EVALUATION OF PAIN ALLEVIATING, ANTI-INFLAMMATORY AND ANTI-RHEUMATIC PROPERTIES OF SELECTED KENYAN MEDICINAL PLANTS

A proposal submitted in fulfillment of requirements for Doctor of Philosophy degree of University of Nairobi, Physiology.

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Signature…………………………… Date………………
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Date of submission…………………………………………………………………….
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CHAPTER ONE

1. INTRODUCTION

Rheumatoid arthritis (RA) is a complex chronic inflammatory condition with a systemic autoimmune disorder that manifests with pain, inflammation and tissue damage in joints, together with anemia. The disease has idiopathic etiology and a rather unusual pathogenesis which makes it difficulty to identify effective therapies (Wood, 2004; Hultqvist et al., 2006; Lindquist 2002). Osteoarthritis (OA) on the other hand is a degenerative joint disease characterized by damage to the articular cartilage, changes in subchondral and marginal bone, synovitis and capsular thickening, usually affecting the weight bearing joints (Bendele, 2001; Schaller et al., 2005).

Conventionally, the treatments of intense and unrelenting pain, such as that resulting from rheumatoid arthritis, joint pain, neuropathic pain or injury is based on opioids, non-steroidal anti-inflammatory drugs (NSAIDs) and drugs inhibiting inflammatory cytokines despite their well documented side effects and prohibitive cost. On the contrary, natural products offer a more accessible and cheaper alternative, and they have even contributed enormously to the development of current therapeutic agents (Setty and Sigal 2005; Lipsy and Tao, 2007). However, most of the commonly used herbal remedies used by the Kenyan TMP have not been systematically validated. In addition, there’s a rapid disappearance of traditional herbal knowledge and increased biodiversity degradation all threatening the loss of our economically valuable cultural heritage (Srithi et al., 2009). All these calls upon for conscious and concerted efforts to collect, document and scientifically validate medicinal plants use in our communities.

The commonly used medicinal plant preparations for the management of OA and RA are usually combined plant extracts, and indeed this may the future direction in the development of
efficacious, safe and cost effective herbal remedies world over (Lee 2000; Chrubasik et al., 2007; Park et al., 2007). These herbal remedies have actually been recognized as effective, safe and cost effective agents and indeed several herbal based traditional medicinal preparations are patented as phytopharmaceutical products (Darshan and Doreswamy 2004). In Kenya, several medicinal preparations are used in the management of osteoarthritis and rheumatoid arthritis and the objective of this study will be to investigate the analgesic, anti-inflammatory and anti-rheumatoid properties of selected medicinal plant preparations/extracts in Machakos/Makueni region of Kenya.

CHAPTER TWO

2. Literature review

Osteoarthritis (OA) and rheumatoid arthritis (RA) are among the most common joint ailments and a leading cause of irreversible joint deformities and functional impairment (Wood, 2004). Moreover, these conditions often lead to both long term morbidity and early mortality (Kelly et al., 1997; Doran et al., 2002) and the economic burdens associated with their management are devastating. RA affects 1-2% of the population is more common in women than in men while osteoarthritis is a common problems affecting over 60% of all people by the age of 50 years (Wood 2004; Schaller et al., 2005; Lawrence et al., 2008).

Over the years, there has been a variety of exciting new therapies for the management of OA and RA with improved patient responses based on clinical signs and symptoms reported (Wood 2004; Setty and Sigal 2005; Caporali et 2008). However, all these therapies have not been without devastating side effects, some of which are life threatening but remain in use only by the terminally ill patients where the safety vs. severity of the condition is the driving force towards their use. For instance, the most commonly prescribed DMARDs are associated with a range of
side effects related to liver and bone marrow toxicity (methotrexate, sulfasalazine, leflunomide, azathioprine, gold salts, D-penicillamine), renal toxicity (cyclosporine A, parenteral gold salts, D-penicillamine), pneumonitis (methotrexate), allergic skin reactions (gold compounds, sulfasalazine), autoimmunity (D-penicillamine, sulfasalazine, minocycline) and infections (azathioprine, cyclosporine A) (Mikuls, 2003; Fleischman et al., 2004; Woods 2004; Majithia and Geraci, 2007; Caporali et al., 2008). TNF and interleukin antagonists have been implicated with serious infections, malignancies, heart failure, chronic demyelinating disorders, lupus-like autoimmune disease, vasculitis, liver abnormalities and hematological abnormalities (Francis 1999; Mohan et al., 2001; Brown et al., 2002; Kroesen et al., 2003; Wood 2004).

Like many other chronic diseases RA and OA are associated a high usage of herbal medicines (Resch et al., 1997; Lipsy and Tao, 2007; Setty and Sigal 2005). According to World Heath Organization statistics, more than 80 percent of the population in developing countries continues to rely on traditional medicine and indigenous knowledge to meet their health needs (Nanyingi et al., 2008). Owing to the fact that traditional medicine is accessible, affordable, culturally acceptable, socially sanctioned, and easy to prepare with little or no side effects, most people prefer it to the exorbitantly priced health care services conventional medicine and practitioners offer, not to mention the hazardous side effects of these medications and their limitations in the domain of holistic health, especially in economically impoverished African societies.

Thus, there is a growing need for safe and effective traditional medicine therapies, an important tool to increase access to health care particularly in poor and marginalized populations. Several plants species are used in the management of these conditions and but very few have been systemically investigated for their efficacy. This study hopes to come up with a valid anti-arthritic and anti-rheumatoid herbal formula.
2.2 Objectives

The general objective will be to investigate the analgesic, anti-inflammatory and anti-rheumatoid properties of selected medicinal plants used by some Kenyan TMP.

2.2.1 Specific objectives:

1. To analyze the diversity of medicinal plants used in the management of RA by different TMP in the selected localities (Machakos/Makueni area). The entire method of preparation, extraction and formulation will also be analyzed and documented.

2. To evaluate the pain alleviating properties of the various plants parts/extracts and preparations using the tail flick and the formalin tests. Analgesic potency will also be analyzed in the formalin test.

3. To investigate the anti-arthritis properties of selected plant extract/preparation in the Complete Freund’s Adjuvant (CFA) arthritis model in rats.

4. To evaluate the anti-rheumatoid properties of the plant extract/preparation in the Collagen-Induced Arthritis model in rats.

5. To evaluate the immunosuppressive effects of the plant extract/preparation in rats.

6. To study the histopathological effects of the anti-arthritis and anti-rheumatoid plant extract/preparation.

7. Perform separation of the most active anti-arthritis/rheumatoid extracts/preparation to elucidate on the biochemical composition of the most active ingredients.
2.3 Hypothesis

The commonly used herbal medicine preparation and plant extracts have significant pain relieving, anti-arthritic and anti-rheumatoid properties and are safe for medicinal usage.

2.4 Justification

Joint pain and rheumatoid arthritis are difficulty and expensive conditions to manage especially in economically impoverished communities where provision of primary healthcare is inexistent or inadequate. There is also a great demand for new anti-rheumatic agents that are able to act on chemical mediators of inflammation, but have fewer toxic effects and cost less than the current treatments that block the actions of TNF-α, IL-1/6 or those of the Disease Modifying Anti-Rheumatic Drugs (DMARDs) (Majithia and Geraci, 2007).

The therefore aims to identify and verify the efficacy of locally used medicinal plant formulations used in the management of the two joint conditions. Individual plant extracts and the medicinal preparation will also be compared for relative potency. The study hopes to come validate the hypothesis that the commonly used herbal preparation/plant extracts are safe, cheaper and effective in the management of rheumatoid arthritis and osteoarthritis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethnobotanical survey

An ethnobotanical survey will be carried out in Machakos and Makueni districts to identify the plants and procedures involved in the making of the medicinal preparation used in the management of RA/OA. Field visits will be done and semi-structured questionnaires
administered to registered traditional herbal practitioners (TMP) to obtain data regarding the diversity of plants used, methods of preparation and formulation, diagnosis, dosage, treatment outcomes and prognostic indicators, recurrence as well as patient satisfaction. A sample questionnaire is shown on appendix 1. Data obtained from the questionnaire will be analyzed using the appropriate methods and the commonly used plants collected for taxonomic identification. Voucher specimens will be deposited at the national museums of Kenya herbarium/UoN herbarium.

3.2 Sample preparation

The plants of interest will be harvested and dried under the shade. They will be processed into powder form before extraction, which will be done in accordance to the methods used by the TMP. For comparative purposes, methanolic, chloroform and metnanolic: diclorolomethanolic extracts will also be prepared by cold extraction. Basically the dry powdered plant material will be macerated in the solvent extractor for 3 days at room temperature and the supernatant filtered in whatman no.1 paper. The residue from the filtration will also be extracted again twice using the same procedure. The filtrates obtained will be combined and then evaporated to dryness under reduced pressure to obtain a lyophilized extract. The percent yield will be calculated as per the equation % yield = Wcrude extract/Wdried plant × 100 where Wcrude extract = weight of crude extract and Wdried plant = weight of dried plant material (Phrompittayarata et al., 2007).

3.3 Evaluation of the anti-nociceptive activity in the Tail-flick test

The anti-nociceptive activity of the plant extract/preparation will be assessed using the tail flick test. This is a phasic pain test that measures tail flick responses to radiant heat stimulation, a spinally integrated nociceptive reflex (Le Bars et al., 2001). The test is simple to perform,
displays minimal inter-animal variability and allows for repeated testing without conditioning effects, making it a useful tool for screening analgesic drugs (Langerman et al., 1995; Le Bars et al., 2001).

The mice will be kept in cages in a well ventilated drought free house with a 12 hour light-dark cycle and provided with food and water *ad libitum*. The test room will be maintained at equal ambient temperature to that of the animal house. A randomized block design will be used based on the sex of the animals. The test stimulus will be preset at a temperature that rises from 35°C to 55°C in ten seconds. The heating will be terminated manually when a tail flick response occurs. Tail flick latency will be scored and a cut off time of 40 sec will be adopted. Testing will be done at -10, 0, 0.5, 1, 2, 3, 4, 5 hours. The average of -10 and 0 (pretreatment scores) will be used as the base line (control) and test material will be administered at time 0. The extract will be administered i.p at two predetermined dosages with a saline treated control group. The person scoring the nocifensive behavior will not be aware of what has been injected into the animals.

### 3.4 The Evaluation of the Analgesic and Anti-Inflammatory Activity in the Formalin Test

The method will be adapted from that described by Rosland *et al* (1990). In this tonic pain test, a 20 μL of 0.5% formalin solution will be injected into the dorsal surface of the hind paw in mice. Scoring will be based on the duration of time spent in nocifensive behavior for 60 min in blocks of five minutes. In this test, the nocifensive behavior will be defined as licking and biting of the formalin injected paw. The mean duration of time spent in pain in the first 10 minutes will be taken as the pain response in the first phase of the formalin test, while the late phase will be taken as between 20 and 45 minutes after injection of the formalin. The observer will not be aware of what has been administered in the animals.
To evaluate for the antinociceptive potency of the antinociceptive activity of the plant extracts/preparation, the effects will be compared with those of morphine, aspirin and dexamethasone. Treatment (with the plant material, vehicle, morphine, naloxone, aspirin and dexamethasone) will be done i.p at two predetermined dosages 30 min before the formalin injection. The percentage inhibition of licking/biting will be calculated the formula: 

\[
\left(\frac{C - T}{C}\right) \times 100; \text{ where } C \text{ represents the vehicle treated control group value for each phase and } T \text{ represents the treated group value for each phase (Dongmo et al., 2005).}
\]

### 3.5 Evaluation of Anti-Arthritic Effect in Adjuvant-Induced Arthritis in Rats

Experimental arthritis will be induced in rats according to the method described by Jia et al (2003). In this chronic pain model, the stimulus is inflammatory reaction in the joints caused by the injected material and involves intradermal injection of *Mycobacterium butyricum* with Freund’s adjuvant into the tails (Bendele, 2001; Le Bars et al., 2001; Yu et al., 2002). Rat adjuvant induced arthritis is widely used experimental model of polyarthritis, periarthritis and periosteitis (Mendele, 2001; Yu et al., 2002; Jia et al., 2003) and the polyarthritis induced is similar to various inflammatory conditions. Furthermore, results from this test are predictive of analgesic and anti-inflammatory activity of the test substance (Bendele, 2001; Cho et al., 2008).

The right footpad of each rat will be injected S.Q with 0.1 ml of CFA (Sigma). The plant material will be administered i.p at three predetermined dose levels, distilled water, and acetylsalicylic acid at 10 mg/kg will be given daily to the treatment groups, control group, and reference group, respectively, starting at 8 days after CFA injection for 14 consecutive days. The edema (paw perimeter) of the left and right hind paws will be evaluated at 2 h, 8, 12, 16, and 24 days post-injection of CFA. Experiments terminated at day 24. The edema inhibition will be
calculated as follows: Inhibition rate (%) = Vc - Vt/Vc × 100, where Vc is the edema value of the control group; and Vt is the edema value of treated group.

3.6 Induction of RA Rats Using the Collagen Induced Arthritis (CIA)

Adult (10-15 wk) male Sprague Dawley rats will be used in these experiments due to their susceptibility to collagen induced arthritis (CIA), the most commonly used animal models of rheumatoid arthritis which involves both humoral and cell-mediated immune components (Trentham, 1982; Cho et al., 2007; Bendele, 2001). The arthritis ensues when rats are immunized against homologous or heterologous type II collagen, and is characterized by marked bone destruction associated with deposition of immune complexes on articular surfaces, bone resorption, periosteal proliferation, synovitis and periartricular inflammation (Mendele 2001; Maruyama et al., 2006). The rats will be housed in groups of 10 and maintained at a temperature of 21 ± 2°C on a 12-hour light/dark cycle (7:00 am to 7:00 pm) and provided with food and water ad libitum.

Collagen II from bovine articular cartilage will be dissolved overnight at 4°C in 0.1M acetic acid at a concentration of 2.5mg/mL. The solution will be emulsified with 1.2 times volume of Complete Freund’s Adjuvant (CFA), and 100 μL of the emulsion administered subcutaneously at the base of the tail of the rats for immunization on day 1 (see figure 1). Booster injection of 100 μL of the emulsion will be given on day 15. The rats will be given the plant extract/preparation orally by galvaging at predetermined dosages from day 0 to 21, 5 days per week. Control rats will be given an equal volume of 2.5% DMSO solution. Their weight and paw diameters will be measured 2 days each week from day 0 to 39.
Scoring will be done by monitored by assessment of several different parameters at various time points before and after induction of arthritis.

### 3.6.1 Macroscopic scoring system:

The extent of paw swelling and edema shall be scored as the method of Paola and Cuzzocrea (2008), with a score ranging from 0 to 3 (0, no swelling; 1, slight swelling and/or erythema; 2, pronounced edematous swelling and 3, severe arthritis and joint rigidity). Each limb shall be graded, thus allowing a maximum score of 12. The arthritic score for each mouse will be the sum of the scores of all four paws (Paola and Cuzzocrea, 2008).

### 3.6.2 Hind paw diameter

To investigate the effects of treatments on edema in arthritic joints, tibiotarsal joint circumferences will be measured using a tape measure and the change in paw size after treatments expressed as percent change in paw diameter (Chillingworth and Donaldson 2003).
3.6.3 Wetting with acetone

This test is used to measure cold allodynia in animal models of pain (Kim et al., 2008). Acetone will be applied five times on the dorsal surface of the hind paw at intervals of more than 2 minutes at room temperature. Brisk withdrawals will be measured and the data expressed as paw withdrawal latency (PWL), which is the number of responses divided by number of trials expressed as a percentage (Kim, 2008). \[ \text{PWL} = \frac{\text{Number of responses}}{\text{Number of trials}} \times 100. \]

3.6.4 Biochemical assay of pro-inflammatory and anti-inflammatory cytokines

Cytokines, a diverse group of polypeptides that are generally associated with inflammation, immune activation, and cell differentiation or death, include Interleukins (IL), Interferon’s (IFN), tumor necrosis factors (TNF), Chemokines and growth factors (Dinarello, 2000; Wong et al., 2008). Moreover, the balance between the effects of pro-inflammatory and anti-inflammatory cytokines thought to be the key determinant in the outcomes of an inflammatory reaction as well as therapeutic efficacies of agents (Wong et al., 2008). In this study, the levels of TNF-\(\alpha\), IL-1 \(\beta\) (pro-inflammatory) and INF-\(\alpha\) (anti-inflammatory) will be analyzed from blood samples collected at different levels of RA (as shown in figure 1) using a rat cytokine ELISA kit (BioPlex cytokine kit, BD, UK) in accordance with the manufacturers instructions. Rat paw tissue exudates collected immediately after sacrificing the animals will also be assayed.

3.6.5 Histopathological grading of Rheumatoid Arthritis

This will be based on the three main features of chronic arthritis (Chillingworth and Donaldson 2003; Krenn et al., 2006). These are enlargement of synovial lining, cellular density of synovial stroma and leukocyte infiltration. Each will be assigned a score of 0-3, with 0 being no change and 3 the high grade synovitis. The arthritic score for each rat will be the sum of the scores of all
four paws. Hind ankle joints will be dissected on day 35, fixed in 10% neutral buffer formalin, decalcified in 10% formic acid-formalin, and embedded in paraffin. The hind ankle joints will be sectioned at 4 µm and stained with haematoxylin and eosin and blind scored for arthritis as shown in appendix 2.

3.7 Statistical analysis

All the data will be expressed as mean ± standard error of the mean (SEM) and statistically compared using one-way analysis of variance (ANOVA). Post hoc comparisons of the differences between the drug-treated groups and controls will be performed using Dunnett’s t-test and P values < 0.05 will be considered significant.

References


**Budget**

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<th>Quantity</th>
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<td>3 years</td>
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<td>45,000</td>
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</tr>
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<td>1000</td>
<td>100,000</td>
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</tr>
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<td>20 lits</td>
<td>12,000</td>
</tr>
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<td>Methanol</td>
<td>3000 per 5 lits</td>
<td>20 lits</td>
<td>12,000</td>
</tr>
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<td>Chloroform</td>
<td>3000 per 5 lits</td>
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<td>12,000</td>
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<td>Cytokine Assay kit</td>
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<td>Transport costs</td>
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<td>100,000</td>
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<tr>
<td>Data storage, handling and analysis</td>
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<td></td>
<td>30,000</td>
</tr>
<tr>
<td>Na-pentobarbitone100ml</td>
<td>5000</td>
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<td>5000</td>
</tr>
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<td>Syringes, Needles, slides, cover slips and other consumables</td>
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<td></td>
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<td><strong>Sub-total</strong></td>
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## Work plan

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<th>Year 3</th>
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<td>1-4</td>
<td>5-8</td>
<td>9-12</td>
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<tr>
<td>Literature review, PhD registration and ethnobotanical survey</td>
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<td></td>
</tr>
<tr>
<td>Plant collection and preparation of extracts</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Antinociceptive and analgesic testing</td>
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<td></td>
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</tr>
<tr>
<td>Induction of CIA in mice and testing for anti-rheumatic properties of herbal extracts</td>
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<tr>
<td>In-vitro analysis and histological analysis of arthritis</td>
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<td>Data entry and cleaning</td>
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<tr>
<td>Data analysis</td>
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<td>Determination of dosage</td>
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<td>Standardization</td>
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<tr>
<td>Thesis writing, submission</td>
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<td></td>
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<tr>
<td>Dissemination of research findings/publication</td>
<td></td>
<td></td>
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</tbody>
</table>

*Note: The table shows the distribution of activities across years and months.*
Appendix 1: Data acquisition questionnaire
Data acquisition questionnaire on plant extract/preparation usage in OA and RA

QUESTIONNAIRE

PART 1: RESPONDENTS DETAILS

Name............................................................... Sex....M/F Age...........Years.

Occupation........................................................

Registered ………….. (Yes/No). If yes, note the registration number/details……………..

Level of education...............................

Location/Residence..........................................................................................................................

Efficacy/Availability/Toxicity Data

Name and part(s) of Plant (Local name)...............................................................used in OA...... RA....

Availability of the plant (abundant/scarce/endangered) ..............................................................

Preparation method(s).............................................................................................................

Formulation used (mixed with)............................................................... in OA…..RA....

Administration form (s).............................................................................................................

Route(s) of application .............................................................................................................

Diagnosis OA .................................. RA .....................................
Approximate dosage...........................................................

Response of Patient Good........................Fair................. Poor......................

Duration of response: Minutes....... Hours........  Days ....... Months...... Years.......

Complication......................................................................................................

Shelf life of the formulation ............................................................................

Treatment outcomes and recurrence ...........................................................

Prognostic indicators ......................................................................................

Patient satisfaction..........................................................................................

Proportion of initial case vs. cases initially reported (and treated) else where .......... ...
..........................................................................................................................

PART 2: RESPONDENTS CONSENT AGREEMENT

I....................................................................................... Hereby agree to participate in this study
with my full consent and conscious and declare that to the best of my Knowledge the information
that I have provided is true, accurate and complete.

Signature/Thumb print.................................................. Date.............
PART 3: RESEARCHER'S DECLARATION

1. The following research will be undertaken with respect to the indigenous knowledge and intellectual proprietary of the herbal practitioners.

2. We will at no given time initiate or conduct practices that are deemed to obtain information from the respondents by intimidation, coercion or false pretence.

3. The respondents will be informed of the intended project prior to questionnaire administration and in confidential to eliminate any degree of conspiracy.

4. We will be no under any obligation to edit or tamper the information provided by the respondents.

5. The information collected will be used for the described research purpose and not any undisclosed intentions.

Signatory Researchers:

1. Dr. Wambugu S.N.............................. Prof. Kanui T.I..........................
Appendix 2: Histological grading of arthritis

1. *Enlargement of the synovial lining cell layer (at X 200).*

<table>
<thead>
<tr>
<th>Points</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The lining cells form one layer.</td>
</tr>
<tr>
<td>1</td>
<td>The lining cells form 2-3 layers.</td>
</tr>
<tr>
<td>2</td>
<td>The lining cells form 5-4 layers, few multinucleated cells may occur.</td>
</tr>
<tr>
<td>3</td>
<td>The lining cells form more than 5 layers, lining might be ulcerated and multinucleated cells may occur</td>
</tr>
</tbody>
</table>

2. *Density of the resident cells (at X200)*

<table>
<thead>
<tr>
<th>Points</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>The synovial stroma shows normal cellularity</td>
</tr>
<tr>
<td>1</td>
<td>The cellularity is slightly increased</td>
</tr>
<tr>
<td>2</td>
<td>The cellularity is moderately increased, multinucleated cells might occur</td>
</tr>
<tr>
<td>3</td>
<td>The cellularity is greatly increased, multinucleated giant cells, pannus formation and rheumatoid granulomas might occur</td>
</tr>
</tbody>
</table>

3. *Inflammatory infiltrate (at X400)*

<table>
<thead>
<tr>
<th>Points</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No inflammatory infiltrate</td>
</tr>
<tr>
<td>1</td>
<td>Few mostly perivascular situated lymphocytes or plasma cells</td>
</tr>
<tr>
<td>2</td>
<td>Numerous lymphocytes or plasma cells, sometimes forming follicle-like aggregates</td>
</tr>
<tr>
<td>3</td>
<td>Dense band-like inflammatory infiltrate or numerous large follicle-like aggregates</td>
</tr>
</tbody>
</table>

Sum 0 or 1 = No synovitis
Sum 2–4 = Low-grade synovitis
Sum 5–9 = High-grade synovitis