e-ISSN: 0975-5160, p-ISSN: 2820-2651

Available online on www.ijtpr.com

International Journal of Toxicological and Pharmacological Research 2024; 14(6); 52-59

Original Research Article

Persea Americana's Anti-Inflammatory and Analgesic Benefits: Insights from in-Vitro and In-Vivo Analysis

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Received: 11-03-2024 / Revised: 12-04-2024 / Accepted: 25-05-2024
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Conflict of interest: Nil

Abstract

Background: Persea americana also known as avocado, belong to family Lauraceae. The leaves and fruits are used as traditional folk medicine to treat inflammation and algesia. The purpose of this research is to evaluate the anti-inflammatory and analgesic activity of hydro-alcoholic extract from persea americana seeds.

Methods: Phytochemically, hydro-alcoholic extract of persea americana seeds gave positive reactions for Proteins, amino acids, Fatty acids, alkaloids, glycosides, flavonoids, tannins, saponins, phenolic, Terpenoids and steroids. The invitro anti-inflammatory potential was accessed by using Protein denaturation method. In vivo anti-inflammatory activity was evaluated by using the carrageenan-induced paw edema method and analgesic activity was evaluated by using eddy's hot plate method.

Result: The present finding exhibited a concentration dependent inhibition of protein denaturation by Persea americana and IC 50 Value found at 305 µg/ml. The hydroalcoholic extract showed significant inhibition on the rat paw volume at 52.40% as compared to standard (diclofenac sodium). The analgesic activity of hydroalcoholic extract was shown significant activity after 60 minutes.

Conclusion: The extract possesses analgesic and anti-inflammatory activity which may be mediate through the phytochemical constituents of the plant which supports its traditional use. Further isolation of active constituent responsible for activity will be isolated.

Keywords: anti-inflammatory, analgesic, hydro-alcoholic extract, Persea americana seeds.

Introduction

Inflammation is the body's exaggerated response to remove foreign substances, infections, or irritants and facilitate tissue repair (Karch & MS, 2002). Major symptom of inflammation is Rubor (redness), Tumour (swelling), Calor (heat) and Dalor (pain) (Supriyatna et.al., 2015). Persea americana Mill., a member of the Lauraceae family, is grown in tropical and subtropical climates worldwide and commonly known as "Avacoado" (Kendir & Köroğlu, 2018). P. americana contains fatty acid and lipids, carbohydrates, protein, additionally it also contains phytosterols, terpenoids (monoterpenes, triterpenes, sesquiterpenes) flavonoids, carotenoids and alkaloids. Avocados are rich in fatty acid and lipids such as monosaturated fatty acid, polysaturated fatty acid and saturated fatty acid (Araújo et al., 2020). Bioactivities of P. americana has been reported such as antidiabetic (Cardoso et.al, 2016), antihypertensive, hypercholesteraemic, antimicrobial and antioxidant. (Lu et al., 2012).

The purpose of this research is to evaluate the anti-inflammatory and analgesic activity of hydro-alcoholic extract from persea americana seeds.

Material & method

Plant Material

With the recent scientific breakthroughs globally, there is an increasing interest in investigating the medicinal properties of plants. The interest in these substances arises from their remarkable pharmacological properties, low toxicity, and commercial viability. The library search will yield material regarding the utilisation of traditional medicinal herbs in a certain geographic area. This measure will ensure the availability of plants for the purpose of harvesting. From the literature review it was extracted that Persea americana is having significant activity. So, for this research purpose Persea americana seeds was selected and for this research study.

Collection and authentication of seed

Persea americana was purchased from local market of Ujjain Madhya Pradesh, Seeds were extracted from the fruit and dried. The seeds were identified and authenticated by Dr. Narendra Kumar (HOD of

Agriculture, Oriental University, Indore). A voucher specimen [OCPR/ P'Cognosy/2023/16] was deposited in the herbarium of Pharmacognosy department, Faculty of pharmacy, Oriental University, Indore, MP.

Extraction of Seed

The dried seeds were powdered and 50gm powder was extracted with mixture of alcohol and water in 1:1 ratio (hydroalcoholic) using Soxhlet apparatus for 48 hrs at 50-60ºC. Extract was collected and filtered, resultant solution was concentrated to get thick and concentrated extract. The extract was evaporated to dryness and percentage yield was calculated. The dry extract was kept in vacuum desiccator for further use.

Physico-chemical Analysis

The powdered plant components underwent physiochemical examination. The different variables considered were carried out (WHO.2002; Ayurvedic Pharmacopeia, 2008) in dry powder of P. americana. The initial phytochemical analysis of all the plant extracts was conducted according to the established qualitative methodologies. Chemical tests were conducted to detect the presence of alkaloids, flavonoids, carbohydrates, glycosides, steroids, and tannins (Khandelwal 2006).

Pharmacological Activity

Anti-inflammatory activity - Inflammation is a typical defensive reaction to tissue damage resulting from physical injury, harmful chemicals, or microbial pathogens. Inflammation is an intricate biological process that is often linked to pain. It encompasses various events, including the augmentation of vascular permeability, elevation of protein denaturation, and modification of cell membranes. (Vogel & Maas, 2006).

Invitro method for determination of anti-inflammatory activity with Inhibition of Protein Denaturation method

Principle - The denaturation of proteins is extensively documented and is mostly caused by the inflammatory process, particularly in disorders such as arthritis. Therefore, the prevention of protein denaturation, which was first believed to be the primary way that NSAIDs worked according to Mizushima [1964] before the discovery of their ability to inhibit cyclooxygenase [Chandra S, 2012], could potentially have a significant impact on the anti-inflammatory activity of NSAIDs. The drug's capacity to hinder the process of heat and hypotonic protein denaturation may play a role in its anti-inflammatory actions.

Requirement - Phosphate Buffer (pH 7.4), Phosphate Buffer (pH 6.8) Standard Drug – Diclofenac sodium (Gift sample received from zydus health care, Indore) was used as reference drug. Standard drug was diluted with phosphate buffer of 7.4 pH to get the concentration of 10 µg/ml. 10 mg of extract was dissolved in 1 ml of Dimethyl formamide (DMF) and then further diluted up to 10ml of with phosphate buffer of 7.4 pH to get the concentration of 1000µg/ml. Further this stock solution (1000µg/ml) was diluted to get the 100, 200, 300, 400 and 500 µg/ml concentration.

Procedure - In a 10 ml volumetric flask, added 0.4 ml of egg albumin (from fresh hen's egg), 5.6 ml of Phosphate buffer (pH 6.8) and 4 ml of different concentration of extract (100, 200, 300, 400, 500 and 1000 µg/ml) to get final reaction mixture. All the final test solutions of extracts were incubated at 37±2ºC for 15 minutes and then these reaction mixtures were boiled on water bath at 70ºC for 5 minutes. Following the cooling of the reaction mixture mentioned above, the level of turbidity was quantified at a wavelength of 660 nm using a SHIMADZU UV 1800 spectrophotometer. Blank Solution – The reaction mixture of control does not contain any drug or extract solution.

Observation – After taking absorbance percentage of inhibition of denaturation of protein was calculated with respect to control and standard. The percentage inhibition was calculated by following formula:

$$\frac{\text{Absorbance of Control} - \text{Absorbance of diffent concentration of extract}}{\text{Absorbance of Control}} \times 100$$

The absorbance was calculated in triplicate, and the IC50 of the test extract, which inhibits protein denaturation, was determined by regression analysis using the Ms-Excel programme. All results are displayed in **Table 5 and in figure 5.**

In-vivo method for determination of anti-inflammatory activity

Animal selection - Wistar Albino rats (180 – 200 gm), of both genders, were used for experimental work. The cages were positioned in the experimental room 24 hours before to the test for the purpose of acclimatisation. The animals were provided with a regular laboratory condition at room temperature of 26 ± 2℃ and subjected to a 12-hour light/dark cycle. The animals were given standard diet ad libitum and water. The experimental protocol was approval by the Institutional Animal Ethics Committee (IAEC), Faculty of Pharmacy, Oriental University, Indore. [IAEC:1877/PO/Re/S /16/CCS EA/2023/012].

Acute Toxicity Studies –

Acute toxicity studies were carried out as per OECD Guideline No. 425recommended by CCSEA. Animals were divided into two groups (n = 6). Prior to the trial, the animals underwent an overnight fasting period. The hydroalcoholic extract was

administered orally in doses of 500 mg/kg and 1000 mg/kg of body weight to both the groups of rats and observed for 14 days for mortality and physical or behavioural changes. (Chaudhari et al., 2012) All observations were discussed in **Table 6**.

Carrageenan Induced Acute Paw Edema Method (Goyal R.K. 2006)

Principle - This approach depends on the capacity of anti-inflammatory drugs to suppress the swelling induced in the hind paw of rats following the administration of a phlogistic substance called carrageenan. The injecte

paw's volume is monitored both prior to and following the injection of irritants. The paw volume of the treated animals is being compared to that of the control group. A plethysmograph was used for the purpose of quantifying the volume of a paw. (Goyal, R. K., 2006).

Requirement – Wistar Rats (180 – 200gm), 0.1% w/v solution of carrageenan in normal saline was prepared. Calculated dose of Extract (100 mg/kg) and Diclofenac sodium (5 mg/kg) was dissolved in normal saline.

Procedure – Total four groups of six animal were used for the experimental activity. First group was marked as normal control and normal saline was given orally. Remaining groups were injected with 0.1% w/v carrageenan in normal saline for induction of acute inflammation into right hind paw of each rat into sub-plantar region. After one-hour, oral administration of hydroalcoholic extract (100 mg/kg body weight) and diclofenac sodium (5 mg/kg body weight) was administered. The Paw volume was measured by plethysmometer at before and after carrageenan administration till 0, 1, 2 and 3 hours. All observations were summarised in table 11. The percentage inhibition was calculated by using the following formula -

$$\%\ \text{of anti} - \text{inflammatory activity} = \frac{Vc - Vt}{Vc} \times 100$$

Where, Vt = increase in rat paw volume with test compounds.

Vc = increase in paw volume in control rats.

Analgesic Activity (Eddy's Hot Plate Method)

Principle **-** The hot plate test is a straightforward behavioural screening method used to estimate the impact of test chemicals on the pain sensitivity threshold. The underlying idea is that when animals are placed on a heated surface, they will exhibit negative responses to the stimulation. Initially, cats clean their paws by licking them, and then they make evident efforts to escape the stimulus by jumping. Substances that alter the nociceptive threshold will either prolong the time it takes for an individual to lick or jump (indicating analgesic effects) or shorten it (indicating hyperalgesic responses). ((Eddy & Leimbach, 1953; Vogel H, **2006**).

Requirement – Normal saline, Wistar Rats (180 – 200gm), Calculated dose of Extract (100 mg/kg) and Ibuprofen (5 mg/kg) was dissolved in normal saline.

Procedure **–** Rats were divided into three groups containing 6 rats in each group. The temperature of Eddy's Hot plat was maintained at 55±1ºC and rats were placed individually, and its basal reaction time was noted. Licking of paw and jumping response was taken as index of reaction to heat. The first group was served as control and only normal saline was given orally. After noting the initial reaction time, standard drug (ibuprofen 5mg/kg) and hydroalcoholic extract of 100 mg/kg was given to each rat by intra peritoneal route. Then each rat was placed onto the eddy's hot plate and noted the paw licking or jump response. Rats with baseline latencies of more than 5 sec or less than 40 seconds were eliminated from the study. The post treatment reaction time was recorded at 0, 30, 60, 90 and 120 minutes. The rats were removed from hot plate immediately after they exhibited jumping. (Hu et al., 2008). All results were summarised in table 9 and figure 10.

Statistical Analysis – Results are displayed as mean and SEM. One way ANOVA was used to determine the significance of difference between control & treated groups. ($p>0.05$).

Result & discussion

Recently, there has been significant interest in natural chemicals obtained from plants, such as steroids, phenolic compounds, terpenoids, and flavonoids, due to their many pharmacological properties, including analgesic and anti-inflammatory effects. The investigation of potential health benefits associated with certain flavonoids, triterpenoids, and steroids has generated a rising interest in their analysis. To address this, the purpose of this study was to evaluate the invitro and in vivo anti-inflammatory and analgesic properties of hydroalcoholic extracts derived from Persea americana (Avacado) seed.

Identification of Physical appearance and extractive values of Persa americana.

The powdered drug of Persea Americana (Avacado) seed was used for this research study, and hydroalcoholic extract in ration 1:1 of alcohol and water using Soxhlet's apparatus. Extractive values, physical appearance and percentage yield were summarised in **Table** 1.

Table 1: Physical appearance and Extractive value of extract.

S. No.	Particulars	Observation
1.	Plant Used	Persea Americana (Avacado)
2.	Part	Seeds
3.	Yield	26.2 %
4.	Colour of extract	Brown
5.	Texture of extract	Dry and Semi solid

I. Physico-chemical constant

The physico-chemical parameters such as loss on drying, total ash, acid insoluble ash and water-soluble ash of seeds of Persea americana were analysed as:

1. Loss on drying – Loss on drying was calculated and result was summarised in table 2.
2. Ash value - Ash values are useful for assessing the quality and purity of a crude medicine, particularly when the substance is in powdered form. The purpose of ashing crude drugs. The purpose is to eliminate any remnants of organic substances that could potentially disrupt an analytical measurement. (Khandelwal, Cokate). Total ash, Acid insoluble ash and Water-soluble ash of seeds of Persea americana was performed and results are summarised in **Table 2.**

Table 2: Physic-chemical parameter of crude powder of seed of Persea americana.

S. No.	Physicochemical standards	Result in % w/w	Standard % w/w (Ayurvedic Pharmacopoeia, 2009)
1	Loss on Drying	7.25	NMT 10%
2	Total Ash	5.24	NMT 6%
3	Acid insoluble ash	2.28	NMT 3%
4	Water soluble ash	10.68	NMT 12%
5	Loss on drying	5.25 %	NMT 10%

NMT = not more than

II. Phytochemical screening - The initial phytochemical investigation identified many active constituents in diverse extracts, including carbohydrates, proteins, amino acids, fats and oils, steroids, terpenoids, glycosides, alkaloids, tannins, and other phenolic compounds. Results are tabulated in **Table 3.** Positive mark (+) marks indicate presence of phytoconstituents Negative (-) mark indicate the absence of phytoconstituents.

Table 3: Phytochemical screening of hydroalcoholic extract of Persea americana

S.No.	Phytoconstituents	Chemical test	Observation and result
1.	Alkaloids	Dragendorff 's Test	++
		Mayer's Test	+
		Hager's Test	+
		Wagner's Test	+
2.	Carbohydrates	Molisch's test	++
		Benedict's Test	++
		Fehling's Test	++
3.	Protein & Amino Acid	Biuret test	++
		Millon's Test	++
		Ninhydrin Test / Hydrolysis test	++
		Xanthoproteic test	++
		Warming test	++
4.	Glycoside	Modified Bontrager's test	+
		Legal's test (Cardiac glycoside)	-
		Keller-Killiani test	-
		Coumarin Glycoside	-
5.	Saponins	Froth Test	+
		Foam Test	+

6.	Steroids and Triterpenoids (Phytosterols)	Salkowski's test	++
		Liebermann Buchard test	++
7.	Phenols	Ferric chloride test	++
		Potassium Mercuric Iodide Test	++
8.	Tannins	Phenazone Test	+
		Gelatin test	+
9.	Flavonoids	Alkaline reagent test	++
		Shinoda Test	++
		Zinc hydrochloride Test	++
10.	Diterpenes	Copper acetate test	+

Where; (-) Negative, (+) Positive, (++) Highly presence.

Pharmacological activity

Invitro method of determination of anti-inflammatory activity (protein denaturation method)

The process of denaturation of proteins is a widely known factor that leads to inflammation. The production of auto antigens in specific inflammatory illnesses may result from the denaturation of proteins within the body. According to Mizushima and Kobayashi (1968), inflammatory medicines such as salicylic acid and phenylbutazone have demonstrated a capacity to cause protein denaturation when the dosage is increased. Agents capable of inhibiting protein denaturation would be valuable for the development of anti-inflammatory drugs (Sakat et al., 2010).

Table 4: Effect of extract on protein denaturation.

S. No.	Concentration (µg/mL)	(%) Inhibition	IC50 Value
1.	Control	-	305 µg/ml
2.	100 µg/ml	35.66%	
3.	200 µg/ml	42.28%	
4.	300 µg/ml	49.88%	
5.	400 µg/ml	59.37%	
6.	500 µg/ml	63.84%	
7.	1000 µg/ml	76.82%	
8.	Diclofenac Sodium (10 µg/ml)	49.23%	

In present research work, the in vitro anti-inflammatory effect of hydroalcoholic extract of P. Americana was evaluated against denaturation of egg albumin protein. The results were summarized in **Table 4**. The present finding exhibited a concentration dependent inhibition of protein denaturation by extract in rage of 100 to 1000 µg/ml. Diclofenac sodium (10 µg/ml) was used as standard drug. IC 50 value was found at 305 µg/ml as shown in Figure 1.

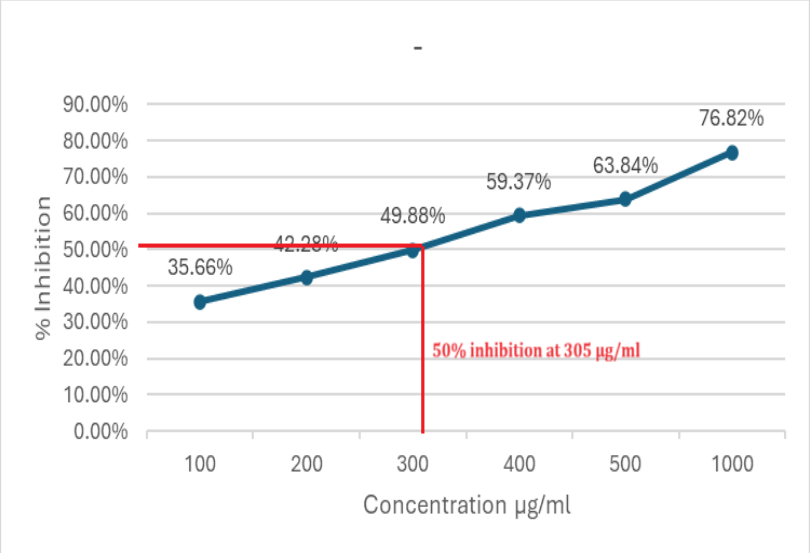

Figure 1: Graph Showing IC50 value and % Inhibition of protein denaturation.

Hence, In the present study the protein denaturation method was selected for invitro assessment of anti-inflammatory activity of hydroalcoholic extract of Persea americana. Denaturation of tissue proteins is a well-documented factor that contributes to inflammatory and arthritic disorders. The production of auto antigens in certain arthritic illnesses may result from the denaturation of proteins within the body. Agents capable of inhibiting protein denaturation would be valuable for the development of anti-inflammatory drugs. (Opie, 1962; Umapathy et al., 2010).

In vivo method of anti-inflammatory activity

Acute toxicity – From the acute toxicity study, LD50 dose for extract was found 1000 mg/kg body weight. The ED 50 of compound was calculated as $1/10^{th}$ of LD50. Hence, the therapeutic dose was taken as 100 mg/kg of body weight.

Carrageenan induced rat paw edema method

The induction of inflammation in the rat paw by carageenan is a well-established model for studying the genesis of edoema and hyperalgesia. This model has been widely used to evaluate the anti-edemal effects of drugs that are known to be responsive to cyclooxygenase inhibitors. (Chaudhari et. al, 2012) The treatment of Carageenan in rats through the sub-plantar route leads to a characteristic biphasic edema. The initial phase, occurring within 0-2 hours, is caused by the release of histamine and serotonin. The second phase of swelling, lasting for 2-6 hours, is attributed to the production of prostaglandins and bradykinin-like chemicals (Pitchaiah et al., 2012). According to reports, the use of a COX-1 inhibitor can decrease the initial and subsequent stages of swelling formation. Prostaglandins, proteases, and lysosomes are believed to be the mediators of the second phase. Clinically effective steroidal and NSAIDs treatments have a significant impact on the second phase of edoema development. (Yang et al., 2010)

The result was displayed in **Table 5** obtained with extract and diclofenac showed significant inhibition of inflammatory edema first and second hours after carrageenan induction. The effect of hydroalcoholic extract of P. americana at 100 mg/kg showed potency and percentage inhibition was found to be 52.40% as compared to diclofenac sodium (5 mg/kg) and percent inhibition found to be 61.28%.

Table 5: Effect of hydroalcoholic extract in carrageenan induced rat paw volume

Group No	Treatment and dose	Change in Paw Volume in ml (±SEM)				% inhibition after 3 h
		0 h	1 h	2 h	3 h	
Control	Normal Saline	0.180 ±0.042	0.179±0.060	0.180±0.050	0.180±0.040	-
Toxic control	Carrageenan (0.1%)	0.338±0.076	0.588±0.041	1.482±0.221	1.552±0.241	-
Standard (Diclo-fenac Sodium)	5 mg/kg	0.340±0.061	0.458±0.020	0.546±0.220	0.601±0.150	61.28
Hydroalcoholic Extract (P. American)	100 mg/ kg	0.332±0.036	0.360±0.027	0.319±0.048	0.158±0.062	52.40

Values are mean ± SEM, n = 6

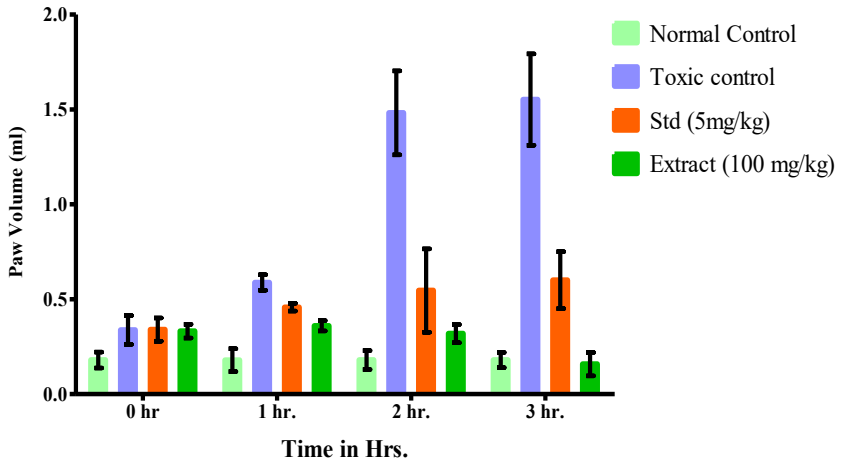

Figure 2: Graph representing Change in paw volume

Analgesic Activity – (eddy's Hot Plate method) - Most inflammatory conditions are commonly linked to pain as a secondary phenomenon, according to reports. In this work, we assessed the central analgesic efficacy of certain drugs using the Hot Plate method. Pain is regulated by various intricate mechanisms, such as the opiate, dopaminergic, descending nor adrenergic, and serotonergic systems. The analgesic effect produced by certain synthesised compounds may be due to a central mechanism that involves these receptor systems or a peripheral mechanism that inhibits prostaglandins, leukotrienes, and other endogenous molecules that play a major role in inflammation and pain. (Mali et al., 2013)

The effect of hydroalcoholic extract (100 mg/kg) and ibuprofen (5 mg/kg) were evaluated for central analgesic activity. **Table 6** displayed the result on thermal stimulus induced pain in rats. Pre-treatment with ibuprofen produced significant changes in paw licking or jump response time in early phase of pain, while hydroalcoholic extract has not shown any significant changes in paw licking time. (Chaudhari et. al, 2012). The effect of analgesia induced by hydroalcoholic extract was observed at 60-minute time interval.

Table 6: Effect of hydroalcoholic extract on paw licking or jump response on hot plate.

Group No	Treatment and dose	Paw Licking Latency in seconds (Mean ± SEM)				
		0 Min	30 Min	60 Min	90 Min	120 Min
Control	Normal Saline	1.32±0.05	3.10±0.18	3.71±0.21	4.18±0.26	5.12±0.29
Standard (Ibuprofen)	5 mg/kg	2.33±0.11	3.26±0.24	9.78±0.10	17.4±0.09	22.2±0.10
Hydroalcoholic Extract (P. American)	100 mg/ kg	4.32±0.03	6.93±0.02	8.98±0.18	11.2±0.19	13.4±0.26

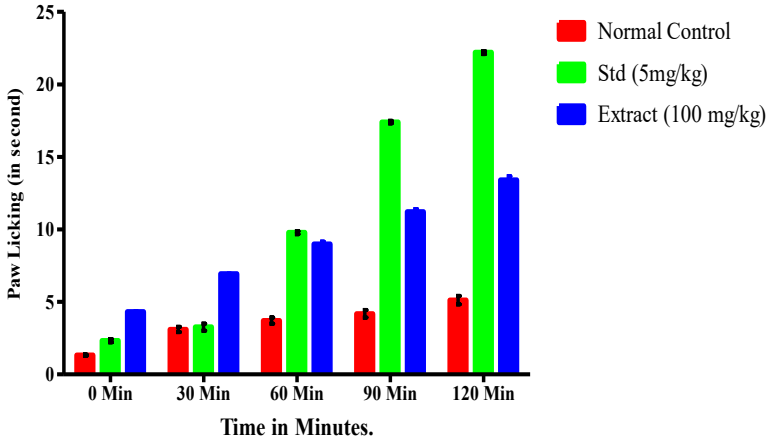

Figure 3: Graph representing Paw licking response.

Conclusion

In conclusion, the seed extract of Persea americana which contains alkaloids, phytosterols, lipids and fatty acids may be responsible for anti-inflammatory potential. The analgesic effect of Persea americana is not mediated much. However, the exact mechanism and active compounds involved for this potential will be clarified in future studies.

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