# Anti-inflammatory and wound healing activity of methanol extracts of Azadirachta indica A. Juss, Lawosinia inermis L and Achyranthus aspera L in in vitro models

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**Research Article**

**Keywords:**

**Posted Date:** April 20th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1518005/v1

## Abstract

# Background

Lymphoedema is a pathological process that results from damage, infection, blockage, or genetic defects in the lymphatic system. The main causes of lower limb lymphoedema in Ethiopia are lymphatic filariasis (LF) and podoconiosis. Many natural products are being used for management of wound and inflammatory conditions in Ethiopia. Few of these products have been systematically evaluated using *in vitro* tests.

# Objective

The objective of this study was to conduct *in vitro* cytotoxicity assays, anti-inflammatory, and wound healing activity tests on selected Ethiopian plant extracts.

# Methods

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cytotoxicity of the plant extracts. For the anti-inflammatory activity, cyclooxygenase enzymes 1 (COX-1), cyclooxygenase enzymes 2 (COX-2) and 15-lipoxygenase enzyme inhibitory assay was used. Antioxidant activities were conducted using DPPH assay. Quantification of cell multiplication based on the measurement of BrdU incorporation during deoxyribonucleic acid (DNA) synthesis, was used to measure deoxyribonucleic acid (DNA) /cell proliferation.

# Results

*Azadirachta indica* A. Juss (82%) and *Lawosinia inermis* L (87.6%) showed significant inhibition against COX-1 at 25 mg/ml, while *Achyranthus aspera* L (91.7%) and *A. indica* (95%) were effective in inhibiting COX-2 at the same concentration. Similarly, *L. inermis* L inhibited 88.8% of COX-2 at the same concentration. *L. inermis* (74.8%) showed the highest inhibitory activity against 15-LOX, followed by *A. asper* (73.1%) and *A. indica* (66%) at 25mg/ml. All the tested extracts showed free radical scavenging activity at 10 mg/ml. *A. indica* and *L. inerms* have shown better stimulation of human epidermal keratinocytes, with optimal stimulation observed between 2.5 mg/ml and 5 mg/ml of the extracts.

# Conclusion

Plant extracts or compounds that inhibited these enzymatic inflammatory activities may contain potential drivers or templates for the development of effective anti-inflammatory drugs. Antioxidants that maintain non-toxic ROS levels in wound tissue can improve the wound healing process.

# Introduction

Ethiopia has a wide range of natural products traditionally used to treat different disease conditions. About 80% of humans and 90% of domestic animals rely on medicinal plants for their health care (1). In Ethiopia, traditional medicines are associated with the societies' aesthetic, historic, social or spiritual values, and are relatively low in cost (1).

Herbal products have demonstrated healing properties, are recognized as important sources of therapeutics, and are used to treat a range of conditions like swelling, burns, wounds and systemic diseases. These have attracted the scientific community to study the active constituents of plants with medicinal properties in order to develop drugs. (2).

Inflammation is a local reaction of living tissue to pathogenic microorganisms, chemical substances, or parasites. The purpose of the inflammatory response is to bring inflammatory mediators such as leukocytes and plasma proteins that normally circulate in the blood to the site of infection or tissue damage in order to eliminate the causative agent and initiate healing (3). When a cell is injured, cytokines such as tumor necrosis factor and IL-1 from leukocytes, monocytes and macrophages are released as a response to pain, thereby alerting the body to increase blood flow around the injury area. This initiates the production of cyclooxygenase (COX) and 5-lipoxygenase (5-LO) leading to synthesis of prostaglandins and leukotrienes, respectively (2).

Generally, inflammation is beneficial to the body. However, it may cause damage if it cannot remove the causative agents or is inappropriately directed against the host. Wounds are physical injuries that cause the skin to open or break, and wound healing is critical to restore anatomical continuity and skin function. Wound healing is the biological reaction to injury that results in the wound being reduced and closed. A complex process involving cellular and biochemical interactions restores the functional barrier (4). Inflammation induces formation of edema due to leakage of body fluid and proteins as well as leukocyte collection and accumulation at the sites of inflammation (5). Prostaglandins are hormones that are produced by all types of cells in the body at the time of injury. Once they are present in the intracellular space, they induce fever, inflammation, and pain. The cascade of inflammation pathway leads to the secretion of more cellular mediators of inflammation whose outcome is cellular destruction, and then restoration of tissue structure and function(5).

In lymphoedema, chronic interstitial fluid accumulation due to damage of the lymphatic system results in inflammatory cascades and adipose cell differentiation activation which leads to progressive inflammation, and this change is the most important in the pathophysiology of lymphoedema. Formation of fibrosis and chronic inflammation are the hallmarks of lymphoedema (6).

Response to inflammation is the beginning of processes which happen simultaneously to promote wound healing. The main biomolecule elements to the response to inflammation in skin restoration are leukocytes with infiltrate the injury sites. They are not the only cells that combat invasive microbes, but they are also engaged in tissue breakdown and development. As a result, an abnormal entrance or

activation of infiltrating leukocytes into injured tissue can have a substantial impact on cell migration, cell multiplication, formation of different types of cells, and the quality of wound healing. Realizing the significance and the complicated role of the inflammatory response to wound repairs will provide strategies to manage diseases characterized by abnormal tissue remodeling(7).

Poor venous drainage, inadequate blood supply, increased skin tension and infection are some of the local factors that impair wound healing (8). Lesions of the skin of lymphoedematous limb favor the entry of micro-organisms into the tissues. Micro-organisms may invade the epidermis and subcutaneous tissues and results in swelling, erythema, and severe pain. Patients with secondary lymphoedema are more prone to develop cellulitis (9).

The resolution of the inflammatory response is required for successful tissue repair after injury. Previous research identified, nuclear factor-erythroid factor 2-related factor 2 (Nrf-2), a keratinocyte growth factor-1 target, as a novel transcription factor regulating the inflammatory response to repair (7).

The arachidonic acid pathway is a major component of the inflammatory pathway. Arachidonic acid is secreted from injured cellular and tissue membranes and converted into prostaglandins and thromboxane by cyclooxygenase (COX) enzyme. COX has three known isomers. These are cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2), and the new isomer, cyclooxygenase 3 (COX-3). Inhibition of COX-1 in the gastrointestinal mucosal layer is related to the side effects of non-steroidal anti-inflammatory drugs, and selective inhibition of COX-2 is preferable. COX-2 is stimulated at the beginning of inflammation in response to pro-inflammatory mediators and stimuli like endotoxins and cytokines. COX-2 favors synthesis of prostaglandins, which play a role in inflammation, swelling, and pain, after being activated. Lipoxygenases (LOX) are dioxygenase enzymes that are involved in the production of leukotrienes from arachidonic acid, and act as intermediate biomolecules in inflammatory and allergic reactions. These enzymes are responsible for catalyzing incorporation of molecular oxygen into unsaturated fatty acids like linoleic and arachidonic acids. Four main iso-enzymes of LOX enzymes have been identified based on the site of oxidation, namely, 5-Lipoxygenases (5-LOX), 8-Lipoxygenases (8-LOX), 12-Lipoxygenases (12-LOX), and 15-Lipoxygenases (15-LOX). The common substrates for LOX enzymes are linoleic acid and arachidonic acids. The LOX enzyme from soybeans is usually used for the *in vitro* anti-inflammatory assay due to the lack of human LOX enzyme (10).

Metabolism of arachidonic acid by the COX enzymes produces either prostaglandins and thromboxane A2, or, through the LOX pathway, hydroperoxy-eicosatetraenoic acids and leukotrienes (Fig. 1). The LOX enzyme pathway is present in leucocytes as well as in mast cells, neutrophils, eosinophils, monocytes, and basophils. Phospholipase A2 cleaves arachidonic acid from cell membrane phospholipids and metabolizes arachidonic acids to leukotrienes. Leukotrienes act as phagocyte chemo-attractants, which attract innate immune cells to sites of inflammation. Medicinal plants may thus be possible sources of COX-2 and LOX inhibitors with fewer side effects (2).

Multitarget inhibitors that are capable of inhibiting COX-2 and 5-LOX could be more effective anti-inflammatory drugs than any currently available non-steroidal anti-inflammatory drugs (NSAID). They

have the potential to be safer and more effective drugs for the management of inflammation. As a result, the first step toward developing an anti-inflammatory treatment alternative is to identify compounds with distinct mechanisms of action. Screening the natural world, particularly plants, is an important approach to identifying biologically active compounds (10).

Three plant species, namely *L. inermis*, *A. indica* and *A. Aspera* were selected based on information from an earlier systematic review (11). Because of their traditional use in the management of injuries and inflammatory conditions, the methanol extracts of their leaf parts were tested for anti-inflammatory and wound healing activities in *in vitro* models.

The objective of this study was to investigate the cytotoxicity, and the anti-inflammatory and wound healing activities of methanol extracts of *L. inermis, A. indica* and *A. Aspera* extracts in *in vitro* models.

# Methods

# 2.1. Plant material collection and extraction

The leaves of *Lawsonia inermis* (Henna) were collected from Laga Gandisa (approximately 90 32' 59" N and 410 28' 31" E), 53 km from Dire Dawa, Ethiopia. The leaves of *Azadirachta indica* (Neem) were collected from Kurare Goti (approximately 100 7' 15" N and 380 9' 13" E), 209 km northwest of Addis Ababa on the way to Dejen Town, Ethiopia. The leaves of *Achyranthes aspera* (Telenge) were collected from the Nile Gorge (approximately 100 7'51"N, and 380 9'19" E), 210 km northwest of Addis Ababa on the way to Dejen, Ethiopia. No access permit was required from Ethiopian Biodiversity institute for the collection of these plants. Collection of all plant materials was carried out in consultation with a botanist from the Ethiopian Public Health Institute, local people, and traditional healers in the areas. Plant materials were authenticated by a botanist and specimens were archived at the Herbarium of Ethiopian Public Health Institute with voucher numbers of MG-012/05 for *L. inermis*, NA10 for *A. aspera*, and DG-18 for *A. indica*.

# 2.2. Cytotoxicity assay of plant extracts

### Cell culture

Vero cells from cryopreservation were thawed and sub-cultured twice before use for cytotoxicity assay. Cells were sub-cultured in 10% Fetal bovine serum (FBS) medium (Sigma-Aldrich/Merck, Germany). Medium was replaced 2–3 times a week. Confluent cultures were detached using trypsin–EDTA.

### Cell plating

Cells were cultured evenly in 96-wells plates at a density of 2x104 cell per well and let them to attach overnight. Serial dilution of the plant extracts was prepared (0.78 mg/ml – 400mg/ml). After 24 hours of incubation, medicinal plant extracts were introduced (serial dilutions of extracts prepared in media). Then,

experimental plates were incubated in cell culture incubator at 37°C, 5% CO2 for 48 hrs. Cells in media without the extract were used as controls.

### Assay

After 48hrs incubation, cells were rinsed with PBS, and phenol free media were added to the cells. Then, 25 µL of MTT (5 mg/ml) (Thermo-fisher Life Technologies, USA) was added to all wells. Plates were incubated for 4 hours in a humidified cell culture incubator at 37°C. Then, medium with MTT solution was removed from the wells and 100µL of DMSO was added to solubilize the formazan salt. The content in the plate was mixed well on an orbital shaker at room temperature, at 150 cycles/min, for 1 h. Optical density (OD) of all wells were read at a wavelength 590 nm on an ELISA plate reader (BiOTek ELISA microplate reader ELx808). Percentage of cell viability was calculated using the formula written below. Absorbance of each concentration of the extract was subtracted from the respective concentration of extract and control well.

$$\text{Cellviabilty}\,(\%) = \frac{\text{AverageAbsorbacnceinduplicatedextractwell} - \text{Averageblanks}}{\text{Averageabsorbanceincontrolwells}} \times 100$$

Blank wells - wells without media and cells

Control wells - wells with cell and media

## 2.3. Cyclooxygenase 1 and 2 enzymes inhibitory activity assay

Methods used for the cyclooxygenase enzyme1, and cyclooxygenase enzyme 2 inhibitory assays were similar. The cyclooxygenase enzyme activity assay kit (Catalogue No. 701050, Cayman Chemical, Ann Arbor, MI, USA) was used with slight modification. The peroxidase activity was assayed calorimetrically at 590 nm(12).

One hundred sixty microliters of assay buffer and ten microliters of hemin was added to the background wells. Then, 150µl of assay buffer and 10µl of enzymes (COX-1 and COX-2) was added to 100% initial activity wells. Plant extract dilution was done in DMSO (Sigma-Aldrich/Merck, Germany) to yield concentrations from 0.78mg/ml to 25mg/ml. Indomethacin (Sigma-Aldrich/Merck, Germany), serially diluted (3.125µg/ml – 50 µg/ml) was used as positive control. Ten microliters of plant extracts and indomethacin was added to the inhibitor wells, and 1µl of solvent (the same solvent used for dissolving inhibitor) to the 100% Initial Activity wells and background wells. Plates were shaken carefully for a few seconds and incubated for five minutes at 25°C. Twenty microliters (20µl) of the colorimetric substrate solution and arachidonic acid were added to all the wells. Then, plates were shaken carefully and incubated for two minutes at 25°C, Finally, absorbance was measured at 590 nm using a microplate reader (BiOTek ELISA microplate reader ELx808). All tests were done in three replicas. The percent (%) inhibition of COX-1 and COX-2 was determined using the following formula:

$\mathrm{PercentInhibition} = \frac{(\mathrm{AEA}-\mathrm{AIA})}{\mathrm{AEA}}\mathrm{x100}$ Where:

Enzyme test activity absorbance (EAA)= (Abs of enzyme test - Blank abs).

AIA (activity inhibition test absorbance) = (Abs Inhibition Test – Blank Abs). In addition, absorbance of each concentration of the extracts were subtracted from the respective concentration of EAA.

COX-2 selectivity index (SI) values were also calculated using the formula:$\mathrm{SI} = \frac{\mathrm{IC50(COX-1)}}{\mathrm{IC50(COX-2)}}$

# 2.4. Lipoxygenase enzyme inhibitory assay

The LOX inhibitory activity of methanol extracts was measured using a 15-LOX inhibitor screening kit (Novus Biological, Bio-techne Ltd, Abingdon, UK) using the manufacturer's instructions. Plant extracts were dissolved in methanol and serially diluted from 0.78 mg/ml to 25 mg/ml. In the blank wells of the plate, 100µl of 1X assay buffer was added; in positive control wells, 90µl and 10µl assay buffer; and to the 100% activity wells, 90µl 15-LOX and 10µl methanol. To the inhibitor wells of the plate, 90µl 15-LOX and 10µl serially diluted plant extracts and nordihydroguaiaretic acid (0.625µM-100µM) were added. Then, plates were kept for five minutes at room temperature. After incubation, reactions were initiated by adding 10µl of linoleic acid to all wells, and plates were put on a plate shaker for 10 minutes. Then, 100µl of chromogen was added to all the wells to stop enzyme catalysis and develop the reaction, and plates were covered with a plate cover and placed on a plate shaker for five minutes. Assays were performed in duplicate on the same day. The assay plate was read at 495 nm with a microplate reader (BiOTek ELISA microplate reader ELx808) within 2 minutes. $IC_{50}$ values were determined using GraphPad prism, and the percentage of inhibition of each extract and NDGA (Merck Life Science UK Ltd, UK) was determined using the below equation:

$$\mathrm{Percentageinhibition} = \frac{(\mathrm{Initialactivity} - \mathrm{Inhibitoractivity})}{\mathrm{Intialactivity}}\mathrm{x100}$$

Where initial activity = The 100% activity well - Blank wells

In addition, absorbance of each concentration of the extract was subtracted from the respective concentration of extract and control well.

# 2.5. The 2,2 diphenyl 1 picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging capacity of the methanolic extracts of *L. inermis* L, *A. aspera* L, and *A. indica* A. Juss extracts were determined using the 2,2 diphenyl 1 picrylhydrazyl (DPPH) ((Sigma-Aldrich/Merck, Germany) assay. The assay was performed in 96-well microplates using the procedures previously described (13) with minor adjustments. One hundred microliter (100 µL) of different extract concentrations in methanol (0.078mg/ml to 10mg/mL) were added to 100 µL of 0.04% methanolic DPPH solution. Plates were kept for 30 mins in a dark place at ambient temperature, and the optical density was

measured at 540 nm using an ELISA plate reader (BiOTek ELISA microplate reader ELx808). Ascorbic acid (0.078mg/ml to 10 mg/mL) and Trolox (Sigma-Aldrich/Merck, Germany) (0.5mg/ml – 0.25µg/ml) were used as positive controls, methanol as a negative control and extract without DPPH as blank. To evaluate the anti-radical scavenging efficacy, the $IC_{50}$ (defined as the concentration of plant extracts that resulted in 50% reduction of the DPPH color) was determined.

The percent DPPH radical scavenging activity was determined using the below formula:

$$\mathrm{DPPHscavengingactivity}\,(\%) = \frac{(\mathrm{Ac} - \mathrm{As})}{\mathrm{Ac}} \times 100$$

Where the Ac = absorbance of control = DPPH + Methanol without sample] and,

As = the absorbance of sample = [DPPH + Sample (extract/standard)].

# 2.6. Cell proliferation assay

DNA synthesis is an extremely popular and widely used method to study cell proliferation. Determining the synthesis of new DNA is a precise way to assess cell multiplication in individual cells or in cell populations. Direct measurement of new DNA synthesis using BrdU proliferation ELISA involves the incorporation of a labelled nucleoside into genomic DNA.

Human Epidermal Keratinocytes obtained from Sigma-Aldrich/Merck($2x10^5$ cells per well) were plated together with methanolic extracts of *L. inermis* L, *A. aspera* L, and *A indica* A. Juss extracts (78µg/ml – 10mg/ml) in 96-well plates in a final volume of 200 µl/well and incubated in a humidified atmosphere at 37°C. Twenty microliter per well (20 µl/well) BrdU labeling solution (Sigma-Aldrich/Merck, Germany) was added and cells were re-incubated for an additional 2hrs at 37°C. Labelling medium was withdrawn, and 200 µl/well FixDenat (Sigma-Aldrich/Merck, Germany) was added to the cells to fix it, and incubated for 30 mins at 25°C.Fix Denat solution was removed thoroughly, and 100µl/well anti-BrdU-POD (Sigma-Aldrich/Merck, Germany) working solution was added and incubated for 90 min at 25°C. Antibody conjugate was removed, and plate wells were rinsed three times with washing solution, and removed by tapping. One hundred microliter substrate solution per well (100µl/well) was added after removal of washing solution, and plates were incubated at 25°C (5–30 min). Then, 25µl of 1M $H_2SO4$ (Sigma-Aldrich/Merck, Germany) was added to all wells and incubated for 1 min on a plate shaker (300 rpm). Absorbance of the samples was measured using an ELISA reader (BiOTek ELISA microplate reader ELx808) at 450nm within 5 minutes. Data analysis and interpretation of the absorbance (OD) reading. $IC_{50}$ was determined using GraphPad prism and percent cell replication (DNA replication) was calculated using the following formula:

$$\mathrm{Percentcellreplication} = \frac{(\mathrm{Initialactivity} - \mathrm{stimulationactivity})}{\mathrm{Intialactivity}}\mathrm{x}100$$

Where initial activity = The 100% activity well - Blank wells

### 3. Statistical analysis

Graphs of quantitative data and values were calculated using GraphPad Prism (GraphPad Prism version 8.4.1(676). $IC_{50}$ and $EC_{50}$ values for the assay parameters (COX-1, COX-2, 15-LOX, radical scavenging activity, cell proliferation and cytotoxicity) were determined using the 'log concentration vs. response' of the 'non-linear regression analysis' function. The samples were tested at five to seven different concentrations in duplicates and triplicates. For the cyclooxygenase and lipoxygenase inhibitor assays, data from three separate experiments were used, while for cytotoxicity, DPPH assay and cell proliferation assays, data from two separate experiments were used. In addition, percent inhibition and multiplication were calculated for inhibitory and stimulatory activities of plant extracts.

# Results

# 4.1. Cytotoxicity activity

MTT assay was performed using Vero cells by treating the cells with different concentration of methanol extracts. Vero cell lines were obtained from the National Veterinary Institute (NVI), Bishofitu, Ethiopia.

The assay was based on the reduction of yellow tetrazolium MTT to insoluble formazan-blue crystals by mitochondrial succinate dehydrogenase enzymes. Only viable cell with active mitochondria reduces significant amounts of MTT, and the plate reader's absorbance value is directly proportional to cell viability. The assay was performed as a percentage of relative cell viability against various concentrations of the plant extracts (14).

*A. aspera* L and *A. indica* L are less toxic at higher concentration with $IC_{50}$ of 40.8mg/ml and 27.7mg/ml, respectively. *L. inermis* showed higher toxicity with $IC_{50}$ = 25.15 mg/ml. More than 80% of the cells were viable in the three methanol extracts at a concentration of 12.5mg/ml (Fig. 2).

# 4.2. Anti-inflammatory activity

# 4.2.1. COX 1 and COX 2 enzyme inhibitory assay

Methanol extracts of three medicinal plants were tested for COX-1 and COX-2 enzyme inhibitory activity, and dose dependent inhibitions were observed for all the extracts. $IC_{50}$ was calculated using GraphPad prism for the crude plant extracts based on dilution series. The $IC_{50}$ value of indomethacin was determined by testing at different concentrations (3.125 µg/ml – 50 µg/ml), and 50 µg/ml was used as a positive control in the assay (Fig. 3).

Compared to indomethacin, *A. indica* A. Juss (82%) and *L. inerms* L (87.6%) showed significant inhibition against COX-1at 25 mg/ml, with $IC_{50}$ of 1.6 mg/ml and 3.9 mg/ml, respectively. *A. aspera* L (91.7%) and *A. indica* (95%) were effective in inhibiting COX-2 ($IC_{50}$=4.86 mg/ml and 5.67mg/ml) at 25 mg/ml (Fig.6). Similarly, *L. inermis* L inhibited 88.8% of COX-2 at the same concentration. Indomethacin (50 µg/ml)

showed 92% and 95% inhibition against COX-1 and COX-2, respectively (Figs.4 and 5). Regarding the selectivity index of COX-2, *A. aspera* and *L. inermis* had higher values than indomethacin (Table 1).

Table 1
Selectivity index of methanol extract leaves of plant species and indomethacin

| Methanol extracts of leaves plant species | $IC_{50}$ | | COX-1/COX-2 (SI) |
|---|---|---|---|
| | COX-1 | COX-2 | |
| *Achyranthus aspera* L (mg/ml) | 6.4 | 4.8 | 1.33 |
| *Lawsonisa Inermis*L (mg/ml) | 3.9 | 7.69 | 0.5 |
| *Azadirachta indica* A.Juss (mg/ml) | 1.6 | 5.67 | 0.28 |
| Indomethacin (µg/ml) | 2.53 | 2.66 | 0.95 |

# 4.2.2. 15-Lipoxygenase inhibitory activity

The results described in Fig. 7 showed that methanol extracts of *L. inermis. A. indica, and A. aspera* demonstrated moderate inhibition against 15-lypoxygenase enzyme. Among the three plant extracts, the methanol extracts of leaves of *L. inermis* had the highest inhibitory activity at 25mg/ml (74.8%), followed by *A. asper* (73.1%). *A. indica* showed the lowest activity against 15-LOX (66% inhibition) at the same concentration (Fig. 7). Nordihydroguaiaretic acid (NDGA) used as a standard drug exhibited 95.6% inhibition of 15-LOX at 30µg/ml (Fig. 8).

# 4.3. Wound healing activity.

# 4.3.1. DPPH radical scavenging activity

Antioxidant activities of the leaves of methanol extracts *L. inermis* L, *A. aspera* L, and *A. indica* A. Juss were assessed using DPPH radical scavenging activity, i.e., colorimetric method. All the tested extracts showed the best free radical scavenging activity at 10mg/ml as indicated by their percent inhibitions (93.8%, 95%, 93.4%, respectively) and $IC_{50}$values, which are comparable inhibitory activity to that of ascorbic acid and Trolox, used as standard in this study (Fig. 9). *L. inermis* L and *A. aspera* L had the best antioxidant activity with $IC_{50}$ values of 0.22mg/ml and 0.14mg/ml, respectively. The radical scavenging activity of *A. indica* was dose dependent, i.e., the lower the concentration, the lower the radical scavenging activity. Ascorbic acid and Trolox are known potent antioxidants and had the highest DPPH scavenging activity with $IC_{50}$ value of 0.07mg/ml and 0.04 mg/ml, respectively.

# 4.3.2. Cell proliferation activity

Quantification of cell multiplication based on the measurement of BrdU incorporation during DNA synthesis was used to measure percent of DNA/cell proliferation. The stimulating effect of the methanol

extracts of plants on Human Epidermal Keratinocytes (HEK) were dose dependent. At higher concentrations, inhibitory activities of the methanol extracts were observed. *A. indica* and *L. inermis* showed better stimulation of human epidermal keratinocytes, while optimal stimulation was observed between 2.5 mg/ml and 5 mg/ml (Fig. 10). At 10 mg/ml, *A. indica* showed a 50.1% increase in human epidermal keratinocyte multiplication, followed by *L. inermis* and *A. aspera* at 49.3% and 44%, respectively. The lowest $EC_{50}$value was determined for *A. indica* (0.89 mg/ml), and *A. aspera* (1.78 mg/ml), while the $EC_{50}$ value for *L. inermis* was 2.25 mg/ml.

# Discussion

Previous research has reported important results that showed the therapeutic importance of medicinal plants. There is a growing need to identify bioactive compounds with anti-inflammatory, antimicrobial, and wound healing activities which could be a future drug candidate (15). As a result, these medicinal plants will have immense importance in limb care (lymphoedema).

Cytotoxicity assays were conducted for the three methanol extracts in the *in vitro* models, and all concentrations used were found to be safe to the cell lines used in these experiments. However, all plant extracts showed varying level of toxicity to Vero cells which might be attributable to their differences in phytochemical constituents(16). According to our results, *A. aspera* and *A. indica* had the lowest toxicity to Vero cell lines while *L. inermis* was more toxic which may be associated with the levels of biomolecules such as alkaloids and saponins in the extracts(17).

The anti-inflammatory activity of the plant extracts was determined using two parameters, cyclooxygenase, and 15-lipoxygenase inhibition assay. Both inhibition of LOX and COX are important in the management of chronic inflammatory conditions. Metabolism of arachidonic acid by COX enzymes leads to the secretion of prostaglandins and thromboxane that mediate pain and inflammation-related edema. The LOX pathway utilizes arachidonic acid to produce leukotriene, including the leukocyte chemoattractant LTB4 (18). All methanol extracts of *A. aspera, A. indica* and *L. inermis* were shown to inhibit both cyclooxygenase and 15-lypoxygenase enzymes.

All the methanol extracts tested were effective in inhibiting COX-2, a key enzyme in catalyzing the secretion of prostaglandins, thromboxane, and levuloglandins. Prostaglandins are known to have an effect against almost all known physiological and pathological processes through reversible interactions with G protein-coupled membrane receptors. Levuloglandins are a new category of products that seem to function through irreversible covalent bonding with a variety of proteins(19). *L. inermis* and *A. indica* were effective in inhibiting COX-1. Similarly, Jacob *et al* (2015) report that *L. inermis* showed dual inhibition of both LOX and COX enzymes, which is important in the reduction of chronic inflammatory conditions(20) and agrees with the current finding. In addition, ethanol and methanol extracts of *L. inermis* have been shown to inhibit inflammation in the carrageenan-induced rat paw edema model(21, 22). In our previous

work, alkaloids, terpenoids, saponins, anthraquinones, tannins, phenols, steroids and flavonoids of plant origin were the major bioactive secondary metabolites isolated from the three methanol extracts which have been described to have significant anti-inflammatory activity (23). Flavonoids, present in both *A. indica* and *L. inermis*, have been reported to have a dual inhibitory activity of cyclooxygenase and lipoxygenase, known to inhibit the biosynthesis of prostaglandins(23).

In another study, Attiq *et al* (2018) reported that alkaloids and terpenoids which are present in *L. inermis* and *A. aspera* have been shown to inhibit COX-1 and COX-2-mediated PGE2 secretion in *in vitro* models. Further, alkaloids and terpenoids have been shown to inhibit PG2 and COX-2 through inhibition of cellular nuclear factor Kappa B (NF-kB) activity. In a similar study, terpenoids and flavonoids were reported to have the ability to reduce the secretion of pro-inflammatory cytokines such as IL-1beta, IL-6 and TNF-alpha (24). *A. aspera* was more selective to COX-2 than COX-1 with a higher value, i.e., 1.33, which was comparable to indomethacin. Selective COX-2 inhibitors such as Coxibs are preferred to decrease the incidence of gastrointestinal hemorrhage and ulceration upon long-term intake(25). The anti-inflammatory compounds with better COX-2 selectivity index have been shown to have low side effects (26).

Many inflammatory diseases are due to the lipoxygenase enzyme families such as 5-LOX, 8-LOX, 12-LOX, and 15-LOX enzymes. The isomeric enzyme 15-LOX is a major enzyme implicated in the synthesis of leukotrienes from arachidonic acids. Bio-active leukotrienes act as promoters for numerous pro-inflammatory and allergic reactions, therefore inhibition of leukotriene synthesis by 15-LOX is considered to be one of the treatment approaches to regulate inflammation (27).

The anti-LOX enzyme activity of the plant extracts was measured by the inhibition of linoleic acid which is a substrate for soybean lipoxygenase (15-LOX) enzyme. According to previously reported findings, methanol extract of *L. inermis* and *A. aspera* have been shown to have moderate activity (66-74.8%) inhibition) in the inhibition of 15-LOX (12). In another study, leaves of methanol extracts of *A. aspera* showed 70% inhibition of lipoxygenase enzyme, which agrees with the current finding (28). Mzindle (2017) reported that the aqueous and methanol extracts of *A. aspera* were shown to control the release of various mediators in both the early and late stages of inflammation, and were observed to have wound healing potential (29). In addition, methanol extract of *A. aspera* has shown significant activity in Carrageenan-induced paw edema in the rat model (30, 31). There is a direct relationship between anti-LOX activity and presence of plant secondary metabolites such as phenols and flavonoids in plant extracts. Furthermore, different studies have implicated oxygen free radicals in blocking the process of arachnoid acid metabolism by inhibiting lipoxygenase (LOX) enzyme activity (32).

Chaibi *et al* (2017) described the anti-inflammatory activity of methanol extract of *L. inermis*, which showed the methanol extract to be superior in inhibiting the LOX family, 5-LOX with $IC_{50}$ value of 51 ± 0.23 mg/L(33). Schumacher *et al* (2011) reported that the methanol extract of *A. indica* inhibited the TNF-a-activated NF-kB pathway at 240 µg/ml, which indicated the anti-inflammatory activity of the crude extract (34). Plants with antioxidant properties can also have anti-inflammatory activities, because lipoxygenase

is reported to be sensitive to antioxidants due to its inhibition of substrate (lipid hydrogen peroxide) formation that required for lipoxygenase catalysis (35).

Wound healing is a complex and ongoing process which includes homeostasis, re-epithelization, granulation, tissue reformation, and remodeling of the extracellular matrix. Even though wound healing can take place spontaneously without assistance, external factors such as wound infection have focused attention on wound healing (36). Many medicinal plants have been claimed to be useful for wound healing in Ethiopian traditional medicine, and some of the traditional medicines in use have been used for the management of dermatological disorders (11).

All methanol extracts exhibited dose dependent DPPH antioxidant activity and their free radical scavenging activity was correlated to the content of flavonoids, phenols and terpenoids (37). Methanol extract of leaves of *A. aspera* was effective in scavenging DPPH free radicals, which is in agreement with those previously reported findings(36, 38–40). Further, Fikru *et al* (2012) reported the considerable antioxidant and antimicrobial activity of methanol extract of *A. aspera*, and its wound healing properties. The wound healing activity could be due to its role in promoting fibroblast adhesion or reducing xenobiotic-induced leukocyte hyperactivity and inflammatory damage (41). Similarly, *L. inermis* was shown to have high antioxidant and wound healing potential, in agreement with previously reported findings (42–44). In another study, Alzohairy (2016) reported significant antioxidant activity of *A. indica*, and revealed that azadirachtin and nimbolide were the main compounds with radical scavenging activity and reductive potential (45).

Oxidative stress and free radicals have been implicated in impaired wound healing (46). Different reactive oxygen species and their degradation product are generated during the healing of cutaneous wounds, causing oxidation of biomolecules and ability to damage numerous molecules in the cell membrane (47, 48). In addition, high level reactive oxygen species have the capacity to inflict peroxidation of membrane lipids, aggression of tissue membranes and proteins, or harm to DNA and enzymes via oxidation of low-density lipoproteins (LDL) (47). Thus, decrease of antioxidant ability results in redox imbalance, which is a major cause of nonhealing wounds (49).

A previously established *in vitro* cell proliferation assay method was used to measure percent human epidermal keratinocytes in a monolayer cell model. The methanol extracts of leaves of *L. inermis*, *A. aspera* and *A. indica* demonstrated moderate activity in stimulating proliferation of human epidermal keratinocytes. The concentration that showed optimal stimulation was between 2.5mg/ml and 5 mg/ml. Plant secondary metabolites, triterpenes, are known to stimulate cell proliferation, which positively influences the wound healing effect of the methanol extracts of leaves of *L. inermis, A. aspera*, and *A. indica* (50).

Fikru *et al* (2012) reported that 5% and 10% ointment-based methanol extracts of *A. aspera* stimulated high DNA and protein content of granulation tissue in animal models, implying cellular multiplication and suggesting an increase in the synthesis of collagen, a predominant tissue in wound healing (51). In another study reported by Rekik *et al* (2019), *L. inermis* oil promoted wound healing via cell proliferation

in animal models (43). Furthermore, the methanol extract of *L. inermis* also has antibacterial and anti-oxidant activity (52), which could contribute to wound healing. In another animal study, stem bark of *A. indica* was shown to increase the tissue DNA content of plant extract-treated wounds, indicating cell proliferation. In addition, there was considerable increase in the protein and hydroxyproline content of plant extract-treated wound tissues, which is an indication of fibroblast cells and epithelial cells migration, and synthesis of extracellular matrix in *A. indica* extract-treated mice (53).

# Conclusion

Plant extracts or compounds that inhibit enzymatic inflammatory activities may contain potential drivers or templates for the development of effective anti-inflammatory drugs for the management of lymphoedema. More work needs to be done to correctly characterize the compounds responsible for anti-inflammatory activity in these plant species and understand their mechanisms of action. The results of the present study confirmed the presence of biologically active molecules in these plant extracts, which can inhibit the LOX/COX enzymes involved in the biosynthesis of pro-inflammatory leukotrienes and prostaglandins. Oxidative stress must be considered in the inflammatory process of wound healing and chronic wound treatment. Antioxidants that maintain non-toxic ROS levels in wound tissue can improve wound healing.

# Declarations

### Ethics approval

Ethical approval was obtained from the Brighton and Sussex Medical School, Research Governance and Ethics Committee (ER/BSMS9DY2/1), and the Scientific and Ethics Committee of CDT Africa (004/19/CDT), College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. In addition, all methods were performed in accordance with the relevant national and international guidelines and regulations.

**Consent for publication**: Not applicable.

**Availability of data and materials:** All data generated or analyzed during this study are included in this published article.

**Competing interests:** The authors declare that they have no competing interests

### Funding

This research was funded by the National Institute for Health Research (NIHR) Global Health Research Unit on NTDs at BSMS using UK aid from the UK Government to support global health research. NIHR was not involved in the design of the study, collection, analysis, interpretation of data or in writing the

manuscript. The views expressed in this publication are those of the author (s) and not necessarily those of the NIHR or the UK Department for Health and Social Care.

### Authors' contributions

DN, GD, EM, BAL, and AF were involved in conceptualization and design of the study, and in analysis and interpretation of the data. DN wrote the first draft of the manuscript and DN, GD, EM, BAL, and AF critically reviewed the manuscript for intellectual content. All authors have read and approved the final manuscript.

### Acknowledgments

We would like to thank CDT-Africa, Addis Ababa University for the field support, Ethiopian Public Health Institute, Vaccines and Diagnostics Directorate, and Food and Nutrition Research Directorate for allowing us to use their laboratory. Our special gratitude to Clare Callow, Grit Gansch and Manuela McDermid for supporting the procurement of laboratory supplies. Special thanks to Dr. Masresha Tessema, and Mr. Meseret Woldeyohanise for their support during laboratory work.

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# Figures

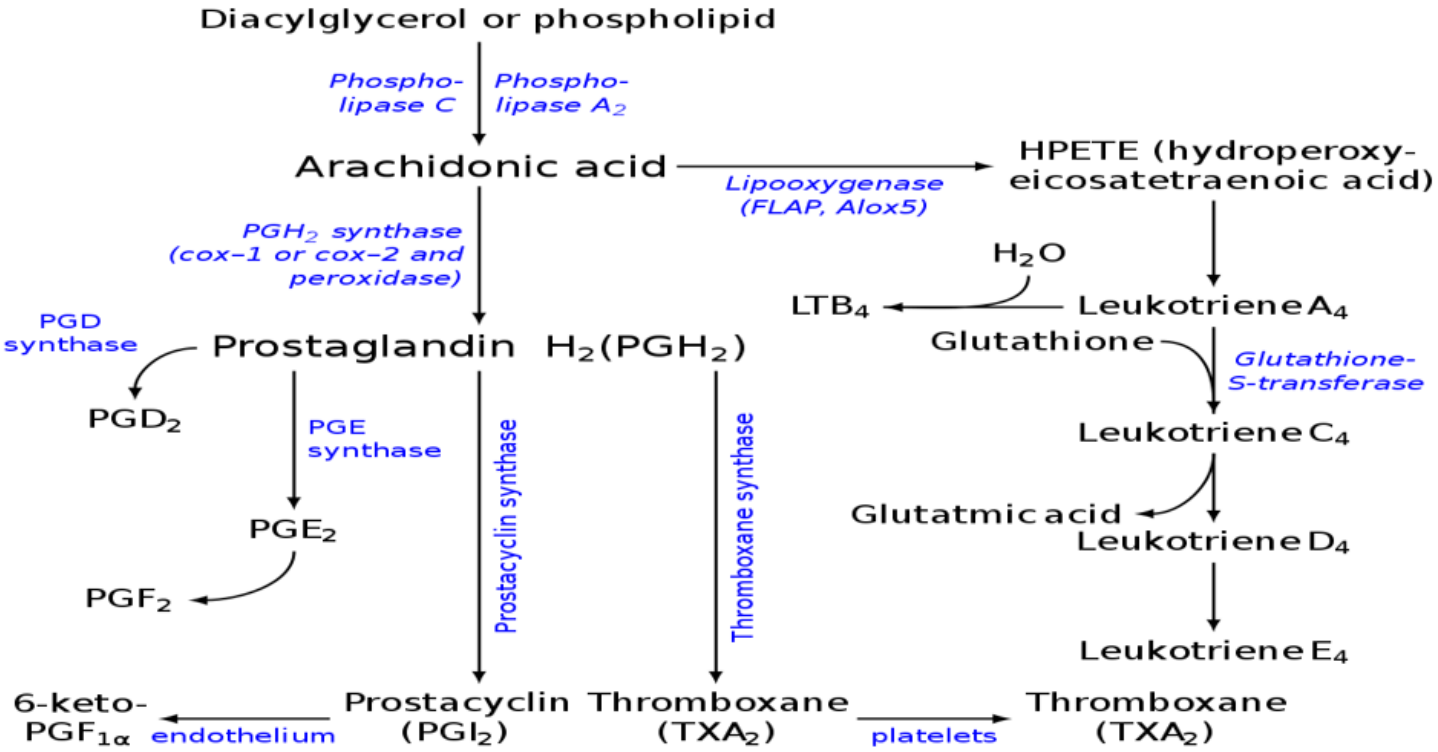
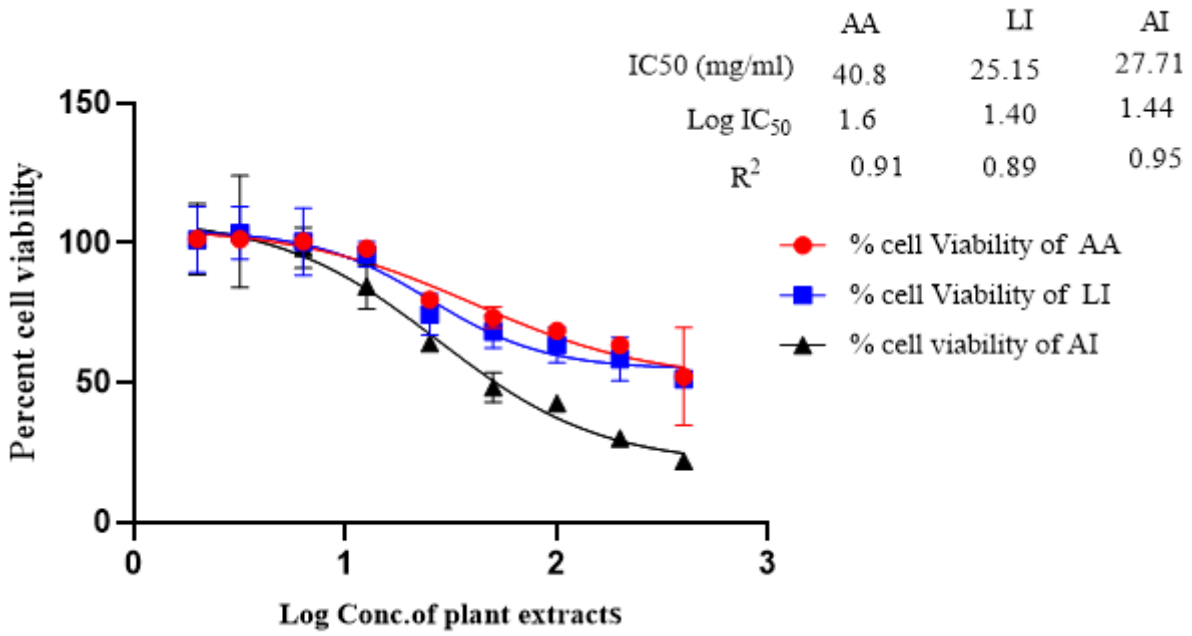



**Figure 1**

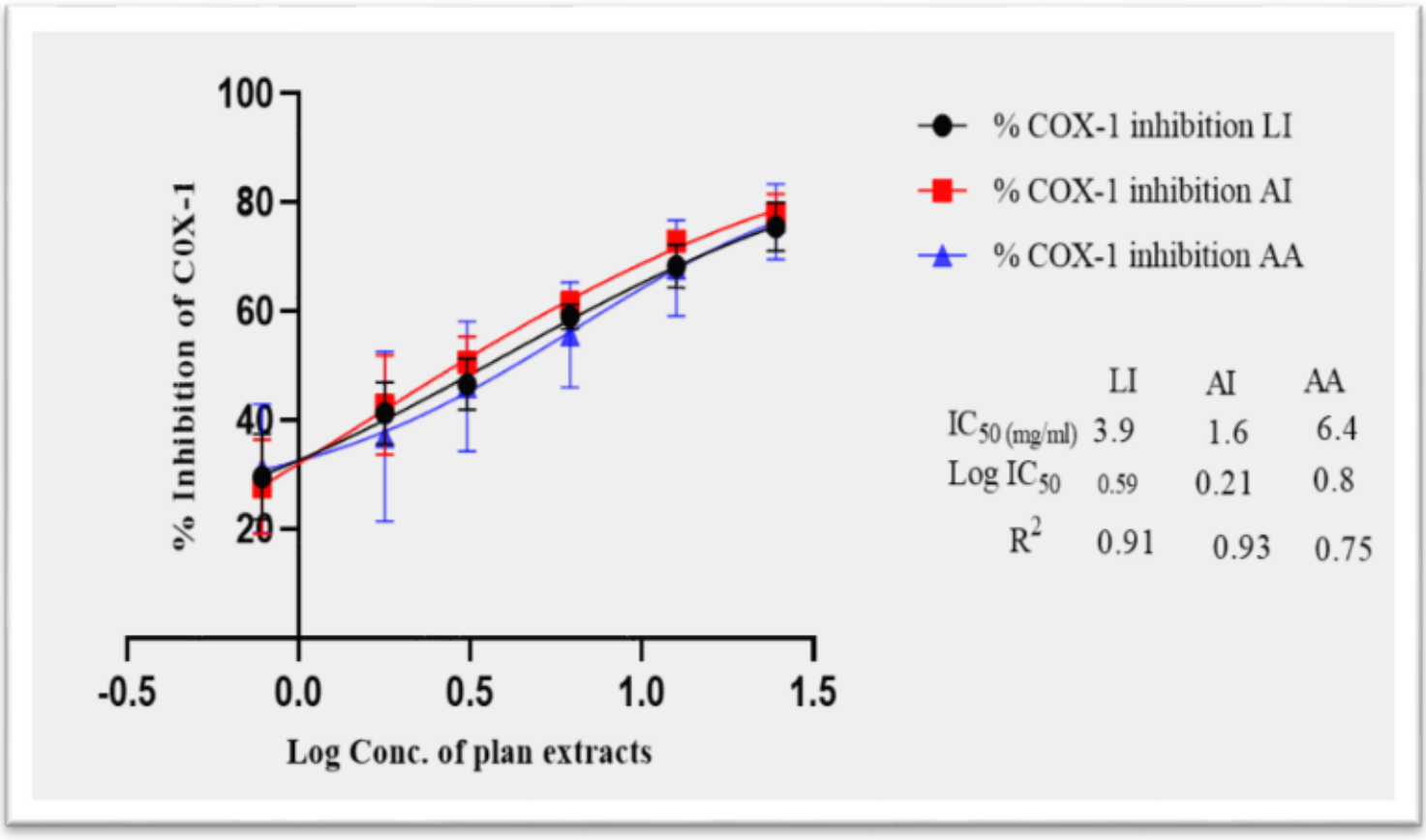
Eicosanoid synthesis (Source: https://en.wikipedia.org/wiki/Eicosanoid#/media/File:Eicosanoid_synthesis.svg )




## Figure 2

Cytotoxicity assays of methanol extract of leaves of L. inermis, A. indica and A. aspera against Vero cell line

AA = Acyranthus asper, AI= Azadiractha indica, LI = Lawsoniainermis




## Figure 3

Percent inhibition of methanol extract of *L. inermis, A. indica and A. aspera* against COX-1 enzyme. *Acyranthus asper*, AI= *Azadiractha indica*, LI = *Lawsonia inermis*, COX-1 = cyclooxygenase enzyeme-1

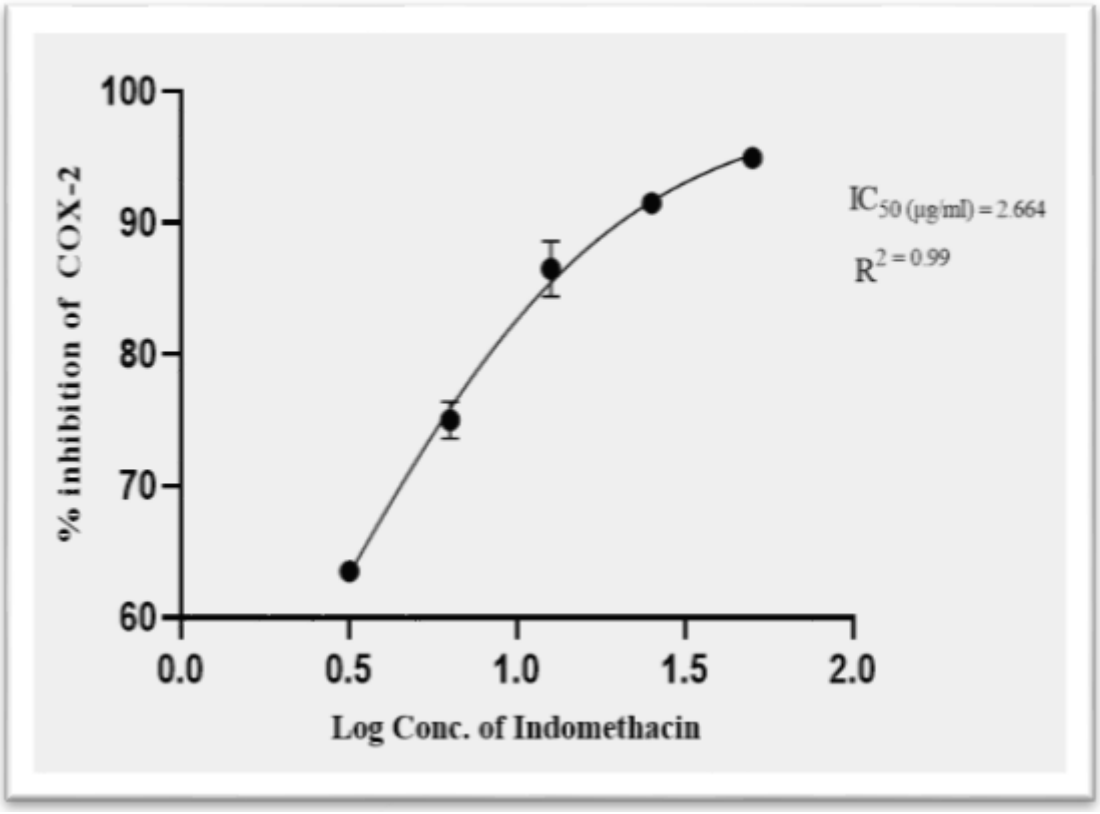
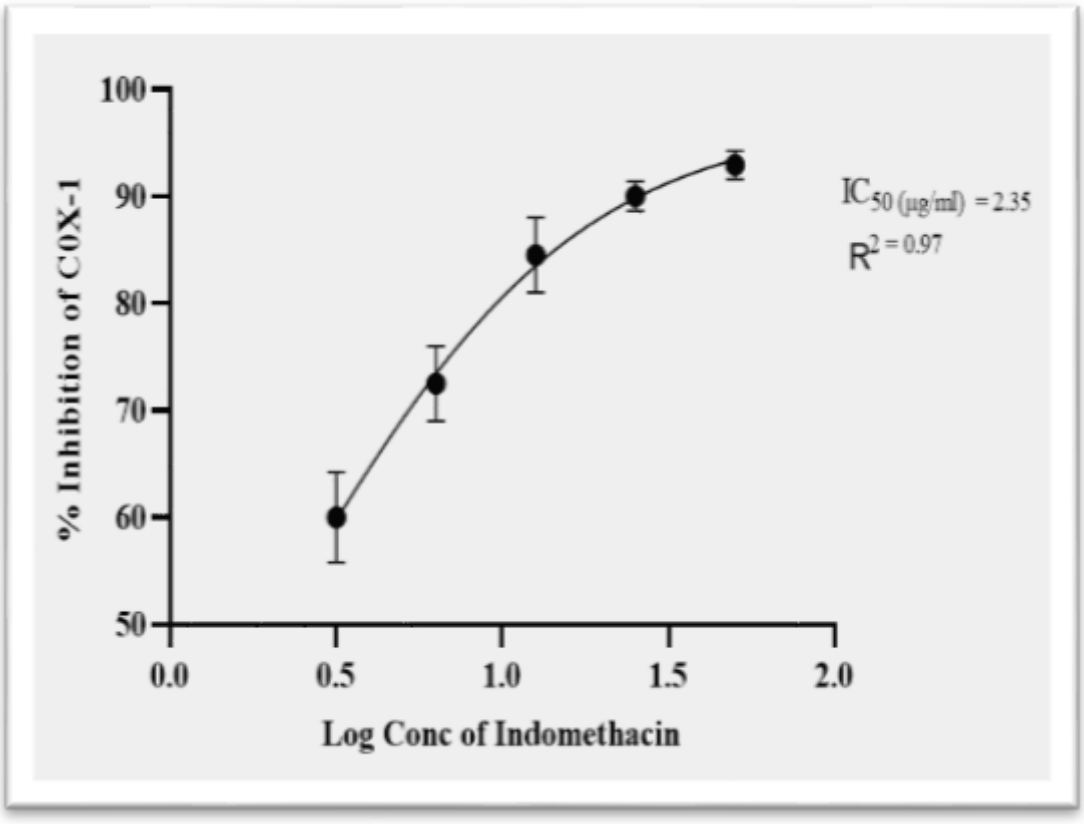


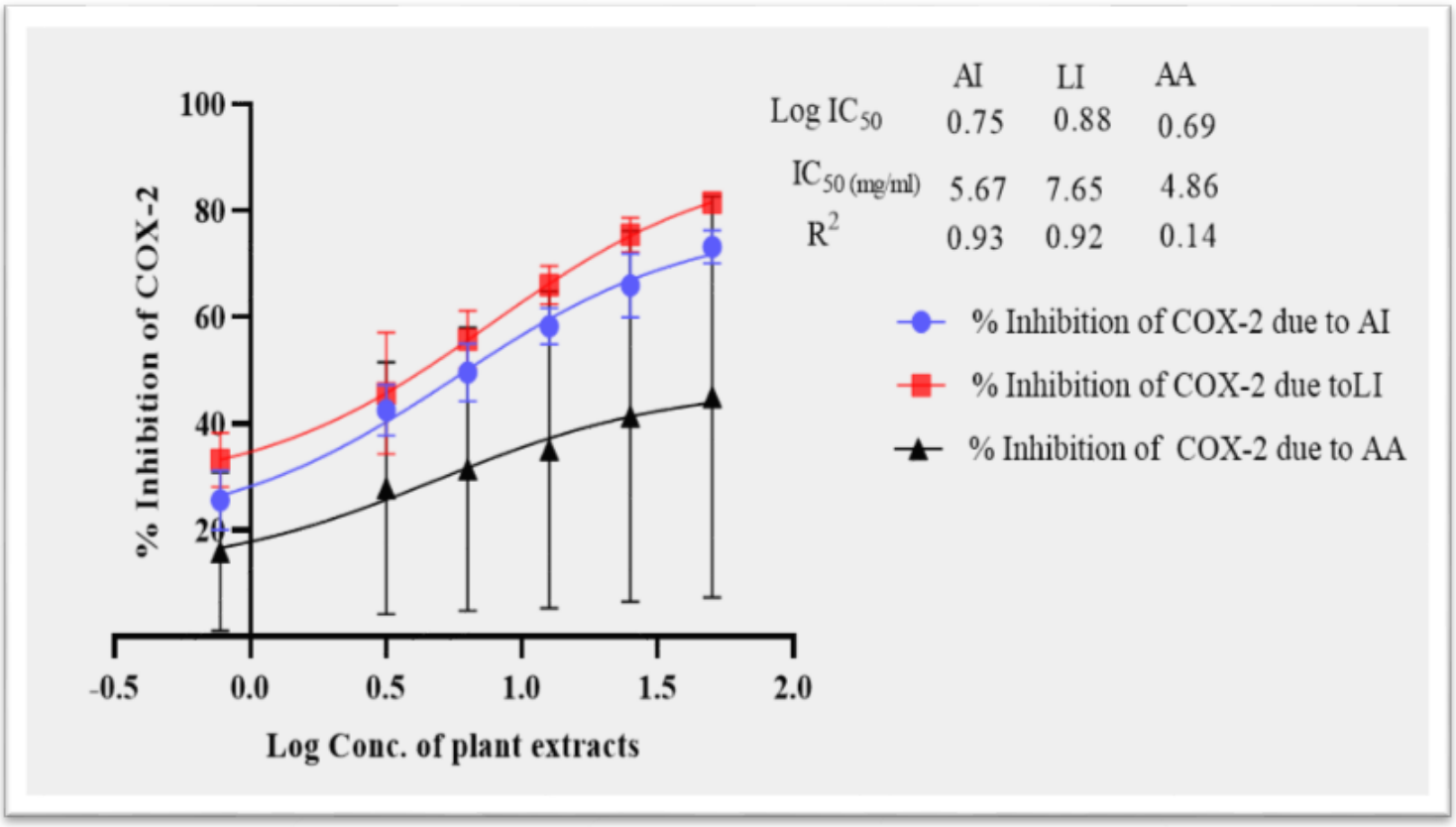

**Figure 4**

Percent inhibition of indomethacin against COX-2 enzyme



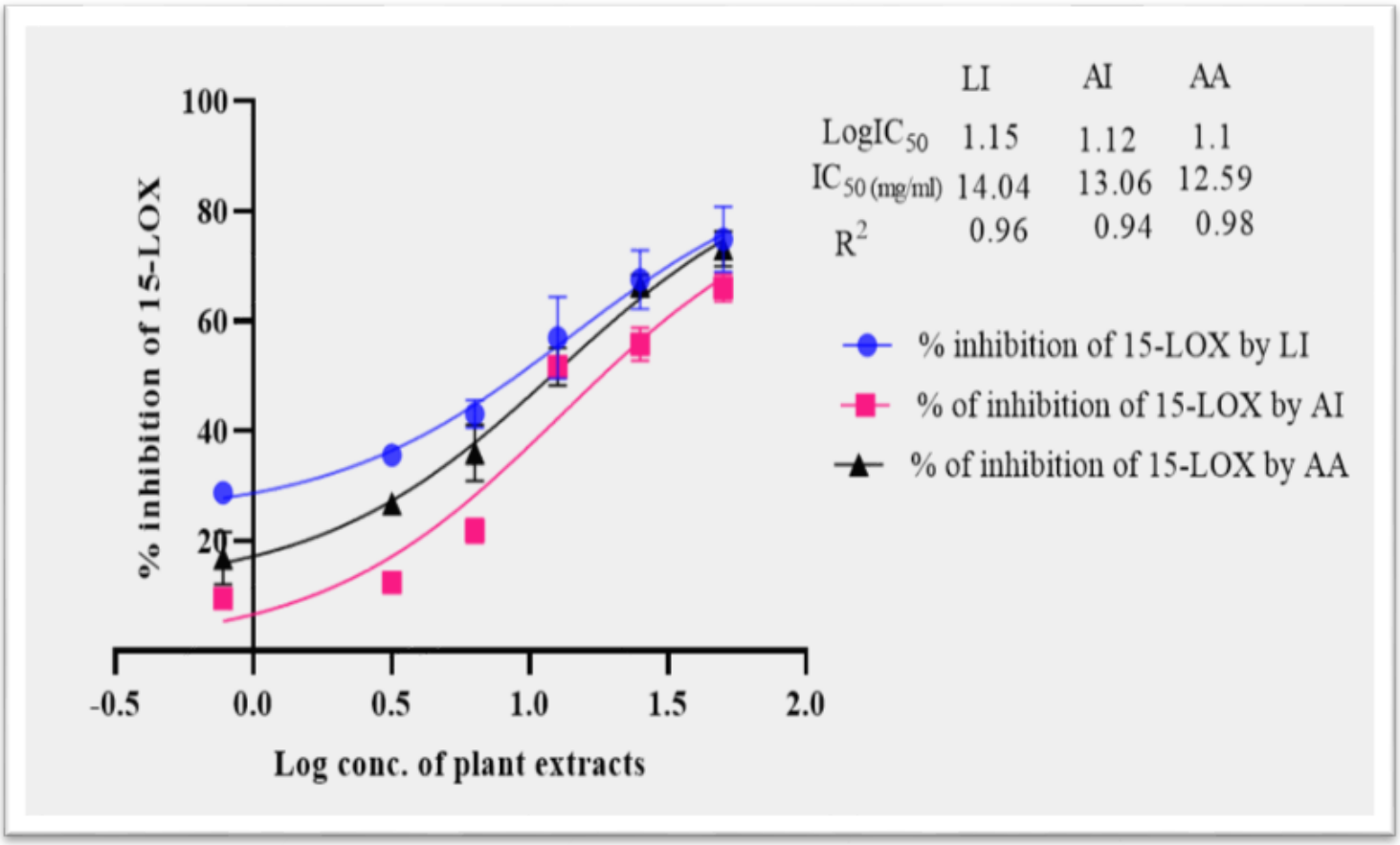
## Figure 5

Percent inhibition of indomethacin against COX-1



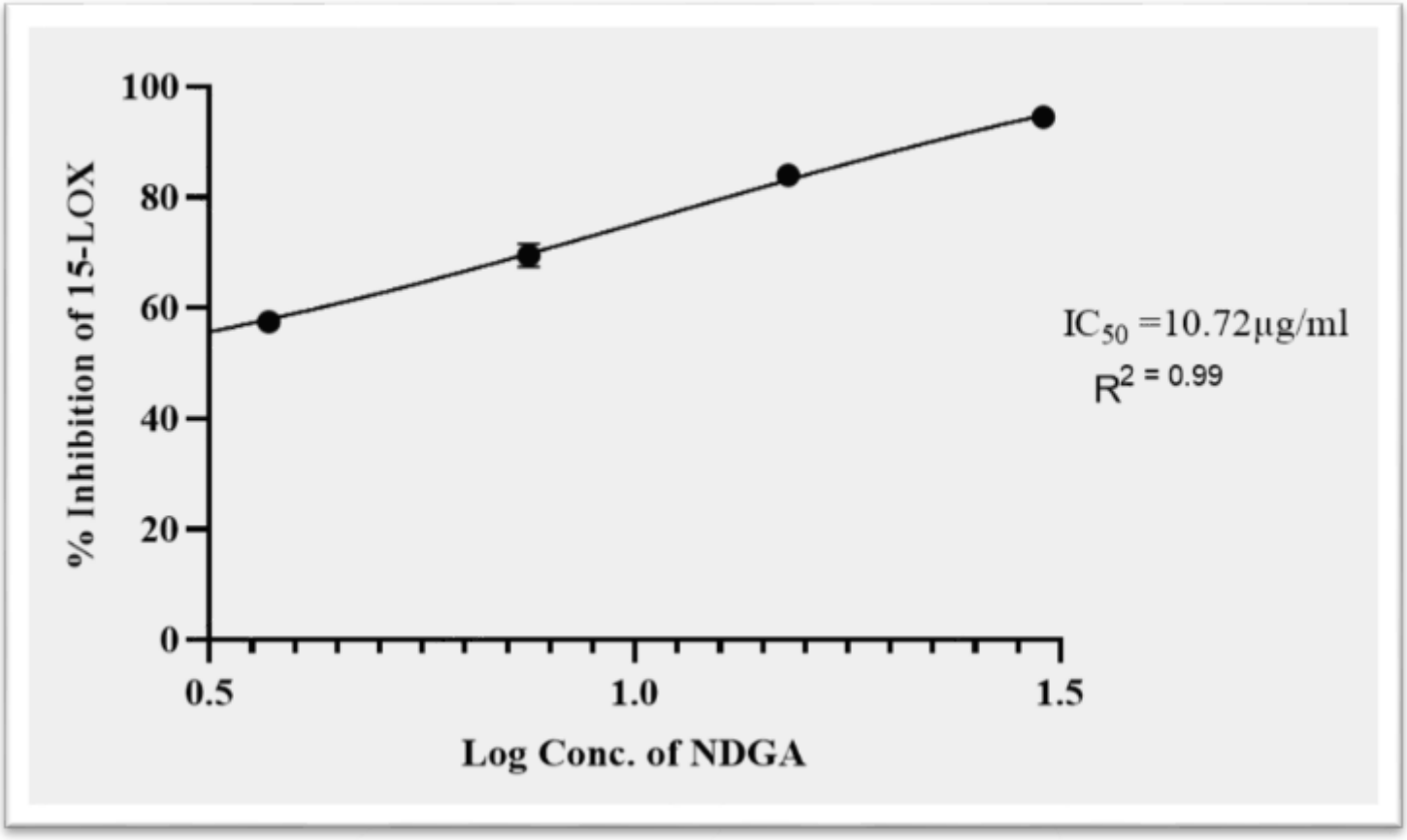

## Figure 6

Percent inhibition of methanol extracts of *L. inermis, A. aspera*, and *A. indica* against COX-2 COX-2 = cyclooxygenase enzyeme-2 AA = *Acyranthus asper,* AI= *Azadiractha indica*, LI = *Lawsonia inermis*




## Figure 7

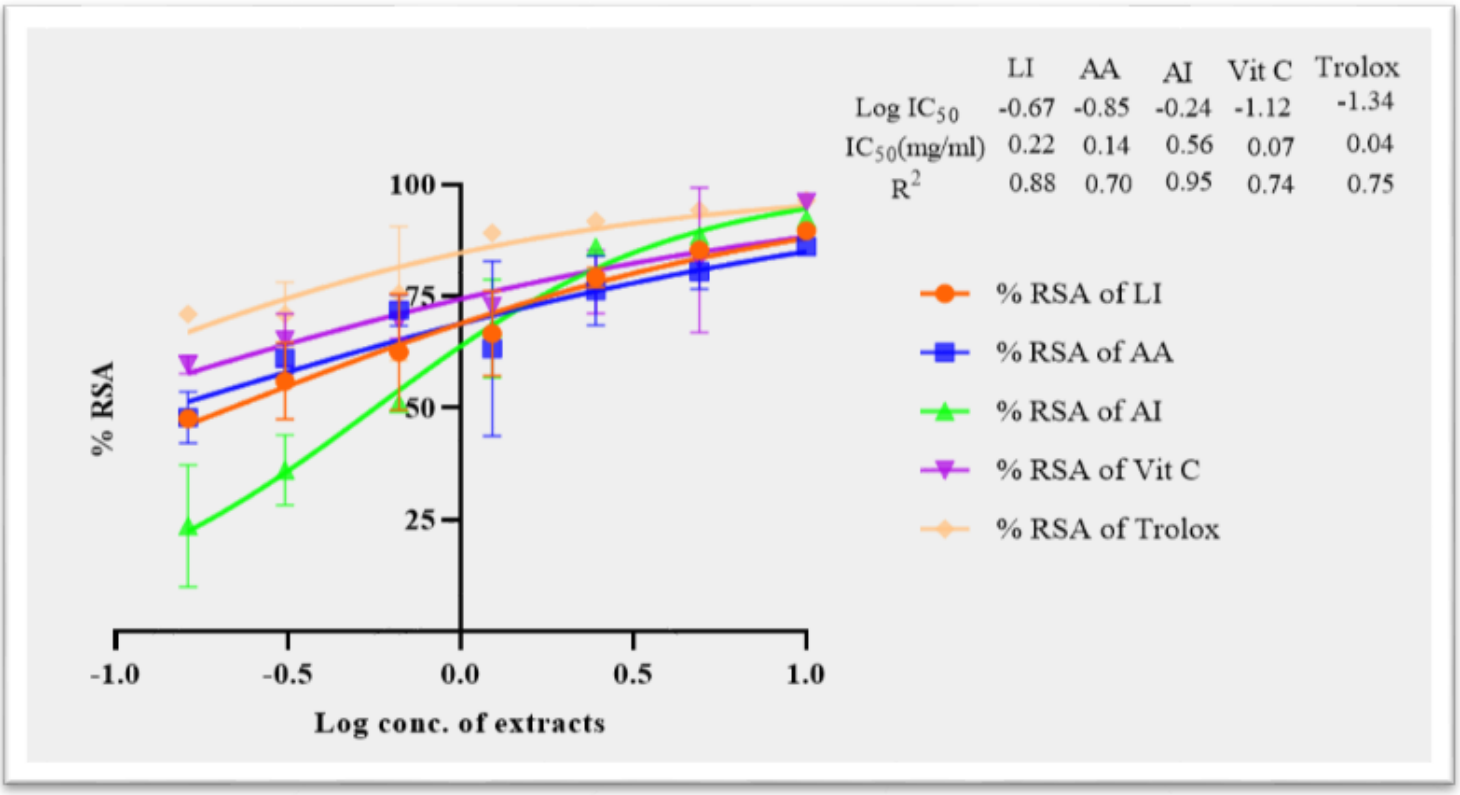
15-Lipoxygenase inhibitory activity of methanol extracts of L. inermis, A. indica and A. aspera AA = *Acyranthus asper*, AI= *Azadiractha indica*, LI = *Lawsonia inermis*, 15-LOX = 15-lipooxygenase enzyme




## Figure 8

Nordihydroguaiaretic acid inhibition of 15-LOX at different concentrations

15-LOX = 15-lipooxygenase enzyme, NDGA = Nordihydroguaiaretic




## Figure 9

Antioxidant activity of leaves of methanol extracts L. inermis L, A. aspera L, and A. indica A Juss

RSA = Radical scavenging activity, AA = *Acyranthus asper*, AI= *Azadiractha indica*, LI = *Lawsonia inermis,* Vit C=vitamin C

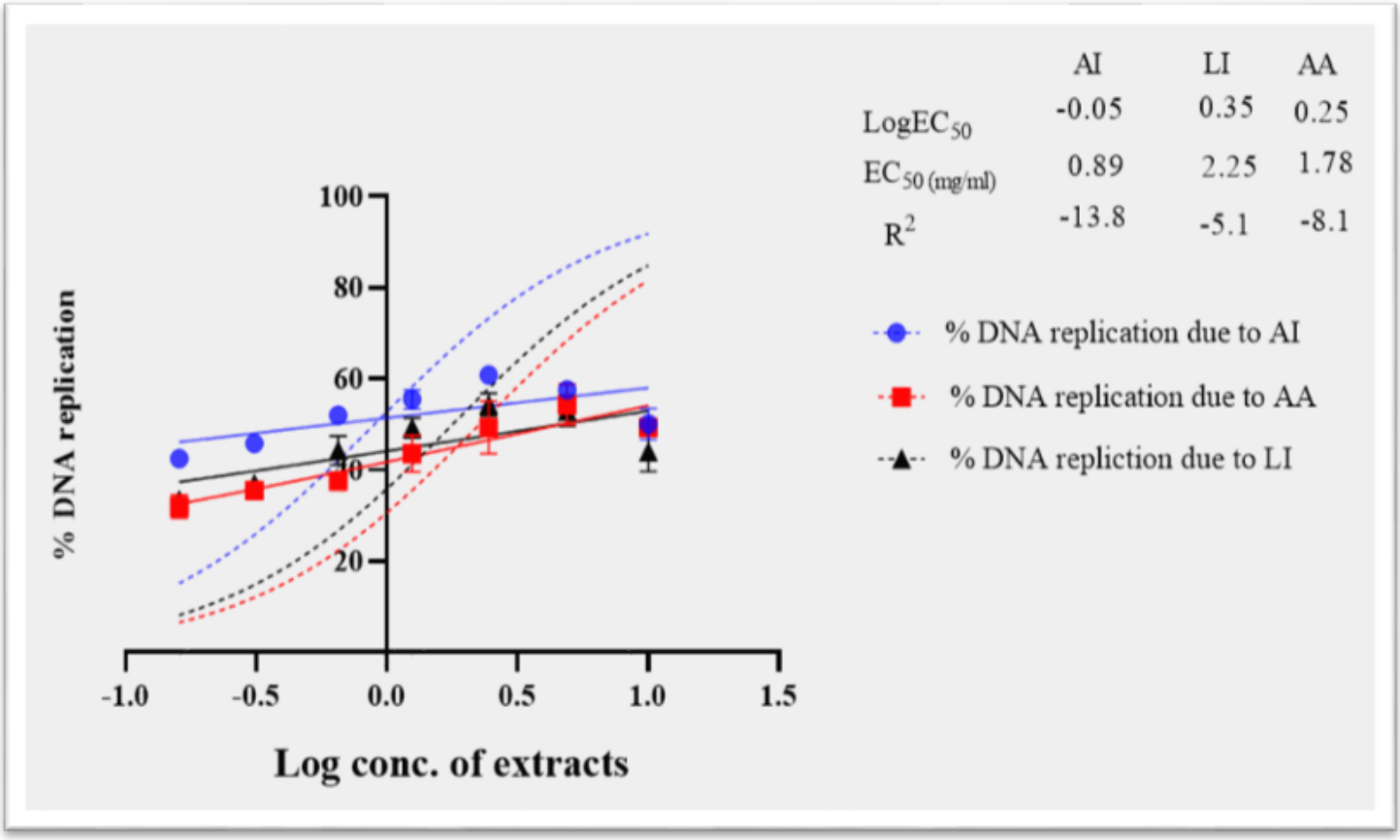



**Figure 10**

Percent DNA replication activity versus Log con. of extracts of Methanol extract of leaves of L. inermis, A. indica, and A. aspera

DNA = Deoxyribonucleic acid, AA = *Acyranthus asper*, AI= *Azadiractha indica*, LI = *Lawsoniainermis*