THE ANALGESIC PROPERTIES OF *AZADIRACHTA INDICA* (NEEM) IN MICE

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DECLARATION

I hereby declare that this dissertation is my original work and that to the best of my knowledge it has not been presented for an award of degree in any other institution.

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Date:30/9/2013

SUPERVISOR APPROVAL

This dissertation has been submitted with my approval as University supervisor.

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DEDICATION

To my family for giving me full support every time I needed it and for helping me meet the expenditures incurred during the project work.

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ACRONYMS

ALE *Azadirachtaindica* leaf extract

I.P. Intraperitoneally

N.R No Response

ABSTRACT

Pain is among the most common symptoms leading patients to consult a physician in the USA.The National Centre for Health Statistics estimates that about 25% of the US population has chronic or recurrent pain, and 40 % state that the pain has a moderate or severe degrading impact on their lives. Pain is therefore a very common condition. The major strategies of pain management include pain relieving medications,physical or occupational therapy and complementary therapies (such as acupuncture and massage). However,there is need to come up with other means of managing pain. Herbal medicines are therefore becoming increasingly popular in the management of pain , although research on herbal medicines is still in the early phases.

Themain objective of this study was to investigate the analgesic and antinociceptive properties of extracts of *Azadirachta indica* in mice. Assessment of analgesic potency was done using the hot plate method.The purpose of the experiment was to demonstrate whether leaf and bark extracts *of Azadirachta indica* exhibited any analgesic effects on mice. The drug was administered intraperitoneally in three different concentrations to three groups of mice and observations made on the duration taken for the animals to respond to the heat stimulus.Positive and negative control groups of mice were also set up using morphine and saline respectively administered to the mice.

The end point was be taken as the time taken for the mouse to stand on its hind legs and lick its front paws.Naloxone was then given to study the mechanism of analgesia,that is, to demonstrate central origin of analgesia,given that it is an opioid antagonist.This was used todemonstrate whether the extract acted through the opioid receptors if the effect is reversed hence analgesia is decreased.The reaction time was takenat intervals of 5 minutes for 1 hour after administration of drug. Phytochemical tests were also carried out on both the bark and leaf extracts.

Dose dependent analgesic activity was observed for the *A. indica* extracts. The *Azadirachta indica* extracts exhibited mild analgesic activity, with the leaf extract showing more activity than the bark extract. The extract worked through opioid receptors.

CHAPTER ONE

1.1 INTRODUCTION

Pain is anunpleasant feeling that is related to real or potentialtissue damage or a damage that is defined similarly.From many points of view,the pain is a common symptom intended for seeking aid. International Association for the study of pain (IASP) defines pain as 'an upleasant emotional situation which is originating from a certain area, which is dependent or non-dependent on tissue damage and which is related to past experience of the person in question.'

There has been an increase in the amount of knowledge available with regards to causes and management of pain as well as developments in technology in this field.However many patients still experience pain. There is therefore need for the patients and caregivers to seek for different alternatives in pain management. For this reason inaddition to the pharmacological treatment options for pain management, today, non-pharmacological treatment options and complementary medical attempts are being used.

Herbal medicines can be considered as part of these non-pharmacological methods available for pain management.Herbal medicine is the use of the chemical constituents present in roots, leaves, seeds and flower parts of the herbs for treatment.This increased interest in medicinal herbs has led to scientific studies of their therapeutic potencies and safety profiles.Some of the reasons encouraging this shift include the toxicity of newer drugs and their costs.

Plant remedies have been a major source of medication for treatment of diseases all over the world for centuries. Presently it is estimated that more than half of the world's population use herbal remedies or have used them. *Azadirachta indica* is one such herbal drug. It is reported to possess hypolipidaemic, hypoglycemic, immunostimulant, hepatoprotective, anti-inflammatory and antifertility properties. A previous study has demonstrated possible antinociceptive actions of the leaf extracts in mice using glacial acetic acid induced writhing(Khanna N et al,1995). This study was therefore undertaken to demonstrate this antinociceptive action in mice using the hot plate method.

1.2 LITERATURE REVIEW

1.2.1Description

The neem or margosa tree, also called Indian lilac, is an evergreen, or deciduous fast growing plant which may reach a height of 25 meters. It thrives mainly in tropical climates with an annual rainfall of 400 to 800m and an extended dry season. Neem can tolerate severe drought and poor, shallow even saline soils.The fruits are produced in drooping panicles usually once or twice a year. They are oval fruits 1.4-2.4 cm long and have when ripe a yellowish sweet pulp hat encloses a brown seed kernel, embedded in hard white shell.The leaves which may be used for pest control, are usually medium green, unpaired pinnate, and may reach a length of 30 cm. The asymmetric serrate leaflets number 7 o 17 and are up to 7 cm long. The fragrant flowers are white and small.

Fig 1:Photo of growing plant

Fig 2:Photo of growing plant

1.2.2 GEOGRAPHICAL DISTRIBUTION

Azadirachta indica is a tree belonging to the Meliaceae (Mahogany) family. It is native to parts of southern and southeastern Asia. Today, the plant also occurs in tropical and subtropical areas of Africa, America and Australia.In India it is popularly known as Indian neem/Margosa tree or Indian lilac. The Sanskrit name for the neem tree is 'Arishtha' meaning reliever of sickness. In Kenya it is commonly known as 'Mwarubainne'.

1.2.3 MEDICINAL USES

Neem (*Azadirachta indica*) is perhaps the most useful traditional herbal medicine in India. Each part of the neem tree has a medicinal property and is thus commercially exploitable. In the last few decades there has been a lot of progress in the biological activity and medicinal properties of neem, such that it has now become a valuable source of unique natural products for development of medicines against various diseases and also for development of industrial products.

The medicinal properties of the plant has been studied by several workers. The antipyretic effect antimalarial, antitumor, antiulcer, antidiabetic , antifertility and effect on the central nervous systemhave previously been studied. Antimicrobial activity has also been studied. The antimicrobial activity of the seed oil against a variety of pathogens has also been reported.(Khattak SG et al,1985)

The antifungal effects were reported for example against polyporous wood rot, and against *Trichophyton mentagrophytes* in concentration of 125 µg/ml. (Vohra SB 1992)

The present study was aimed at find out the analgesic properties of neem extracts

1.2.4 CHEMICAL CONSTITUENTS

More than 125 compounds have been isolated from various parts of neem. The compounds have been divided into two major classes: isoprenoids and others. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin, nimbin, salanin and azadirachtin.

The non isoprenoids include proteins, carbohydrates, sulphurous compounds, flavonoids,glycosides, tannins etc.

1.3 JUSTIFICATION OF THE STUDY

Various parts of neem tree have been used traditionally in ayurvedic medicine in India since time immemorial. Neem oil, bark and leaf extracts have been used therapeutically as folk medicine to control leprosy, intestinal helminthiasis,respiratorydisorders,constipation,rheumatism,syphilitic sores and as a health promoter. Neem oil has also been used to control skin infections.

Previous studies have shown that neem has anti-inflammatory,antipyretic and analgesic activities.The chloroform extract of stem bark is effective against rat paw oedema. Inflammatory stomatitis in children is cured by the bark extract. Antipyretic activity has also been reported in neem oil (Okpanyi SN 1981).

The plant has also been shown to possess analgesic activity mediated through opioid receptors in laboratory animals. A previous study has also been done to demonstrate the analgesic effects of the plant in rats through oral administration.Previous work in mice has employed the use of glacial acetic acid induced writhing as a measure of analgesia(P. Kholsa, 2000). However, in this study the hot plate method was used to demonstrate analgesia

No significant studies have been done to follow up on and further investigate the analgesic effect of the Kenyan species hence presenting a considerable knowledge gap.

CHAPTER 2

_OBJECTIVES OF THE STUDY

General objectives

1.To perform the phytochemical screening and study the analgesic effects of *Azadirachta indica.*

Specicific objectives

1.To carry out phytochemical studies of the plant extract .

2.To assess the analgesic properties of *Azadirachta indica* by the hot plate method.

3.To determine possible mechanism of analgesia through the effect of opioid antagonist naloxone.

CHAPTER 3

METHODOLOGY

3.1 MATERIALS AND APPARATUS

1. Methanol

2. Oven

3. Fresh leaf and bark material from *Azadirachta indica*

4. Needle and syringes

5. Hot-plate

6. A rotary evaporator

7. Mice

8. Naloxone hydrochloride

9.Milling machine

10.Distilled water

3.2 METHODOLOGY

The plant material was collected from Nairobi and dried under shade.The identification was done using a plant sample from the herbarium. The main part collected was the leaves and fresh bark.Thedried materials(leaves and bark) was ground into a powder using a grinding machine.The pulverized material was then weighed and the weight recorded.

EXTRACTION

The material obtained was then soaked with 600ml of methanol for 48 hours after which sequential extraction was used to obtain the constituents from the plant.The filtrate was reduced using a rotaryevaporator then dried in an oven to obtain the extract which was then weighed. This was done separately for both the bark and the leaf portions.

The leaf extract obtained was divided into two portions each for both phytochemistry and test for analgesia. All extracted samples were stored in the fridge so as to maintain their potency.

3.3 PHYTOCHEMICAL TESTS

These were carried out on a portion of the leaf extract and bark extract.

3.3.1 Test for alkaloids-The Dragendorff's reagent test

Approximately 50 mg of the dried extract was extracted by boiling in water with 2 ml of 1 % sulphuric acid for 2-3 minutes then filtered and excess sulphuric acid neutralized with dilute ammonia solution.

About 5 ml of chloroform was added to the filtrate,the chloroform layer separated and washed with some distilled water.The separated chloroform layer was then evaporated to dryness and the residue obtained was dissolved in 0.2 ml of 1% sulphuric acid. Lastly,one drop of the Dragendorff's reagent was added and observations made for precipitation.

3.3.2 Test for cardiac glycosides-The Kedde's test

About 0.5 g of the dried extract was extracted with 5 ml of 70% alcohol by heating in a water bath for 2-3 minutes cooled and filtered.To the filtrate,5 ml of distilled water was added followed by 3 drops of lead sub acetate solution and 10% sulpuric acid drop wise. This was to precipitate any excess lead ions. Then the solution was filtered and extracted in two successive portions of chloroform. Then these two extracts were combined and washed with 1 ml of distilled water. The extract was then evaporated to dryness and into it; a drop of 90% alcohol and two drops of 3,5-dinitrobenzoic acid in 90% alcohol were added. The solution was then made alkaline with the addition of 20% NaOH solution and observations made for any precipitation.

3.3.3 Test for saponins

Saponins of both the steroid and triterpenoid groups respond to the following tests

a) A little of the powdered plant was put in a test tube,water added and shaken .A persistent froth suggests that saponins are present.

b) About 0.2 g of the dried extract was extracted with 10 ml of warm water, filtered while retaining the filtrate. 2 ml of 1.8% NaCl solution was added to two test tubes and to one of the test tubes 2 ml of distilled water was added and to the other 2 ml of the extract. The concentration of NaCl in each test tube was isotonic with blood serum.One drop of blood was added to each test tube and were inverted gently to mix the contents. Haemolysis in the tube containing the extract and not the control would indicate the presence of saponins.

3.3.4 Test for tannins

About 0.5 g of the obtained powder was put in 4ml ethanol then boiled in water,cooled and filtered. The filtrate is then divided into three 1 ml portions and treated as follows.

Tube1. A few drops of ferric chloride were added

Tube 2. Approximately 0.5 ml of 10% potassium dichromate solution was added

Tube 3. Approximately 0.5 ml of lead sub acetate solution was added

In each tube, observations for color change and/or precipitation was made and recorded.

3.4 ASSESSMENT OF ANALGESIC POTENCY BY HOT PLATE METHOD

The study was carried out in a total of nine mice of either sex.The mice were kept on a standard diet and water.

A mouse was placed on a hot plate maintained at constant temperature between 50 and 55 □C .The end point was taken as the time taken for the mouse to stand on its hind legs and lick its front paws.Only mice that reacted within 30 seconds were used. A cut off time was also established to prevent thermal injury to the mice. The initial reaction time was then recorded.

The mice were divided into three goups. Group 1 (negative control) was given saline 2mg/kg i.p. The second group (positive control) was given morphine 50mg/kg i.p. For the next group the leaf extract was given at increasing concentrations. 10mg/kg,30mg/kg and 100mg/kg of leaf extract was used.

The same procedure was repeated using 30 mg/kg of bark extract.

3.5 DETERMINATION OF MECHANISM OF ANALGESIA

Naloxone was administered at 1 mg/kg thirty minutes before administration of the *Azadirachta indica* extract. Observations were made at 5 minute intervals over a period of 1 hour.The mean reaction time was then calculated for each group.

CHAPTER 4:

RESULTS AND DISCUSSION

4.1 Weights of the materials

TABLE 1: WEIGHT OF PULVERIZED MATERIAL

	Leaf material	Bark material
Weight of flask + extract (g)	137.38	78.46
Weight of flask (g)	42.25	40.15
Weight of extract (g)	95.01	38.31

TABLE 2: WEIGHT OF EXTRACT

	Leaf material	Bark material
Weight of flask + extract (g)	55.38	49.33
Weight of flask (g)	49.21	47.32
Weight of extract (g)	6.17	2.01

The %yield for the leaf material was 6.49 %

The % yield for the bark material was 5.29%

4.2: Phytochemical tests

The results for the various tests carried out are shown in table 3

Table 3:Results for Phytochemical Tests on leaf and bark extract.

Test	Observation	Inference1 (bark extract)	Inference1 (leaf extract)
1.Saponins			
a)shake with water	Frothing but not persistent	Saponins present	Saponins present
b)Haemolysis test	Haemolysis present	Saponins present	Saponins present
2.Tannins			
a)Ferric Chloride	Dirty green precipitate	Tannins present	Tannins present
b)lead sub acetate	Green yellow precipitate	Tannins present	Tannins present
c)Potassium dichromate	Green precipitate	Tannins present	Tannins present
3.Glycosides			
a)kedde's test	Purple color	Glycosides present	Glycosides present
b)Keller Killiani's test	Light green color	Glycosides present	Glycosides present
4.Alkaloids			
a)Dragendroff test	Orange red precipitate	Alkaloids absent	Alkaloids absent
b)Vital morin test	Purple color	Alkaloids absent	Alkaloids absent

Both the bark and leaves contained glycosides, tannins and saponins but no alkaloids. Similar results was therefore obtained for both the leaf extract and bark extract of *Azadirachta indica*. The result reveals that the plant extracts owing to their phytochemical constituents, may give the reported pharmacological activities.

4.3: ANALGESIC ACTIVITY

The time in seconds taken for each mouse to stand up and lick its front paws was measured using a stopwatch and recorded at intervals of 15 minutes for a period of 60 minutes as shown in table 4.

TABLE 4:RESULTS FOR ASSESSMENT OF ANALGESIC POTENCY BY HOT PLATE METHOD

TIME(MIN)	POSITIVE CONTROL	NEGATIVE CONTROL	LEAF EXTRACT (10mg/kg)	LEAF EXTRACT (30mg/kg)	LEAF EXTRACT (100mg/kg)	BARK EXTRACT
0	14	9	8	10	9	9
5	15	7	12	14	15	9
10	19	10	13	13	15	8
15	21	9	15	16	16	10
20	20	8	14	14	17	10
25	25	8	11	12	16	9
30	25	10	10	15	14	10
35	27	12	12	11	18	10
40	25	8	13	14	15	10
45	NR	7	14	18	18	14
50	NR	10	15	16	17	12
55	NR	11	16	16	16	12
60	NR	10	17	17	NR	NR

Response time was in seconds.

NR=No response.

4.4: Mechanism of analgesia

Naloxone is an opioid receptor antagonist hence it would compete with the extract for binding to the opioid receptors .This therefore results in a decrease in the analgesic effect as shown below in table 6

TABLE 6: RESULTS FOR ANALGESIC EFFECT AFTER ADMINISTRATION OF NALOXONE

TIME 1 (MIN)	POSITIVE CONTROL	NEGATIVE CONTROL	ALE E(30mg/kg)	ALE + NALOXONE
0	14	9	10	10
5	15	7	14	10
10	19	10	13	11
15	21	9	16	12
20	20	8	14	13
25	25	8	12	11
30	25	10	15	12
35	27	12	11	10
40	25	8	14	10

45	NR	7	18	13
50	NR	10	16	12
55	NR	11	16	14
60	NR	10	17	NR

The graphical representation of the results is shown in figures 3, 4 and 5

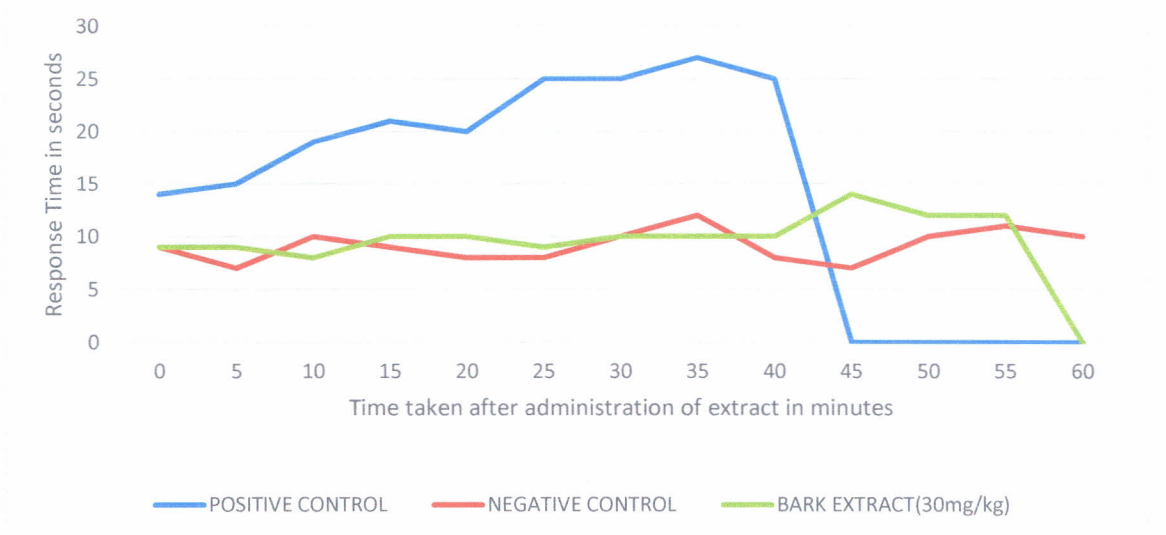

Figure 3: Graph showing analgesic effect of bark extract

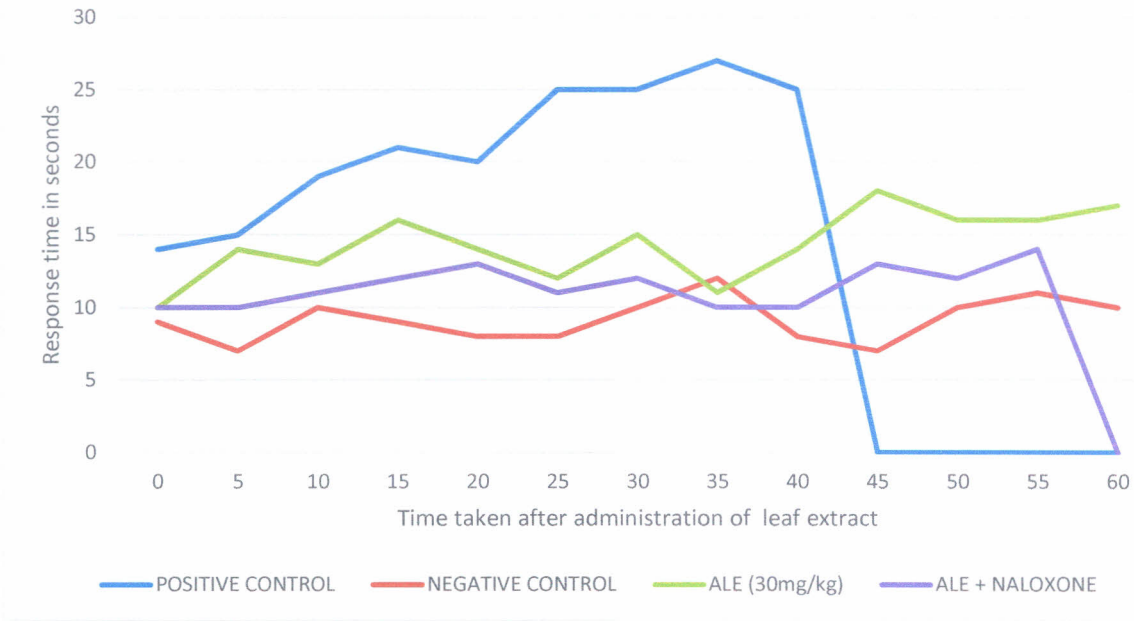

Figure 4: Graph showing analgesic effect of leaf extract after administration of Naloxone

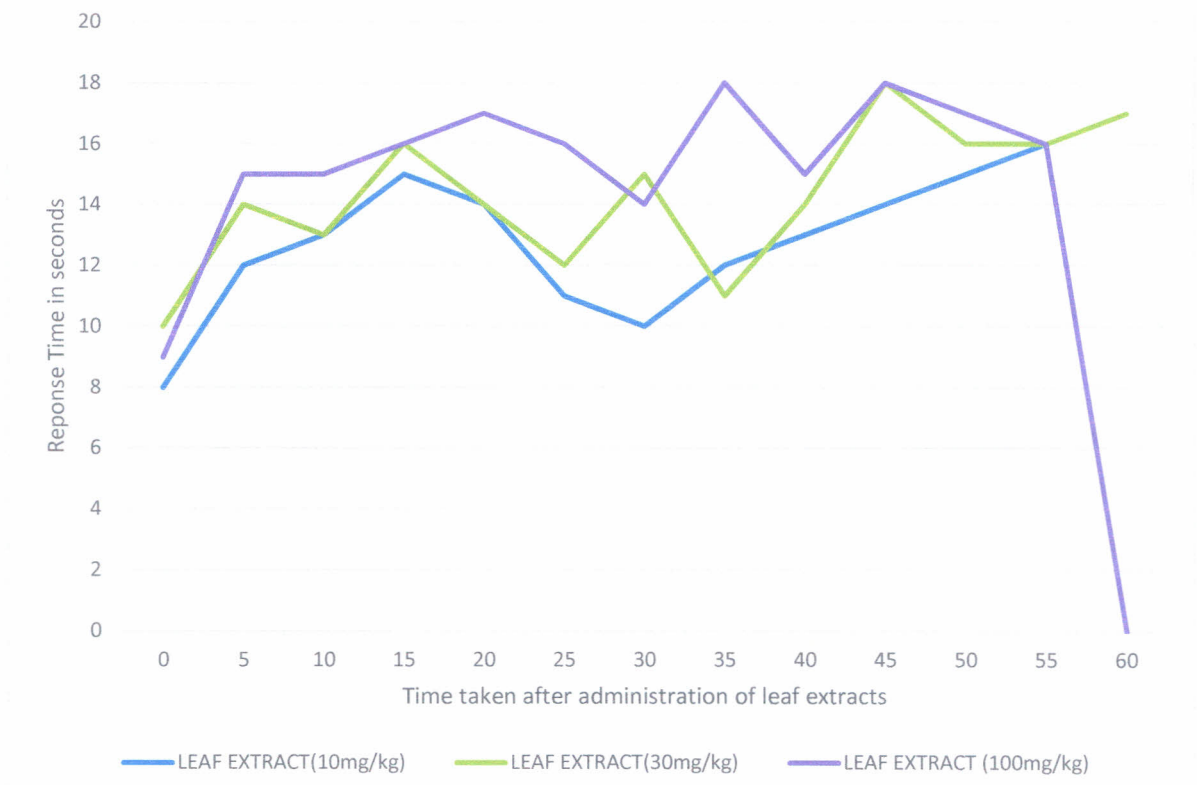

Figure 5:Graph showing analgesic effect of leaf extracts at different concentrations

Figure 6: Photo of Hot plate setup

DISCUSSION

The positive control used was morphine which is a potent analgesic. Its potency increased with time until a point beyond which no more response was recorded.

The negative control used was normal saline which has no effect on the mice and showed a normal response.

The longer the time taken to respond to the heat stimulus the greater the analgesic effect. Peak analgesic effect for the leaf extract was observed 45 minutes after administration of the *A. indica* leaf extract. From the data obtained analgesic effect rose gradually with time until it reached a peak after 45 to 55 minutes .Beyond this time the analgesic effect observed gradually began to decline. Generally, the results also demonstrated the limited duration of analgesic action of the extracts which was within the first 55 minutes.

Both leaf and bark extracts showed mild analgesic activity but bark extract had less activity as compared to methanol extract. Both extracts had activity when compared with the negative control but the activity was not as much as for the positive control hence suggesting mild activity. The leaf extract had a much higher analgesic effect than the bark extract. The bark extract had very mild activity compared to the controls used.

Pretreatment with the opioid antagonist naloxone partially reduced the analgesic effect of A.induca. This indicates the involvement of endogenous opioid peptides in mediation of antinociceptive action of *A.indica*. However, since the analgesic effect is only partially reduced after administration of naloxone, some other non opioid mechanisms may also be involved. There is a possibility that the *A.indica* extracts may be modulating some other neurotransmitters involved in pain sensitivity. This may further confirm the results obtained by other related experiments ,such as the findings reported by Khanna et al.

4.5 LIMITATIONS OF THE STUDY

a. Lack of availability or access to literature needed, this was a major problem faced during the literature review.

b. The reagents and materials required for lab work were expensive or not readily available hence this limited the study. Access to adequate number of lab animals was also a major limitation

c.Idiosyncratic reactions in the lab animals could have led to less accurate results. Individual variations in response to the drug was therefore a major challenge.

d.The process involved in collection of the materials for the project were cumbersome

e.There was a limitation in the number of methods available for assessing analgesia

CHAPTER 5

5.1 CONCLUSIONS

A. indica has various components as shown by phytochemical tests. These components include tannins, glycosides and saponins. The various components present are responsible for the reported biological activity of the plant extract.

Dose dependent analgesic activity was observed for the *A. indica* extracts. The *Azadirachta indica* leaf extracts exhibited considerable analgesic activity and this explains its folkloric use in management of pain. Analgesia could be mediated via opioid receptors as well as via some other neurotransmitters due to the partial inhibitory effects obtained after pre-treatment with naloxone.

5.2 RECOMMENDATIONS

Having demonstrated some mild analgesic activity, further research is necessary to identify the specific biological compound(s) responsible for this action. This may involve isolation, structure elucidation and proper characterization of the various components in a bid to improve on their potency, safety and efficacy .Plants from geographical areas where the plant is used medicinally should be collected and their phytochemical studies carried out including toxicity tests and this may lead to important sources of pharmaceutical products. The toxicity of the plant was not conducted thus need for future research for both animals and humans to evaluate its toxicity profile.

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