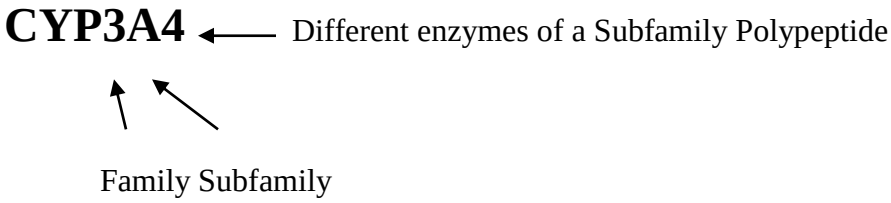
1.3 Pharmacokinetic (PK) herb-drug interactions

These interactions occur when a herbal drug changes the absorption, distribution, metabolism, protein binding or excretion of a drug that results in altered levels of the drug or its metabolites. Most of the current evidence of pharmacokinetic drug interactions involves metabolizing enzymes and drug transporters. Although drug interactions can involve enzymes such as glutathione S-transferases and uridine diphosphoglucuronyl transfereases (UGTs), most herbal-drug interactions are related to oxidative metabolism by the cytochrome P-450 system (CYP) or by the effect of a herbal drug on the efflux drug transporter P-glycoprotein. The most common pharmacokinetic interactions usually involve either the inhibition or induction of the metabolism of drugs catalyzed by the important enzymes, cytochrome P450 (CYP) (Wanwimolruk and prachayasittikul, 2014). 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).

1.4 Role of Cytochrome P450 Enzymes in Drug Interactions:

The CYP450 enzymes are the major catalyst involved in the metabolism of drugs. CYP450 is a superfamily of hemoproteins, containing 57 genes responsible for the oxidative metabolism as well as metabolic activation of the vast majority of xenobiotics (drugs, dietary components and pollutants) and endogenous substrates (e.g., steroids, cholesterol and bile acids). Modulation (either inhibition or enhancement) of CYP-mediated drug metabolism upon concurrent administration of other drugs or exposure to some exogenous agents is considered as the most common factor responsible for drug interactions. In CYP naming first no. after CYP denotes family, alphabet denotes subfamily and the no. after subfamily denotes different enzymes of a subfamily polypeptide.

Figure 1: Nomenclature of CYP enzymes



In the human liver, there are at least 18 distinct CYP450 isozymes while only 10 isoforms from families 1, 2, and 3 (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2F1, and CYP3A4) are responsible for the hepatic metabolism of most of the drugs. P450 enzymes in animal species are different from humans. However, similarities can be found for some specific CYP isoforms.

Table 1: Some CYP enzymes in man, mouse, rat, dog and monkey.

Family	Subfamily	Human	Mouse	Rat	Dog	Monkey
CYP1	A	1A1, 1A2	1A1, 1A2	1A1, 1A2	1A1, 1A2	1A1, 1A2
	B	1B1	1B1	1B1	1B1	1B1
CYP2	A	2A6 2A7 2A13	2A4 2A5 2A12 2A22	2A1 2A2 2A3	2A13 2A25	2A23 2A24
	B	2B6 2B7	2B9 2B10	2B1 2B2 2B3	2B11	2B17
	C	2C8 2C9 2C18 2C19	2C29 2C37 2C38 2C39 2C40 2C44 2C50	2C6 2C7* 2C11* 2C12* 2C13* 2C22 2C23	2C21 2C41	2C20 2C43

			2C54 2C55			
	D	2D6 2D7 2D8	2D9 2D10 2D11 2D12 2D13 2D22 2D26 2D34 2D40	2D1 2D2 2D3 2D4 2D5 2D18	2D15	2D17** 2D19** 2D29** 2D30** 2D42**
	E	2E1	2E1	2E1	2E1	2E1
3	A	3A4 3A5 3A7 3A43	3A11 3A13 3A16 3A25 3A41 3A44	3A1/3A23 3A2* 3A9* 3A18* 3A62	3A12 3A26	3A8

** strain specific

* gender difference

In the preclinical development of new drugs, animal models are commonly used to predict the metabolic behavior of new compounds in human. In the early stages of drug development to avoid or minimize toxic drug–herb interactions, it is important to identify drugs that can interact with herbs using proper *in vitro* and *in vivo* models. However the humans are different from animals in isoform composition, expression and catalytic activities of drug metabolizing enzymes. The species-specific isoforms of CYP1A, CYP2C, CYP2D and CYP3A shows significant interspecies differences in catalytic activity which should be considered while extrapolating metabolism data from animal models to human. Selection and search of an optimal and exact animal species to mimic drug metabolism in man is still a challenge in a drug

development program. The species used in metabolism studies should have possible metabolism-linked toxicity end points. The use of human recombinant CYP proteins and expression systems could not be the accurate method. The most commonly used species in metabolism studies are mouse, rat, rabbit, dog and monkey in lesser extent guinea pig and hamster. All of them are not perfect in their CYP profile compared to man (Martignoni et al, 2006).

Herbal/dietary constituents may be metabolized by CYPs to nontoxic metabolites and excreted, but the formation of toxic metabolites is possible. The mechanism for herbal toxicity remains elusive, but there are accumulating data suggesting the role of the formation of reactive metabolites/intermediates through the bioactivation of major herbal constituents in herbal toxicity and carcinogenicity. It has been hypothesized that the resultant reactive metabolites following herbal bioactivation covalently bind to cellular proteins and DNA, leading to toxicity via multiple mechanisms such as direct cytotoxicity, oncogene activation and hypersensitivity reactions.

Xenobiotics, drugs and a variety of naturally occurring dietary or herbal constituents can interact in several ways with the CYP450 system (Lehmann, 1998) resulting in altered drug clearance and effect (Rendic, 2002).

• A compound may be a **substrate** of one or several CYP isoforms. If the main isoform is saturated, it becomes a substrate for the secondary enzyme(s).

• A compound can be an **inducer** of a CYP isoform, either of the one is a substrate for, or may induce several different enzymes at the same time. The process of induction increases the rate of metabolism of substrates of that enzyme.

• A compound may also be an **inhibitor** of CYP450 enzymes. There are several mechanisms of inhibition, and a compound may inhibit several isoforms including others than those for which it is a substrate (Zhou, 2003).

On the other hand, if the substrate is a prodrug activated by CYP-mediated metabolism, inhibition of its metabolism can reduce its effects and induction can either enhance or reduce its effects and toxicity, depending on the effects of induction on the further metabolism or excretion of the active metabolite.

Mechanisms of CYP450 inhibition:

Drug metabolism could be inhibited by different mechanisms including:

(i) reversible enzyme inhibition,

(ii) reduction of enzyme available for metabolism by irreversible inhibition or suppression of its synthesis

(iii) reduction in the supply of enzyme cofactor(s).

(iv) Inhibition of drug metabolism could result in an increase in drug plasma concentration (which may result in drug toxicity) and a decrease in the concentration of its metabolites, which could be clinically significant in cases of active or toxic metabolites.

Mechanisms of CYP450 induction:

Drug metabolism could be enhanced by different mechanisms including:

(i) an increase in the amount of enzyme available for metabolism (induction), which could be achieved by transcriptional activation, mRNA or protein stabilization

(ii) an activation of enzyme metabolic activity, which is different from induction in that the amount of the enzyme available for metabolism is not altered but its catalytic activity is stimulated in the presence of the activator

(iii) an increase in the supply of enzyme cofactor when it is the rate limiting step of metabolism.

Induction may increase the amount of P450 present and enhance the speed of oxidation and clearance of a drug. Prediction of the time-course of enzyme induction is difficult because of several factors, including the drug half-life and enzyme turnover, which determine the time-course of induction. The time-course of induction depends on the time required for enzyme degradation and new enzyme production.

1.5 I*n vitro* models

In vitro approaches are important as they provide background, as well as anticipatory knowledge, for *in vivo* predictions and are efficient regarding cost and time. Several *in vitro* approaches can be used to predict herb-drug interactions. These approaches includes subcellular fractions, such as liver microsomes, cytosols and homogenates, precision-cut liver slices, isolated and cultured hepatocytes or liver cell lines and cDNA-expressed enzymes (Rodrigues, 1994; Gustavsen, 2016). The use of primary cultures of human hepatocytes (PCHH) in *in vitro* models, are necessary to make better predictions of drug–herb interactions in humans (Venkataramanan et al, 2003; Wentworth, 2000). Few in vitro techniques enlisted below (Turpeinen, 2006),

***Human-derived in vitro* techniques**

1. *Primary hepatocytes*

Good in vitro – in vivo correlations in the metabolic activity of a number of drugs have been demonstrated and consequently, currently cultured human hepatocytes are the most recommended tools to study CYP-mediated metabolism and induction. Hepatocyte cultures can be prepared from whole livers and surgical wedge biopsies. The maintenance of normal cellular physiology and intercellular contacts in hepatocytes requires special matrix configurations and demanding technical abilities. Although several cryopreservation applications have been developed, the time for efficient use of a single hepatocyte batch is still quite short

2. *Immortalised cell lines*

The most common ones for metabolism studies are probably the liver-derived HepG2 and BC2 cell lines and the lung-derived line A549. In order to increase the expression patterns and levels of DMEs (drug metabolising enzyme), a number of genetically engineered cell lines and co-culture systems have been developed. However, most of these approaches have failed or been shown to posess a very restricted metabolic capacity. Very recently a human hepatoma-derived cell line HepaRG was introduced and demonstrated to possess several liver-specific functions and morphological resemblance to normal human hepatocytes.

3. *Liver slices*

Precision-cut liver slices retain a wide range of enzymatic activities and closely resemble the organ level of the liver. Liver slices are also suitable for induction studies. If a whole cell metabolism needs to be studied for short periods, liver slices represent a valuable tool.

4. *Subcellular fractions*

Liver samples can be used directly as homogenates or subcellular fractions, including microsomes, can be prepared. Liver homogenate contains all phase I and II enzymes. Microsomes are derived from endoplasmic reticulum after homogenisation and differential ultracentrifugation and contain CYPs and UGTs. Together with liver homogenates, microsomes are the most widely used *in vitro* system for drug metabolism studies.

5. *cDNA-expressed CYPs*

Isolated heterogeneous human CYP enzymes, expressed as single enzymes at a time from cDNA in bacterial, yeast, and mammalian cells have been commercially available for several years. Recombinant CYPs have been adopted as frontline tools in early drug development.

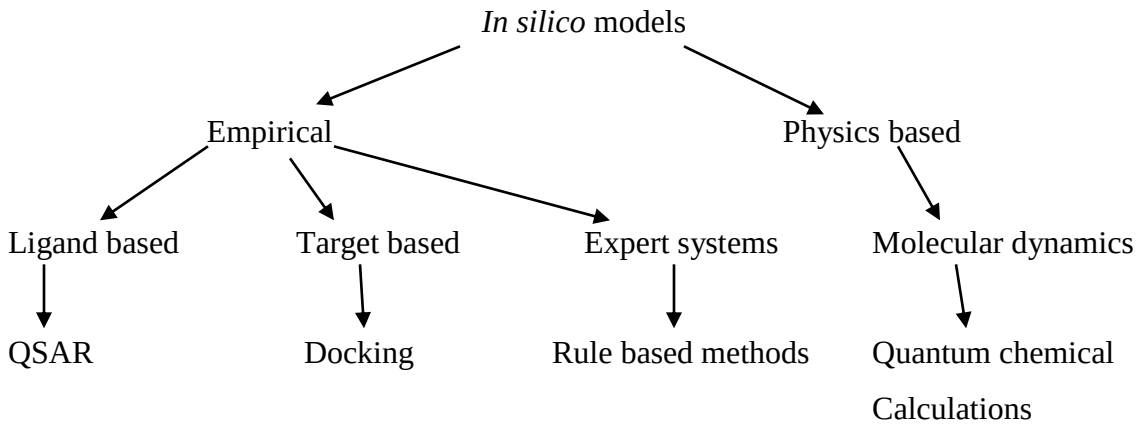
6. *Novel cell-based technologies*

Preliminary studies concerning the expression, inhibition, and regulation of CYPs in bioartificial liver systems, stem cell-derived cultures and assays have already been published, making these technologies a quite interesting possibility for future drug metabolism studies.

Computational in silico methods

There is an increasing use of *in silico* methods to study CYPs, Phase II enzymes, P-gp and their interactions with xenobiotics, including herbs (Ekins and Wrighton, 2001; Raunio, 2015).

Figure 2: Classification of *in silico* methods



1.6 Arthritis

Arthritis (from Greek arthro-, joint + -itis, inflammation; plural: arthritides) is a form of joint disorder that involves inflammation of one or more joints (Wollenhaupt J. *et al*, 1998). The major complaint by individuals who have arthritis is joint pain. Pain is often a constant and may be localized to the joint affected. The pain from arthritis is due to inflammation that occurs around the joint, damage to the joint from disease, daily wear and tear of joint, muscle strains caused by forceful movements against stiff, painful joints and fatigue. There are over 100 different forms of arthritis. The most common form, osteoarthritis (degenerative joint disease), is a result of trauma to the joint, infection of the joint, or age. Other arthritis forms are rheumatoid arthritis, psoriatic arthritis, and related autoimmune diseases. Septic arthritis is caused by joint infection. There are several diseases where joint pain is primary and it is considered as the main feature. Generally when a person has "arthritis" it means that they have one of these diseases, which include:

1. Ankylosing spondylitis

2. Gout and pseudo-gout

3. Juvenile idiopathic arthritis

4. Osteoarthritis

5. Rheumatoid arthritis

6. Septic arthritis

7. Still's disease

But, from all these Rheumatoid arthritis and osteoarthritis are the two major forms of arthritis affecting the large number of people in world (Becker, 2005).

1.7 Rheumatoid arthritis (RA):

RA is an autoimmune disease that involves inflammation of the synovium (a thin layer of tissue lining a joint space) with progressive erosion of bone, leading in most cases to misalignment of the joint, loss of function, and disability. That means the body's defense system (called the immune system) attacks its own joints and organs. This can cause swelling of the synovium, the tissue that lines the joint. Both genetic and environmental factors are involved in the initiation and progression of the disease. Today, about 0.5% to 1% of the population is affected by RA. Disease onset generally occurs between 30 and 55 years of age and women are often more affected than men.

1.8 Animal Models used in rheumatoid arthritis study (Chaudhari et al, 2014)

1. Collagen Type II Induced Arthritis (CIA) In Rats

CIA model is standard animal model of RA. There are three different cartilage-derived proteins are responsible for induction of arthritis in rats i.e. collagen type II, collagen type XI, and cartilage oligomeric matrix protein. To study mechanism of immune response to auto antigen which are generally involved in human disease, it is an excellent model. Induction of collagen arthritis in many strains of rats by immunizing them with type II collagen emulsified in Incomplete Freund's Adjuvant (IFA). After the induction of disease there is development of both cellular and humeral immune response to type II collagen, which can be passively transferred by sensitized spleen and lymph node cells as well as IgG antibodies to type II collagen.

2. Complete *Freunds Adjuvant Induced (CFA)* Arthritis in rats

Freund's complete adjuvant induced arthritis in rat model is the best and most widely used experimental model for arthritis. Increased levels of TNFα, interferon γ (INFγ), IL1, IL6 and IL17A mRNA have been detected in this type of model. This model is sensitive to anti inflammatory and immune inhibiting medicines and best for the study of phathophysiological and pharmacological control of inflammation process as well as for the evaluation of anti-nociceptive potential of drug.

3. Carrageenan* Induced *Paw* Edema *in rats

Carrageenan (CRR), a sulphated polysaccharide, is most commonly used in pain models. CRR produces acute and chronic inflammatory responses. The acute response appears to be similar to rheumatoid arthritis lesions, which are characterized by sustained cellular emigration. Hydrolyzed carrageenan induces inflammation by inhibiting deoxyribonucleic acid synthesis it also effects on chromium release and cell morphology retarded cell growth and eventually caused cell death.

4. Formaldehyde Induced Arthritis

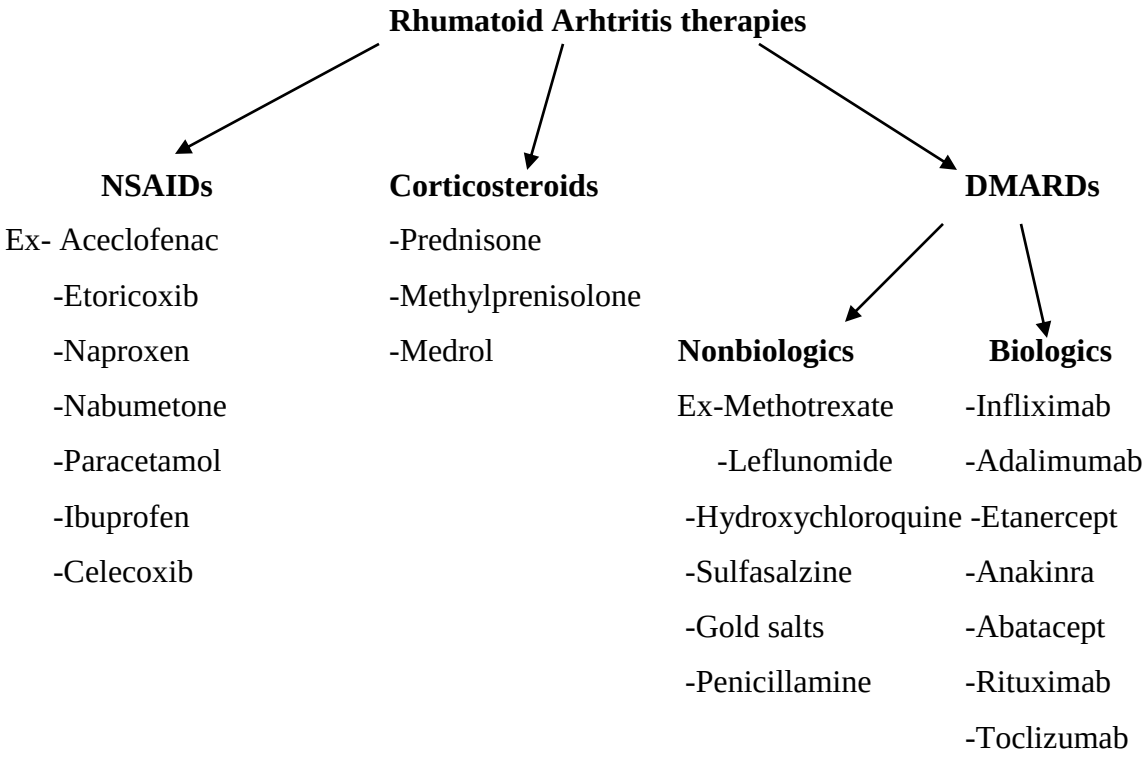
The development of edema in the paw of the rat after injection of formaldehyde is due to the release of histamine, serotonin and the prostaglandin like substances at the site of injection. Formaldehyde induces arthritis by denaturing protein at the site of administration, which produces immunological reaction against the degraded product.

1.9 Treatment Strategies in RA:

The major aim and the challenge of the treatment of RA is symptom relief, prevention of disability and achieving sustained disease remission to provide a good quality of life. Historically, most patients with rheumatoid arthritis were treated according to a sequential strategy, beginning with a nonsteroidal anti-inflammatory drug. NSAIDs are helpful for providing partial relief of pain and stiffness at this early stage. When prescribing NSAIDs, the

gastrointestinal, renal and cardiovascular status of the patient should be considered and patients should be advised of and monitored for adverse effects (AEs). After diagnosis, patients can be treated with disease-modifying antirheumatic drugs (DMARDs) with or without biologic agents with the goal of achieving disease remission and avoiding structural damage (Burgos-Vargas et al., 2013). The major groups of drugs used include nonsteroidal anti-inflammatory drugs, disease modifying antirheumatic drugs (DMARDs), corticosteroids and biological agents (Mahajan et al., 2006). Glucocorticoids are potent suppressors of the inflammatory response in many diseases, including RA. Glucocorticoids are DMARDs in that they have been found to provide clinical benefit, especially during the first 6 months after onset of RA, decreasing the radiologic progression of joint damage. Low-dose oral glucocorticoids (≤10 mg prednisone/day or equivalent) and local injections of glucocorticoids are highly effective for relieving symptoms in patients with active RA, and they may be used as a bridge therapy until DMARDs become effective, which may take several months. However, due to their limited disease-modifying effects, glucocorticoids should be combined with DMARDs and cannot be used as the primary or sole treatment. Patients using glucocorticoids should be carefully monitored for AEs. Biologic agents such as adalimumab, etanercept and infliximab, along with nonbiologic disease modifying antirheumatic drugs are used for substantial effectiveness in lowering disease activity, reducing joint erosions, and improving the quality of life of patients with RA (Harrold, 2012).

Figure 3: Treatment Strategies in RA



i. Disease modifying antirheumatic drugs (DMARDs): These drugs are the first drugs usually tried in patients with RA. They are prescribed in addition to rest, strengthening exercises and anti-inflammatory drugs.

Methotrexate is the most commonly used DMARD for RA. Leflunomide (Arava) and chloroquine may also be used. These drugs may have serious side effects, so one should need frequent blood tests when taking them.

ii. Anti-inflammatory medications: These include aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and naproxen.

iii. Antimalarial medications: This group of medicines includes hydroxychloroquine and is usually used along with methotrexate. It may be benefited after weeks or months.

iv. Corticosteroids: These medications work very well to reduce joint swelling and inflammation but because of long-term side effects, corticosteroids should be taken only for a short time and in low doses when possible.

v. Biologic Agents: Biologic drugs are designed to affect parts of the immune system that play a role in the disease process of RA. They may be given when other medicines for RA have not found to be useful. Most of them are given either under the skin (subcutaneously) or into a vein (intravenously).

There are different types of biologic agents:
White blood cell modulators include: abatacept and rituximab
Tumor necrosis factor (TNF) inhibitors include: adalimumab, etanercept, infliximab, golimumab, and certolizumab.

Interleukin-6 (IL-6) inhibitors: tocilizumab (Actemra)

Biologic agents can be very helpful in treating rheumatoid arthritis. However, people taking these drugs must be watched very closely because of serious risk factors like Infections from bacteria, viruses and fungi, Leukemia, lymphoma and/or Psoriasis

vi. Surgery: Occasionally, surgery is needed to correct severely damaged joints. Surgery may include:

Removal of the joint lining (synovectomy)

Total joint replacement in extreme cases; may include total knee, hip replacement, ankle replacement, shoulder replacement and others

vii. Physical Therapy: Range-of-motion exercises and exercise programs prescribed by a physical therapist can delay the loss of joint function and help keep muscles strong.
Sometimes therapists will use special machines to apply deep heat or electrical stimulation to reduce pain and improve joint movement. Joint protection techniques, heat and cold treatments, and splints or orthotic devices to support and align joints may be very helpful. Frequent rest periods between activities, as well as 8 to 10 hours of sleep per night, is recommended.
viii. Nutrition: Some people with RA may have intolerance or allergies to certain foods. A balanced nutritious diet is recommended. It may be helpful to eating foods rich in omega-3 fatty acids (fish oils).

Although conventional treatment of rheumatological conditions is generally considered to have improved in terms of effectiveness, the use of non-steroidal anti inflammatory drugs (NSAIDs), second line therapies and corticosteroids have all been associated with adverse reactions. For this reason, many patients suffering from chronic, musculoskeletal disorders look for complementary and alternative medicine (CAM) methods for symptomatic relief (Boisset 1994). In the initiation of RA the activation of auto reactive T cells and the recruitment of these T cells along with other leukocytes into the joints have been involved. A variety of mediators of inflammation has been produced by these leukocytes which induces synovial inflammation and eventually cause tissue damage in the joints. In consequences, these mediators become potential targets for therapeutic agents for arthritis. Many of these mediators can be targeted by natural products, including herbal mixtures belonging to traditional or CAM (Venkatesha, 2011). Also as per the research, the people suffering from chronic pain, as in RA, and those dissatisfied with current treatment are very likely to seek alternative treatments. An estimated 60–90% of persons with arthritis use CAM. Among the most widely used treatments are chiropractic and herbal therapies (Soeken. et al 2002). Thus, some of the herbal medicines used for RA are discussed as follows:

ix. Herbal drugs:

• **γ - Linolenic Acid: -** Borage seed oil, Borago officinalis, Evening primrose oil, Oenothera blennis, Blackcurrant seed oil, Ribes nigrum.

Herbs used in treatment of RA: - *Harpagophytum procumbens* (Devil's claw), *Ocimum americanum* (American basil), *Ocimum basilicum* (Common basil), *Ocimum sanctum* (Tulsi/ Holy basil), *Salix fragilis* (Crack willow), *Salix purpurea* (Purple willow), *Salix daphnoides* (Violet willow), *Salix alba* (White willow), *Tripterygium wilfordii* (Lei gong teng), *Uncaria tomentosa* Cat's claw, *Urtica diocia* (Stinging neetle), *Capsaic*in, *Curcumin* (diferuloyl methane), *Feverfew* (Tanacetum parthenium), *Andrographis paniculata*, *Boswellia serrata, Vitis vinifera, Zingiber officinale, Allium cepa, Aralia cordata, Camellia sinensis, Gentiana macrophylla, Sinomenium acutum, Turpinia arguta, Cibotium barametz, Magnolia officinalis, Paeonia lactiflora, Triphala guggulu, Celastrus aculeatus, Cynodon dactylon, Helenium microcephalum, Trewia polycarpa*

• **Food supplements:-** Fish oil, selenium supplements, extracts from New Zealand green lipped mussel.

• **Marketed Formulations:-** Rumalaya, Mahayogiraj guggul, Gokshuradi guggul, Joint Aid plus.

Among the herbs used, *Andrographis Paniculata Nees* is commonly used herbal medicine for treatment of inflammatory diseases. Andrographolide (3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5, 8a-dimethyl-2-methylene-1-napthalenyl]ethylidene] dihydro-4-hydroxy-2(3H) furanone) is the major diterpenoid constituent of the plant *Andrographis paniculata Nees* (Family Acanthaceae), which has been traditionally employed for centuries in Asia and Europe for the treatment of several diseases, including inflammatory diseases(Abu-Ghefreh et al, 2009). It is official in the Indian Pharmacopoeias and is a prominent component in at least 26 Ayurvedic formulas (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 *Andrographis Paniculata* 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). Various reports suggests that inflammatory responses are a critically a part of pathophysiology of several diseases including septic shock, cancer, atherosclerosis, rheumatoid arthritis and diabetes. So the manipulation of inflammatory responses may allow for prevention of serious diseases or symptoms (Shen et al, 2013). In 2013, Hidalgo and his co-workers performed a clinical trial of *Andrographis paniculata* standardized extract (30% andrographolide) which showed effectiveness for symptom relief and reduce serological parameters in patients with RA and the data supported a long term treatment similar to other DMARDs. The concurrent use of herbal medicines along with prescription drug may lead to drug interaction. Thus, there is a need to evaluate these interactions for the safety of the patients and to provide a mean for the rational use of herbal drugs in the treatment of arthritis. The present study was designed to evaluate the possible interaction of *Andrographis Paniculata Nees* and NSAIDs like Etoricoxib, Naproxen and Nabumetone when given concomitantly.

1.9 Literature review:

1.9.1. *Andrographis paniculata Nees*

Classification

Kingdom : Plantae

Division: Angiospermae

Class: Dicotyledoneae

Order: Tubiflorae

Family : Acanthaceae

Genus: Andrographis

Species: *paniculata Nees.*

Active constituent:

Andrographolide R = H neoandrographolide R = H andrographiside R = Glc andropanoside R = OH

Biological name: *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae)

Description: Stem quadrangular. Leaves opposite, lanceolate, apex acuminate,petiole short. Inflorescence racemose; flowers whitish, small. Fruits capsule, linear - oblong,erect, acute at both ends. Seeds numerous, small and glabrous.

Plant material of interest: Dried aerial parts.

Mechanism of action: Andrographolide inhibits NK-kappa β binding to DNA in vitro, reducing expression of a variety of inflammatory proteins, including COX-2. In rat neutrophils, it appears to exert anti-inflammatory action via a decrease in gene expression required for neutrophil adhesion and transmigration.

Pharmacology: It increases biliary flow and liver weight in rat. Andrographolide produces a significant dose dependent choleretic effect, as evidence by increase in bile flow, bile salt and bile acids in conscious rats and anaesthetised guinea pigs. It shows hepatoprotective action. It improves non-specific immune response. The immune response may be specifically directed at a microbial invader already present in the body, or generally, strengthening the immune system in preparation against future infections. Andrographis strongly stimulates phagocytosis and the production of specific antibodies.

Clinical Indications: Antipyretic, antiinflammatory, analgesic, antihepatotoxic, antidiabetic, antimalarial, antibacterial, anti fertility, and immunosupressive.

Side effects: Gastric discomfort, vomiting and loss of appetite.

Dose: 600 – 1200 mg daily.

Drug Interaction:

1. Extracts of Herba Andrographidis may have a synergistic effect with isoniazid.
2. It increases the elimination of theophylline.

Storage condition: Store in a well-closed container, protected from light and moisture.

Andrographolide:

Pubchem CID : 5318517

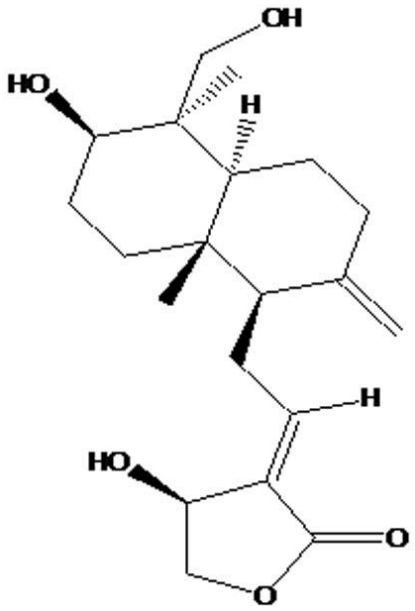
IUPAC name- (3E,4S)-3-[2-[(1R,4aS,5R,6R,8aS)-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylidene-3,4,4a,6,7,8-hexahydro-1H-naphthalen-1-yl]ethylidene]-4-hydroxyoxolan-2-one

MW: 350.449200 g/mol

MF: $C_{20}H_{30}O_5$

Structural formula:

Figure 4: Chemical structure of Andrographolide



A. Anti-inflammatory and anti-rheumatoid activities of *Andrographis paniculata Nees*:

1. *In vitro* and *in vivo* anti-inflammatory effects of andrographolide (Abu-Ghefreh *et al*. 2008).

Results provided evidence that andrographolide is an effective anti-inflammatory drug that is active both *in vitro* and *in vivo* and affects non-specific inflammation as well as inflammation resulting from antigen–antibody interaction. It is as effective as the steroid drug – dexamethasone. It primarily acts by inhibiting the expression of mRNA for inflammatory cytokines. In the mouse model of allergic lung inflammation, the drug not showed a strong inhibitory effect on the generation of the inflammatory cytokines – TNF-α and GM-CSF, and abolished the accumulation of critical effecter cells in the bronchial lavage fluid. Thus, andrographolide has the potential to be used in the treatment of inflammatory diseases, including those arising from antigen–antibody interaction.

2. Efficacy of an *Andrographis paniculata* composition for the relief of rheumatoid arthritis symptoms: a prospective randomized placebo-controlled trial. (Burgos et al., 2009).

Andrographolide gave the reduction of the production of pro-inflammatory mediators, such as COX-2, iNOS and cytokines. The molecular mechanism of andrographolide implied the reduction of the activation of transcription factors as NF-κB, AP-1, STAT3, NFAT and the inhibition of intracellular signaling pathways. *Andrographis paniculata* standardized extract (30% andrographolide) in clinical trials showed effectiveness for symptom relief and reduce serological parameters in patients with RA, and the data supported a long term treatment similar to other DMARDs.

3. **Andrographolide reduces IL-2 production in T-cells by interfering with NFAT and MAPK activation. (Carreta et al., 2009).**

The study determined that andrographolide reduced IL-2 production in Jurkat cells stimulated with phorbol myristate acetate and ionomycin (PMA/Ionomycin). It observed that andrographolide reduced NFAT luciferase activity and interfered with its nuclear distribution, with these effects being linked to an increase in c-jun-N-terminal kinase (JNK) phosphorylation. Additionally, reduction of NF-κB activity in Jurkat cells treated with andrographolide was observed. Western blotting analysis demonstrated that andrographolide decreased ERK1 and ERK5 phosphorylation induced by anti-CD3 or PMA/Ionomycin. The study concluded that andrographolide can exert immunomodulatory effects by interfering with NFAT activation and ERK1 and ERK5 phosphorylation in T-cells.

4. **Andrographolide a New Potential Drug for the Long Term Treatment of Rheumatoid Arthritis Disease. (Hidalgo et al., 2005a).**

Andrographolide showed a reduction of the production of pro-inflammatory mediators, such as COX-2, iNOS and cytokines. The molecular mechanism of andrographolide implied the reduction of the activation of transcription factors as NF-κB, AP-1, STAT3 and NFAT and the inhibition of intracellular signaling pathways. A standardized patented *Andrographis paniculata* extract (Paractin®, 30% andrographolide) in clinical trials showed effectiveness for symptom relief and reduce serological parameters in patients with Rheumatoid Arthritis, and the data supported a long term treatment similar to other DMARDs.

5. **Andrographolide interferes with binding of nuclear factor-kappa B to DNA in HL-60-derived neutrophilic cells. (Hidalgo et al., 2005b).**

The study analyzed the effect of andrographolide on the activation of NF-kB induced by platelet-activating factor (PAF) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) in HL-60 cells

differentiated to neutrophils. Andrographolide inhibited the NF-kB-luciferase activity induced by PAF. Andrographolide reduced the DNA binding of NF-kB in whole cells and in nuclear extracts induced by PAF and fMLP. Andrographolide reduced cyclooxygenase-2 (COX-2) expression induced by PAF and fMLP in HL-60/neutrophils. The study concluded that andrographolide exerted its anti-inflammatory effects by inhibiting NF-kB binding to DNA, and thus reducing the expression of proinflammatory proteins, such as COX-2.

6. **Study of anti-inflammatory activities of the pure compounds from *Andrographis paniculata* (burm.f.) Nees and their effects on gene expression. (Parichatikanond et al., 2010).**

The results revealed that andrographolide exhibited the highest efficacy. Therefore, changes in the levels of mRNA transcripts by andrographolide (AP 2) were further measured using human cDNA microarrays. The molecular response to *Andrographis Paniculata* was complex and mediated by various processes. Among the altered gene expressions, the genes involved in immune and inflammation processes were selectively down-regulated, such as cytokines and cytokine receptors (TNFSF14, TNF, TNFRSF6, and IL1A), chemokines (CCL8 and CXCL11), JAK/STAT signaling (JAK3 and STAT5A), TLRs family (TLR4 and TLR8) and NF-κB (NFKB1). Expression of some genes was validated using RT-PCR. The results demonstrated that dehydroandrographolide (AP 1), andrographolide (AP 2) and neoandrographolide (AP 3) exhibited the anti-inflammatory effect by interfering COX and inflammatory cytokines and the underlying mechanisms of andrographolide (AP 2) may be related to down-expression of genes involved in inflammatory cascade.

7. **Effect of an extract of *Andrographis paniculata* leaves on inflammatory and allergic mediators *in vitro* (Chandrasekaran *et al*. 2010).**

The extract of *Andrographis paniculata* containing at least seven phytochemical constituents showed significant anti-inflammatory and anti-allergic properties in the models investigated in this study. Considering its dual inhibitory activity on inflammatory and proinflammatory mediators, the study proposed that a combination of these two mechanisms is responsible for the overall activity of the extract. Based on its inhibitory activity towards LTB4 and PGE2 products, Andrographis paniculata leaves (AP) could be designated as a dual inhibitor. As a novel finding, this study demonstrated that AP inhibits A23187 induced LTB4 production. The extended comprehension of the molecular mechanisms involved in the anti-inflammatory and anti-allergic

effects of this extract and its diterpenoid constituents may lead to an improved phytomedical treatment of inflammatory and allergic disorders at an early phase. AP illustrated significant alleviation of pro-inflammatory, inflammatory, and allergic mediators. However, no inhibition was observed against histamine release. This outcome has been summed up to deduce that AP is fairly potent in attenuating the inflammation by inhibiting proinflammatory (NO, IL-1 beta and IL-6), inflammatory (PGE2 and TXB2) and allergic (LTB4) mediators.

B. Reported pharmacokinetic methods for *Andrographis paniculata Nees*:

1. **Pharmacokinetic Study of *Andrographis paniculata* Tablets in Healthy Thai Male Volunteers. (Wangboonskul et al., 2006).**

Pharmacokinetics of andrographolide, main active compound contained in *Andrographis paniculata* product, was investigated in healthy Thai male volunteers. The bio-analytical method using HPLC for the determination of andrographolide in plasma was developed and validated following US FDA and Thailand guidelines for the bioavailability and bioequivalence studies. Blood samples were pretreated by using C-18 solid phase cartridges before injecting into the HPLC. However, an individual peak was detected in all plasma samples obtained from the volunteers taking *Andrographis paniculata* products. The study concluded that the saturated metabolism process of andrographolide possibly which occurred at high dose.

2. **Pharmacokinetic study of *andrographis paniculata* chloroform extract in rats. (Naidu et al., 2009).**

The study developed an automated blood sampling method to evaluate the oral bioavailability of *Andrographis paniculata* chloroform extract (APCE) in conscious freely moving rat. The blood samples were collected from tail vein after APCE was administered orally at a single dose of 1 g/kg. Acetonitrile was used to extract the Andrographolide (AGN) and 14-deoxy -11, 12-didehydroandrographolide (DDA) from plasma samples after centrifugation at 3000 rpm for 10 min at 5 ºC. A 20 µl aliquot of supernant was passed through a membrane filter before injection into an HPLC/UV system. The analytes were separated by Nucleosil C18 (250 × 4.6 mm i.d., particle size 5µm; Phenomenex) at ambient temperature and eluted with methanol: water (65: 35 v/v) pH 2.8 adjusted H3PO4) with a flow rate of 1.0 mL/min. The UV detector was set at 210 nm. The concentration–response relationship from the present method indicated linearity over a concentration range of 10 to 30 000 ng/mL. Biological fluids were thereby sampled following a

dose of evodiamine (1 g/kg, p.o.). The oral bioavailability was estimated to be about 0.1% in the conscious rat system.

3. Determination of andrographolide in human plasma by high-performance liquid chromatography/mass spectrometry (Gu et al., 2007).

In this study, a rapid method based on high-performance liquid chromatography/electrospray–mass spectrometry (HPLC/ESI-MS) method for the quantitative determination of andrographolide (AND) in human plasma was developed and validated. A liquid–liquid extraction (LLE) procedure was selected to isolate AND from biological matrixes. Isosorbide-5-mononitrate (IS-5-MN) was selected as the internal standard (IS). This validated method can be used to assess the bioavailability and pharmacokinetics of the drug.

4. **Pharmacokinetic and oral bioavailability of andrographolide from *Andrographis paniculata* fixed combination Kan Jang in rats and human (Panossian *et al*. 2000).**

The pharmacokinetics of andrographolide (AND) in rats following intravenous and oral (the main route) administration were studied. The bio-availability of AND was investigated at two levels of dosage. It was found that AND is rapidly and almost totally (91 %) absorbed into the blood after oral administration of *Andrographis paniculata* extract (APE) at a therapeutic dose (20 mg/kg). It has been shown that AND has a high affinity for human serum albumin and 61.2 % of AND was bound to serum proteins. Only 40 % of AND can be absorbed into the tissues and blood cells of the organism. In the phase of absorbtion, the concentration of AND in the blood was not, practically, changed during the first 1-1.5 hours and increased to maximal level (548 ng/ml) 2 hours after administering Kan Jang.

C. Reported herb-drug interactions for *Andrographis paniculata Nees*:

1. Herb–drug interaction of *Andrographis paniculata* extract and andrographolide on the pharmacokinetics of theophylline in rats (Chien *et al*. 2010).

Study developed a HPLC/UV method for the simultaneous determination of five major compounds in *Andrographis paniculata* extract (APE). Based on the results, pretreatment of andrographolide (AG) increased elimination of theophylline, and chronic use of *Andrographis paniculata* could elevate the concentration of theophylline in the blood. The study suggested that there should be a warning for the interaction with APE and its herbal ingredients with CYP1A2 substrates. Studies were suggested to clarify the effect of other herbal ingredients of APE on

theophylline. People taking preparations of *Andrographis paniculata* should be alerted to the possibility of herb–drug interaction.

2. The effect of Kan Jang extract on the pharmacokinetics and pharmacodynamics of warfarin in rats. (Hovhannisyan *et al*. 2006)

The aim of this study was to determine whether concomitant treatment of rats with Kan Jang (a standardized fixed combination of extracts from *Andrographis paniculata* and Eleutherococcus senticosus) and warfarin would lead to an alteration in the pharmacological effects of warfarin. The concentration of warfarin in blood plasma was measured by capillary electrophoresis using 50mM borate buffer (pH 9.3) as mobile phase with simultaneous detection of warfarin at 208.1 and 307.5 nm. Prothrombin time in blood plasma was measured using thromboplastin reagent. The concomitant application of Kan Jang and warfarin did not produced significant effects on the pharmacokinetics of warfarin and practically no effect on its pharmacodynamics.

D. Reported CYP activities for *Andrographis paniculata Nees*:

1. Effects of *Andrographis paniculata* extract and Andrographolide on hepatic cytochrome P450 mRNA expression and monooxygenase activities after *in vivo* administration to rats and *in vitro* in rat and human hepatocyte cultures (Pekthong *et al*. 2008).

The ability of *Andrographis paniculata* extract (APE) and Andrographolide (AND) to modulate hepatic CYP expression was examined *in vivo* in rats and *in vitro* in rat and human hepatocyte cultures. After *in vivo* administration, APE at dose levels of 0.5 g/kg/day (i.e. 5 mg/kg/day AND equivalents) and at 2.5 g/kg/day (i.e. 25 mg/kg/day AND equivalents) and AND at dose levels of 5 and 25 mg/kg/day significantly decreased CYP2C11 activity. In primary cultures of rat and human hepatocytes, treatment with AND 50μM and APE-containing 50μM AND also resulted in significant decreases in CYP2C expression and activity. In addition, in human hepatocytes, treatment with APE and AND 50μM resulted in a decrease in CYP3A expression and activity. In conclusion, this study suggested that AND and APE could cause herb–drug interactions in humans through modulation of CYP2C9 and CYP3A4 expression and activities. The study clearly documented the potential for APE and its pure constituent AND to decrease CYP2C expression *in vivo* in rat and *in vitro* in rat and human hepatocytes. Both CYP2C and CYP3A are responsible for the metabolism of a wide variety of commonly worldwide-prescribed medications. An increased blood plasma concentration of drugs that may result from decreased

activity of these enzymes has the potential to seriously adverse reactions in patients concurrently taking *Andrographis paniculata*.

2. Differential inhibition of rat and human hepatic cytochrome P450 by *Andrographis paniculata* extract (APE) and andrographolide (AND) (Pekthong *et al*. 2007).

This *in vitro* study with rat and human liver microsomal CYPs, APE inhibited the catalytic activities of both rat and human liver microsomal CYP1A2, CYP2C and of human liver microsomal CYP3A4. Based on *Ki* and IC50 values, these results suggested that APE could act as an anticarcinogen in humans because of its specific inhibitory effect on CYP1A2 activity. The inhibitory effect of APE on CYP3A and 2C9 activities cannot be excluded to cause drug–drug interactions, especially for CYP2C9 since its expression level in human liver is low and because it is known to metabolize several narrow therapeutic index drugs. Further study was in progress to fully assess the safety of AND and APE in term of CYP inhibition, after *in vivo* administration to rat and comparison of the *in vitro* effects in rat and human hepatocyte cultures.

3. Impact of *Andrographis paniculata* crude extract on mouse hepatic cytochrome P450 enzymes (Jarukamjorn *et al*. 2006).

Impact of *Andrographis paniculata* crude extract on mouse hepatic cytochrome P450 enzymes was evaluated by assessment of the P450 content and P450-associated activities. A significant increase in EROD and PROD activities suggested for the first time that *Andrographis paniculata* might effectuate hepatic cytochrome P450 enzymes of which CYP1A1 and CYP2B are the responsive P450 isoforms.

4. Strong synergistic induction of CYP1A1 expression by andrographolide plus typical CYP1A inducers in mouse hepatocytes (Jaruchotikamol *et al.* 2007).

The effects of andrographolide, the major diterpenoid constituent of *Andrographis paniculata*, on the expression of cytochrome P450 superfamily 1 members, including CYP1A1, CYP1A2 and CYP1B1 as well as on aryl hydrocarbon receptor (AhR) expression in primary cultures of mouse hepatocytes were investigated in comparison with the effects of typical CYP1A inducers, including benz[a]anthracene, β-naphthoflavone and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Andrographolide significantly induced the expression of CYP1A1 and CYP1A2 mRNAs in a concentration dependent manner, as did the typical CYP1A inducers, but did not induce that of CYP1B1 or AhR. Interestingly, andrographolide plus the typical CYP1A inducers synergistically induced CYP1A1 expression and the synergism was blocked by an AhR antagonist, resveratrol.

The CYP1A1 enzyme activity showed a similar pattern of induction. This was the first report that showed that andrographolide has a potency to induce CYP1A1 enzyme and indicates that andrographolide could be a very useful compound for investigating the regulatory mechanism of the CYP1A1 induction pathway. In addition, the findings suggested the preparing advice for rational administration of *Andrographis paniculata*, according to its ability to induce CYP1A1 expression.

5. Different AhR binding sites of diterpenoid ligands from *Andrographis paniculata* caused differential CYP1A1 induction in primary culture in mouse hepatocytes (Chatuphonprasert *et al*. 2011).

In the conducted study, influence of the four diterpenoids on CYP1A1 mRNA expression was investigated in primary cultured mouse hepatocytes. Diterpenoid constituents of *Andrographis paniculata* including Andro and DHA were found to induce CYP1A1 expression by themselves and to enhance BNF-induced expression. Molecular docking analysis of diterpenoids with mouse AhR-LBD could predict their potency on induction of CYP1A1. Furthermore, binding affinity of BNF to AhR-LBD was strengthened in the presence of Andro or DHA by the analysis, the potential of which was correlated with the degree of the enhancement of CYP1A1 mRNA induction. Exposure to environmental carcinogens in daily life is common, some of which are activated by CYP1A1, *Andrographis paniculata* used as health supplement or alternative medication, therefore, should be concerned in term of herb–drugs interactions on risk of carcinogenesis, according to its ability to induce CYP1A1 expression.

6. Gender-associated modulation of inducible CYP1A1 expression by andrographolide in mouse liver (Jarukamjorn *et al*. 2010).

The study described confirmation of an enhancing effect of andrographolide on the CYP1 family in vivo in the PAH-responsive C57BL/6 mouse. Andrographolide did not alter CYP1 expression in the PAH-nonresponsive DBA/2 mouse. The enhanced expression induced by andrographolide was observed in male C57BL/6 mice, but not in intact or ovariectomized females, or in orchiectomized male mice. However, treatment with testosterone restored the effect in both orchiectomized males and ovariectomized females. These observations indicate a male hormone-related system to be a crucial mediator of the modulation of CYP1 expression by andrographolide. Precautions should be taken regarding the use of *Andrographis paniculata* as

an alternative medication or health promotion, according to its distinctive characterization on sexually dimorphic modulation of CYP1A1 expression.

7. Synergistic increases of metabolism and oxidation–reduction genes on their expression after combined treatment with a CYP1A inducer and andrographolide (Chatuphonprasert *et al*. 2009)

The results revealed that andrographolide is a potent inducer of CYP2A4, and CYP2B9. Interestingly, andrographolide had a markedly synergistic effect on CYP1A1 and CYP1B1 in combination with a typical CYP1A inducer, β-NF. A risk assessment of andrographolide or *Andrographis paniculata* should be conducted, since the expression of genes, especially drug metabolism-related genes, might be affected. Among P450 isoforms, andrographolide by itself induced CYP1A1, CYP2A4, CYP2B9 and CYP2B10 expression. Synergistic expression of CYP1A1 and CYP1B1 mRNA was confirmed by quantitative RT-PCR. These observations suggested that drug interaction and risk assessment with the use of andrographolide or *Andrographis paniculata* should be elucidated.

8. *Andrographis paniculata*: a review of aspects of regulatory mechanisms of hepatic CYP1A enzymes (Jarukamjorn *et al.* 2008).

The crude extract of *Andrographis paniculata* has exhibited capability to increase hepatic CYP1A enzymes including ethoxyresorufin and methoxyresorufin activities, in accord with the inductive effects conveyed by andrographolide. Strong synergistic induction of CYP1A1 by co-treatment with andrographolide and a typical CYP1A inducer as well as a robust increase of CYP1A1 by andrographolide in which the induction was blocked by an AhR antagonist resveratrol, affirmed participation of AhR-mediated transcription activation on andrographolide-induced CYP1A1 expression. Therefore, some advice and precautions for rational use of *Andrographis paniculata* and/or andrographolide including some associated risks are of clear concern. Moreover, investigations not restricted to the AhR-mediated pathway revealing the synergistic mechanism of andrographolide on CYP1A1 induction as well as influences of other confounding substances in this herb and andrographolide analogues, which may contribute modulatory effects on regulation of P450s machinery, are needed and are yet to be elucidated.

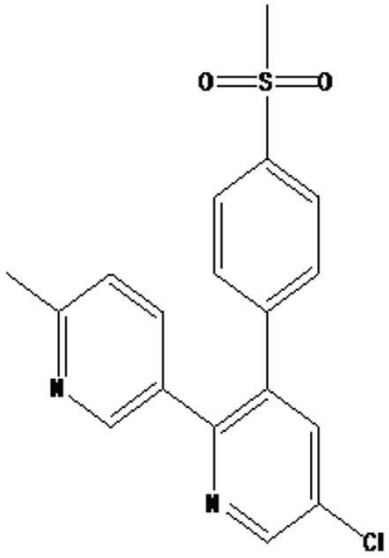
9. Cytochrome P450 induction properties of food and herbal-derived compounds using a novel multiplex RT-qPCR in vitro assay, a drug–food interaction prediction tool (Koe et al., 2014).

A multiplex RT-qPCR was developed to examine CYP1A2, CYP2D6, and CYP3A4 induction properties of compounds from food and herbal sources. Cytochrome induction can lead to serious drug–drug or drug–food interactions, especially if the co-administered drug plasma level is critical as it can reduce therapeutic effects and cause complications. Using this optimized multiplex RT-qPCR, cytochrome induction properties of andrographolide, curcumin, lycopene, bergamottin, and resveratrol were determined. Andrographolide, curcumin, and lycopene produced no significant induction effects on CYP1A2, CYP2D6, and CYP3A4. However, bergamottin appeared to be a significant in vitro CYP1A2 inducer. Resveratrol was found to be a weak in vitro CYP1A2 inducer. Examining the cytochrome induction properties of food and herbal compounds help complement CYP inhibition studies and provide labeling and safety caution for such products.

1.9.2. Etoricoxib:

Structural formula:

Figure 5: Chemical structure of Etoricoxib



Chemical name(IUPAC): 5-chloro-2-(6-methylpyridin-3-yl)-3-(4-methylsulfonylphenyl)pyridine

Molecular formula: $C_{18}H_{15}ClN_2O_2S$

Molecular weight: 358.8

CAS: 202409-33-4

λmax: (acetonitrile-phosphate buffer): 238, 280 nm

Appearance: White solid

Solubility: Etoricoxib is freely soluble in methanol, tetrahydrofuran, dimethyl sulfoxide, methyl ethyl ketone, dimethyl formamide, and chloroform. Etoricoxib is soluble in isopropyl acetate, ethanol and toluene, sparingly soluble in 2-propanol, and practically insoluble in water.

Melting Point: 127-128°

Therapeutic category: Anti-inflammatory; analgesic

Storage: -20º C

Half–life: 22 hr

Protein binding: Etoricoxib is extensively protein bound, primarily to plasma albumin, and has an apparent volume of distribution of 120 L in humans.

Mechanism of action: Etoricoxib selectively inhibits cyclooxygenase 2 (COX-2).

Pharmacodynamics: Etoricoxib is a COX-2 selective inhibitor (approximately 106 times more selective for COX-2 inhibition over COX-1). Currently it is approved in more than 60 countries worldwide but not in the US, where the Food and Drug Administration (FDA) require additional safety and efficacy data for etoricoxib before it will issue approval.

Metabolism: Etoricoxib is extensively metabolised with <1% of a dose recovered in urine as the parent drug. The major route of metabolism to form the 6'-hydroxymethyl derivative is catalyzed by CYP enzymes. CYP3A4 appears to contribute to the metabolism of etoricoxib *in vivo*. *In vitro* studies indicate that CYP2D6, CYP2C9, CYP1A2 and CYP2C19 also can catalyse the main metabolic pathway, but their quantitative roles *in vivo* have not been studied. Five metabolites have been identified in man. The principal metabolite is the 6'-carboxylic acid derivative of etoricoxib formed by further oxidation of the 6'-hydroxymethyl derivative. These principal metabolites either demonstrate no measurable activity or are only weakly active as COX-2 inhibitors. None of these metabolites inhibit COX-1.

Absorbtion: Bioavailability is 100% following oral administration.

Side effects: Weakness, fatigue, dizziness, headache, diarrhea, nausea, indigestion (dyspepsia), stomach pain or discomfort, heartburn, swelling of the legs due to fluid retention (oedema), increased blood pressure, Palpitations (unpleasant sensations of irregular and/or forceful beating of the heart).

Dose: 60 mg -120 mg daily.

Drug – drug interactions: CYP3A4 inhibitors or inducers; rifampicin, ethinyloestradiol; oral salbutamol and minoxidil. Antidepressant SSRIs and venlafaxine may increase risk of bleeding. Risk of side effects increased with concomitant use of aspirin, ciclosporin, ketorolac or other NSAIDs. Lithium and methotrexate, coumarins, phenindione, phenytoin and sulphonylureas

Literature Survey for Etoricoxib:

A. Anti-inflammatory and anti-rheumatoid activities of Etoricoxib:

1. Etoricoxib for arthritis and pain management. (Brooks and Kubler, 2006).

Selective cyclooxygenase (COX)-2 inhibitors, plays an important role in the pharmacologic management of arthritis and pain. Clinical trials have established the efficacy of etoricoxib in osteoarthritis, rheumatoid arthritis, acute gouty arthritis, ankylosing spondylitis, low back pain, acute postoperative pain, and primary dysmenorrhea. Comparative studies indicated at least similar efficacy with etoricoxib versus traditional NSAIDs. Etoricoxib was generally well tolerated in these studies with no new safety findings during long-term administration. The gastrointestinal, renovascular and cardiovascular tolerability profiles of etoricoxib have been evaluated in large patient datasets. The data suggested that etoricoxib is an efficacious alternative in the management of arthritis and pain, with the potential advantages of convenient once-daily administration and superior gastrointestinal tolerability compared with traditional NSAIDs.

B. Reported pharmacokinetic methods for Etoricoxib:

1. Determination of etoricoxib in human plasma using automated on-line solid-phase extraction coupled with LC-APCI/MS/MS. (Dalmora et al., 2008).

A validated liquid chromatography-tandem mass spectrometry method with atmospheric pressure chemical ionization (LC-APCI/MS/MS) was reported for the determination of etoricoxib in human plasma using antipyrin as internal standard, followed by on-line solidphase

extraction. The method was performed on a Luna C18 column and the mobile phase consisted of acetonitrile:water (95:5, v/v)/ammonium acetate (pH 4.0; 10 mM), run at a flow rate of 0.6 mL/min. The method was linear in the range of 1-5000 ng/mL (r2>0.99). The lower limit of quantitation was 1 ng/mL. The recoveries were within 93.72-96.18%.

2. **Absorption, metabolism, and excretion of etoricoxib, a potent and selective cyclooxygenase-2 inhibitor, in healthy male volunteers. (Rodrigues et al., 2003).**

Etoricoxib (100 µCi/dose) was administered to six healthy male subjects (i.v., 25 mg; p.o., 100 mg). The plasma clearance and the harmonic mean half-life was found to be 57 ml/min and 24.8 h resp. Etoricoxib accounted for the majority of the radioactivity (~75%) present in plasma following both i.v. and p.o. doses. Radiochromatographic analysis of the excreta revealed that etoricoxib was metabolized extensively, and only a minor fraction of the dose (<1%) was excreted unchanged. Etoricoxib is well absorbed in healthy male subjects and is metabolized extensively via oxidation (6'-methyl oxidation >1'-N-oxidation) and the metabolites are excreted largely in the urine.

3. Determination of two COX-2 inhibitors in serum and synovial fluid of patients with inflammatory arthritis by ultra performance liquid chromatography–inductively coupled plasma mass spectroscopy and quadrupole time-of-flight mass spectrometry. (Gika et al., 2009)

Two sulphur-containing COX-2 inhibitors celecoxib and etoricoxib were determined in the serum and synovial fluid of inflammatory arthritis patients using a sensitive ultra performance liquid chromatography–inductively coupled plasma mass spectroscopy (UPLC/ICPMS) method. Confirmation of the identity of the analytes in the samples was also performed by electrospray quadruple time-of-flight mass spectrometry in positive electrospray ionisation mode. The two COX-2 inhibitors were extracted from serum and synovial fluid following dilution with acetate buffer (pH 5) and liquid–liquid extraction (LLE) into ethyl acetate. Extracted samples were then analysed using UPLC/ICPMS with sulphur specific detection. The UPLC/ICPMS method was applied to the analysis of samples from patients receiving either 200 mg/day of celecoxib (2×100 mg), 90 mg/day etoricoxib or placebo.

4. **Development and validation of an HPLC method for analysis of Etoricoxib in human plasma. (Mandal et al., 2006).**

Determination of etoricoxib in human plasma was performed by a simple high-performance liquid chromatographic method. An aliquot quantity of 1 ml plasma sample was taken and 10 µl internal standard was added and mixed. Saturated borate solution of 0.3 ml was added to it and mixed for 1 minute followed by liquid-liquid extraction with ethyl acetate. Organic layer was separated and evaporated to dryness under nitrogen atmosphere at low temperature (below 50°). Residue was reconstituted with 150 µl of mobile phase. During the whole procedure the samples were protected from light. The assay was performed on Hypersil BDS, C18 (150×4.6 mm, 5 µ particle size) column, using 10 milimol ammonium acetate buffer:acetonitrile = 65:35 v/v as mobile phase with ultra violet detection at 235 nm. Stability study showed that after three freeze-thaw cycles the loss of three quality control samples were less than 10%. Samples were stable at room temperature for 12 h and at -20° for 3 months. Before injecting onto HPLC system, the processed samples were stable for at least 8 h. The method was used to perform bioequivalence study in human.

5. **Validated liquid chromatographic ultraviolet method for the quantitation of Etoricoxib in human plasma using liquid–liquid extraction. (Ramakrishna et al, 2005).**

A simple, sensitive and specific HPLC method with UV detection (284 nm) was developed and validated for quantitation of Etoricoxib in human plasma. Following a single-step liquid–liquid extraction with diethyl ether/dichloromethane (70/30, v/v), the analyte and internal standard (Zaleplon) were separated using an isocratic mobile phase of water/acetonitrile (58/42, v/v) on reverse phase Waters symmetry® C18 column. A linear range of 5–2500 ng/mL was established. Stability of Etoricoxib in plasma was more than 90%, with no evidence of degradation during sample processing (autosampler) and 30 days storage in a freezer. This validated method is sensitive and simple with between-batch precision of less than 6% and was used in pharmacokinetic studies.

6. Single- and Multiple-Dose Pharmacokinetics of Etoricoxib, a Selective Inhibitor of Cyclooxygenase-2, in Man. (Agrawal et al, 2003).

The single- and multiple-dose pharmacokinetics of etoricoxib was examined in two clinical studies. Single-dose pharmacokinetics including dose proportionality, absolute bioavailability of the highest dose-strength (120-mg) tablet and the effect of a high-fat meal on the bioavailability

of the tablet were investigated in a two-part, open, balanced crossover study in two panels of healthy subjects (12 per panel). Steady-state pharmacokinetics was investigated in an open-label study in which 24 healthy subjects were administered 120-mg single and multiple (once daily for 10 days) oral doses of etoricoxib tablets. The pharmacokinetics of etoricoxib were found to be consistent with linearity through doses at least twofold greater than the highest anticipated clinical dose of 120 mg. Etoricoxib administered as a tablet was rapidly and completely absorbed and available; the absolute bioavailability was estimated to be 100%. A high-fat meal decreased the rate of absorption without affecting the extent of absorption of Etoricoxib which suggests the dosing of Etoricoxib irrespective of food. Steady-state pharmacokinetics of Etoricoxib, achieved following 7 days of once-daily dosing, were found to be reasonably predicted from single doses.

7. Determination of etoricoxib in human plasma by liquid chromatography–tandem mass spectrometry with electrospray ionization. (Brautigam et al, 2003).

A validated liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was developed for the determination of etoricoxib in human plasma with phenazone as internal standard. The plasma samples were extracted by solid-phase extraction using polymer-based cartridges. Chromatography was carried out on a short, narrow bore RP C column (3032 mm). Detection was achieved by a Sciex API 3000 triple quadrupole mass 18 spectrometer equipped with a turbo ion spray source working in positive ion mode. The respective mass transitions used for quantification of etoricoxib and phenazone were m/z 359.2→280.2 and m/z 189.0→104.1. The analytical method was validated over the concentration range 0.2–200 ng/ ml. The method is applicable to pharmacokinetic studies in humans.

8. Stability indicating high performance liquid chromatographic assay for the pharmacokinetics of cyclooxygenase (COX-2) inhibitor etoricoxib in rats. (Radwan et al., 2009).

An accurate, sensitive and simple high-performance liquid chromatography (HPLC) assay with UV detection was developed and validated for the simultaneous determination of Etoricoxib and its internal standard (IS) Flurbiprofen in plasma. Plasma samples were cleaned up by protein precipitation followed by solvent evaporation and reconstitution with the mobile phase, an aliquot of the resulted solution were injected into the chromatograph. Peaks were eluted from a Novapak-C8 column using a mobile phase consisting of acetic acid: triethylamine: acetonitrile:

water (0.02: 0.01: 41: 59.97, v/v), pH 4.0 at flow rate of 1 ml/min. The detection wavelength was 245 nm. 200 µL of plasma were required for Etoricoxib determination. The run time was 10 min with etoricoxib and IS eluted in 3.8 and 7.2 min, respectively. The assay was applied to determine the pharmacokinetics of Etoricoxib in rats after 15 mg/kg oral dose. Etoricoxib plasma concentrations time profile follows two compartmental open models with fast distribution and slow elimination phases. This assay was being utilized in determining etoricoxib pharmacokinetics in animals to monitor its interactions with other drugs or food supplements in the laboratory.

C. Reported herb-drug interactions for Etoricoxib:

1. Pharmacokinetics and Cardiovascular Effect of Etoricoxib in the Absence or Presence of St. John's Wort in Rats. (Radwan et al, 2012).

The effect of chronic administration of Etoricoxib (EXB), in the absence or presence of St. John's Wort (SJW), on its pharmacokinetic parameters and blood pressure was investigated in rats. Rats were divided into 3 groups; each group received daily different oral treatment for 3 weeks. Rats blood pressures were monitored initially, after 1 and 3 weeks of treatment and after 1 week of discontinuing dosing of both drugs. EXB pharmacokinetic parameters in the absence or presence of SJW were calculated after 3 weeks. SJW was significantly affected EXB pharmacokinetic parameters. The steady state peak plasma concentration and terminal half-life were reduced by 32 % and 91 %, respectively, due to a more than 3 fold increase in its apparent clearance which is a concentration and time dependent effect. EXB was significantly increased ($P < 0.001$) rat blood pressure while, co-administration of EXB and SJW was not significantly affected ($P > 0.05$) as compared to the control. Monitoring blood pressure of patients anticipated taking EXB for extended period should be advised. The co-administration of SJW with EXB should be avoided since SJW would greatly reduce EXB concentrations by inducing its metabolism.

D. Reported CYP activities for Etoricoxib:

1. **Role of human liver cytochrome P4503A in the metabolism of etoricoxib, a novel cyclooxygenase-2 selective inhibitor. (Kassahun et al., 2001).**

Etoricoxib was shown to be metabolized via 6'-methylhydroxylation (M2 formation) when incubated with NADPH-fortified human liver microsomes. Similar to in vivo data, 1'-*N'*-oxidation was a relatively minor pathway. Over the etoricoxib concentration range studied (1–1300 mM), the rate of hydroxylation conformed to saturable Michaelis-Menten kinetics (apparent *K*m = 186 ± 84.3 mM; *V*max = 0.76 ± 0.45 nmol/min/mg of protein; mean ± S.D., *n* = 3 livers) and yielded a *V*max/Km ratio of 2.4 to 7.3 ml/min/mg. This in vitro *V*max/*K*m ratio was scaled, with respect to yield of liver microsomal protein and liver weight, to obtain estimates of M2 formation clearance (3.1–9.7 ml/min/kg of b.wt.) that agreed favorably with in vivo results (8.3 ml/min/kg of b.wt.) following i.v. administration of [14C] etoricoxib to healthy male subjects. Cytochrome P450 (P450) reaction phenotyping studies—using P450 form selective chemical inhibitors, immunoinhibitory antibodies, recombinant P450s, and correlation analysis with microsomes prepared from a bank of human livers—revealed that the 6'-methyl hydroxylation of etoricoxib was catalyzed largely (~60%) by member(s) of the CYP3A subfamily. By comparison, CYP2C9 (~10%), CYP2D6 (~10%), CYP1A2 (~10%), and possibly CYP2C19 played an ancillary role. Moreover, etoricoxib (0.1–100 mM) was found to be a relatively weak inhibitor (IC_{50} > 100 mM) of multiple P450s (CYP1A2, CYP2D6, CYP3A, CYP2E1, CYP2C9, and CYP2C19) in human liver microsomes.

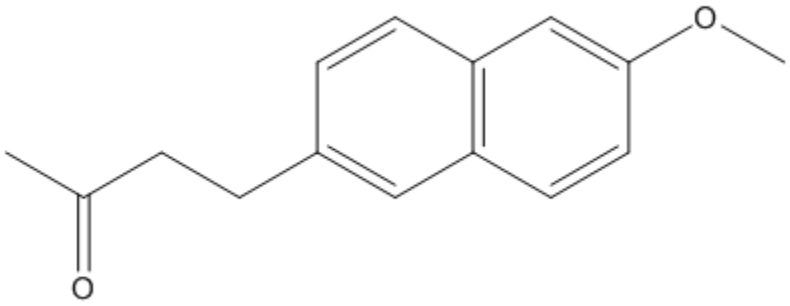
2. **In Vitro Metabolism Considerations, Including Activity Testing of Metabolites, in the Discovery and Selection of the COX-2 Inhibitor Etoricoxib (MK-0663). (Chauret et al., 2001).**

Characterization of the metabolites of the etoricoxib (MK-0663 and L-791,456) produced in vitro indicate formation of an N-oxide pyridine and hydroxymethyl pyridine that can further be glucuronidated or oxidized to an acid. Significant turnover was observed in human hepatocytes. Several CYPs were involved in the oxidative biotranformations and from in vitro studies; etoricoxib was not a potent CYP3A4 inducer or inhibitor. Based on an in vitro whole blood assay, none of the metabolites of etoricoxib inhibited COX-1 or contributed significantly to the inhibition of COX-2.

1.9.3. Nabumetone:

Structural formula:

Figure 6: Chemical structure of Nabumetone



Chemical name (IUPAC): 2-Butanone,4-(6-methoxy-2-naphthalenyl)-.
4-(6-Methoxy-2-naphthyl)-2-butanone.

Molecular formula: $C_{15}H_{16}O_2$

Molecular weight: 228.2863

CAS: 42924-53-8

λmax: 261 nm in methanol.

Appearance: White or almost white, crystalline powder.

Solubility: Practically insoluble in water, freely soluble in acetone, slightly soluble in methanol.
Melting Point: 80-81ºC

Therapeutic category: Anti-inflammatory, antineoplastic agent.

Storage: Protected from light.

Half–life: Approximately 23 hours for the active metabolite, 6MNA. Increased in patients with renal insufficiency.

Protein binding: The active metabolite, 6MNA, is more than 99% bound to plasma proteins.

Mechanism of action: The parent compound is a prodrug, which undergoes hepatic biotransformation to the active component, 6-methoxy-2-naphthylacetic acid (6MNA), that is a potent inhibitor of prostaglandin synthesis, most likely through binding to the COX-2 and COX-1 receptors.

Pharmacodynamics: Nabumetone is a naphthylalkanone. It is a non-selective prostaglandin G/H synthase (a.k.a. cyclooxygenase or COX) inhibitor that acts on both prostaglandin G/H synthase 1 and 2 (COX-1 and -2). Prostaglandin G/H synthase catalyzes the conversion of arachidonic acid to prostaglandin G2 and prostaglandin G2 to prostaglandin H2. Prostaglandin H2 is the precursor to a number of prostaglandins involved in fever, pain, swelling, inflammation, and platelet aggregation. The parent compound is a prodrug that undergoes hepatic biotransformation to the active compound, 6-methoxy-2-naphthylacetic acid (6MNA). The analgesic, antipyretic and anti-inflammatory effects of NSAIDs occur as a result of decreased prostaglandin synthesis.

Metabolism: Undergoes rapid biotransformation to the principal active metabolite, 6-methoxy-2-naphthylacetic acid (6MNA). Approximately 35% of a 1000 mg oral dose of nabumetone is converted to 6MNA and 50% is converted into unidentified metabolites which are subsequently excreted in the urine.

Absorbtion: Well absorbed from the gastrointestinal tract. Coadministration of food increases the rate of absorption and subsequent appearance of 6MNA (the active metabolite) in the plasma but does not affect the extent of conversion of nabumetone into 6MNA.

Side effects: Diarrhea, dyspepsia, and abdominal pain, heme positive stools, dizziness, headache, edema, constipation, skin rash, nausea, and flatulence.

Dose: The recommended starting dose is 1,000 mg taken as a single dose with or without food. Some patients may obtain more symptomatic relief from 1,500 mg to 2,000 mg per day. Nabumetone tablets can be given in either single or twice-daily dose. Dosages greater than 2,000 mg per day have not been studied. The lowest effective dose should be used for chronic treatment. Patients weighing under 50 kg may be less likely to require dosages beyond 1,000 mg; therefore, after observing the response to initial therapy, the dose should be adjusted to meet individual patients' requirements.

Drug – drug interactions: Acenocoumarol, Alendronate, Anisindione, Colesevelam, Cyclosporine, Dicumarol, Ginkgo biloba, Methotrexate, Telmisartan, Timolol, Trandolapril, Treprostinil, Warfarin.

Literature Survey For Nabumetone:

A. Anti-inflammatory and anti-rhumatoid activities of Nabumetone:

1. **Nabumetone: therapeutic use and safety profile in the management of osteoarthritis and rheumatoid arthritis. (Hedner et al., 2004).**

This is a review article about Nabumetone which itself is non-acidic and after following absorption, it undergoes extensive first-pass metabolism to form the main circulating active metabolite (6-MNA) which is a much more potent inhibitor of preferentially cyclo-oxygenase (COX)-2. The three major metabolic pathways of nabumetone are O-demethylation, reduction of the ketone to an alcohol, and an oxidative cleavage of the side-chain occurs to yield acetic acid derivatives. Essentially no unchanged nabumetone and < 1% of the major 6-MNA metabolite are excreted unchanged in the urine from which 80% of the dose can be recovered and another 10% in faeces. Nabumetone is clinically used mainly for the management of patients with osteoarthritis (OA) or rheumatoid arthritis (RA) to reduce pain and inflammation. Compared with COX-2 selective inhibitors, nabumetone exhibits similar anti-inflammatory and analgesic properties in patients with arthritis and there is no evidence of excess GI or other forms of complications to date.

B. Reported pharmacokinetic methods for Nabumetone:

1. **Clinical pharmacokinetics of nabumetone. The dawn of selective cyclo-oxygenase-2 inhibition? (Davies, 1997.)**

   Nabumetone and its metabolites bind extensively to plasma albumin. Nabumetone is eliminated following biotransformation to 6-MNA, which does not undergo enterohepatic circulation and the respective glucoroconjugated metabolites are excreted in urine. Substantial concentrations of 6-MNA are attained in synovial fluid, which is he proposed site of action in chronic inflammatory arthropathies. A smaller area under the plasma concentration-time curve (AUC) is evident at steady state as compared with a single dose; this is possibly due to an increase in the volume of distribution and saturation of protein binding. Renal failure significantly reduces 6-MNA elimination but steady-state concentrations of 6-MNA are not increased, possibly because of nonlinear protein binding. Elderly patients with osteoarthritis demonstrate decreased elimination and increased plasma concentrations of nabumetone as compared with young healthy volunteers. Rheumatic disease activity also influences 6-MNA plasma concentrations, as patients with more active disease and lower serum albumin concentrations demonstrate a lower area under the plasma concentration versus time curve. A reduced bioavailability of 6-MNA in patients with severe hepatic impairment is also evident. Dosage adjustment may be required in the elderly, patients with active rheumatic disease and those with hepatic impairment, but not in patients with mild-to-moderate renal failure.

2. **An overview of the clinical pharmacokinetics of nabumetone. (Hyneck, 1992).**

   This review stated, 80% of nabumetone is absorbed, and food, milk, and aluminum antacids increase the rate, but not the extent, of absorption. It is rapidly metabolized in the liver to 6-methoxy-2-naphthylacetic acid (6MNA), the major circulating active metabolite. At steady state, the time to maximum plasma concentration for 6MNA is 1 to 4 h. 6MNA has a very low clearance rate and long half-life (about 24 h). In general, steady state plasma concentrations of 6MNA increase in proportion with increases in the dose of nabumetone administered. Steady state plasma concentrations of 6MNA in the elderly are slightly higher than in healthy volunteers; however, this does not appear to be clinically significant.

Preliminary studies in patients with impaired renal function indicate that the pharmacokinetics is not altered in patients with mild to moderate disease.

3. **Identification and determination of phase II nabumetone metabolites by high-performance liquid chromatography with photodiode array and mass spectrometric detection. (Nobilis et al., 2004).**

   In this work, various high-performance liquid chromatography (HPLC) methods were employed in the evaluation of xenobiochemical experiments leading to the identification and determination of phase II nabumetone metabolites. Optimal conditions for the quantitative enzymatic deconjugation of phase II metabolites were found for the samples of minipig bile, small intestine contents and urine. Comparative HPLC analyses of the samples of above-mentioned biomatrices and of the same biomatrices after their enzymatic treatment using β-glucuronidase and arylsulfatase afforded the qualitative and quantitative information about phase II nabumetone metabolites. Hereby, three principal phase II nabumetone metabolites (ether glucuronides) were discovered in minipig's body fluids and their structures were confirmed using liquid chromatography (LC)–electrospray ionization mass spectrometric (MS) analyses.

4. **Analysis of nabumetone in human plasma by HPLC Application to single dose pharmacokinetic studies. (Kobylinska et al., 2003).**

   A simple and sensitive high performance liquid chromatography method for the determination of nabumetone in human plasma was described. The procedure involved liquid- liquid extraction with ethyl acetate and reversed-phase chromatography with fluorimetric detection (excitation 230 nm, emission 356 nm). The suitability of the method was shown for pharmacokinetic studies.

5. **Extractionless determination of 6-methoxy-2-naphthylacetic acid, a major metabolite of nabumetone, in human plasma by high-performance liquid chromatography. (Jager et al., 2000).**

   Following oral administration of the prodrug nabumetone, the major metabolite 6-methoxy-2-naphthylacetic acid (6-MNA) was determined in human plasma. Minimal sample preparation was followed by reversed-phase liquid chromatography and UV detection, affording high sample throughput. The lower limit of quantification (LLOQ) was 70 ng/ ml,

at a signal-to-noise ratio of 8:1. The assay method displayed good correlation (*r*=0.997), and suggested its application in pharmacokinetic and bioequivalence studies.

6. **Simultaneous analysis of naproxen, nabumetone and its major metabolite 6-methoxy-2-naphthylacetic acid in pharmaceuticals and human urine by high-performance liquid chromatography. (Mikami et al., 2000).**

   A high-performance liquid chromatographic (HPLC) method for simultaneous determination of naproxen (NAP), nabumetone (NAB) and its major metabolite, 6-methoxy-2-naphthylacetic acid (6-MNA), was developed for the application to pharmaceuticals and human urine. Isocratic reversed-phase HPLC was employed for quantitative analysis using triethylamine and 1-heptanesulfonic acid sodium salt (HSA) as ion-pair reagents. Urine samples were purified by solid-phase extraction using Bond-Elut Certify II cartridges containing reversed-phase and anion exchange functionalities. The HPLC assay was carried out using a Wakosil ODS 5C18 column (5 mm, 150_4.6 mm, i.d.). The mobile phase consisted of 0.5 g of HSA dissolved in 1000 ml of a mixture of acetonitrile, water and triethylamine (500:500:1, v:v) adjusted with phosphoric acid to pH 3. The calibration curves of NAP and NAB showed good linearity in the concentration range 32–160 μg/ml with UV detection (270 nm) for pharmaceuticals. In the low concentration ranges (8–96 ng of NAP per ml, 24–288 ng of NAB per ml and 5.6–67.2 ng of 6-MNA per ml), the calibration curves were also obtained with fluorimetric detection (excitation 280 nm, emission 350 nm) for biological fluids. The procedure described in this study was rapid, simple, selective, and is suitable for routine analysis of pharmaceuticals and pharmacokinetic studies in human urine samples.

7. **Comparative biotransformation and disposition studies of nabumetone in humans and minipigs using high-performance liquid chromatography with ultraviolet, fluorescence and mass spectrometric detection. (Nobilis et al., 2003).**

   The disposition of the non-steroidal anti-inflammatory drug (NSAID) nabumetone after a single oral dose administration of nabumetone tablets to humans and minipigs was investigated. Standards of the metabolites were prepared using simple synthetic procedures and their structures were confirmed by NMR and mass spectrometry. A simple HPLC method for the simultaneous determination of nabumetone, 6-MNA and the other metabolites was developed, validated and used for xenobiochemical and pharmacokinetic studies in humans

and minipigs and for distribution studies in minipigs. Naproxen was chosen as the internal standard (I.S.), both UV (for higher concentrations) and fluorescence detection (for very low concentrations) was used. The identity of the nabumetone metabolites in biological samples was confirmed using HPLC-MS experiments. Pharmacokinetics of nabumetone, 6-MNA and 6-HNA (6-hydroxy-2-naphthylacetic acid) in human and minipig plasma was evaluated and compared. The concentration levels of nabumetone metabolites in urine, bile and synovial fluid were also evaluated.

8. **Simplex optimization of the variables affecting the micelle-stabilized room temperature phosphorescence of 6-methoxy-2-naphthylacetic acid and its kinetic determination in human urine. (Pulgarin et al., 2005).**

This article reported the kinetic determination of 6-methoxy-2-naphthylacetic acid (6-MNA), the major metabolite of nabumetone, from micelle-stabilized room temperature phosphorescence (MS–RTP) measurements made by using the stopped-flow mixing technique. This methodology allows one to determine analytes in complex matrices without the need for a tedious separation process. It also shortens analysis times substantially. The proposed method used simplex methodology to optimize the chemical and instrumental variables affecting the phosphorescence. It was applied to the determination of 6-MNA in human urine.

9. **Simple and rapid determination of the active metabolite of nabumetone in biological fluids by heavy atom-induced room temperature phosphorescence. (Pulgarin et al., 2005a).**

A simple, selective and sensitive heavy atom induced-room temperature phosphorimetric method was described for the determination of 6-methoxynaphthylacetic acid, main metabolite of nabumetone, in biological fluid. This technique enables to determine analytes in complex matrices without the need for a tedious prior separation process. Optimized conditions for the determination were 0.12M TlNO3. An amount of 0.025M sodium sulfite and pH 7.5 (adjusted with 0.1M sodium hydrogen phosphate-dihydrogen phosphate buffer solution). The delay time, gate time and time between flashes were 180, 1500μs, and 5 ms, respectively. The maximum phosphorescence signal appeared instantly and the intensity was measured at λex = 328.4 nm

and λem = 544.4 nm. This method was successfully applied to the analysis of 6-methoxynaphthylacetic acid in human serum and urine.

10. **High-performance liquid chromatographic determination of a new anti-inflammatory agent, nabumetone, and its major metabolite in plasma using fluorimetric detection. (Ray and Day, 1984).**

This paper described a high-performance liquid chromatographic assay (HPLC) with fluorescence detection, developed for the determination of nabumetone and its major metabolite (II) in plasma. Separations were performed on a 5-pm Ultrasphere ODS reversed-phase column (25 cm X 4.6 mm I.D.) with a Lichrosorb RP-8 guard column. The compounds were eluted with a mobile phase of methanol in 0.05 mol/l sodium acetate buffer (pH 3.0), 70:30, v/v. The column temperature was 40°C and the flow rate was 1 ml/min.

11. **A review of analytical techniques for determination of oxicams, nimesulide and nabumetone. (Starek and Krzek, 2009).**

This review submitted the use of various analytical techniques for the determination of oxicams, nimesulide and nabumetone. This review covered the time period from 1990 to 2008 during which over 200 analytical methods including all types of chromatographic, spectrophotometric and voltammetric techniques were reported. Presented application concerned analysis of chosen NSAIDs from pharmaceutical formulations and biological samples.

C. Reported herb-drug interactions for Nabumetone: NIL

D. Reported CYP activities for Nabumetone:

1. **Metabolism of nabumetone (BRL 14777) by various species including man. (Haddock et al., 1984).**

Radiotracer methodology was used to study the metabolic fate of 4-(6-methoxy-2-naphthyl)-butan-2-one (nabumetone) after oral administration to rats, mice, rabbits, dogs, rhesus monkeys and healthy human subjects. Parent compound was not detected in plasma and urine and the major circulating metabolite in all species was identified as 6-methoxy-2-naphthylacetic acid, a compound known to possess anti-inflammatory activity. Metabolites were mainly excreted in urine from which four principal metabolites were isolated and identified by mass spectrometry and independent synthesis. Pathways involving O-demethylation, reduction of the ketone group

and oxidation of the butanone side-chain to acetic acid occurred in all species, but the ratios of the metabolic end-products tended to be species dependent. In the rat about half of the administered nabumetone was oxidized to the pharmacologically active acid metabolite.

2. **A Predominate Role of CYP1A2 for the Metabolism of Nabumetone to the Active Metabolite, 6-Methoxy-2-naphthylacetic Acid, in Human Liver Microsomes. (Turpeinen et al., 2009).**

In the present study, several in vitro approaches were used to identify the cytochrome P450 (P450) enzyme(s) responsible for 6-MNA formation. 6-MNA activity correlated strongly with both CYP1A2- mediated phenacetin *O*-deethylation activity and CYP1A2 protein content (r = 0.85 and 0.74, respectively; $p < 0.0001$ for both). Minor fractions were catalyzed by recombinant P450s CYP1A1, CYP2B6, CYP2C19, CYP2D6, and CYP2E1. Experiments with P450-selective chemical inhibitors and monoclonal anti-P450 antibodies showed that furafylline, a mechanism based inhibitor CYP1A2, and anti-CYP1A2 antibody markedly inhibited 6-MNA formation, whereas inhibitors for other P450s did not show significant inhibitory effects. Taken together, these studies indicate that the formation of the active metabolite of nabumetone, 6-MNA, is predominantly catalyzed by CYP1A2 in Human Liver Microsomes with only minor contribution of other P450s.

3. **In Vitro Characterization of the Cytochrome P450 Isoforms Involved in the Metabolism of 6-Methoxy-2-napthylacetic Acid, an Active Metabolite of the Prodrug Nabumetone. (Matsumoto et al., 2011).**

The cytochrome P450 (CYP) isoforms that catalyze the oxidation metabolism of 6-methoxy-2-napthylacetic acid (6-MNA), an active metabolite of nabumetone, were studied in rats and humans. Using an extractive reversed-phase HPLC assay with fluorescence detection, monophasic Michaelis–Menten kinetics was obtained for the formation of 6-hydroxy-2-naphthylacetic acid (6-HNA) in liver microsomes of rats and humans. The CYPs responsible for metabolism of 6-MNA in liver microsomes of rats and humans were identified using correlation study, recombinant CYP supersomes, specific CYP inhibitors and antibodies. Among 14 recombinant rat CYPs examined, CYP2C6, CYP2C11 and CYP1A2 were involved in the metabolism of 6-MNA. These findings suggested that CYP2C9 has the highest catalytic activity

of 6-MNA metabolism in humans. In contrast, metabolism of 6-MNA was suggested to be mediated mainly by CYP2C6 and CYP2C11 in rats.

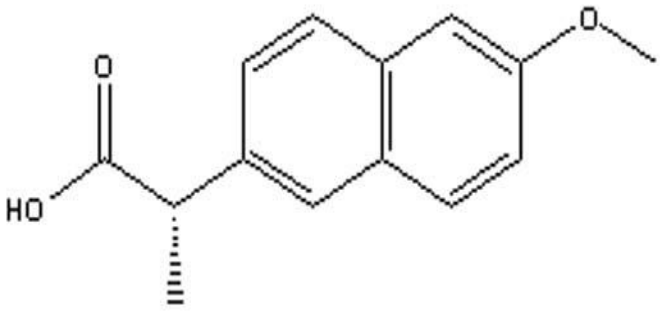
4. Carbon-Carbon Bond Cleavage in Activation of the prodrug Nabumetone. (Varfaj et al., 2014).

A CYP1A2-catalyzed carbon-carbon bond cleavage reaction is required for conversion of the prodrug nabumetone to its active form, 6-methoxy-2-naphthylacetic acid (6-MNA). Despite worldwide use of nabumetone as an anti-inflammatory agent, the mechanism of its carbon-carbon bond cleavage reaction remained obscure. With the help of authentic synthetic standards, this study reported that the reaction involved 3-hydroxylation, carbon-carbon cleavage to the aldehyde, and oxidation of the aldehyde to the acid, all catalyzed by CYP1A2 or less effectively by other P450 enzymes. The data indicated that the carbon-carbon bond cleavage is mediated by the ferric peroxo anion rather than the ferryl species in the P450 catalytic cycle. CYP1A2 also catalyzes O-demethylation and alcohol to ketone transformations of nabumetone and its analogues.

1.9.4. Naproxen

Structural formula:

Figure 7: Chemical structure of Naproxen



Chemical name (IUPAC): (2S)-2-(6-methoxynaphthalen-2-yl) propanoic acid

Molecular formula: $C_{14}H_{14}O_3$

Molecular weight: 230.26

CAS: 22204-53-1

λmax: 271 nm in methanol

Appearance: White or almost white, crystalline powder.

Solubility: Practically insoluble in water, soluble in alcohol and in methanol at low pH and freely soluble in water at high pH.

Melting Point: 153.82 °C

Therapeutic category: Anti-inflammatory; analgesic

Storage: It should be stored in well-closed containers at 15 - 30 deg C and the containers should be light resistant.

Half–life: The elimination half-life of Naproxen ranges from 12 to 20 hours.

Protein binding: Plasma protein binding was 98% for naproxen.

Mechanism of action: The mechanism of action of the Naproxen anion, like that of other NSAIDs, is not completely understood but may be related to prostaglandin synthetase inhibition.

Pharmacodynamics: Naproxen is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. The mechanism of action of the naproxen anion, like that of other NSAIDs, is not completely understood but may be related to prostaglandin synthetase inhibition.

Metabolism: Naproxen is extensively metabolized in the liver to 6-desmethylnaproxen. Approximately 95% of the drug is excreted in urine as unchanged naproxen (less than 1%) and 6-desmethylnaproxen (less than 1%) and their glucuronide or other conjugates (66-92%).

Absorbtion: Naproxen is rapidly and completely absorbed from the gastrointestinal tract with an in vivo bioavailability of 95%.

Side effects: heart attack, stroke, high blood pressure, heart failure from body swelling (fluid retention), kidney problems including kidney failure, bleeding and ulcers in the stomach and intestine, low red blood cells (anemia), life-threatening skin reactions, life-threatening allergic reactions, liver problems including liver failure, asthma attacks in people who have asthma.

Dose: 250-500 mg orally twice a day.

Drug – drug interactions: Angiotensin Converting Enzyme (ACE)-inhibitors/Angiotensin Receptor Blockers, Antacids and Sucralfate, Aspirin, Cholestyramine, Diuretics, Lithium, Methotrexate, Warfarin, Selective Serotonin Reuptake Inhibitors (SSRIs). Naproxen is highly

bound to plasma albumin; it thus has a theoretical potential for interaction with other albumin-bound drugs such as coumarin-type anticoagulants, sulphonylureas, hydantoins, other NSAIDs, and aspirin.

Literature Survey for Naproxen:

A. Anti-inflammatory and anti-rhumatoid activities of Naproxen

1. NO-naproxen modulates inflammation, nociception and downregulates T cell response in rat Freund's adjuvant arthritis. (Cicala et al., 2000).

Freund's adjuvant (FA) arthritis was induced in rats by a single intraplantar injection into the right hindpaw of 100 ml of mycobacterium butirricum (6 mg ml^{-1}). The effect of equimolar doses of naproxen (1, 3 and 10 mg kg^{-1}) and NO-naproxen (1.5, 4.5 and 16 mg kg^{-1}) was evaluated using two dosage regimen protocols: (i) preventive, starting oral administration of the drugs at the time of induction of arthritis and for the following 21 days (day 1 ± 21); (ii) therapeutic, starting oral administration of the drugs 7 days after adjuvant injection and for the following 14 days (day 7 ± 21). Hindpaw swelling (days 3, 7, 11, 14, 17, 21) and nociception (days 15 and 21) were measured. On day 22 rats were sacrificed, draining lymph nodes were removed and T cells isolated. In vitro proliferation of T cells following stimulation with concanavalin A (0.5 ± 5 mg ml^{-1}) was measured using a tritiated thymidine incorporation assay. IL-2 receptor expression on T cells was measured by FACS analysis. Naproxen and NO-naproxen showed similar activity in reducing oedema formation in the non-injected (controlateral) hindpaw. Both drugs showed anti-nociceptive effect. NO-naproxen was anti-nociceptive at a dose of 4.5 mg kg^{-1} and inhibited T cell proliferation while naproxen showed the same extent of inhibition only at a dose of 10 mg kg^{-1}. Inhibition of T cell proliferation was well correlated with reduced IL-2 receptor expression on T cells.

In addition, NO-naproxen reduced both IL-1β and TNFά plasma levels whilst naproxen reduced IL-1 β levels only. In conclusion, both naproxen and NO-naproxen reduced inflammation and nociception associated with arthritis. In addition NO-naproxen interferes to a larger extent with cellular mechanism involved in T cell activation in rat adjuvant arthritis indicating that introduction of the NO moiety in the naproxen structure increased the effect at the level of the immune system.

2. **Pharmacokinetic/Pharmacodynamic Modeling of Antipyretic and Anti-Inflammatory Effects of Naproxen in the Rat. (Josa et al., 2001).**

Pharmacokinetic/pharmacodynamic modeling was used to characterize the antipyretic and anti-inflammatory effects of naproxen in rats. An indirect response model was used to describe the antipyretic effects of naproxen after short intravenous infusions. The model assumed that basal temperature (*T*a) is maintained by the balance of fever mediators given by a constant (zero order) rate of synthesis (*K*syn), and a first order rate of degradation (*K*out). An inhibitory *E*max model adequately described the inhibition of IR(t) by naproxen. A more complex model was used to describe the anti-inflammatory response of oral naproxen in the carrageenin-induced edema model. After carrageenin injection, the additional synthesis of mediators was described by IR(t). Such mediators induced an inflammatory process, which is governed by a first order rate constant (KIN) that can be inhibited by the presence of naproxen in plasma. The sigmoidal Emax model also well described the inhibition of KIN by naproxen. Estimates for IC50 [concentration of naproxen in plasma eliciting half of maximum inhibition of IR(t) or KIN] were 4.24 and 4.13 mg/ml, for the antipyretic and anti-inflammatory effects, respectively.

3. **Naproxen-PC: A GI safe and highly effective anti-inflammatory. (Lichtenberger et al., 2008).**

As naproxen has been demonstrated to be associated with the lowest cardiovascular adverse events in comparison with both COX-2 selective inhibitors and conventional NSAIDs, development of a Naproxen-PC formulation for evaluation in animal models and clinical trials has been done. An oil-based formulation of naproxen and triple strength soy lecithin provided excellent GI protection in both: 1) an acute NSAID-induced intestinal bleeding model in rats pretreated with L-NAME that are intragastrically administered a single dose of naproxen (at a dose of 50 mg/kg) vs the equivalent dose of Naproxen- PC; and 2) a more chronic model (at a naproxen dose of 25 mg/kg BID) in rats that have pre-existing hind paw inflammation (induced with a intradermal injection of Complete Freund's Adjuvant/CFA). Both models demonstrated the superior GI safety of Naproxen-PC vs naproxen while this novel formulation had significant anti-inflammatory efficacy to reduce hind paw edema and the generation of PGE2 in the collected joint synovial fluid. So Naproxen-PC induced significantly less GI injury and bleeding in two rodent model systems while maintaining anti-inflammatory and COX-inhibitory activity.

B. Reported pharmacokinetic methods for Naproxen:

1. Determination of naproxen and its metabolite O-desmethylnaproxen with their acyl glucuronides in human plasma and urine by means of direct gradient high performance liquid chromatography. (Vree, T.B., 1992).

Naproxen is metabolized in humans by 0-demethylation, and by acyl glucuronidation to the 1-0-glucuronide. Naproxen, its metabolite and the conjugates measured directly by gradient high-performance liquid chromatographic analysis without enzymic deglucuronidation. The glucuronide conjugates were isolated by preparative chromatography from human urine samples. Mild acidic hydrolysis of one urinary conjugate resulted in naproxen. This conjugate was also formed by alkaline isomerization of isolated naproxen acyl glucuronide, indicating that the structure of this urinary conjugate must have been naproxen isoglucuronide (4-O-glucuronide).

Mild acidic hydrolysis of another urinary conjugate resulted in 0-desmethylnaproxen. This conjugate was also formed by alkaline isomerisation of isolated 0-desmethylnaproxen acyl glucuronide, indicating that the structure of this urinary conjugate must have been 0-desmethylnaproxen isoglucuronide (4-0-glucuronide). A pharmacokinetic profile of naproxen was performed and some preliminary pharmacokinetic parameters of naproxen obtained from two human volunteers were given.

2. Determination of naproxen in human urine by solid-phase microextraction coupled to liquid chromatography. (Aresta et al., 2005).

An SPME–LC–UV method for the determination of the non-steroidal anti-inflammatory drug (NSAID) naproxen and, after hydrolysis, its glucuronide in human urine samples was developed for the first time using a carbowax/templated resin (CW/TPR-100)-coated fiber. The procedure required a very simple sample pre-treatment, an isocratic elution, and provides a highly selective extraction. All the aspects influencing adsorption (extraction time, temperature, pH and salt addition) and desorption (desorption and injection time and desorption solvent mixture composition) of the analyte on the fibre have been investigated.

3. Directly coupled HPLC-NMR and HPLC-MS approaches for the rapid characterisation of drug metabolites in urine: application to the human metabolism of naproxen. (Sidelmann et al., 2001).

Naproxen (6-methoxy-a-methyl-2-naphthyl acetic acid) was administered as the S-enantiomer and was metabolised in vivo to form its demethylated metabolite which is subsequently

conjugated with b-D-glucuronic acid as well as with sulfate. Naproxen is also metabolised by phase II metabolism directly to form a glycine conjugate as well as a glucuronic acid conjugate at the carboxyl group. In this study, the metabolism of naproxen was investigated in urine samples with a very simple sample preparation using a combination of directly-coupled HPLC-1H NMR spectroscopy and HPLC-mass spectrometry (MS). A buffer system was developed which allows the same chromatographic method to be used for the HPLC-NMR as well as the HPLC-MS analysis. The combination of these methods was complementary in information content since the NMR spectra provide evidence to distinguish isomers such as the type of glucuronides formed, and the HPLC-MS data allow identification of molecules containing NMR-silent fragments such as occur in the sulfate ester.

4. **High-performance liquid chromatographic determination of naproxen, ibuprofen and diclofenac in plasma and synovial fluid in man. (Blagbrough and Daykin, 1992).**

High-performance liquid chromatographic assay procedures was developed for naproxen, ibuprofen and diclofenac in human plasma and synovial fluid samples. A single liquid-liquid extraction procedure was used to isolate each compound from acidified biological matrix prior to the quantitative analysis. A spherisorb ODS column (12.5 cm x 4.6 mm I.D.) was used for all the chromatography. Naproxen was eluted with a mobile phase of methanol-Sorensen's buffer at pH 7 (37:63, v/v). Ibuprofen and diclofenac were eluted using mobile phases of methanol-water at pH 3.3 (65:35, v/v and 63:37, v/v, respectively). Diphenylacetic acid was used as the internal standard for the assay of naproxen and flurbiprofen was used in the analysis of ibuprofen and diclofenac. The assays were used in clinical studies of the three drugs in osteo- and rheumatoid arthritis patients.

5. **Pharmacokinetics and Effects of Alkalization During Oral and Intravenous Administration of Naproxen in Horses. (Cagnardi et al., 2011).**

The pharmacokinetics of naproxen (NAP), after both intravenous (iv) and oral administration of 10 mg/kg body weight (BW), was investigated in horses under normal metabolic conditions and in horses whose conditions were modified by the iv administration of 250 mg/kg BW of sodium bicarbonate (NaHCO3). The hypothesis that blood and consequent urinary alkalization could modify NAP pharmacokinetics was evaluated. Drug quantification was performed on serum and urine using High Performance Liquid Chromatography (HPLC) with ultraviolet-visible

detection. After iv administration, NAP was rapidly distributed ($t_{1/2}$ά: 0.71 ± 0.43 iv NaHCO3 and 0.55 ± 0.62 hours No NaHCO3), whereas its elimination was quite slow ($t_{1/2}$β: 6.74 ± 0.41 hours), particularly in iv NaHCO3 animals ($t_{1/2}$β: 8.95 ± 1.37 hours).After oral treatments, NAP was more rapidly absorbed and elimination was slower in iv NaHCO3 animals ($t_{1/2}$λz: 17.50 ± 6.66 vs. 7.17 ± 0.91hours). The oral bioavailability of NAP was approximately 87% and 77% in No NaHCO3 and iv NaHCO3, respectively. Urinary excretion of the drug as a parent compound was low. The alkalization procedure did not anticipate the elimination of the acidic drug as expected, but it also influenced the absorption of the drug that was administered orally. The dosage scheme of 10 mg/kg BW iv or orally seems to be appropriate to produce an anti-inflammatory effect for 12 to 24 hours.

6. **Profiling urinary metabolites of naproxen by liquid chromatography–electrospray mass spectrometry. (Aresta et al., 2006).**

This work described a liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) approach for the characterization of naproxen and *O*-6-desmethylnaproxen glucuronides. The method was found to be fast and efficient and permitted to individuate ά and β isomers of both naproxen and *O*-6-desmethylnaproxen glucuronides. The procedure could be potentially extended to the characterization of other drug metabolites.

7. **Quantitative gas-liquid chromatographic analysis of naproxen, 6-O-des-methyl-naproxen and their conjugates in urine. (Wan and Matin 1979).**

A quantitative gas-liquid chromatographic method was developed for the analysis of naproxen, 6-O-des-methyl-naproxen and their conjugates in urine.

8. **Quantitative Determination of naproxen in plasma by a simple High-Performance Liquid Chromatographic Method. (Broquaire et al., 1981).**

A high-performance liquid chromatographic method for the determination of naproxen in plasma was based on the single extraction of the drug from acidified plasma with Chloroform using P-naphthalene acetic acid as internal standard. The chromatographic system consisted of a column packed with Spherisorb ODS (5 pm); the mobile phase was acetonitrile-phosphoric acid (pH 3) (45: 55, v/v). The method accurately measured plasma naproxen concentrations down to 1 pg/ml using 100 µl of sample, with no interference from endogenous compounds. The method described was very suitable for routine clinical and pharmacokinetic studies.

9. Simultaneous quantitative determination of naproxen, its metabolite 6-0-desmethylnaproxen and their five conjugates in plasma and urine samples by high performance liquid chromatography on dynamically modified silica. (Andersen and Hansen, 1992a).

The glucuronides of the anti-inflammatory drug naproxen and its metabolite 6-0-desmethylnaproxen have been produced on a preparative scale by enzymatic synthesis. 6-0-Desmethylnaproxen, the glycine conjugate of naproxen and the 0-sulphate of 6-0-desmethylnaproxen were prepared by chemical synthesis. Naproxen and the purified metabolite and conjugates were used as standards for the analytical investigation of the metabolic pattern of naproxen in humans. A reversed-phase high-performance liquid chromatographic method based on bare silica dynamically modified with cetyltrimethylammonium ions has been developed. The system was optimized to give a separation of naproxen, 6-0-desmethylnaproxen and five conjugates. Using this method it was also possible to deduce the relationship between the amount of the intact ether-glucuronide and acyl-glucuronide of 6-0-desmethylnaproxen.

C. Reported herb-drug interactions for Naproxen:

1. Synergistic effect of the interaction between naproxen and citral on inflammation in rats. (Ortiz et al., 2010).

In this study, examination of the effects on inflammation and gastric injury in rats resulting from the interaction between naproxen and citral was examined. Naproxen, citral, or fixed-dose naproxen–citral combinations were administered orally and their anti-inflammation (carrageenan-induced paw edema) and gastric damage were assessed in rats. The pharmacological interaction type was evaluated by the isobolographic analysis. Naproxen, citral, or combinations of naproxen and citral produced anti-inflammatory effects. The sole administration of naproxen produced significant gastric damage, but this effect was not obtained with either citral or combinations. ED30 values were estimated for the individual drugs, and isobolograms were constructed. The results indicated that a synergistic interaction underlies the anti-inflammatory effect. The data suggested that the naproxen–citral combination can interact and to produce minor gastric damage and may have therapeutic advantages for the clinical treatment of inflammation.

D. Reported CYP activities for Naproxen:

1. Cytochromes P450, lA2, and 2C9 are Responsible for the Human Hepatic O-Demethylation of R* and S-Naproxen. (Miners et al., 1996).

A preliminary report implicated cytochrome P450 (CYP) 2C9 in the human liver microsomal 0-demethylation of S-naproxen, suggesting that this pathway may be suitable for investigation of human hepatic CYP2C9 in vitro. Kinetic and inhibitor studies with human liver microsomes and confirmatory investigations with cDNA-expressed enzymes were performed to define the role of CYP2C9 and other isoforms in the O-demethylation of R- and S-naproxen. All studies utilized a newly developed sensitive and specific HPLC assay that measured the respective 0-desmethyl metabolites of R- and S-naproxen in incubations of human liver microsomes and in COS cell lysates. The data demonstrated that CYP2C9 and CYPlA2 together account for the majority of human liver R- and S-naproxen O-demethylation, precluding the use of either R- or S-naproxen as a CYP isoform-specific substrate in vitro and in vivo.

2. Hypothesis

RA is an autoimmune disease that involves inflammation of the synovium with progressive erosion of bone, leading in most cases to misalignment of the joint, loss of function, and disability. Most of the patients with rheumatoid arthritis were treated according to a sequential strategy, beginning with a nonsteroidal anti-inflammatory drug. Nonsteroidal anti-inflammatory drugs (NSAIDs) commonly used in RA treatment are ibuprofen, etoricoxib, nabumetone and naproxen. The people suffering from chronic pain in RA and those dissatisfied with current treatment are very likely to seek alternative treatments, and an estimated 60–90% of persons with arthritis use CAM (traditional or complementary and alternative medicine).

Andrographis paniculata (Acanthacae) commonly known as king of bitters is a frequently used herb in Ayurvedic formulations and has several biological activities including hepatoprotective, antioxidant, antivenom, antifertility, inhibition of replication of the HIV virus, antimalarial, antifungal, antibacterial, antidiabetic, suppression of various cancer cells and principally anti-inflammatory properties. Andrographolide is one of the active constituent of *Andrographis paniculata Nees* (AP) and has been reported to have antiarthritic effect.

As per the previous studies, AN induces CYP1A2 activity. According to few literature, AN inhibits CYP1A2. APE has CYP1A2 inhibitory activity *in vitro*. ETO is predominantly (60 %) metabolized by CYP3A4 in humans and also by CYP1A2, CYP2D6, CYP2C9, and CYP2C19 to lesser extent. As CYP3A4 is not present in rats, the possible pathway of metabolism of ETO in rats may be through CYP1A1 and CYP1A2. CYP1A2 plays an important role in the metabolism of NAB to the active metabolite 6-MNA along with other enzymes like CYP2C6, CYP2C11 in rats and CYP2A6, CYP2C9, CYPC19, CYP2D6, CYP3A4 in human. 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.

Thus the hypothesis was any substance influencing the CYP1A2 enzyme is likely to affect the metabolism of ETO, NAB and NP, which should be studied.

3. Need of work

Most commonly used NSAIDs and COX-2 inhibitors in RA treatment are ibuprofen, etoricoxib, nabumetone and naproxen. *Andrographis paniculata* (Acanthacae) is a frequently used herb in Ayurvedic formulations having anti-inflammatory properties. Andrographolide is one of the active constituent of *Andrographis paniculata Nees* (AP) and has been reported to have antiarthritic effect. Several anti-inflammatory and anti-arthritic polyherbal formulations consisting *Andrographis Paniculata* as a major ingredient are available in local Indian markets.

In treatment of arthritis it is common practice that, along with the DMARDs and NSAIDs, herbal formulations are either taken with or without knowledge of health care provider by the patients for better therapeutic effects. Herb-drug interactions may result in various synergistic/beneficial as well as antagonistic/undesirable effects.

Etoricoxib (ETO), nabumetone (NAB) and naproxen (NP) belongs to class of NSAIDs, widely used in the treatment of rheumatoid arthritis for mild to moderate pain relief. Many studies have been reported previously for pharmacokinetic and pharmacodynamic interaction between few herbs and conventional drugs. Unfortunately, not a single attempt has been done to investigate pharmacokinetic and pharmacodynamic interaction of AP and it's one of the major constituent AN with ETO, NAB and NP after oral administration in rats.

Previously various analytical and bioanalytical methods were reported in literature for the determination of andrographolide alone and in combination with other drugs. Similarly, seldom analytical and bioanalytical methods were previously reported in literature for the determination of ETO, NAB and NP. However, no analytical method was available for simultaneous estimation of AN with ETO/NAB/NP.

So there was a need to develop a new validated HPLC method for simultaneous determination of AN with ETO/NAB/NP in rat plasma and application of the developed method for pharmacokinetic study in rats. Investigation of the possible herb-drug interactions of these compounds through comparing their pharmacokinetic profiles after oral administration in rats was needed. Study of single and combined effects of these drugs on

pharmacodynamic profiles in FCA induced rheumatoid arthritis in rats should be evaluated and compared to support the pharmacokinetic interaction.

So to avoid any possible herb-drug interaction, investigation of pharmacokinetic and pharmacodynamic effects on co-administration of AN and APE with ETO, NAB and NP would be needed.

4. AIM AND OBJECTIVE

The aim of this study was to investigate pharmacokinetic and pharmacodynamic interaction of pure Andrographolide and *Andrographis Paniculata Nees* extract with Etoricoxib or Nabumetone or Naproxen in wistar rats.

Objective:

1. To establish analytical method for simultaneous determination of Andrographolide and selected synthetic drug (ETO/NAB/NP) in rat plasma.
2. To validate the developed method as per USFDA guidelines.
3. To apply the developed method for the pharmacokinetic study.
4. To evaluate the pharmacokinetic interaction of Andrographolide and extract of *Andrographis Paniculata* with selected drugs (ETO/NAB/NP).
5. To evaluate the pharmacodynamic interaction of Andrographolide and extract of *Andrographis Paniculata* with selected drugs (ETO/NAB/NP).
6. To correlate the pharmacokinetic and pharmacodynamic study results for the establishment of herb-drug interaction.

5. PLAN OF WORK

- Literature Review
- Selection of drug candidates (Herbal & Synthetic)
- Identification of animals for experimental procedures
- Development of protocol
- Approval of Animal Ethics committee for experimental work
- Procurement of chemicals and reagents
- Procurement of animals for experimental procedures
- Stabilization of animals as per requirement of the study
- Preparation of dosing solution as per protocol
- Analytical Methods
    - Development and optimization of analytical method to determine drug from plasma
    - Optimization of extraction procedure of drug from plasma
    - Validation of analytical method as per USFDA guidelines
    - Dosing of experimental animals as per schedule
    - Collection of blood samples after specific time intervals as mentioned in protocol
    - Separation and storage of plasma samples

- Pharmacokinetic Study
- Determination of pharmacokinetic parameters from analytical data
- Interpretation and statistical analysis of pharmacokinetic data
- Evaluation of the herb-drug interaction through the pharmacokinetic study.

➢ Pharmacodynamic Study

- Comparative anti arthritic activity using FCA induced arthritic model in rats.
- Dosing of experimental animals as per schedule
- Evaluation of different parameters like change in paw volume, mechanical nociceptive threshold, mechanical hyperalgesia, histopathology and hematological parameters.
- Interpretation and statistical analysis of pharmacodynamic data
- To evaluate the herb-drug interaction through the pharmacodynamic study.

➢ Correlation of the pharmacokinetic and pharmacodynamic study results for the establishment of herb-drug interaction.

➢ Zeroing of data, documentation and publication.

6. Herb drug interaction of *Andrographis paniculata Nees* on Pharmacokinetic and pharmacodynamic of Etoricoxib

6.1 Experimental

6.1.1. Pharmacokinetic study in rats

6.1.1.1. Chemicals and reagents:

Etoricoxib was obtained as gift sample from Cadila Healthcare Ltd. Ahmedabad and celecoxib (IS) was obtained as generous gifts from Cipla Pharmaceutical Pvt. Ltd. Mumbai. 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) (≥ 30% Andrographolide). High purity deionized water was obtained from Millipore, Milli-Q (Bedford, MA, USA) water purification system.

6.1.1.2. 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, ETO and internal standard celecoxib (IS). A series of working standard solutions were obtained by further diluting the stock solutions in methanol. The IS working solution (250 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 400 µL of blank plasma to yield final concentrations of 1, 5, 10, 15, 20, 25, 30, 40, 50 and 60 µg/ mL. The quality control (QC) samples were similarly prepared at concentrations of 1, 10 and 60 µg/mL for the low, medium and high concentration QC samples, respectively. All solutions were kept refrigerated (-80ºC) and brought to room temperature before use.

6.1.1.3. Sample preparation:

20 µl of each solution of AN and ETO were added into 400 µl drug free plasma separately. To each calibration standard, 20 µl of internal standard celecoxib (250 µg/ml) solution was added and vortexed

for 2 min. 1.2 ml of chloroform 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 mobile phase, 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, 10 and 60 µg/mL as a single batch at each concentration.

6.1.1.4. 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 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, ETO and celecoxib (I.S.) from other endogenous substances in plasma sample. Mobile phase consisted of the mixture of solvent A (Acetonitrile) and solvent B (Water) in the ratio of 55:45 (v/v) for 35 minutes at a flow rate of 0.5 mL/min. The concentration of AN was determined by using the peak area ratio of pure AN to I.S. The concentration of AN in APE was determined by using the peak area ratio of AN in APE and I.S. Similarly, the concentration of ETO was determined by using the peak area ratio of ETO and I.S. Each calibration curve was analyzed individually by using least square weighted linear regression. Typical equations of the calibration curves of AN and ETO were y = 0.5951x - 0.0191 and y = 1.5942x + 0.0289 respectively with good correlation coefficient (r^2= 0.9943 and 0.9901 respectively). Intra and inter batch precisions were within limits (R.S.D. < 15 %) and accuracy was in between the range 85 to 115 %. Further validation of the method was done according to the US Food and Drug Administration (FDA) guidelines for selectivity, matrix effects, recovery and stability (Guidance for Industry: Bioanalytical Method Validation, 2001, Jian-kang L. et al, 2013, Chang H. et al, 2012, Wang Q. et al, 2012).

6.1.1.5. Method validation:

The method was validated according to the Food and Drug Administration (FDA) guidelines for selectivity, linearity, precision, accuracy, matrix effects, recovery and stability.

6.1.1.5.1. 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.

6.1.1.5.2. 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 analyte to IS versus analyte concentration.

6.1.1.5.3. 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).

6.1.1.5.4. 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 250 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.

6.1.1.5.5. 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.

6.1.1.6. 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.

6.1.1.7. Drug administration and blood sampling:

Experimental animals were randomly divided into five groups of 18 animals of each as follows, oral administration of Group 1- pure AN alone (60 mg/kg, p.o.), Group 2-APE alone (200mg/kg, p.o.), Group 3- ETO alone (10 mg/kg, p.o.), Group 4-co-administration of AN with ETO (60 mg/kg+10 mg/kg, p.o.) and Group 5-co-administration of APE with ETO (200 mg/kg+10 mg/kg, p.o.). After drug administration, 18 animals were further subdivided into three groups with six animals each. In first sub group blood samples were collected at 0, 0.75, 4 and 10 h, in second sub group blood samples were collected at 0.25, 1, 6, and 12 h and in third group at 0.5, 2, 8 and 24 h. 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 blood and stored at −80 ◦C until further analysis.

6.1.1.8. 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_{max}) and time reaching C_{max} (t_{max}) were read directly from the observed individual plasma concentration-time data. All data were expressed as mean ± standard deviation (SD). The area under the curve (AUC_{0-t}) was calculated using the linear-trapezoidal rule, with extrapolation to infinity ($AUC_{0-\infty}$) from the last detectable concentration using the terminal elimination rate constant (k_e) calculated by linear regression of the final log-linear part of the drug concentration–time curve. Apparent elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/k_e$, total body clearance (Cl) as dose/$AUC_{0-\infty}$, and apparent volume of distribution (Vd) as Cl/k_e. Differences between groups were evaluated by one-way ANOVA (Bonferroni post-test). The differences were considered to be significant at *P<0.05, **P<0.01, ***P<0.001.

6.1.2. Pharmacodynamic study:

6.1.2.1. Arthritis assessments:

The animals were divided into fifteen groups of six animals each as follows Group A-vehicle control group, Group B-arthritic control group, Group C-standard group (10 mg/kg ETO, p.o). Group D to F arthritic animals treated with APE 100 mg/kg, 200 mg/kg and 400 mg/kg p.o.; Group G to I arthritic animals treated with APE 100 mg/kg, 200 mg/kg and 400 mg/kg co-administered with 10 mg/kg ETO, p.o., Group J to L arthritic animals treated with AN 30 mg/kg, 60 mg/kg and 120 mg/kg p.o., Group M to O arthritic animals treated with AN 30 mg/kg, 60 mg/kg and 120 mg/kg co-administered with 10 mg/kg ETO, 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, Zhang R. et al, 2009). 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.

6.1.2.2. 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.

6.1.2.3. 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.

6.1.2.4. 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.

6.1.2.5. 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.

6.1.2.6. Hematology analysis:

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

6.1.2.7. Statistical analysis:

The data was analyzed by two way ANOVA followed by Bonferroni test. The values of $P < 0.05$ were considered statistically significant.

6.2. Results

6.2.1. 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) and solvent B (Water) in the ratio of 55:45 (v/v) with run time 35 minutes.

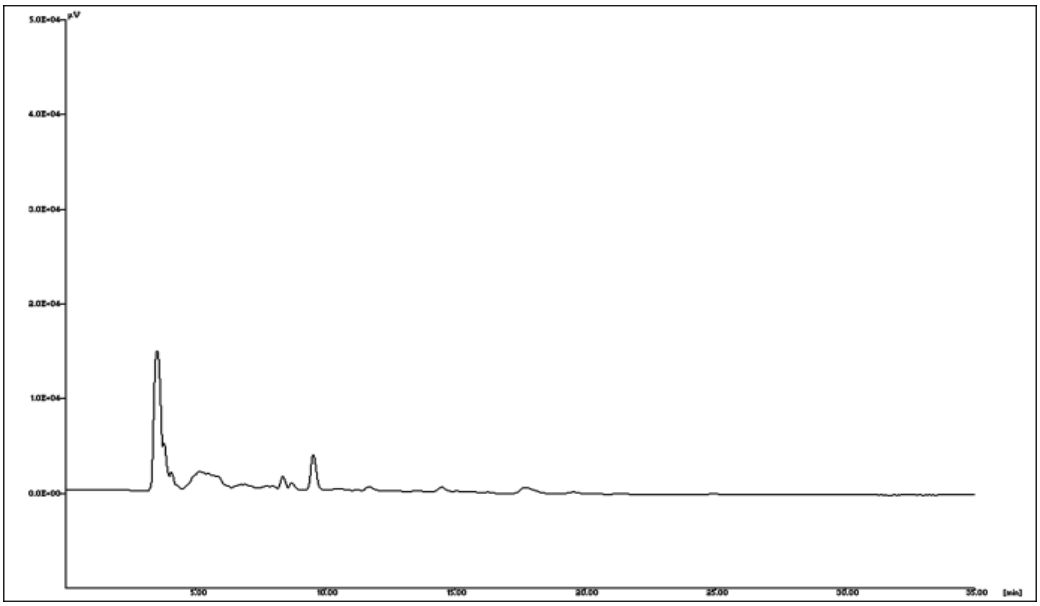
6.2.2. Method validation:

The method was validated according to the USFDA guidelines for selectivity, linearity, precision, accuracy, matrix effects, recovery and stability.

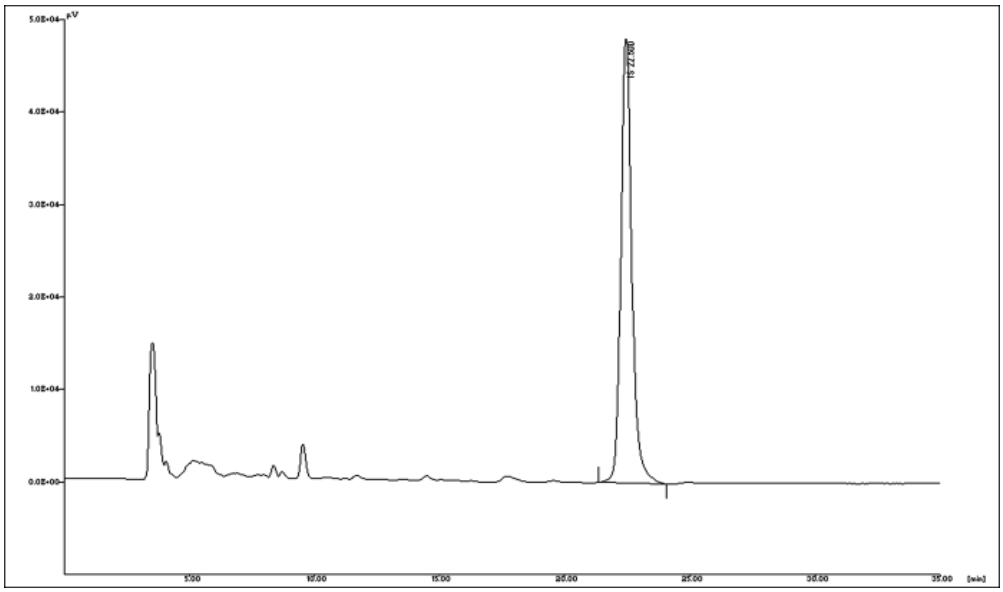
6.2.2.1. Selectivity:

The retention time of AN, ETO and IS was 6.5±1, 10.5±1 and 22.3±1 min, respectively (figure 8).

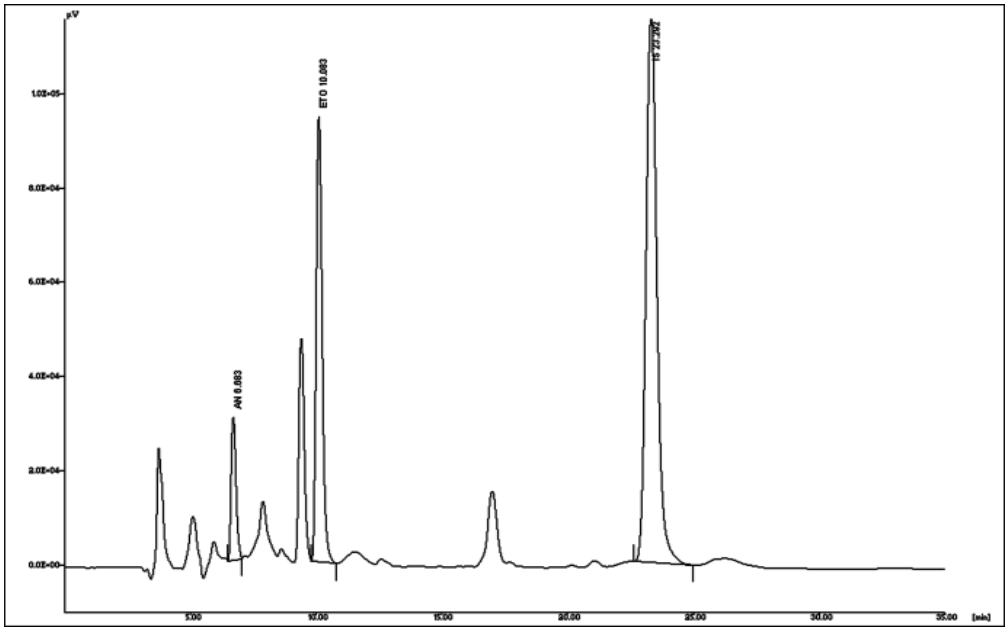
Figure 8: Representative chromatograms of AN, ETO and IS in rat plasma.



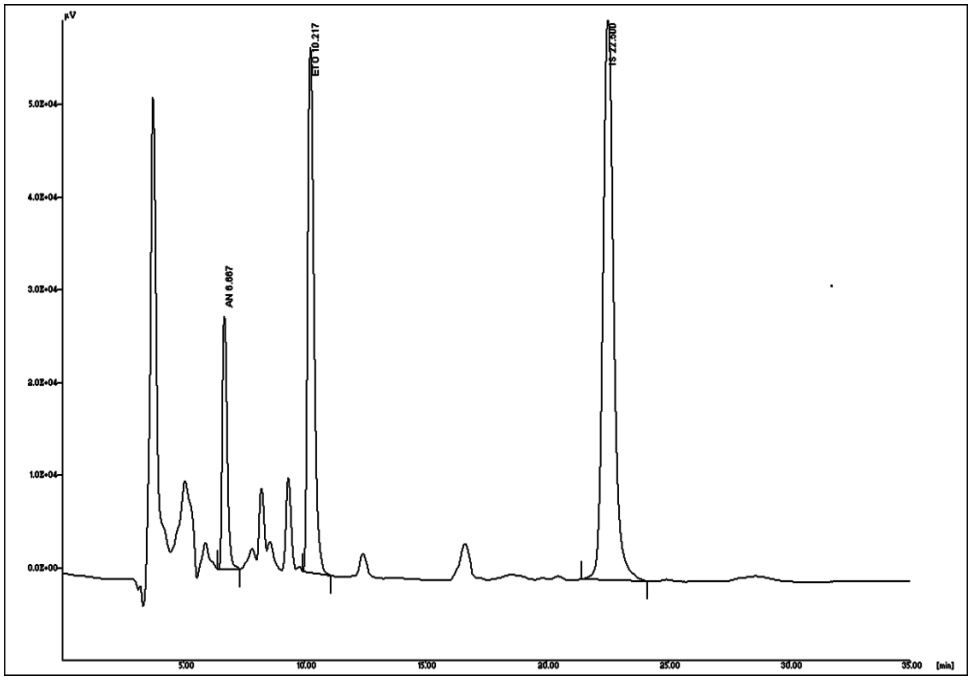
(A) Chromatogram of a blank plasma sample



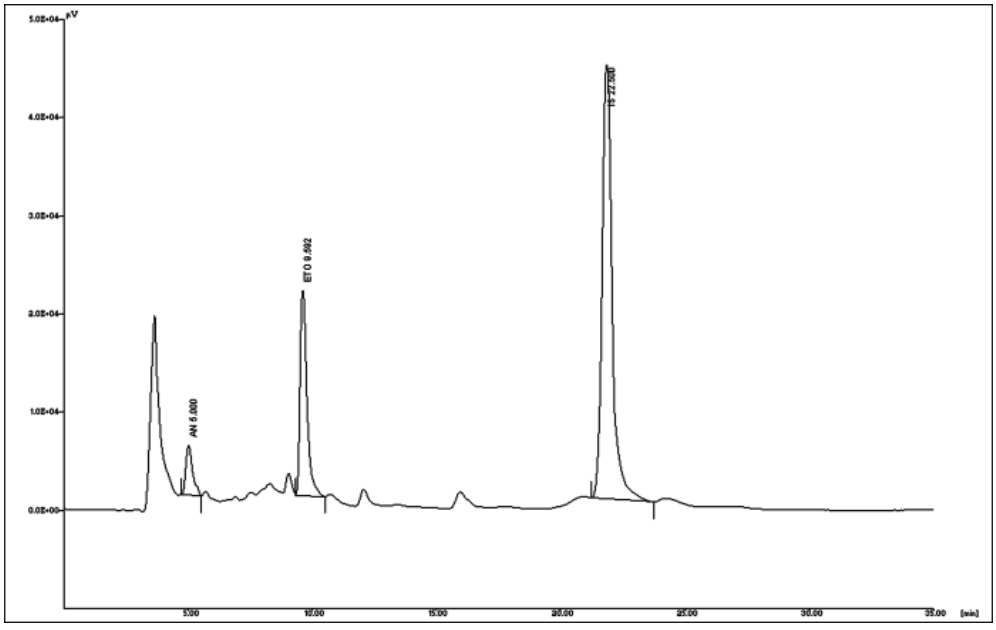
(B) Chromatogram of a blank plasma sample with I. S.



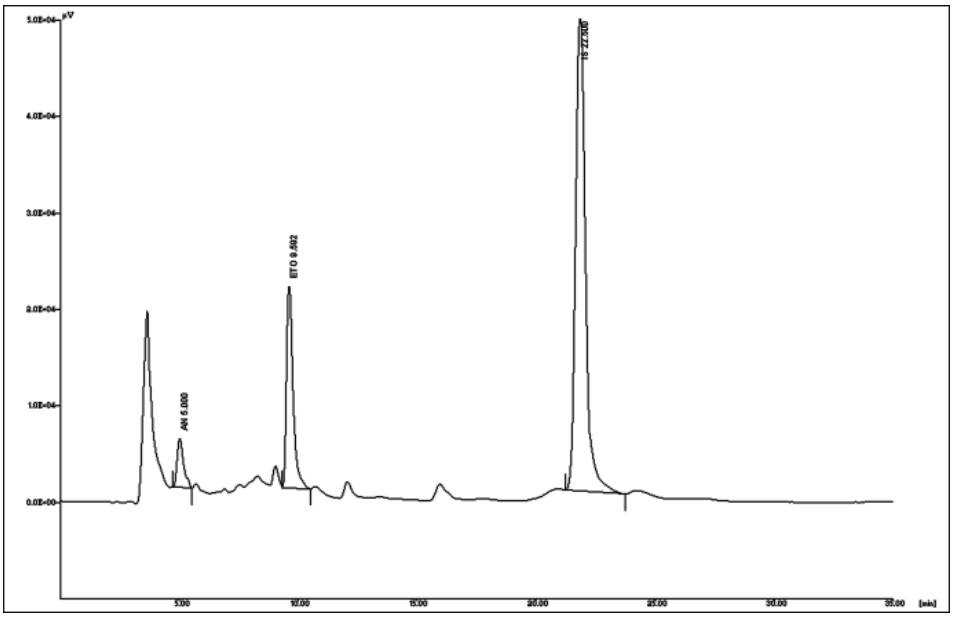
(C) Chromatogram of a blank plasma sample with pure AN (5µg/ml), ETO (5µg/ml) and I. S.



(D) Chromatogram of a blank plasma sample with AN in APE (5µg/ml), ETO (5µg/ml) and I. S.



(E) Chromatogram of a plasma sample obtained from rat at 4 hr after oral administration of 60 mg of pure AN and 10 mg of ETO

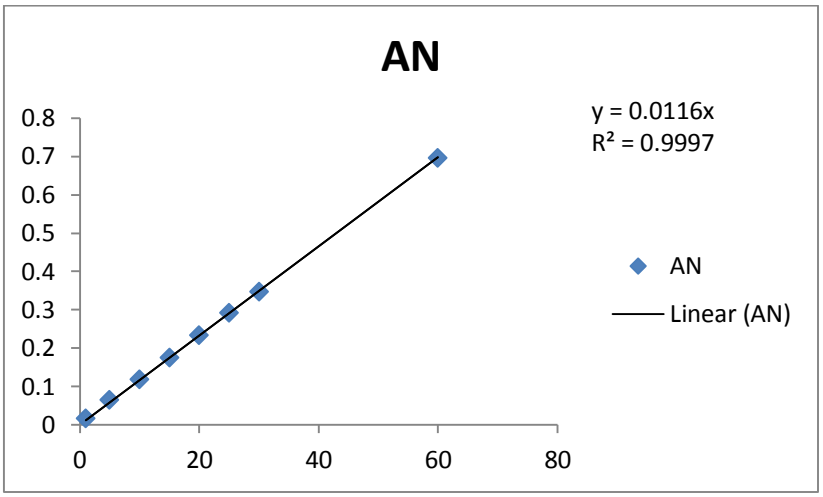


(F) Chromatogram of a plasma sample obtained from rat at 6 hr after oral administration of 200 mg of APE and 10 mg of ETO.

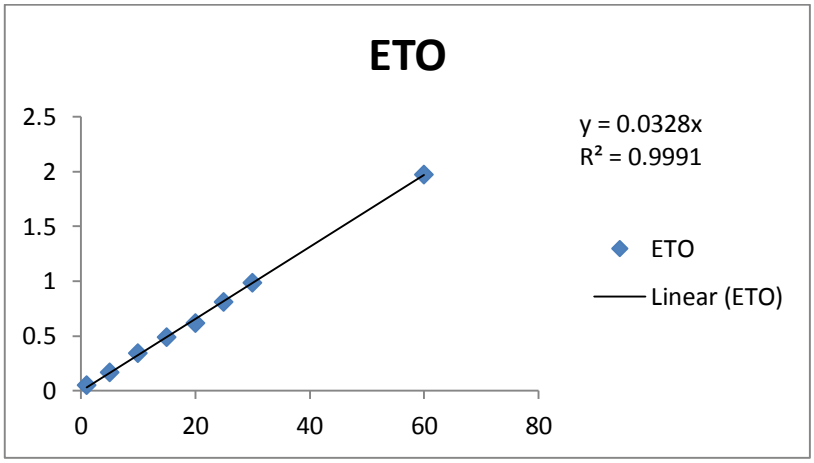
6.2.2.2. Sensitivity and linearity:

The lower limit of quantitation (LLOQ) for andrographolide and etoricoxib was 1 µg/mL. Calibration standards were prepared by spiking the appropriate amounts of the standard solutions into 400 µL of blank plasma to yield final concentrations of 1, 5, 10, 15, 20, 25, 30, 40, 50 and 60 µg/ mL. Each calibration curve was analyzed individually by using least square weighted linear regression. Typical equations of the calibration curves of AN and ETO were $y = 0.0116x$ and $y = 0.0328x$ respectively with good correlation coefficient (r^2= 0.9997 and 0.9991 respectively) (Figure 9).

Figure 9: Calibration curves of AN and ETO



(A) Calibration curve of AN



(B) Calibration curve of ETO

6.2.2.3. Accuracy and precision

Table 2 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 precision. In this assay, the intra-day precision was less than 6.97 % for each QC level of AN and 6.82 % for ETO. The inter-day precision was less than 6.19 % for each QC level of AN and 7.71 % for ETO.

Table 2: Intra and inter-batch precision and accuracy for determination of AN and ETO in rat plasma.

Sample	Nominal concentration (µg/mL)	Intra day			Inter day		
		Measured concentration (µg/mL) (mean ± S.D.)	RSD (%)	RE (%)	Measured concentration (µg/mL) (mean ± S.D.)	RSD (%)	RE (%)
AN	1	1.04 ± 0.07	6.97	-4.2	1.04 ± 0.06	6.19	-4
	10	10.05 ± 0.45	4.47	-5.8	10.12 ± 0.41	4.05	-12.4
	60	60.06 ± 0.48	1.60	-6.4	59.92 ± 0.53	1.79	7.2
ETO	1	1.03 ± 0.0701	6.82	-2.8	1.03 ± 0.07	7.20	-3
	10	10.10 ± 0.40	4.02	-10.6	10.13 ± 0.78	7.71	-12.6
	60	59.94 ± 0.56	1.87	5.4	59.92 ± 0.29	0.96	7.8

6.2.2.4. 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 3.

Table 3: Extraction recovery of AN and ETO in rat plasma (n = 5).

Sample	Nominal concentration (µg/mL)	Peak area[a] (n=5) (A) (Mean ± S.D.)	Peak area[b] (n=5) (B) (Mean ± S.D.)	Extraction Recovery[c] (%) (A/B)
AN	1	28342 ± 703.6	28229 ± 593.5	100.40
	10	181444 ± 4455	181374 ± 3765	100.03
	60	1072364 ± 10398	1070034 ± 9786	100.21
ETO	1	85724 ± 674.6	86095 ± 728.7	99.56
	10	535046 ± 3739	536324 ± 4380	99.76
	60	3062422 ± 54768	3059088 ± 43504	100.1
I.S.	250	1600044 ± 94624.5	1572478 ± 56663.28	101.75

[a] Standards spiked before extraction.

[b] Standards spiked after extraction.

[c] 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).

6.2.2.5. Stability:

All stability QC samples were analyzed in three replicates. The results indicated that each analyte had an acceptable stability under those conditions, as shown in Table 4.

Table 4: Stability of AN and ETO in rat plasma (n = 3).

Sample	Nominal concentration (µg/mL)	Sample conditions			
		Bench top stability[a]	Auto-sampler stability[b]	Freeze-thaw stability[c]	45 days storage stability[d]
AN	1	1.58 ± 0.05	1.56 ± 0.0321	1.58 ± 0.0351	1.56 ± 0.0435
	10	10.20 ± 0.1761	10.22 ± 0.1721	10.16 ± 0.1517	10.12 ± 0.1571
	60	59.71 ± 0.1939	59.97 ± 0.2631	59.96 ± 0.2959	59.91 ± 0.3593
ETO	1	1.69 ± 0.0173	1.69 ± 0.0152	1.68 ± 0.0208	1.69 ± 0.01
	10	10.53 ± 0.0907	10.57 ± 0.1305	10.65 ± 0.1552	10.63 ± 0.0251
	60	59.90 ± 0.3365	59.87 ± 1.0442	59.17 ± 1.1616	59.92 ± 1.1052

[a] Exposed at ambient temperature (25 ◦C) for 2 h.

[b] Kept at ambient temperature (25 ◦C) for 12 h.

[c] After three freeze‑thaw cycles.

d Stored at −80 ◦C for 45 days.

6.2.3. 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 10 and the pharmacokinetic parameters are shown in Table 5. AN in the APE, pure AN alone and ETO were absorbed into the circulatory system and reached its peak concentration approximately 8, 10 and 4 h respectively after administered individually. Decrease in t_{max} of AN in APE and pure AN on co-

administration with ETO to 6 and 8 h was observed. No change in t_{max} of ETO on co-administration with pure AN was observed whereas t_{max} of ETO on co-administration with APE was increased to 6 h. The C_{max} of AN and ETO were decreased significantly after co-administration of AN and APE with ETO than AN, APE and ETO individually administered groups.

Co-administration of pure AN with ETO significantly ($P < 0.05$) decreased the AUC_{last}, AUC_{∞}, $AUMC_{last}$, $AUMC_{\infty}$, $t_{1/2}$, MRT $_{last}$ and MRT_{∞} of AN and ETO when compared to AN and ETO alone groups. There was significant increase in CL of ETO and AN, significant decrease in Vd of AN while increase in Vd of ETO was observed in co-administered group when compared to respective alone groups.

Co-administration of APE with ETO significantly ($P < 0.05$) decreased the AUC_{last}, AUC_{∞}, $AUMC_{last}$, $AUMC_{\infty}$, $t_{1/2}$, MRT $_{last}$, MRT_{∞} of ETO when compared to ETO alone group. Whereas for AN in APE the AUC_{last}, $AUMC_{last}$, $t_{1/2}$ and MRT $_{last}$ was increased and $AUMC_{\infty}$, Vd and MRT_{∞} was decreased significantly after co-administration with ETO after comparison with APE alone group. There were no significant changes observed in AUC_{∞}, CL and MRT $_{last}$ of AN in APE in co-administered and alone groups.

Table 5: Results of pharmacokinetic parameters of all the experimental groups

Parameters	**ETO alone group (Group 1)**	**Co-administration of ETO with**	
		AN (Group 2)	**APE (Group 3)**
C_{max} (µg/ml)	18.34 ± 0.04	11.52 ± 0.07 [c]	10.86 ± 0.07 [c]
t_{max} (h)	4	4	6
AUC $_{0-t}$ (h/µg/ml)	238.00 ± 0.75	128.90 ± 0.52 [c]	104.30 ± 0.37 [c]
AUC $_{0-\infty}$ (h/µg/ml)	301.50 ± 0.30	137.50 ± 0.31 [c]	112.80 ± 0.28 [c]
AUMC $_{0-t}$ (h/h/µg/ml)	2286 ± 9.05	870.9 ± 0.69 [c]	1987 ± 0.47 [c]
AUMC $_{0-\infty}$ (h/h/µg/ml)	4763 ± 25.29	24410 ± 110.4 [c]	9192 ± 14.52 [c]
$t_{1/2}$ (h)	43.43 ± 0.23	19.19 ± 0.09 [c]	19.12 ± 0.10 [c]
Vd (L/kg)	0.48 ± 0.00	0.55 ± 0.00	0.76 ± 0.00 [c]
MRT $_{0-t}$ (h)	9.64 ± 0.02	8.13 ± 0.03 [c]	7.09 ± 0.02 [c]
MRT $_{0-\infty}$ (h)	15.83 ± 0.05	9.56 ± 0.02 [c]	8.87 ± 0.02 [c]
CL(L/h/kg)	0.035 ± 0.00	0.07 ± 0.00 [c]	0.08± 0.00 [c]

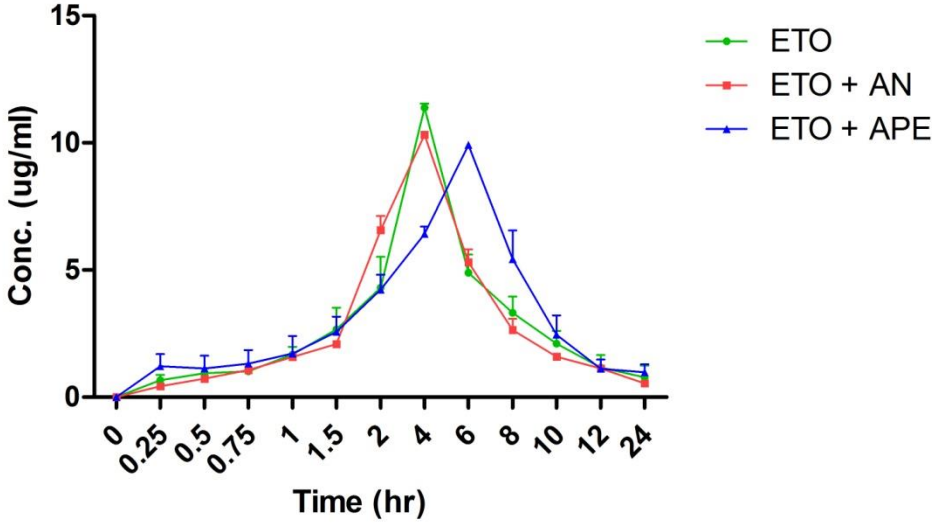
Differences between groups were evaluated by one-way ANOVA (Bonferroni post-test). Data are expressed as mean ± S.E.M.; n=6 rats per group. [c]P<0.001 when compared to group 1.

Where oral administration of, Group 1- ETO alone (10 mg/kg, p.o.),

Group 2-co-administration of AN with ETO (60 mg/kg+10 mg/kg, p.o.) and

Group 3-co-administration of APE with ETO (200 mg/kg+10 mg/kg, p.o.).

Figure 10: Mean concentration-time curve of ETO pharmacokinetic study



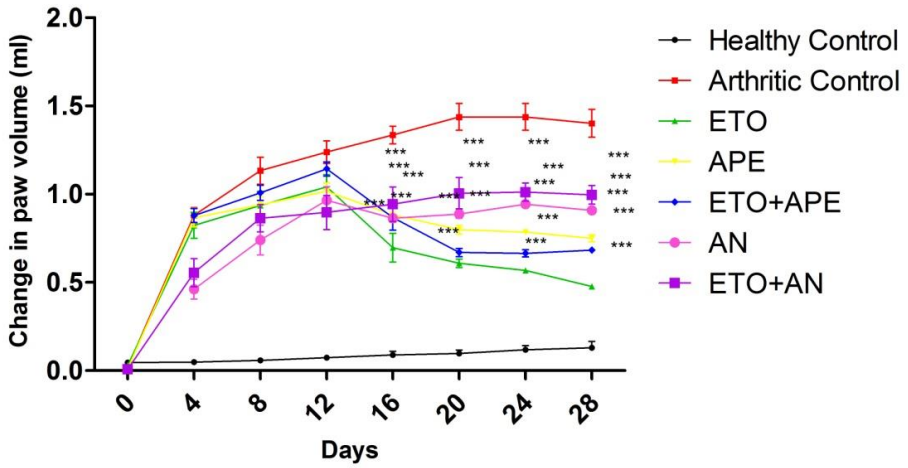
Mean concentration-time curve of ETO alone (10 mg/kg, p.o.), after co-administration of ETO with pure AN (60 mg/kg+10 mg/kg, p.o.) and with APE (200 mg/kg+10 mg/kg, p.o.)

6.2.4. Pharmacodynamic studies (Anti arthritic studies):

6.2.4.1. 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. Treatment with APE (100, 200 and 400 mg/kg) and AN (30, 60 and 120 mg/kg) from day 12 onwards significantly ($P < 0.05$) decreased the paw volume, which was observed till end of the study. Out of three tested doses of APE and AN, APE 200 mg/kg and AN 60 mg/kg were found to be more effective. Hence these doses were used for drug interaction studies. On administration of APE+ETO (200+10 mg/kg) and AN+ETO (60+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 graph is given in Figure 11.

Figure 11: Effect of ETO, APE, ETO+APE, AN and ETO+AN on paw volume.



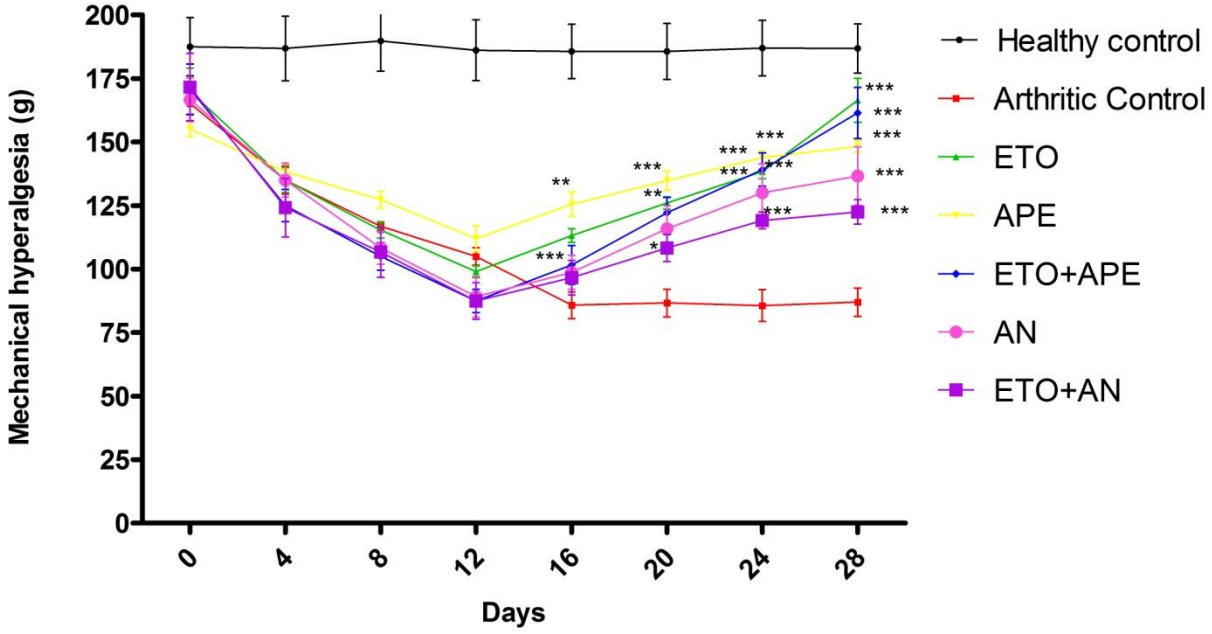
Effect of ETO (10 mg/kg), APE (200mg/kg), ETO+APE (10+200 mg/kg), AN (30 mg/kg) and ETO+AN (10+30 mg/kg) on paw volume. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

6.2.4.2. 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 (100, 200 and 400 mg/kg) and AN (30, 60 and 120 mg/kg) from day 12, there was a significant increase in pain threshold from day 16 onwards which was observed till end of the study (P < 0.05, P < 0.01, P < 0.001 respectively). Out of three tested doses of APE and AN, APE 200 mg/kg and AN 60 mg/kg were found to be more effective. Hence these doses were used for drug interaction studies. On administration of APE+ETO (200+10 mg/kg) and AN+ETO (60+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 (P < 0.01, P < 0.001 respectively). Co-administered and alone groups with

significant change on mechanical withdrawal threshold were further compared with each other and the graph is given in Figure 12.

Figure 12: Effect of ETO, APE, ETO+APE, AN and ETO+AN on mechanical withdrawal threshold.

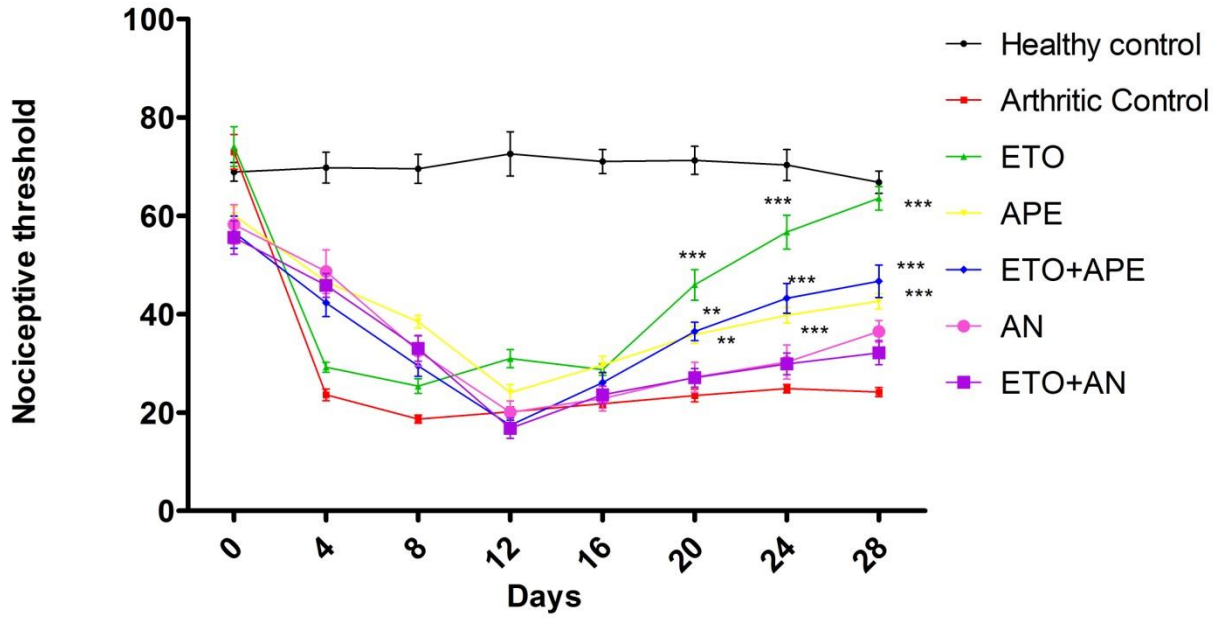


Effect of ETO (10 mg/kg), APE (200mg/kg), ETO+APE (10+200 mg/kg), AN (30 mg/kg) and ETO+AN (10+30 mg/kg) on mechanical withdrawal threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

6.2.4.3. Effect on nociceptive threshold:

There was a significant decrease in mechanical withdrawal threshold observed in all the animals treated with FCA. Administration of APE (100, 200 and 400 mg/kg) and AN (30, 60 and 120 mg/kg) significantly improved the mechanical withdrawal threshold from day 16 when compared to arthritic control. Out of three tested doses of APE and AN, APE 200 mg/kg and AN 60 mg/kg were found to be more effective. Hence these doses were used for drug interaction studies. On administration of APE+ETO (200+10 mg/kg) and AN+ETO (30+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 change on nociceptive threshold were further compared with each other and the graph is given in Figure 13.

Figure 13: Effect of ETO, APE, ETO+APE, AN and ETO+AN on nociceptive threshold.



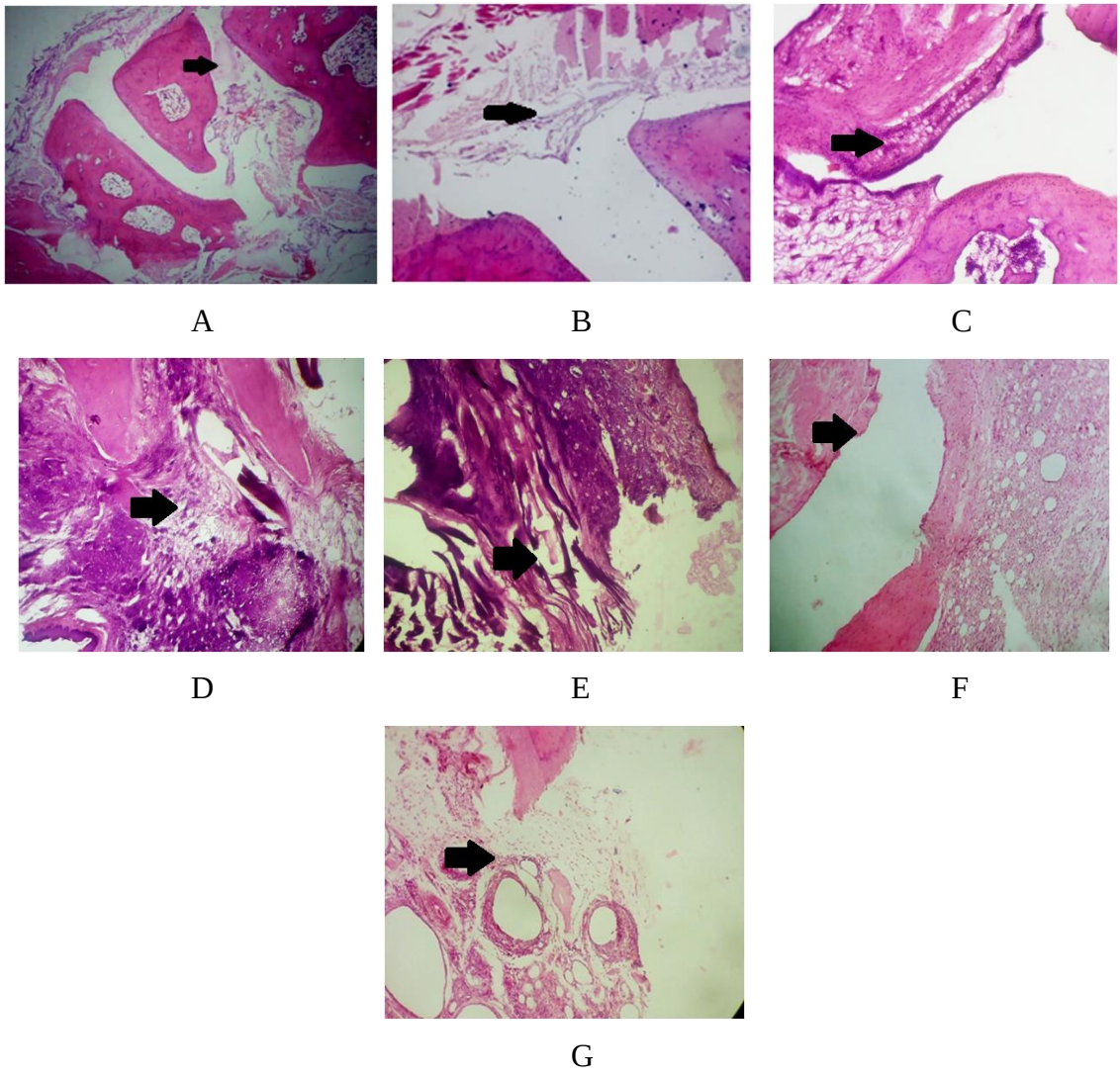
Effect of ETO (10 mg/kg), APE (200mg/kg), ETO+APE (10+200 mg/kg), AN (30 mg/kg) and ETO+AN (10+30 mg/kg) on nociceptive threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

6.2.4.4. 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. Minimal infiltration of inflammatory cells of inflammatory cells and fibrous tissue was observed with ETO (10 mg/kg) treated group. Moderate infiltration of inflammatory cells and fibrous tissue proliferation were observed with group treated with APE (200 mg/kg). Mild infiltration of inflammatory cells and minimal fibrous tissue proliferation were observed in APE (400 mg/kg) treated group and severe infiltration of inflammatory cells and minimal fibrous tissue proliferation were observed in APE (100 mg/kg) treated groups. Similarly, minimal infiltration of the inflammatory cells and prominent blood vessels in AN (30 mg/kg) treated group, no infiltration of any inflammatory exudates in AN (120 mg/kg) treated groups, and moderate infiltration of inflammatory cells in AN (30

mg/kg) treated group were observed compare to control group. Mild infiltration of inflammatory cells and minimal fibrous tissue proliferation were observed for ETO+APE (10+200 mg/kg) and ETO+AN (10+30 mg/kg) treated groups (Figure 14).

Figure 14: Histopathological representation of tibiotarsal joints of animals of ETO anti arthritic study.



A B C D E F G

Histopathological representation of tibiotarsal joints of A. Healthy control animal. (joint bone with no infiltration of inflammatory cells exudates in joint tissue). B. Arthritic control animal. (joint bone surrounded by fibrous tissues with normal bone marrow cavity). C. ETO 10 mg/kg treated animal. (Joint bone with minimal infiltration of inflammatory cells and fibrous tissue). D. APE 200 mg/kg treated animal. (Moderate infiltration of inflammatory cells and fibrous tissue proliferation). E. ETO+ APE (10+200 mg/kg) treated animal. (Moderate infiltration of inflammatory cells and minimal fibrous tissue

proliferation). F. AN 30 mg/kg treated animal. (Moderate infiltration of inflammatory cells and fibrous tissue proliferation.) G. ETO + AN (10+30 mg/kg) treated animal. (Moderate infiltration of inflammatory cells and fibrous tissue proliferation). Stain: H & E 10×

6.2.4.5. Effects on hematological parameters of 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<0.001) reverted to near normal levels in Etoricoxib 10 mg/kg and ETO+APE 200 mg/kg treated animals (Table 6).

Table 6: Effect of APE 200mg/kg, ETO+APE (10+200 mg/kg), AN (30 mg/kg) and ETO+AN (10+30 mg/kg) on hematological parameters in arthritic rats

Group	Normal Control	Arthritic Control	Etoricoxib	APE 200 mg/kg	ETO+APE 10+200 mg/kg	AN 30 mg/kg	ETO+AN 10+30 mg/kg
Hb (gm/100ml)	14.56 ± 0.23	8.86 ± 0.14	13.17 ±0.10	11.00 ±0.15***	12.47 ±0.18***	9.57 ±0.16*	10.14 ±0.06***
WBCs (thousands/µl)	7.763 ±0.16	15.47 ±0.18	10.16 ±0.19	12.80 ±0.12***	11.50 ±0.10***	14.15 ±0.32***	13.55 ±0.12***
RBCs (million/µl)	6.91 ±0.08	3.22 ±0.05	6.07 ±0.10***	5.31 ±0.04***	5.74 ±0.04***	4.30 ±0.05***	4.86 ±0.07***
Platelets (lacks/ µl)	9.31 ±0.10	18.20 ±0.17	10.60 ±0.14***	12.60 ±0.10***	11.59 ±0.11***	14.61 ±0.13***	13.44 ±0.14***
ESR (mm/hr)	8.41 ±0.19	16.35 ±0.75	9.83 ±0.21*	11.11 ±0.13***	10.37 ±0.12***	12.26 ±0.15***	12.90 ±0.12***

*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*

6.3: Discussion

RA is an autoimmune disease causing inflammation of the synovium with progressive erosion of bone, misalignment of the joint, loss of function and disability. Due to its articular manifestations, multiple organ systems may get affected and may result in shortened life expectancy with increased deaths due to cardiovascular disease, infection, and cancer. RA mostly affected population with age between 40 -50 and women are three times more prone than men (Dulai et al., 2012). As inflammatory responses are critically a part of pathophysiology of RA, the manipulation of inflammatory responses may allow for prevention of the disease or symptoms. The major groups of drugs used include nonsteroidal anti-inflammatory drugs, disease modifying antirheumatic drugs (DMARDs), corticosteroids and biological agents (Mahajan et al., 2006). Most of the patients with RA were treated at the beginning with NSAIDs. NSAIDs provide help in partial relief of pain and stiffness at early stage of RA. Commonly used NSAIDs in RA treatment are aceclofenac, ibuprofen, etoricoxib, nabumetone and naproxen.

Many people think that all herbs are safe owing to their natural origin. Hence use of herbal products has increased steadily over the past few years. 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). Since ancient times, in various cultures worldwide, inflammatory disorders and related diseases have been treated with plants or plant-derived formulations (Mueller et al., 2010). Apart from an increase in use, evidence for effectiveness and safety of these complementary therapies is limited. 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. Much of CAM therapy has not been well studied and there is no central source for information about the many commonly used herbal remedies. Guidance to inquiring patients by their health care providers is often limited by a lack of familiarity of the practitioner with these compounds. Even if the practitioner has knowledge of the literature, it is lacking in scientifically verified information on the topic (Setty and Sigal, 2005).

Due to suffering from chronic pain and dissatisfaction with allopathic treatment the patients of RA will seek to alternative treatments. Co-administration of allopathic drug with herbal products may

lead to the herb-drug interactions and other side effects in the treatment of RA. Most of the herb-drug interactions are negative in nature, but sometimes interactions may have a beneficial effect on drug therapy (Biffignandi and Bili, 2000). The evidence of interactions between some commonly used herbal products and other dietary supplements and drugs is usually based on known or suspected pharmacological activity, data obtained from in vitro or animal studies or isolated case reports that frequently lack pertinent information. 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).

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 P450. 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 herb-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. Most commonly rats are used for animal studies, but rat is not the appropriate model for CYP studies as human CYPS are mostly absent in the rats. Pigs, minipigs and monkeys are having very much similar CYP activities in the liver microsomes studies and after careful validation can become valid animals for CYP studies. But for inital and first screening purposes these animals can be a costly affair. So for initial studies rat or mouse models can be reliable.

Andrographis paniculata Nees (AP) is a traditionally used medicinal plant in south asia 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. Since 175 BC, Indian pharmacopoeia mentioned that AP is a predominant

constituent of at least 26 Ayurvedic formulations (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 (Carreta 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.

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). ETO is predominantly (60 %) metabolized by CYP3A4 in humans and also by CYP1A2, CYP2D6, CYP2C9, and CYP2C19 to lesser extent (Kassahun K., 2001). As CYP3A4 is not present in rats, the possible pathway of metabolism of ETO in rats may be through CYP1A1 and CYP1A2 (Martignoni M., 2006).

So the hypothesis for the study was any substance influencing the CYP1A2 enzyme is likely to affect the metabolism of ETO in rats. As per hypothesis pharmacokinetic interactions were found between AN with ETO and APE with ETO which was observed by a significant decrease in C_{max}, $t_{1/2}$ and AUC of ETO by APE and AN significantly ($P<0.05$). So quick elimination of ETO was observed with reduced $t_{1/2}$ when co-administered with APE and AN. Co-administration of ETO with AN and APE reduced the systemic exposure level of ETO *in vivo* with decreasing AUC. The data indicates that AN and some ingredients of APE might have reduced the bioavailability of ETO in rats. The pharmacodynamics of enzyme induction is complex. It depends on the half life of the induced enzyme, the pharmacokinetics of the inducing agent, and the relationship between the plasma concentration of the inducer and the induction stimulus. Maximal induction is a gradual process and these levels may be reached after a few days to two weeks after multiple dosing of inducing compound. AN is well known for its CYP1A inducing properties. But the exact time required for induction of CYP1A2 is not mentioned in the available literature and can be more than 24 h (Pekthong D. et al., 2008). So the

reduction of pharmacokinetic parameters may be attributed due to the initiation of inducing properties of AN which can be correlated with the previous report (Chien C. F. et al, 2010). In addition ETO has decreased the C_{max}, $t_{1/2}$ and AUC of pure AN whereas it has not influenced AN in the form of extract but increased t_{max} of ETO in APE suggests some other components present in extract might have interfered with the influence of ETO. Decrease in t_{max} of pure AN and AN in APE in presence of ETO indicates slow absorption and metabolism. Significant ($P<0.05$) increase in Vd and CL suggests need of further studies in tissue distribution and excretion of ETO on co-administration of with AN and APE. So the study observed that co-administration of AN and APE changes the action of metabolic enzymes of ETO.

We hypothesized that co-administration of APE or AN may affect the cytochrome P450 family of enzymes and an herb-drug interaction will impact the pharmacokinetics of ETO. To evaluate further possible herb-drug interaction pharmacodynamic anti-arthritic study has been performed for the groups with co-administration of APE, AN with ETO followed by histopathological and hematological evaluation. In anti-arthritic study, from increase in change in paw volume, decrease in mechanical nociceptive threshold and mechanical hyperalgesia, it was observed that ETO treated group showed better activity than APE+ETO treated group. 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 supports the findings. The reason may be reduction in the pharmacokinetic parameters of ETO by APE. Similarly AN+ETO group showed significantly decrease in activity compared to ETO due to the pharmacokinetic interaction in all parameters. Further even though the dose of AN is equal in both groups treated with APE and AN there was better activity observed with APE treated group. This can be attributed to the synergistic effects of other components present in extract.

The above mentioned pharmacokinetic interactions can be further confirmed by decrease in activity of ETO and AN treated group compared to AN treated group. The study well correlates the pharmacokinetic interactions with pharmacodynamic activity and suggests the reduced activity of ETO on concomitant administration with APE and AN. However further study needed to establish interactions in humans.

7. Herb drug interaction of *Andrographis paniculata Nees* on Pharmacokinetic and pharmacodynamic of Nabumetone

7.1. Experimental

7.1.1. *In vivo* pharmacokinetic study in rats

7.1.1.1. Chemicals and reagents:

Nabumetone was obtained as gift sample from IPCA Laboratories Ltd. Sejvata (M.P.), and Carbamazepine (IS) was obtained as 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) (30 % w/w andrographolide) was procured from Natural Remedies Pvt. Ltd, Bengalore (Batch No. FAPEX/2013110012). Freund's complete adjuvant (FCA) was procured from Sigma Aldrich, USA. High purity deionized water was obtained from Millipore, Milli-Q (Bedford, MA, USA) water purification system.

7.1.1.2. 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, NAB, 6-MNA 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, 20, 25, 50, 100, 150 and 200 µg/ mL for 6-MNA and 0.5, 1, 2.5, 5, 10, 20, 25 and 50 µg/ mL for AN. The quality control (QC) samples were similarly prepared at concentrations of 1, 20 and 200 µg/mL for 6-MNA and 1, 10 and 50 µg/mL for AN for the low, medium and high concentration QC samples, respectively. All solutions were kept refrigerated (-80ºC) and brought to room temperature before use.

7.1.1.3. Sample preparation:

10 µl of each solution of AN, 6-MNA and NAB was added into 320 µL drug free plasma separately. To each calibration standard, 10 µL of internal standard carbamazepine (200 µg/mL) solution was added and vortexed for 2 min; 1.1mL of ethyl acetate was added to each calibration standard and vortexed for 2 min. After centrifugation at 15000 ×g for 40 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 200 µg/mL for 6-MNA and 1, 10 and 50 µg/mL for AN as a single batch at each concentration.

7.1.1.4. 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 × 4 mm2, 5 µm). The mobile phase system was optimized to give a good resolution of AN, NAB, 6-MNA and carbamazepine (internal standard) from other endogenous substances in plasma sample. The mobile phase consisted of 0.01N acetic acid (Solvent A) and acetonitrile (Solvent B). The gradient was as follows: 0 to 9 min 70% A and 30% B, from 10 to 35 min 30% A and 70% B at a flow rate of 0.5mL/min. The detection was performed with a UV detector at a wavelength of 225 nm. The concentration of AN, NAB and 6-MNA was quantified by calculating its standard curve using the peak area ratio of AN, NAB, 6-MNA to IS. Each calibration curve was analyzed individually using least square 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.

7.1.1.5. Method Validation:

7.1.1.5.1. 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.

7.1.1.5.2. 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.

7.1.1.5.3. 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).

7.1.1.5.4. 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.

7.1.1.5.5. 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.

7.1.1.6. Animals:

Female Wistar rats weighing 180–220 g were purchased from National Institute of Biosciences, Pune. 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.

7.1.1.7. Drug administration and blood sampling:

NAB (7.5 mg/kg, p.o.), AN (60 mg/kg) and APE (200 mg/kg) were prepared by suspending with CMC (0.1%) before the administration. Experimental animals were randomly divided into three groups of 18 animals of each as follows, Group 1-NAB (7.5 mg/kg, p.o.), Group 2-co-administration of AN with NAB (60 mg/kg + 7.5 mg/kg, p.o.) and Group 3-co-administration of APE with NAB (200 mg/kg + 7.5 mg/kg, p.o.). After drug administration, 18 animals were further subdivided into three groups with six animals each. Blood samples (1mL each) were collected at 0, 1.5, 4 and 10 h in first subgroup, 0.5, 2, 6 and 12 h in second sub group and 1, 3, 8

and 24 h in third sub group. 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 (BD vacutainer, USA) and centrifuged at 15 000 ×g (Eppendorf centrifuge 5424-R, Germany) for 20 min. Plasma was separated from blood and stored at -80 °C until further analysis.

7.1.1.8. 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_{max}) and time reaching C_{max} (T_{max}) were 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 followed by Dunnett's test and P value of < 0.05 was considered statistically significant.

7.1.2. Pharmacodynamic study

7.1.2.1. Arthritis assessments:

The animals were divided into seven groups of six animals each as follows Group A-vehicle control group (0.1 % CMC), Group B-arthritic control group, Group C-standard group (7.5 mg/kg NAB) p.o., Group D-arthritic animals treated with APE 200 mg/kg (30 % w/w andrographolide) p.o., Group E-arthritic animals treated with APE 200 mg/kg (30 % w/w andrographolide) co-administered with 7.5 mg/kg NAB, 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 7.5 mg/kg NAB, 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 [29-30]. The dosing of the groups C, D, E, F and G 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.

7.1.2.2. 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.

7.1.2.3. 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.

7.1.2.4. 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.

7.1.2.5. 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.

7.1.2.6. Hematology analysis:

The hematological parameters like hemoglobin (Hb), red blood cell (RBC), white blood cell (WBC) and platelets were determined by standardized laboratory method.

7.1.2.7. Statistical analysis:

The data was analyzed by two way ANOVA followed by Bonferroni test. The values of $P < 0.05$ were considered statistically significant.

7.2. Results

7.2.1. Method optimization:

The chromatographic conditions, especially the composition of mobile phase and time gradient 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. The mobile phase consisted of 0.01N acetic acid (Solvent A) and acetonitrile (Solvent B) with run time 35 minutes. The gradient was as follows: 0 to 9 min 70% A and 30% B, from 10 to 35 min 30% A and 70% B.

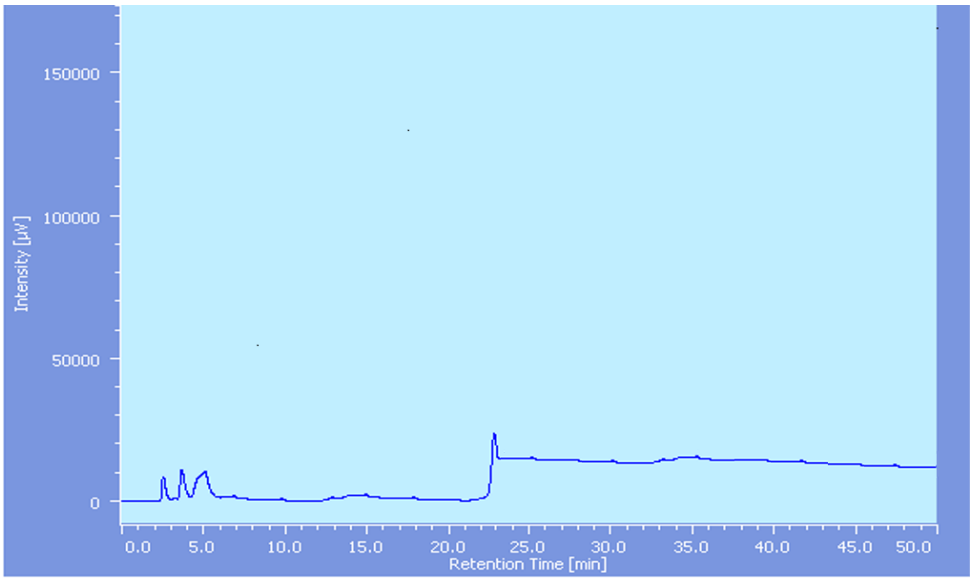
7.2.2. Bioanalytical Method validation:

The method was validated according to the Food and Drug Administration (FDA) guidelines for selectivity, linearity, precision, accuracy, matrix effects, recovery and stability.

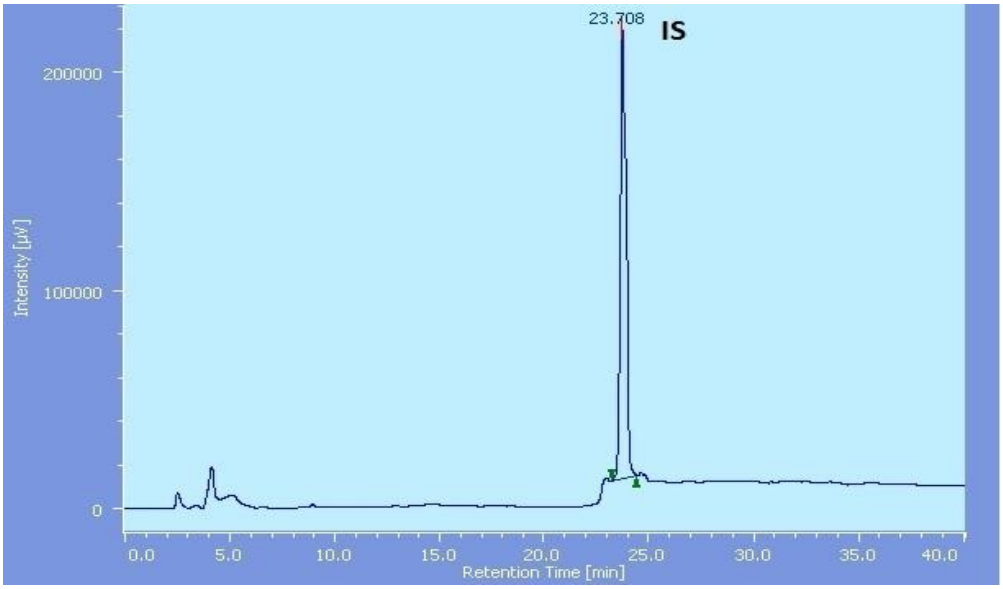
7.2.2.1. Selectivity:

The retention time of 6-MNA, AN, NAB and IS was 6.8±1, 17.6±1, 31.7±1 and 23.7±1 min, respectively (figure 15).

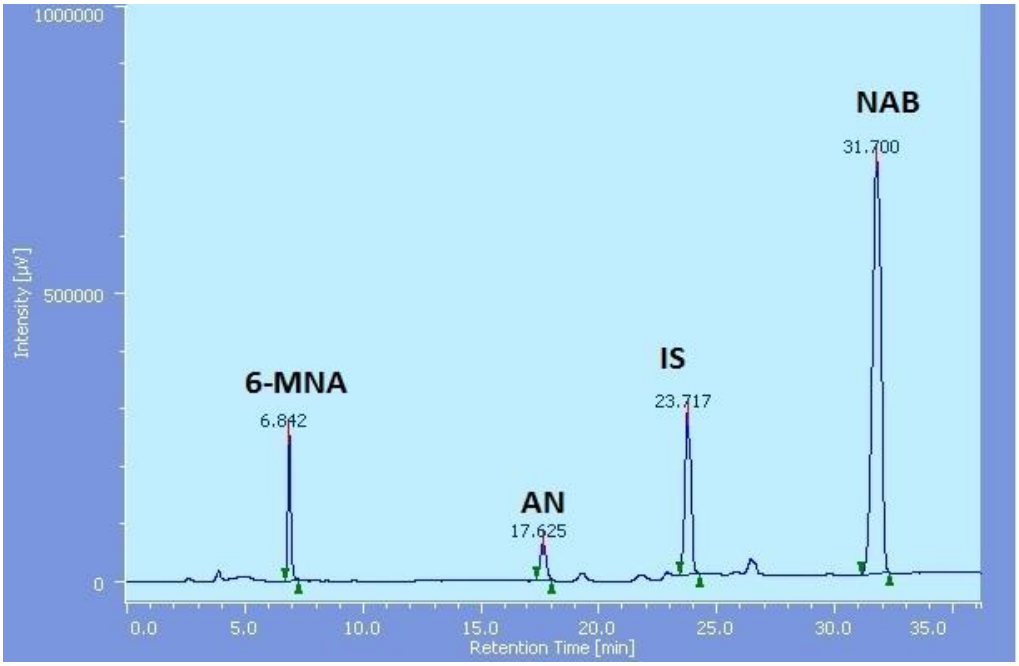
Figure 15: Representative chromatograms of AN, NAB, 6-MNA and IS in rat plasma.



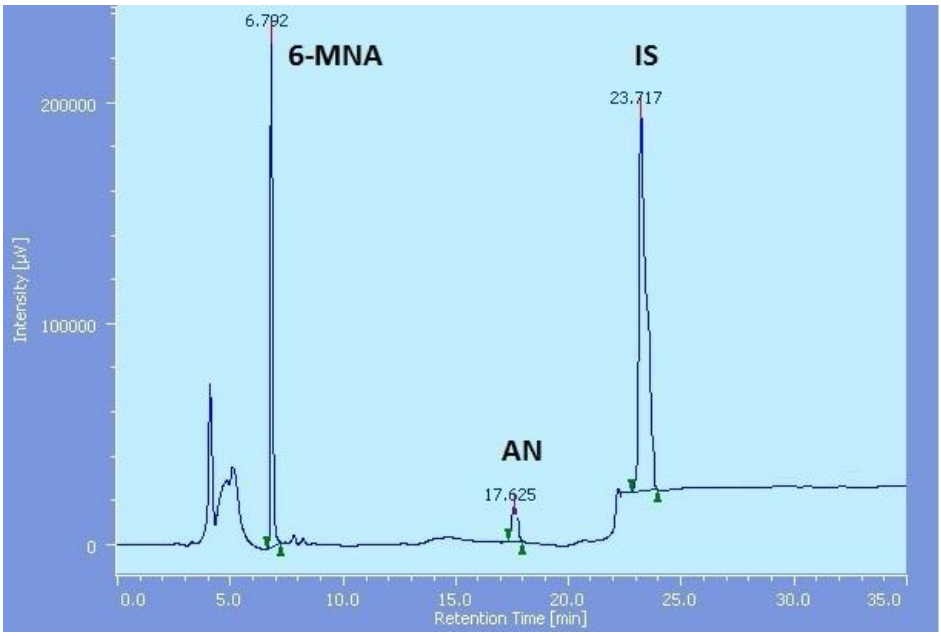
(A) Chromatogram of blank plasma sample



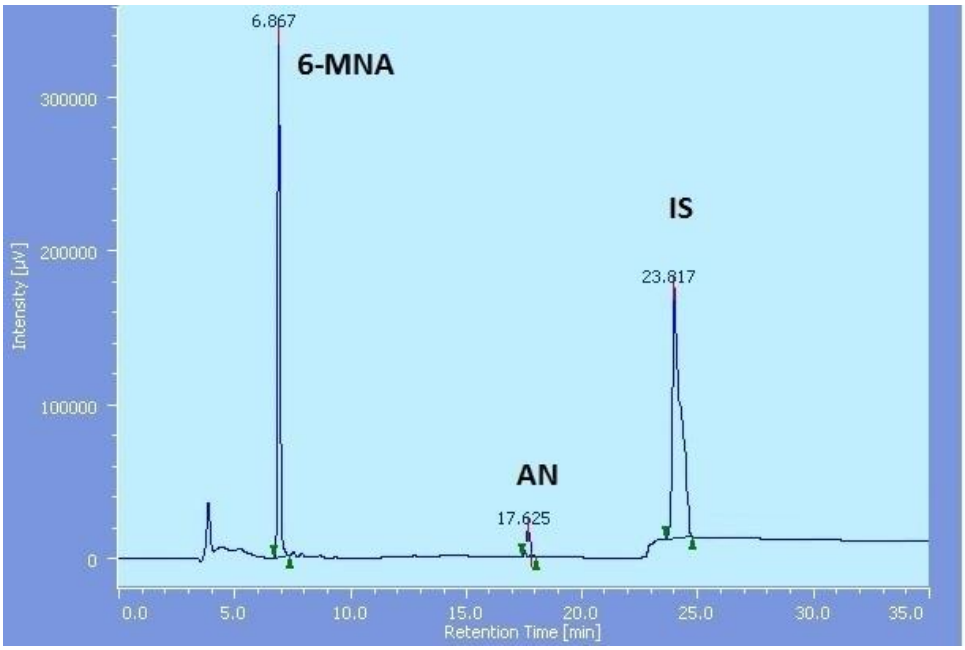
(B) Chromatogram of blank plasma sample with I. S.



(C) Chromatogram of plasma sample spiked with 6-MNA (10 µg/ml), AN (10 µg/ml), NAB (10 µg/ml) and I. S.



(D) Chromatogram of plasma sample obtained from rat at 2 hr after oral administration of 60 mg of AN and 7.5 mg of NAB.

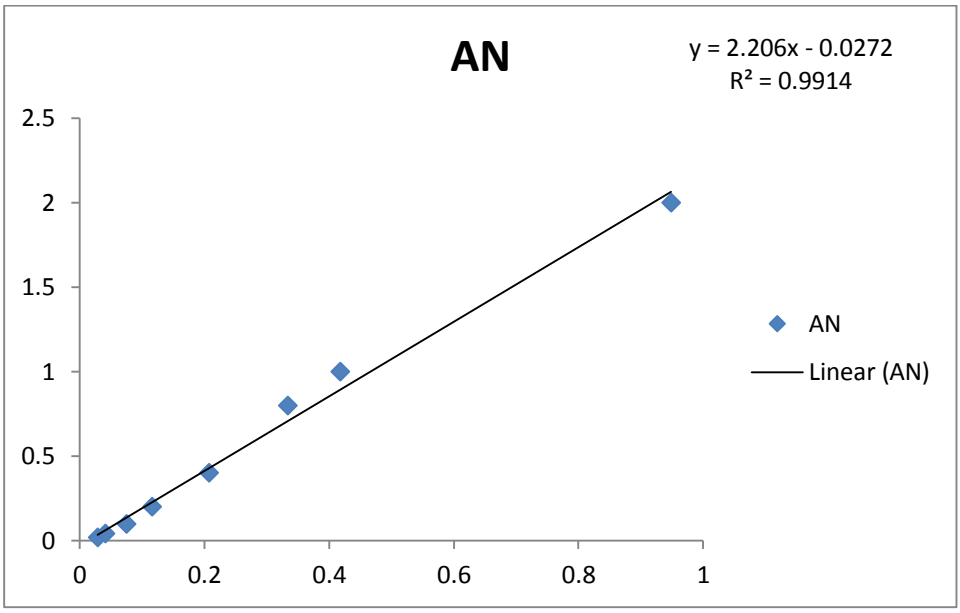


(E) Chromatogram of plasma sample obtained from rat at 1.5 hr after oral administration of 200 mg of APE and 7.5 mg of NAB.

7.2.2.2. Sensitivity and linearity:

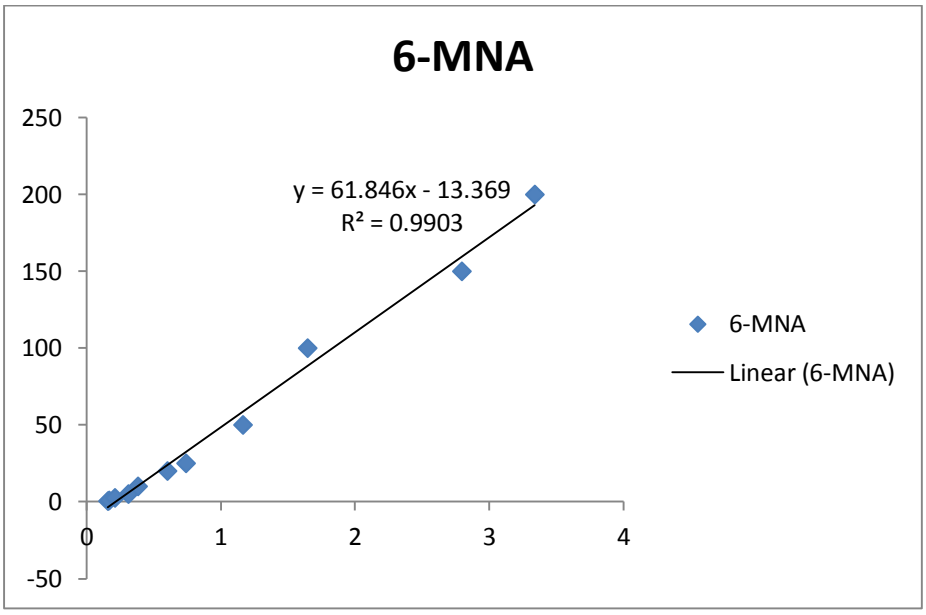
The lower limit of quantitation (LLOQ) for andrographolide and 6-MNA was 1 µg/mL. Calibration standards were prepared by spiking the appropriate amounts of the standard solutions into 320 µL of blank plasma to yield final concentrations of 0.5, 1, 2.5, 5, 10, 20, 25, 50, 100, 150 and 200 µg/ mL for 6-MNA and 0.5, 1, 2.5, 5, 10, 20, 25 and 50 µg/ mL for AN. Typical equations of the calibration curves of 6-MNA and AN were y = 61.846 x - 13.369 and y = 2.206 x - 0.0272 respectively with good correlation coefficient (r^2= 0.9903 and 0.9914 respectively)(Figure 16).

Figure 16: Calibration curves of AN and 6-MNA



(A)

(A) Calibration curve of AN



(B) Calibration curve of 6-MNA

7.2.2.3. Accuracy and precision:

Table 7 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 precision. In this assay, the intra-day precision was less than 10.59 % for each QC level of AN and 12.14 % for 6-MNA. The inter-day precision was less than 9.61 % for each QC level of AN and 10.14 % for 6-MNA.

Table 7: Intra and inter-batch precision and accuracy for determination of AN and 6-MNA in rat plasma.

Sample	Nominal concentration (µg/mL)	Intra day			Inter day		
		Measured concentration (µg/mL) (mean±S.D.)	RSD (%)	RE (%)	Measured concentration (µg/mL) (mean±S.D.)	RSD (%)	RE (%)
AN	1	1.05 ± 0.1032	9.82	5.20	1.05 ± 0.0695	6.57	6.12
	10	10.05 ± 0.2309	2.29	10.59	9.96 ± 0.1417	1.42	9.61
	50	50.38 ± 0.8692	1.72	2.22	49.93 ± 1.1389	2.28	-0.47
6-MNA	1	1.0533 ± 0.0606	5.75	5.85	1.02 ± 0.0657	6.38	3.54
	10	10.21 ± 0.4690	4.59	12.14	10.01 ± 0.2023	2.01	10.14
	50	50.11 ± 1.0208	2.03	0.60	50.48 ± 0.6938	1.37	2.88

7.2.2.4. 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 8.

Table 8: Extraction recovery of AN and 6-MNA in rat plasma (n = 5).

Sample	Nominal concentration (µg/mL)	Peak area[a] (n=5) (A) (Mean ± S.D.)	Peak area[b] (n=5) (B) (Mean ± S.D.)	Extraction Recovery[c] (%) (A/B)
AN	1	202726.4 ± 3785.95	204301.2 ± 3536.84	99.23
	10	892679.4 ± 7561.59	884902.8 ± 8174.14	100.88
	50	3545557 ± 32088.11	3552109 ± 30871.46	99.81
6-MNA	1	810551.8 ± 6765.28	812485.4 ± 4226.15	99.76
	10	2292208 ± 13296.42	2290934 ± 17419	100.05
	50	4553345 ± 33440.02	4545631 ± 36619.18	100.17
	I.S.	1533378 ± 173151.8	1572478 ± 56663.28	97.51

[a] Standards spiked before extraction.

[b] Standards spiked after extraction.

[c] 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).

7.2.2.5. Stability:

All stability QC samples were analyzed in three replicates. The results indicated that each analyte had an acceptable stability under those conditions, as shown in Table 9.

Table 9: Stability of AN and 6-MNA in rat plasma (n = 3).

Sample	Nominal concentration (µg/mL)	Sample conditions			
		Bench top stability[a]	Auto-sampler stability[b]	Freeze-thaw stability[c]	45 days storage stability[d]
AN	1	1.08 ± 0.0984	1.06 ±0.1067	1.09 ±0.1234	0.98 ± 0.0149
	10	10.10± 0.2814	9.95 ± 0.1282	9.92 ± 0.2317	9.92 ± 0.1667
	50	50.49± 1.3409	49.74± 0.7162	49.75± 0.5073	50.10± 1.2586
6-MNA	1	1.02 ± 0.0734	1.03 ± 0.0769	1.08 ± 0.1012	1.06 ± 0.0729
	10	10.38± 0.7866	10.03± 0.0735	10.18± 0.2436	10.07± 0.1199
	50	49.99± 1.0623	50.60± 0.4337	49.80± 1.2479	49.29± 0.5133

[a] Exposed at ambient temperature (25 ◦C) for 2 h.

[b] Kept at ambient temperature (25 ◦C) for 12 h.

[c] After three freeze‑thaw cycles.

[d] Stored at −80 ◦C for 45 days.

7.2.3. Pharmacokinetic study:

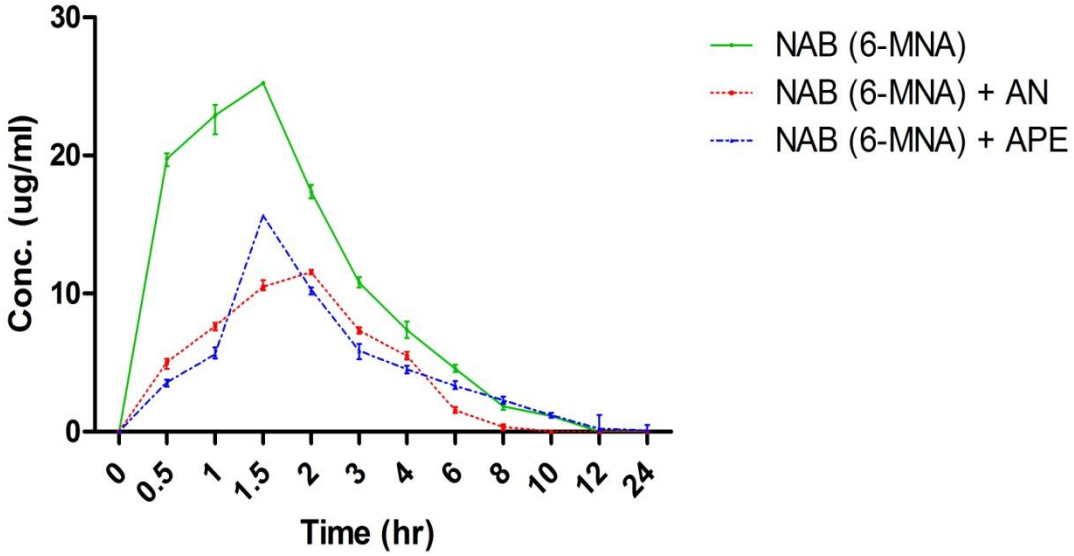
The developed HPLC method was applied successfully to the pharmacokinetic study in the rat plasma for the respective groups. All previously published studies on pharmacokinetics of nabumetone showed its metabolite-6-MNA was detected intact in plasma but the parent compound nabumetone was absent in plasma. The overlaid mean plasma concentration time curves are shown in Figure 17 and the pharmacokinetic parameters are shown in Table 10. 6-MNA was absorbed into the circulatory system and reached its peak concentration approximately 1.5 h after administered individually. T_{max} of group co-administered AN and NAB has been changed to 2 hr but T_{max} of group co-administered APE and NAB was the same. The C_{max} of 6-MNA was decreased significantly after co-administration of AN and APE with NAB compared to group administered with NAB alone. Co-administration of AN with NAB significantly ($P < 0.05$) decreased the $C_{max,}$ AUC_{0-t}, $AUC_{0-\infty}$, $T_{1/2}$, $MRT_{0-t,}$ $MRT_{0-\infty,}$ and Vd of 6-MNA whereas increased T_{max} and CL of 6-MNA in comparison with group treated with NAB alone. Co-administration of APE with NAB significantly ($P < 0.05$) decreased the $C_{max,}$ AUC_{0-t}, and $AUC_{0-\infty}$ of 6-MNA whereas increased the $T_{1/2}$, $MRT_{0-t,}$ MRT0-$_{\infty,}$ Vd and CL of 6-MNA when compared to group treated with NAB alone.

Table 10: Results of pharmacokinetic parameters of all the experimental groups

Parameters	**NAB alone group (Group 1)**	**Co-administration of NAB with**	
		AN (Group 2)	**APE (Group 3)**
C_{max} (µg/ml)	25.22 ±0.016	11.52 ± 0.147***	15.65 ± 0.015***
T_{max}	1.50 ± 0.020	2.01 ± 0.040***	1.48 ± 0.040
AUC $_{0-t}$ (h/µg/ml)	84.47 ± 0.017	38.87 ± 0.014***	54.08 ± 0.021***
AUC $_{0-\infty}$ (h/µg/ml)	88.18 ± 0.519	38.96 ± 0.027***	60.63 ± 0.037***
T $_{1/2}$ (h)	1.92 ± 0.017	0.70 ± 0.010***	9.53 ± 0.016***
MRT $_{0-t}$ (h)	3.18 ± 0.072	2.67 ± 0.017**	9.64 ± 0.012***
MRT $_{0-\infty}$ (h)	2.81 ± 0.018	2.66 ± 0.013***	6.24 ± 0.102***
Vd (l/kg)	0.23 ± 0.006	0.19 ± 0.005***	1.70 ± 0.011***
Cl (l/h/kg)	0.08 ± 0.005	0.19 ± 0.003***	0.12 ± 0.000***

*Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test *P<0.05, **P<0.01, ***P<0.001* when group 2 and 3 compared to group 1.

Figure 17: Mean concentration-time curve of NAB pharmacokinetic study



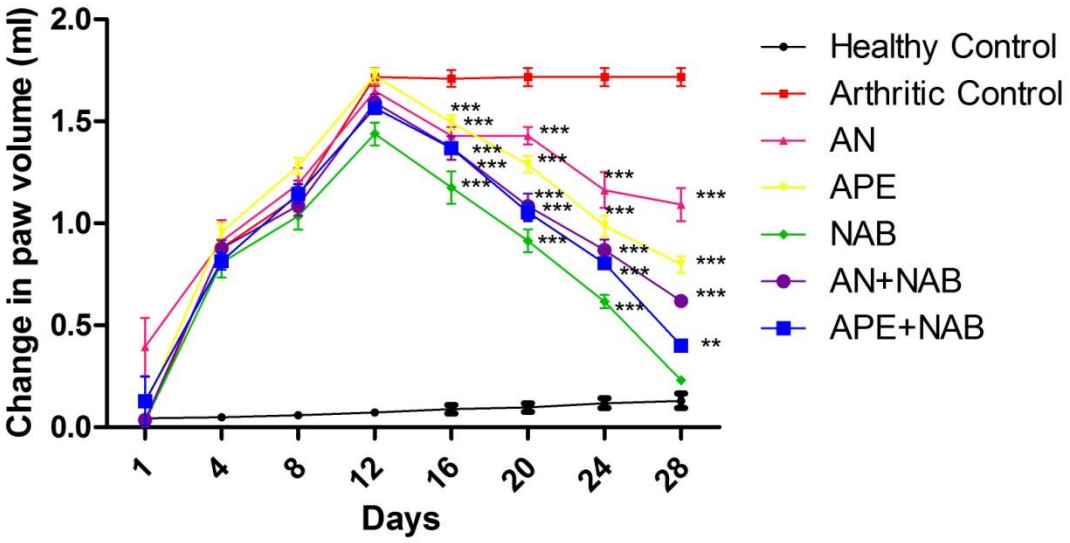
Mean concentration-time curves of NAB (7.5 mg/kg, p.o.) alone, after co-administration of NAB with AN (7.5 mg/kg+60 mg/kg, p.o.) and after co-administration of NAB with APE (7.5 mg/kg +200 mg/kg, p.o.) in rats (n=6).

7.2.4. Pharmacodynamic studies (Anti arthritic studies):

7.2.4.1. 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+ NAB (200+7.5 mg/kg) and AN+ NAB (60+7.5 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 18.

Figure 18: Effect of NAB, AN, APE, NAB +APE, and NAB+AN on paw volume.

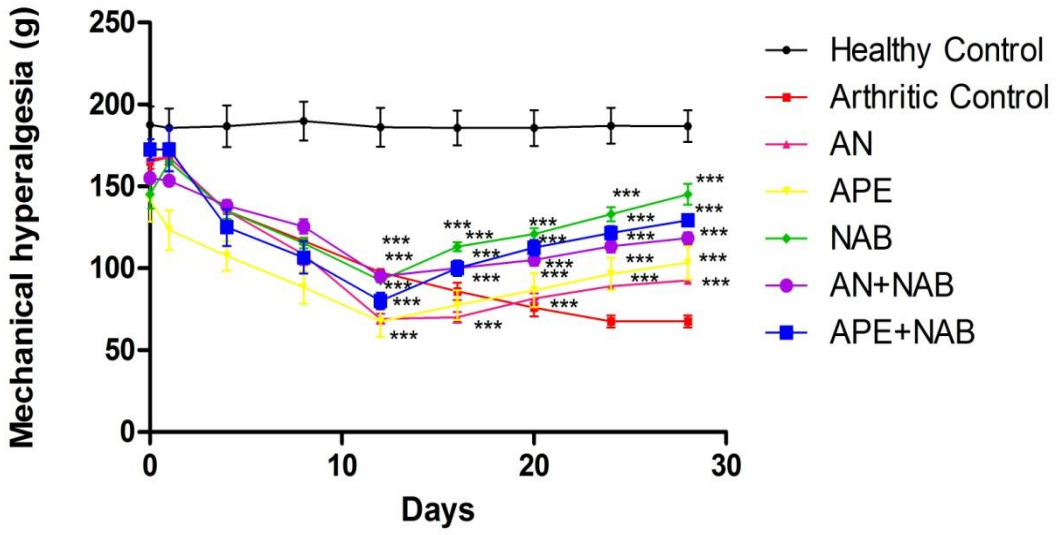


Effect of NAB (7.5 mg/kg), AN (60 mg/kg), APE (200 mg/kg), NAB +APE (7.5+200 mg/kg), and NAB+AN (7.5+60 mg/kg) on paw volume. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

7.2.4.2. Effect on mechanical hyperalgesia:

There was a significant decrease in mechanical withdrawal threshold observed in all the animals treated with FCA. On administration of APE+ NAB (200+7.5 mg/kg) and AN+ NAB (60+7.5 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 (P < 0.05) change on nociceptive threshold were further compared with each other and the results are given in Figure 19.

Figure 19: Effect of NAB, AN, APE, NAB +APE and NAB+AN on mechanical withdrawal threshold.

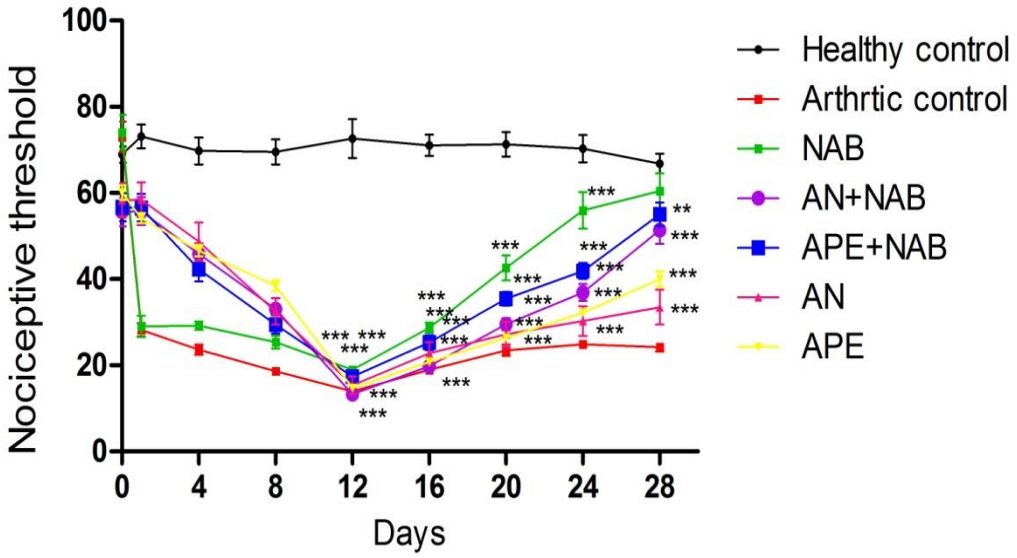


Effect of NAB (7.5 mg/kg), AN (60 mg/kg), APE (200 mg/kg), NAB +APE (7.5+200 mg/kg) and NAB+AN (7.5+60 mg/kg) on mechanical withdrawal threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

7.2.4.3. Effect on nociceptive threshold:

There was significant decrease in pain threshold on administration of FCA which was continued till day 12. On administration of APE+ NAB (200+7.5 mg/kg) and AN+ NAB (60+7.5 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 20.

Figure 20: Effect of NAB, AN, APE, NAB +APE and NAB+AN on nociceptive threshold.

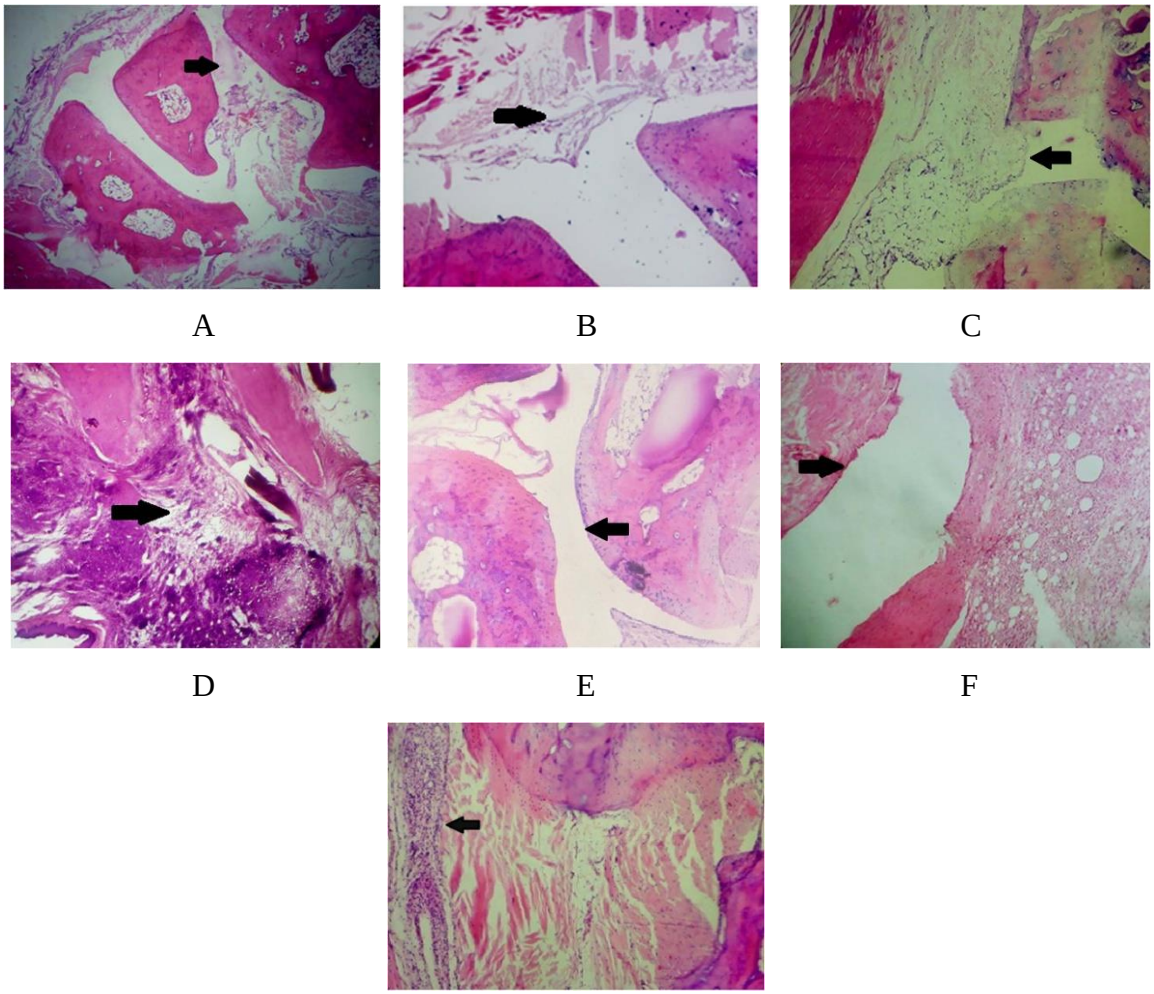


Effect of NAB (7.5 mg/kg), AN (60 mg/kg), APE (200 mg/kg), NAB +APE (7.5+200 mg/kg) and NAB+AN (7.5+60 mg/kg) on nociceptive threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software; *P < 0.05, **P < 0.01, ***P < 0.001 compared to arthritic control.

7.2.4.4. 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. Joint bone surrounded by fibrous tissues no infiltration of inflammatory cells was observed with NAB (7.5 mg/kg) treated group. Moderate infiltration of inflammatory cells and fibrous tissue proliferation was 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. Joint bone with no infiltration of inflammatory cells and fibrous tissue was observed for NAB +APE (7.5+200 mg/kg) treated group and mild infiltration of inflammatory cells and fibrous tissue was observed for NAB +AN (7.5+60 mg/kg) treated group (Figure 21).

Figure 21: Histopathological representation of tibiotarsal joints of animals of NAB antiarthritic study



A B C

D E F

G

Histopathological representation of tibiotarsal joints of **A.** Healthy control animal (Joint bone with no infiltration of inflammatory cells exudates in joint tissue). **B.** Arthritic control animal (Joint bone surrounded by fibrous tissues with normal bone marrow cavity). **C.** NAB 7.5 mg/kg treated animal (Joint bone surrounded by fibrous tissues no infiltration of inflammatory cells). **D.** APE 200 mg/kg treated animal. (Moderate infiltration of inflammatory cells and fibrous tissue proliferation). **E.** NAB + APE (7.5+200 mg/kg) treated animal (Joint Bone with no infiltration of inflammatory cells and fibrous tissue). **F.** AN 60 mg/kg treated animal. (Moderate infiltration of inflammatory cells and fibrous tissue proliferation.). **G.** NAB + AN (7.5+60 mg/kg) treated animal (Mild Infiltration of inflammatory cells and fibrous tissue). Stain: H & E 10 x

7.2.4.5. Effects on hematological parameters of 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<0.001) reverted to near normal levels in NAB 7.5 mg/kg, NAB+AN (7.5+60 mg/kg), and NAB+APE (7.5+200 mg/kg) treated animals as compared to AN and AE alone groups (Table 11).

Table 11: Effect of APE 200mg/kg, NAB+APE (7.5+200 mg/kg), AN (60 mg/kg) and NAB +AN (7.5+60 mg/kg) on hematological parameters in arthritic rats

Group	**Arthritic Control**	**Healthy Control**	**NAB**	**AN (60 mg/kg)**	**APE (200 mg/kg)**	**NAB +AN (7.5+60 mg/kg)**	**NAB +APE (7.5+200 mg/kg)**
Hb (gm/100ml)	8.87 ± 0.14	14.37 ± 0.21***	13.74 ± 0.33***	10.04 ± 0.06***	10.99 ± 0.03***	11.78 ± 0.20***	12.35 ± 0.11***
WBCs (thousands/µl)	15.29 ± 0.18	7.75 ± 0.15 ***	8.46 ± 0.16***	12.07 ± 0.16***	13.39 ± 0.13***	10.92 ± 0.23***	9.37 ± 0.15***
RBCs (million/µl)	3.23 ± 0.05	7.04 ± 0.16***	6.35 ± 0.21***	4.10 ± 0.06***	5.31 ± 0.06***	5.99 ± 0.06***	6.59 ± 0.19
Platelets (lacks/ µl)	17.94 ± 0.09	9.147 ± 0.10***	9.393 ± 0.17***	16.77 ± 0.25***	13.50 ± 0.09***	12.65 ± 0.08***	10.96 ± 0.07***
ESR (mm/hr)	16.34 ± 0.74	8.417 ± 0.19***	9.465 ± 0.08***	13.68 ± 0.32***	12.31 ± 0.07***	11.37 ± 0.08***	10.54 ± 0.08***

*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*

7.3: Discussion

Pharmaceutical products containing biologically active phytochemicals are often defined as herbal or botanical supplements. These are derived or extracted from a broad range of plant sources. Herbal supplements now commonly used in both Eastern and Western countries. Herbal medicines or traditional medicines have a long history of use in the Eastern countries, such as China and India. The use of herbal supplements in the United States has grown at a rapid rate. Herbal supplements can be purchased in several forms. The most common formulations of herbal supplements are extracts. Herbal extracts are crude products usually containing many natural phytochemicals. Previous literature has shown that herbal supplements or herbal medicines can modify drug metabolism mediated by human CYP enzymes. This causes herb-drug interaction which has the clinical consequence of adversely affecting the pharmacokinetics of several drugs. Thus, when dietary supplements and herbs are taken concomitantly with prescription drugs, this can result in serious herb-drug interactions (Wonwilormulk and Prachayasittikul, 2014; Chien et al, 2010).

Rheumatoid arthritis (RA) is the commonest chronic inflammatory arthritis. Most of the patients have a progressive course that eventually leads to considerable functional disability. The treatment of RA is a challenge, the major aims being symptom relief, prevention of disability in order to provide a good quality of life. The major groups of drugs used include nonsteroidal anti-inflammatory drugs, disease modifying antirheumatic drugs (DMARDs), corticosteroids and biological agents (Mahajan et al., 2006; Venkatesha et al., 2010). As popularity of herbal medications has been increased, physicians frequently discover their patients are already using such therapies. Physicians are often not aware of history of CAM therapies unless they directly ask their patients about usage of herbal and dietary supplements. Patients rarely inform use of CAM therapies, as they do not view them as drugs and considers CAM therapies are safe, natural supplements, not drugs. The concomitant use of CAM therapy with prescription or over-the-counter medications may lead to adverse herb–drug interactions (Setty and Sigal, 2005).

In India, many Ayurvedic practitioners are using various plants for the treatment of different types of arthritic conditions. Although the application of these medicaments has a sound tradition and a rational background according to the Indian system of medicine, perhaps it is

essential to investigate the rationality of their use in modern scientific terms. The scientific studies to work out the actual efficacy and other limitations to these drugs would definitely widen their scope for future use if they come out to be really effective. *Andrographis paniculata Nees* (AP) is a traditionally used medicinal plant in south asia 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, antimalarial and anti-inflammatory properties. 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 (Carreta 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). Indian pharmacopoeia contains AP as a predominant constituent of at least 26 Ayurvedic formulations (Jarukamjorn and Nemoto, 2008; Ooi et al., 2011; Qiu et al., 2012; Dhiman et al., 2012; Shen et al., 2013, Carretta et al., 2009). 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.

Cytochrome P450 (CYP) is a group of hemoproteins that play a central role in the oxidative metabolism (phase I) of clinically-used drugs and other xenobiotics. In animals and in man, CYPs can be found in virtually all organs notably the liver, intestine, skin, nasal epithelia, lung and kidney, but also in testis, brain etc. For the evaluation of potential herb-drug interaction, investigation of drug metabolism mediated by cytochrome P450 is essential. The experimental approach is based on animal drug-metabolizing systems and is used to predict kinetics and toxicity in human. However, this interspecies comparison suffers from certain limitations, because in different species, different extent and also different catalytic activity of CYP isoforms has been observed and therefore some caution is required in extrapolation. For initial studies rat or mouse models are more suitable.

CYP1A2 plays an important role in the metabolism of NAB to the active metabolite 6-MNA along with other enzymes like CYP2C6, CYP2C11 in rats and CYP2A6, CYP2C9,

CYPC19, CYP2D6, CYP3A4 in human. 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). CYP1A2 was found to be common in both human and rats. So the hypothesis for the study was any substance influencing the CYP1A2 enzyme is likely to affect the metabolism of NAB in rats.

As hypothesized, pharmacokinetic interaction of AN and APE with NAB was observed by a significant ($P<0.05$) decrease in Cmax, AUC_{0-t} and $AUC_{0-\infty}$ of 6-MNA by APE and AN. Increase in elimination of 6-MNA was observed with reduced $T_{1/2}$ when co-administered with AN. Whereas $T_{1/2}$ of 6-MNA increases on co-administration with APE suggesting decrease in elimination. Increase in CL and decrease in Vd of AN+NAB treated group indicated the increase in elimination. Decrease in MRT support the findings. Co-administration of NAB with AN and standardized APE decreased the systemic exposure level of 6-MNA in vivo with decreasing AUC. The data indicates that AN and some ingredients of APE might have decreased the plasma concentration of 6-MNA in rats. The decrease in Cmax and AUC of 6-MNA is more with AN+NAB treated group compared to APE+NAB treated group. Increase in MRT, Cl and Vd of 6-MNA in APE+NAB treated group reveals that other components of APE other than AN might be interfering in metabolism of NAB. 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). So the effect of AN on CYP1A2 enzyme activity was found to be controversial, and it is difficult to claim the exact mechanism behind the pharmacokinetic interaction observed in this study.

To evaluate further possible herb–drug interaction in vivo anti-arthritic study has been performed in rats by co-administration of standardized APE and AN with NAB followed by histopathological and hematological evaluation. In anti-arthritic study, from increase in change in paw volume, decrease in mechanical nociceptive threshold and mechanical hyperalgesia, it was observed that NAB treated group showed better activity than all other groups and there was no effect for vehicle control group. Further, even though the dose of AN is equal in both groups treated with standardized APE+NAB and AN+NAB, there was better activity observed with APE+NAB treated group. The absence of 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 hematological study of NAB (7.5 mg/kg) support the findings. As the Cmax, AUC_{0-t} and $AUC_{0-\infty}$ of 6-MNA has been decreased on co-administration of NAB with APE and AN, the antiarthritic activity has also been decreased for APE and AN co-administered groups. Increased elimination and reduced plasma concentration of 6-MNA because of AN might have reduced its pharmacological effect. Because of involvement of many other CYPs in the metabolism of NAB and controversial effect of APE and AN on CYP1A2 reported by previous studies the exact reason behind changes observed in pharmacokinetic parameters and pharmacological activity in this study was unclear. However, the present study results suggest the decrease in the Cmax and AUC of 6-MNA with APE and AN treated group is possibly by the inhibition of CYP1A2.

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 (-80ºC) 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.

8.1.1.8. 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_{max}) and time reaching C_{max} (t_{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:

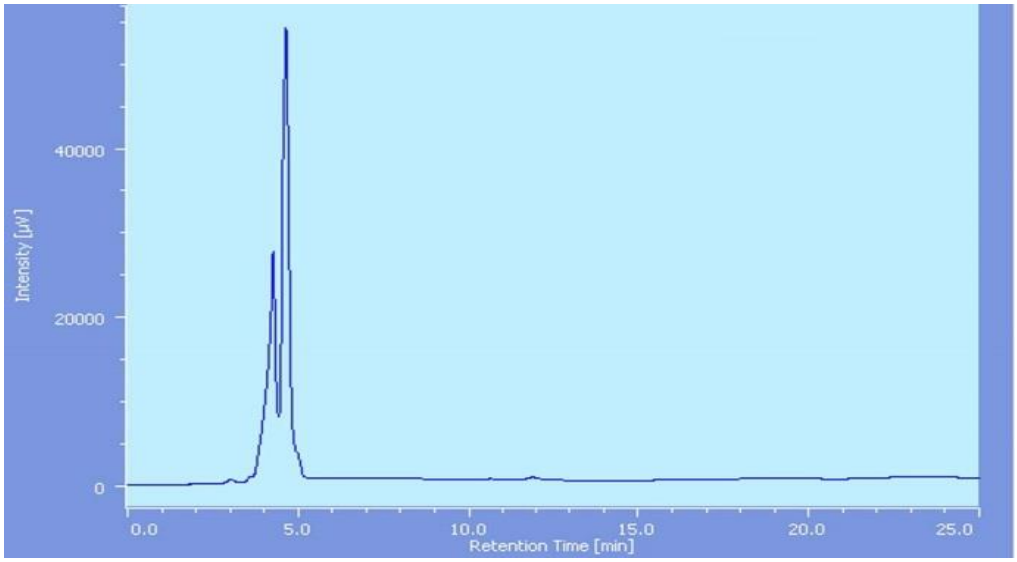
The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for each analyte and the IS as well as a short run time. After comparison of few columns, a Thermo Hypersil ODS column (250 x 4 mm, 5 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:

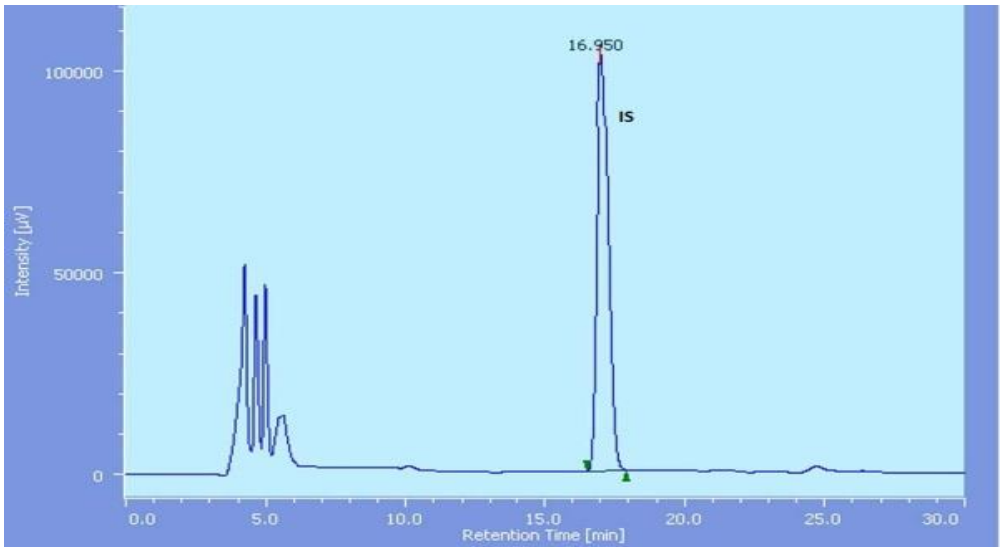
8.2.2.1. Selectivity:

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).

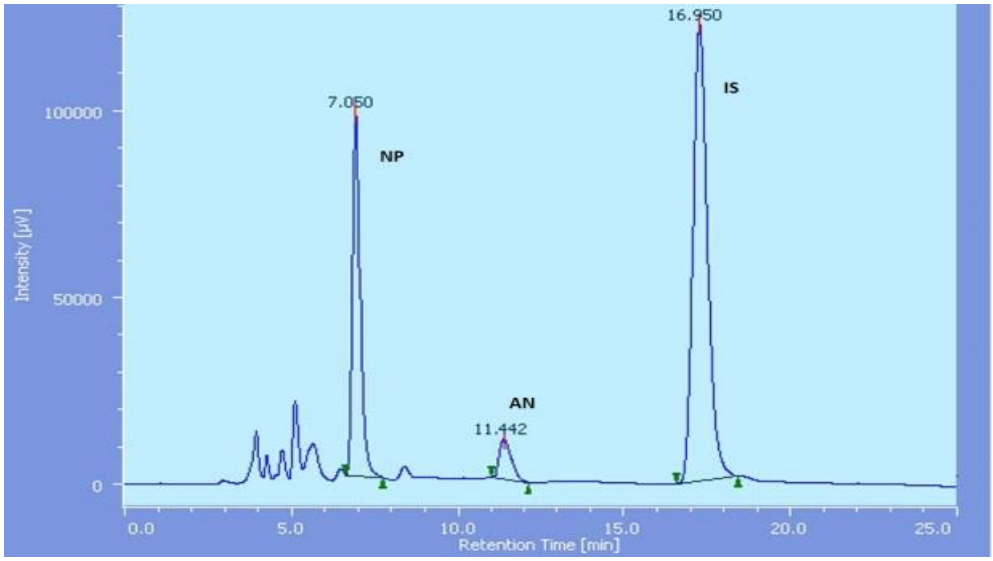
Figure 22: Chromatogram of NP, AN and I.S. in rat plasma



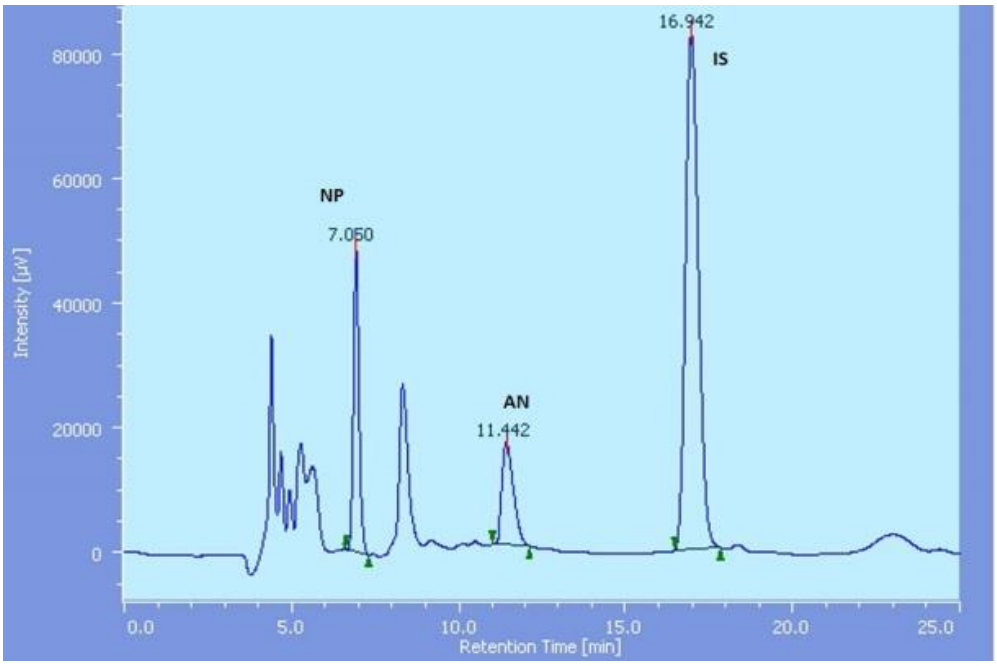
(A) Chromatogram of blank plasma sample



(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.

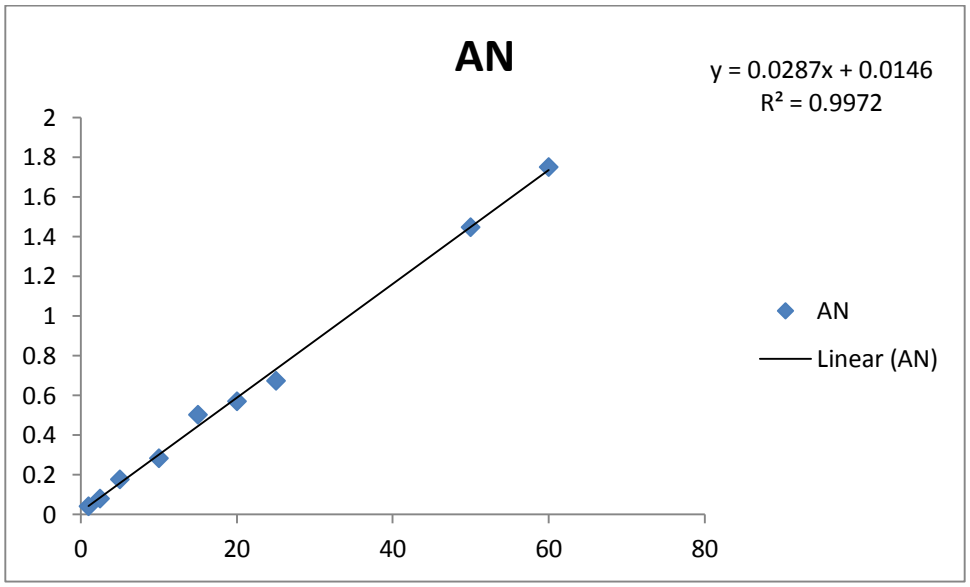


(D) Chromatogram of a plasma sample obtained from rat at 1.5 hr after oral administration of 60 mg of AN and 7.5 mg of NP.

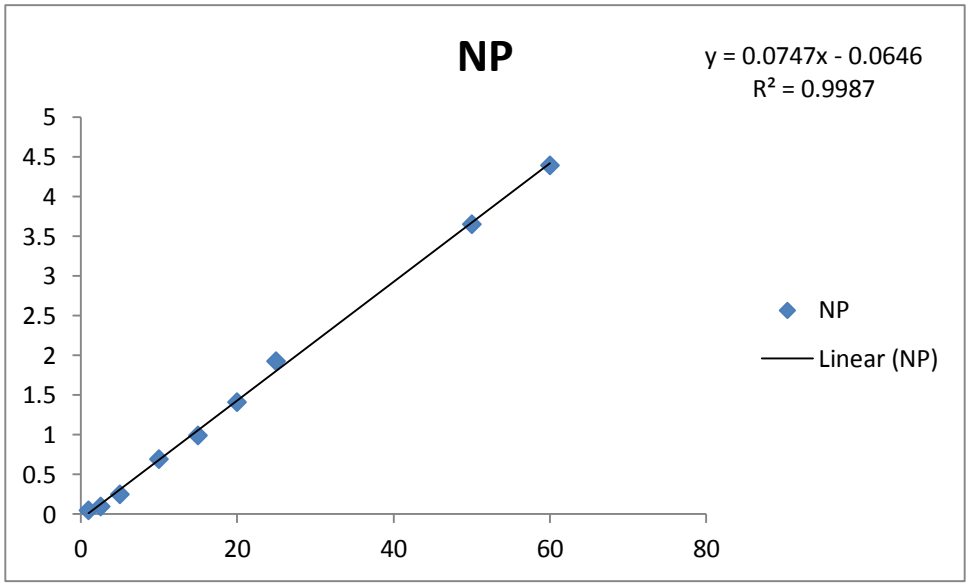
8.2.2.2. Sensitivity and linearity:

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).

Figure 23: Calibration curves of AN and NP



(A) Calibration curve of AN



(B) Calibration curve of NP

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

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.

Nominal concentration (µg/mL)	Intra day			Inter day		
	Measured concentration (µg/mL) (mean±S.D.)	RSD (%)	RE (%)	Measured concentration (µg/mL) (mean±S.D.)	RSD (%)	RE (%)
AN 1	1.05 ±0.06	6.60	-5.85	0.99 ±0.02	2.78	0.96
20	20.00 ± 0.14	0.73	-0.03	19.97 ± 0.17	0.86	2.43
60	59.38 ±0.68	1.15	1.54	60.01 ±0.10	1.53	-1.30
NP 1	1.02 ±0.07	6.90	-2.09	1.05 ±0.06	6.57	-5.52
20	19.95 ±0.13	0.67	4.13	19.93 ±0.16	0.80	6.61
60	60.03 ±0.09	1.51	-3.52	60.02 ± 0.16	1.17	-1.99

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.

Table 13: Extraction recovery of AN and NP in rat plasma (n = 5).

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

[a] Standards spiked before extraction.

[b] Standards spiked after extraction.

[c] 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.

Table 14: Stability of AN and NP in rat plasma (n = 3).

Nominal concentration (µg/mL)	Sample conditions			
	Bench top stability[a]	Auto-sampler stability[b]	Freeze-thaw stability[c]	45 days storage stability[d]
AN 1	0.9977 ± 0.02	1.0259 ± 0.07	1.0786 ± 0.12	1.0091 ± 0.06
20	19.8908 ± 0.10	19.9312 ± 0.17	19.9775 ± 0.16	19.8204 ± 0.06
60	59.6332 ± 0.75	59.4459 ± 1.24	60.1745 ± 0.08	58.8529 ± 0.11
NP 1	1.0661 ± 0.05	1.0126 ± 0.06	1.0375 ± 0.06	1.0177 ± 0.07
20	19.8617 ± 0.11	20.0449 ± 0.14	19.8937 ± 0.09	19.8491 ± 0.11
60	59.7693 ± 0.67	58.8143 ± 0.16	59.5240 ± 1.07	59.0154 ± 0.25

[a] Exposed at ambient temperature (25 ◦C) for 2 h.

[b] Kept at ambient temperature (25 ◦C) for 12 h.

[c] After three freeze‑thaw cycles.

d 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_{max} of groups co-administered AN+NP and APE +NP has been changed to 1.5 hr and 1 h respectively. The C_{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_{max}, T_{max}, AUC_{0-t}, $T_{1/2}$ MRT_{0-t}, CL and increased the $AUC_{0-\infty}$, $MRT_{0-\infty}$ and Vd of NP when compared to NP alone group. Co-administration of APE with NP significantly ($P < 0.001$) decreased the C_{max}, T_{max}, AUC_{0-t},

$AUC_{0-\infty}$,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

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

*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.*

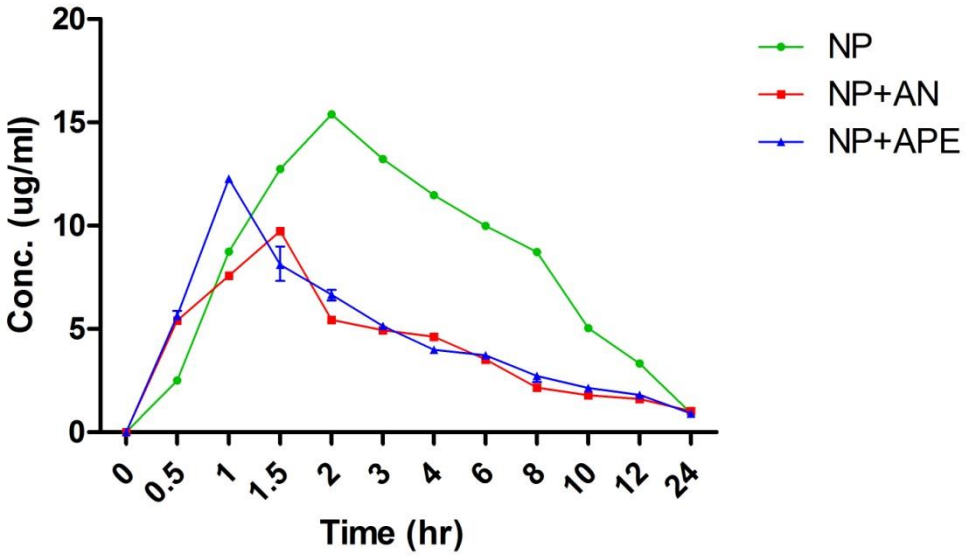
Where oral administration of,

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.).

Figure 24: Mean concentration-time curves of NP pharmacokinetic study



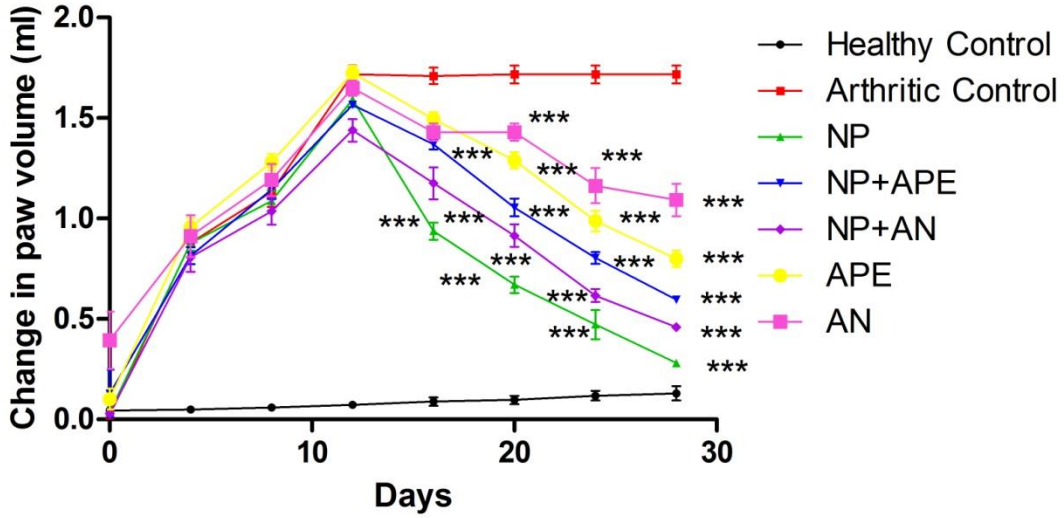
Mean concentration-time curves of NP (7.5 mg/kg, p.o.) alone, after co-administration of AN (7.5 mg/kg+60 mg/kg, p.o.) and after co-administration with APE (7.5 mg/kg +200 mg/kg, p.o.)

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.

Figure 25: Effect of NP, APE, NP + APE, AN and NP + AN on paw volume.

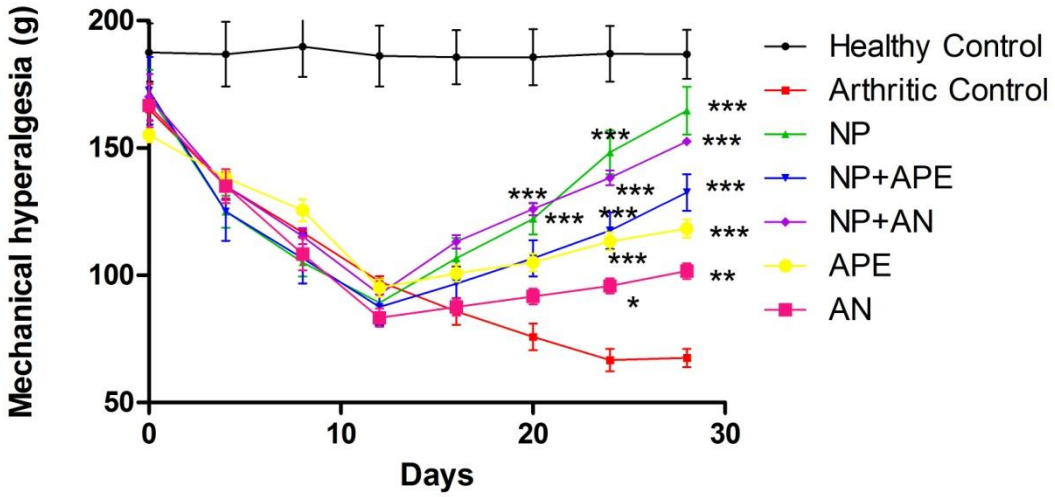


Effect of NP (10 mg/kg), APE (200mg/kg), NP (10 mg/kg) + APE (200 mg/kg), AN (60 mg/kg) and NP (10 mg/kg) +AN (60 mg/kg) on paw volume. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad Instat software;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

Figure 26: Effect of NP, APE, NP + APE, AN and NP +AN on mechanical withdrawal threshold.

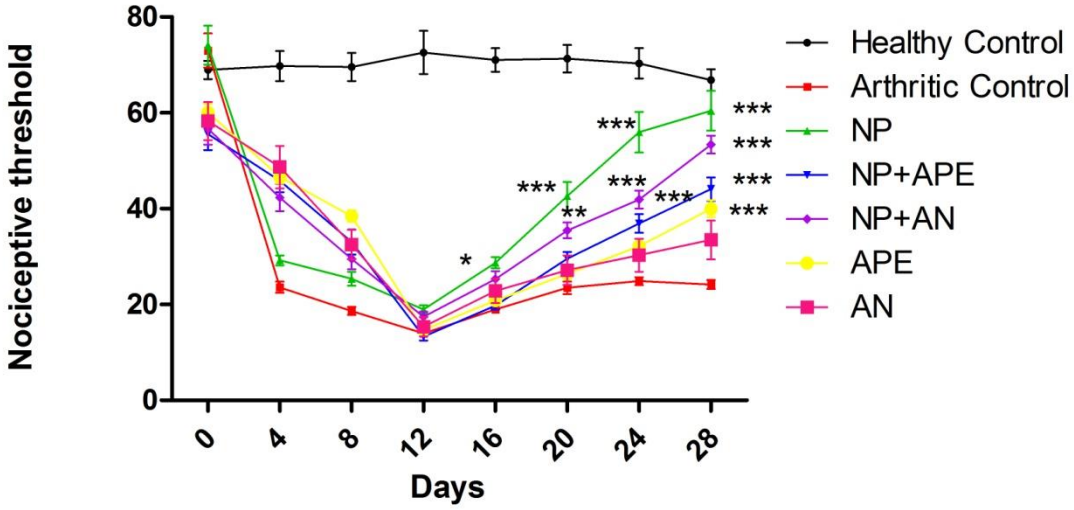


Effect of NP (10 mg/kg), APE (200mg/kg), NP (10 mg/kg) + APE (200 mg/kg), AN (60 mg/kg) and NP (10 mg/kg) +AN (60 mg/kg) on mechanical withdrawal threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using 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.

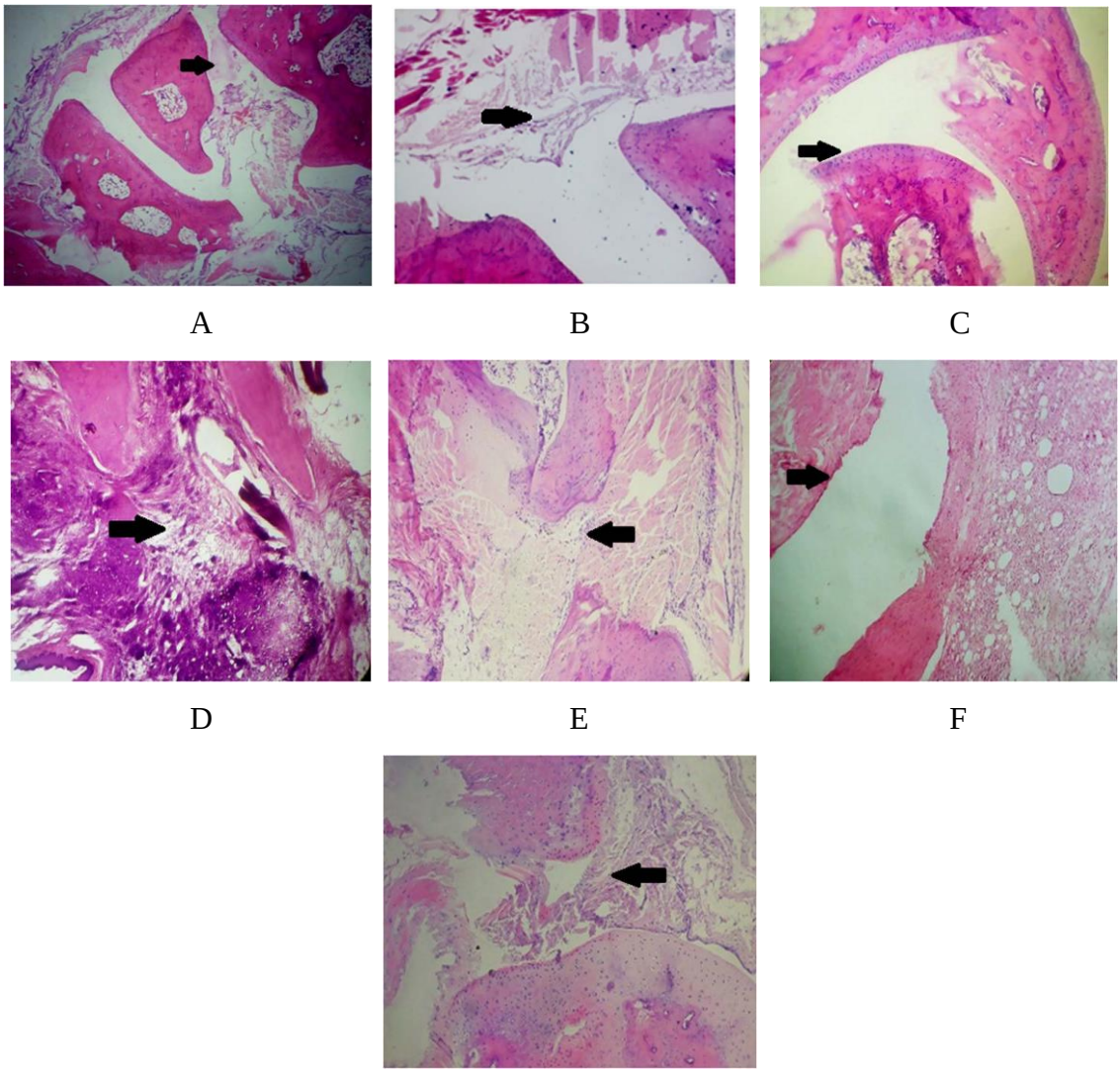


Effect of NP (10 mg/kg), APE (200mg/kg), NP (10 mg/kg) + APE (200 mg/kg), AN (60 mg/kg) and NP (10 mg/kg) +AN (60 mg/kg) on nociceptive threshold. Values are expressed as mean ± SD, n=6 in each group; statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test using Graphpad 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 B C

D E F

G

Histopathological representation of tibiotarsal joints of animals of **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

Group	**Arthritic Control**	**Healthy Control**	**NP**	**AN (60 mg/kg)**	**APE (200 mg/kg)**	**NP +AN (10+60 mg/kg)**	**NP+APE (10+200 mg/kg)**
Hb (gm/100ml)	8.87 ± 0.14	14.37 ± 0.21***	13.59 ± 0.32***	10.04 ± 0.06***	10.99 ± 0.03***	12.37 ± 0.12***	11.75 ± 0.20***
WBCs (thousands/µl)	15.29 ± 0.18	7.75 ± 0.15 ***	8.460 ± 0.16***	12.07 ± 0.16***	13.39 ± 0.13***	9.332 ± 0.1602***	10.89 ± 0.23***
RBCs (million/µl)	3.23 ± 0.05	7.04 ± 0.16***	6.57 ± 0.18	4.10 ± 0.06***	5.31 ± 0.06***	6.35 ± 0.21**	5.97 ± 0.06***
Platelets (lacks/ µl)	17.94 ± 0.09	9.147 ± 0.10***	9.39 ± 0.17	16.77 ± 0.25***	13.50 ± 0.09***	10.94 ± 0.06***	12.62 ± 0.08***
ESR (mm/hr)	16.34 ± 0.74	8.417 ± 0.19***	9.46 ± 0.08	13.68 ± 0.32***	12.31 ± 0.07***	10.50 ± 0.09***	11.31 ± 0.07***

*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 Srilanka 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 (Carreta 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_{max}, T_{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_{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_{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-methyonyl-leucyl-phenylalanine in HL60/neutrophils (Hidalgo et al., 2005), it also showed interaction with arginine and histidine in the cycloxygenase 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.

9. SUMMARY AND CONCLUSION:

Andrographis paniculata Nees (AP) is a traditionally used medicinal plant in Korea, Thiland, China, Japan, South Africa, India, Pakistan and Sri lanka for the treatment of fever, cold, inflammation, diarrhea and other infectious diseases. Since 175 BC, AP along with other plants in polyherbal preparations was recommended in Charak Samhita for treatment of jaundice. Indian pharmacopoeia mentioned that AP is a predominant constituent of at least 26 Ayurvedic formulations. Andrographolide one of the major active component of the plant AP has been extensively used in traditional herbal medicine in China, Southeast Asia, and the Arabian Gulf for the treatment of several diseases, including inflammatory diseases. Traditional use of AP as anti-inflammatory herb has been studied by various scientists and proved the mechanism. Various reports suggests that inflammatory responses are a critically a part of pathophysiology of several diseases including septic shock, cancer, atherosclerosis, rheumatoid arthritis and diabetes. So the manipulation of inflammatory responses may allow for prevention of serious diseases or symptoms.

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. Andrographolide, 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. In clinical trials, *Andrographis paniculata* extract (30% AN) showed effectiveness for symptom relief and reduce serological parameters in patients with Rheumatoid Arthritis.

Several polyherbal formulations consisting AP as a major ingredient as anti-inflammatory and anti-arthritic are available in local Indian markets. In treatment of arthritis it is common practice that, along with the disease–modifying antirhumatic drugs (DMARDs) and nonsteroidal anti-inflammatory drugs (NSAIDs), herbal formulations are either taken with or without knowledge of health care provider by the patients for better therapeutic effects. This may lead to either beneficial or toxic effects. An increasing consumption of medicinal herbs, which are often administered in combination with conventional therapeutic drugs, it is likely that constituents in herbal preparations may be substrates, inhibitors, or inducers of cytochrome P450 enzymes (CYPs) and have an impact on the pharmacokinetics and pharmacodynamics of any co-administered drugs metabolized by this system. Many studies have been reported for interaction

between *Andrographis paniculata Nees* extract and AN with various synthetic drugs. Though CYP1A2 is the common CYP involved in the metabolism of AN, APE, ETO, NAB and NP in rats, unfortunately not a single attempt has been done to investigate interaction of APE and it's one of the major constituent AN with ETO, NAB and NP after oral administration in rats. Previously various bioanalytical methods are reported in literature for the determination of AN alone and in combination with other drugs. Seldom bioanalytical methods were previously reported in literature for the determination of ETO, NAB and NP in rats and humans. However there was no analytical method is available for simultaneous estimation of AN and ETO/NAB/NP. Prompted by the above findings, this study developed new validated HPLC methods for simultaneous determination of AN with ETO/NAB/NP in rat plasma and application of the developed methods for pharmacokinetic study in rats. In the present study we investigated the possible herb-drug interactions of APE and AN with ETO, NAB and NP through comparing their pharmacokinetic profiles and pharmacodynamic after oral administration in rats.

For ETO, a HPLC method with mobile phase consisting of the mixture of solvent A (Acetonitrile) and solvent B (Water) in the ratio of 55:45 (v/v) for 35 minutes at a flow rate of 0.5 mL/min and detection using UV detector at a wavelength of 225 nm for simultaneous determination of AN, ETO and IS was developed and validated as per USFDA guidelines. The developed HPLC method was applied for pharmacokinetic study in rats. After oral co-administration of APE (200 mg/Kg), AN (60 mg/kg) with ETO (10mg/kg) in rats respectively, drug concentrations in plasma were determined using HPLC method. The main pharmacokinetic parameters of Cmax, tmax, t1/2, MRT, Vd, CL, and AUC were calculated by non-compartment model. To evaluate further possible herb-drug interaction anti-arthritic study has been performed in the groups with co-administration of APE and AN with ETO respectively. Change in paw volume, mechanical nociceptive threshold, mechanical hyperalgesia, histopathology and hematological parameters were evaluated to study antiarthritic activity.

Decrease in t_{max} of AN in APE and pure AN on co-administration with ETO was observed. No change in t_{max} of ETO on co-administration with pure AN was observed whereas t_{max} of ETO on co-administration with APE was increased to 6 h. The C_{max} of AN and ETO were decreased significantly after co-administration of AN and APE with ETO than AN, APE and ETO individually administered groups. Co-administration of pure AN with ETO significantly ($P < 0.05$) decreased the AUC_{last}, AUC_{∞}, $AUMC_{last}$, $AUMC_{\infty}$, $t_{1/2}$, MRT_{last} and MRT_{∞} of AN and

ETO when compared to AN and ETO alone groups. There was significant increase in CL of ETO and AN, significant decrease in Vd of AN while increase in Vd of ETO was observed in co-administered group when compared to respective alone groups. In anti-arthritic study, from increase in change in paw volume and decrease in mechanical nociceptive threshold and mechanical hyperalgesia, it was observed that ETO treated group showed better activity than APE+ETO treated group. The results of histopathological and hematological study supports the findings and revealed the dose dependent effects of AN and APE.

For NAB, a HPLC method with mobile phase of 0.01N acetic acid (Solvent A) and acetonitrile (Solvent B) with gradient as follows: 0 to 9 min 70% A and 30% B, from 10 to 35 min 30% A and 70% B at a flow rate of 0.5 mL/min and detection using UV detector at a wavelength of 225 nm was developed and validated as per USFDA guidelines. Pharmacokinetic study in rats was carried out using the developed HPLC method. After oral co-administration of APE (200 mg/Kg), AN (60 mg/kg) with NAB (7.5 mg/kg) in rats respectively, drug concentrations in plasma were determined using HPLC method. The main pharmacokinetic parameters of Cmax, tmax, t1/2, MRT, Vd, CL, and AUC were calculated by non-compartment model. To evaluate further possible herb-drug interaction anti-arthritic study has been performed in the groups with co-administration of APE and AN with NAB respectively. Change in paw volume, mechanical nociceptive threshold, mechanical hyperalgesia, histopathology and hematological parameters were evaluated to study antiarthritic activity.

As per previously published studies on pharmacokinetics of NAB, its active metabolite-6-MNA was detected intact in plasma instead of the parent compound NAB. 6-MNA was absorbed into the circulatory system and reached its peak concentration approximately 1.5 h after administered individually. T_{max} of group co-administered AN and NAB has been changed to 2 hr but T_{max} of group co-administered APE and NAB was the same. The C_{max} of 6-MNA was decreased significantly after co-administration of AN and APE with NAB compared to group administered with NAB alone. Co-administration of AN with NAB significantly ($P < 0.05$) decreased the $C_{max,}$ AUC_{0-t}, $AUC_{0-\infty}$, $T_{1/2}$, $MRT_{0-t,}$ $MRT_{0-\infty,}$ and Vd of 6-MNA whereas increased T_{max} and CL of 6-MNA in comparison with group treated with NAB alone. Co-administration of APE with NAB significantly ($P < 0.05$) decreased the $C_{max,}$ AUC_{0-t}, and $AUC_{0-\infty}$ of 6-MNA whereas increased the $T_{1/2}$, $MRT_{0-t,}$ MRT0-$_{\infty,}$ Vd and CL of 6-MNA when compared to group

treated with NAB alone. From anti-arthritic study, it was observed that NAB treated group showed better activity than all other groups. Increase in change in paw volume, decrease in mechanical nociceptive threshold and mechanical hyperalgesia, absence of 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 hematological study of NAB (7.5 mg/kg) support the findings. Further, even though the dose of AN is equal in both groups treated with standardized APE+NAB and AN+NAB, there was better activity observed with APE+NAB treated group.

For NP, a HPLC method with mobile phase consisting 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 and detection using UV detector at a wavelength of 225 nm was developed and validated as per USFDA guidelines. After oral co-administration of APE (200 mg/Kg), AN (60 mg/kg) with NP (7.5 mg/kg) in rats respectively, drug concentrations in plasma were determined using HPLC method. The main pharmacokinetic parameters of Cmax, tmax, t1/2, MRT, Vd, CL, and AUC were calculated by non-compartment model. To evaluate further possible herb-drug interaction anti-arthritic study has been performed in the groups with co-administration of APE and AN with NP respectively. Change in paw volume, mechanical nociceptive threshold, mechanical hyperalgesia, histopathology and hematological parameters were evaluated to study antiarthritic activity.

NP was absorbed into the circulatory system and reached its peak concentration approximately 2 h after administered individually. T_{max} of groups co-administered AN+NP and APE +NP has been changed to 1.5 hr and 1 h respectively. The C_{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_{max}, T_{max}, AUC_{0-t}, $T_{1/2}$ MRT_{0-t}, CL and increased the $AUC_{0-\infty}$, $MRT_{0-\infty}$ and Vd of NP when compared to NP alone group. Co-administration of APE with NP significantly ($P < 0.001$) decreased the C_{max}, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$ and increased the MRT_{0-t}, $MRT_{0-\infty}$, CL and Vd of NP when compared to NP alone group. In anti-arthritic study from 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.

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.

In conclusion, the results obtained from this study suggested that ETO, NAB and NP with APE and pure AN existed pharmacokinetic herb-drug interactions in rat which is correlated with anti-arthritic study. Significant decrease in Cmax, AUC, $t_{1/2}$ and increase in Vd, CL of ETO was observed after co-administration with pure AN and APE. Co-administration of ETO with APE and pure AN decreased systemic exposure level of each compound *in vivo*. In anti arthritic studies, even though the dose of AN is equal in both groups treated with APE and AN there was better activity observed with APE treated group.

Significant decrease in Cmax, AUC_{0-t} and $AUC_{0-\infty}$ of 6-MNA was observed after co-administration with pure AN and APE. Increase in elimination of 6-MNA was observed with reduced $T_{1/2}$ when co-administered with AN. Increase in CL and decrease in Vd of AN+NAB treated group indicated the increase in elimination. Decrease in MRT support the findings. Whereas $T_{1/2}$ of 6-MNA increases on co-administration with APE suggesting decrease in elimination. Co-administration of NAB with APE and pure AN decreased systemic exposure level of NAB *in vivo*. The decrease in C_{max} and AUC of 6-MNA is more with AN+NAB treated group compared to APE+NAB treated group. Increase in MRT, Cl and Vd of 6-MNA in APE+NAB treated group reveals that other components of APE other than AN might be interfering in metabolism of NAB. In anti arthritic studies, even though the dose of AN is equal in both groups treated with standardized APE+NAB and AN+NAB there was better activity observed with APE+NAB treated group.

Significant decrease in C_{max}, t_{max}, AUC_{0-t}, of NP was observed after co-administration with pure AN and APE. Co-administration of NP with APE and pure AN decreased systemic exposure level of NP *in vivo*. 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 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_{max} and AUC_{0-t} but this decrease is significantly more with AN+NP treated group compared to APE+NP treated group. In anti arthritic studies, even though

the dose of AN was equal in both groups treated with standardized APE and AN there was better activity observed with AN+NAB treated group.

The study observed that co-administration of AN and APE changes pharmacokinetics and pharmacodynamics of ETO, NAB and NP. Further studies should be done to understand the effect of other herbal ingredients of APE on ETO/NAB/NP as well as to predict the herb-drug interaction in humans. Physicians and patients using *A. paniculata* should have the knowledge about its possible herb–drug interaction with ETO, NAB and NP. The study provided valuable information for rational use of herbal remedies in the treatment of arthritis.

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List of publications

Sr. no.	Title	Authors	Journal	Year of Publication	Issue, Volume	Impact factor
1	Pharmacokinetic and pharmacodynamic herb–drug interaction of Andrographis paniculata (Nees) extract and andrographolide with etoricoxib after oral administration in rats	Aishwarya Balap, Bhagyashri Atre, Sathiyanarayanan Lohidasan, Arulmozhi Sinnathambi, Kakasaheb Mahadik	Journal of Ethnopharmacology	2016	183	3.05
2	Pharmacokinetic and Pharmacodynamic Interaction of Andrographolide and Standardized Extract of Andrographis paniculata (Nees) with Nabumetone in Wistar Rats	Aishwarya Balap, Sathiyanarayanan Lohidasan, Arulmozhi Sinnathambi and Kakasaheb Mahadik	Phytotherapy Research	2017	31	2.69
3	Herb-drug interaction of Andrographis paniculata (Nees) extract and andrographolide on pharmacokinetic and pharmacodynamic of naproxen in rats	Aishwarya Balap, Sathiyanarayanan Lohidasan, Arulmozhi Sinnathambi, Kakasaheb Mahadik	Journal of Ethnopharmacology	2017	195	3.05