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Research Article

Anti-inflammatory and Analgesic Activities of Paludose™ Herbal Mixture in Experimental Models

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Abstract
The herbal mixture, Paludose™ is a preparation that is used for the treatment of malaria in Cotonou, Benin Republic. In our previous study, Paludose™ displayed very little antiparasitic effect but prolonged the survival of mice infected with *P. berghei*. This study evaluated its anti-inflammatory and analgesic activities in experimental models. The phenolic constituents of Paludose™ were determined using High-performance liquid chromatography method. The analgesic activities of Paludose™ using acetic acid-induced writhing and the hot plate methods were evaluated in mice. Number of cumulative writhes, pre-dosing, and reaction latency were all recorded. Elucidation of possible mechanism of action was also done. In addition, Wistar rats were used to investigate the anti-inflammatory activity using the carrageenan-induced paw edema model. The paw homogenates of each animal were used for biochemical evaluations. The peak analgesic activity of Paludose™ was obtained at 0.143 mL/kg (83.58 % inhibition) peripherally and at 30 minutes centrally with 41.85% maximum possible effect. However, only naloxone significantly ($p < 0.05$) reversed the effect of Paludose™. Treatment with Paludose™ at 0.143 mL/kg produced a significant inhibition of edema with peak effect seen at 3-hour post-carrageenan injection (68.46 ± 8.46%). Furthermore, the nitrite, malondialdehyde, interleukin-6, and tumor necrosis factor-alpha levels decreased significantly while the level of glutathione was significantly increased at 0.143 mL/kg (Paludose™). Paludose™ is rich in gallic acid, and naringenin and at 0.143 mL/kg exhibit good anti-inflammatory and analgesic activities.

Key Words: Paludose™ herbal Mixture, anti-inflammation, analgesic, rodent

INTRODUCTION

Inflammation is an adaptive biological process that can be induced by microbial infection, and tissue injury which acts by removing the stimuli to initiate the healing process (Shukla *et al*., 2019). Major ill health and impaired quality of life can result from inappropriately triggered and incompletely controlled inflammatory responses. Inflammation is a vital defense mechanism for health (Nathan *et al*., 2010). During acute inflammatory responses, cellular and molecular events and interactions minimize the impending injury and infection in an efficient manner thereby contributing to the restoration of the tissue homeostasis. However, chronic inflammatory diseases are a result of uncontrolled acute inflammation as seen in diabetes, arthritis, cancer, and neurodegenerative disorders as they widely represent a collective burden of economic cost and suffering (Zhou *et al*., 2016). The inflammation process is typically initiated by inducers via stimulating inflammatory cell to produce elevated levels of pro-inflammatory cytokines, including interleukin- 1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) that may affect the functionality of tissues and organs (Ahmed, 2011).

Pain is an unpleasant sensory and emotional feeling that ranges from mild localized discomfort to agony accompanying an existing or impending tissue damage (Swieboda *et al*., 2013). The purpose of pain is to notify the body's defense mechanism to react toward further tissue damage (Galler *et al*., 2021). There is always pain in an inflamed region. In cases of inflammatory process, pain is triggered by normal innocuous stimuli and enhances the response to noxious stimuli. Biochemical mediators such as prostaglandins, bradykinins and substance P acts on the nociceptors causing the sensation released by tissue injury and are often considered the immediate causes of pain. In essence, pain control is an important therapeutic priority (Ezeja *et al*., 2011).

The use of herbal drugs is perhaps the oldest and can be dated back to human history (Yuan *et al*., 2014). In Africa as a continent, the common practice of traditional medicine is the use of herbal plants as they are easily accessible and available

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in the community (Mahomoodally, 2013). For most patients, the traditional healers provide information, counselling, and treatment in a personal manner as well as understanding of their environment (Gurib-Fakim *et al*., 2013). Natural products are important in the development of new drugs and have received increased attention in search of novel drugs in combination with new technology due to the assorted promising biological and pharmacological compounds contained in medicinal plants (Ngo *et al*., 2013; Tasneem *et al*., 2018).

Medicinal plants such as *Cassia alata*, *Zingiber officinale*, *Rosmarinus officinalis*, *Baborago officinalis*, and *Terminalia catappa* used in herbal medicine have been reported to possess anti-inflammatory activities (Sagnia *et al*., 2014; Ghasemian *et al*., 2016; Abiodun *et al*., 2016) while others such as *Curcuma longa*, *Papaver somniferum*, and *Cannabis sativa* have been shown to potentiate analgesic effects in addition to been useful in combination with nonsteroidal anti-inflammatory agents (Klawe *et al*., 2009; Paz-Campos *et al*., 2014; Cavalheiro *et al*., 2021).

Paludose™ is a herbal mixture that is widely used in Cotonou, The Republic of Benin as an antimalarial mixture. It is prepared from medicinal plants and contains aldehyde trans-cinnamic, cinnamyl acetate, eugemol, trans-methoxy cinnamaldehyde, and levomenthol according to the manufacturer. Our previous study on the antimalarial activity, safety, and chemical composition of Paludose™ herbal mixture in a mouse model of *Plasmodium berghei* showed that the herbal mixture appears to be safe but with little antimalarial activity (Abiodun *et al*., 2021).

Conventional anti- inflammatory medicines are effective in the treatment of pain and inflammatory disorders but their prolong use may cause severe adverse effects (Lin *et al*., 2019). Hence, the need for the search and development of a new agent has become imperative. Based on its continuous use in Cotonou for the treatment of malaria, we postulate that it might possess anti-inflammatory and analgesic effects. Thus, this study evaluated the anti-inflammatory and analgesic activities of Paludose™ herbal mixture in experimental models.

MATERIALS AND METHODS

Materials and drugs: Methanol, carrageenan, acetic-acid, diclofenac, naloxone, glibenclamide, L-nitro-arginine (L-NNA), distilled water, normal saline, formalin, gallic acid, rutin, tramadol, Ellman's reagent (5,5'-Dithiobis (2-nitrobenzoic acid) (DNTB) (Alfa Aesar® England), Formaldehyde (BDH®, England), hydrochloric acid (HCl) (BDH®, England), potassium Chloride (KCl) (Sigma-Aldrich®, UK), thiobarbituric acid (TBA) (Sigma-Aldrich®, UK), trichloroacetic acid (TCA) (Sigma-Aldrich® UK), tris-hydroxylamine (Tris-base) (Sigma- Aldrich® UK), folin-ciocalteau's reagent, sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminum chloride hexahydrate ($AlC1_3.6H_2O$), sodium hydroxide (NaOH), dipotassium phosphate (K_2HPO_4), potassium diphosphate (KH_2PO_4), sulfonamide, N-(1-naphthyl) ethyenediamine (NNE), 85% v/v phosphoric acid (H_2PO_4; 97.994 g/mol), naloxone (5 mg/kg), glibenclamide (10 mg/kg), and L-nitro-arginine (10 mg/kg). The Paludose™ herbal mixture was purchased from COPHARBIOTEC general store, Cotonou, Benin.

Phytochemical Composition of Paludose™ Herbal Mixture

Estimation of total phenolic content (TPC): The total phenolic content (TPC) was determined by the spectrophotometric method described by Kim *et al*. (2003). 0.1 mL of Paludose™ herbal mixture was mixed with 0.1 mL of folin-ciocalteau's phenol reagent. After 5 minutes, 1 mL of 7% Na_2CO_3 solution was added to the mixture followed by the addition of 1.3 mL of deionized distilled water and mixed thoroughly. The mixture was kept in the dark for 90 minutes at 25 ºC, after which the absorbance was read at 750 nm. The TPC was determined from extrapolation of calibration curve which was made by preparing gallic acid solution. The estimation of the phenolic compound was carried out in triplicate. The TPC was expressed as milligrams of gallic acid equivalent (GAE) per 1 mL of Paludose™ herbal mixture. The equation of calibration curve was y = 0.0066x-0.0135, where R^2 = 0.9996.

Estimation of total flavonoid content (TFC): Total flavonoid content was determined following a method by Park *et al*. (2015). In a test, 1 mL of Paludose™ herbal mixture, 3.4 mL of 30% methanol, 0.15 mL of $NaNO_2$ (0.5 M) and 0.15 mL of $AlC1_3.6H_2O$ (0.3 M) were mixed. After 5 minutes, 1 mL of NaOH (1 M) was added. The solution was mixed well and absorbance was read at 506 nm. It was measured against the reagent blank rutin standard solution 0 to 100 mg/mL under the same procedure as earlier described. The total flavonoid content was expressed as milligrams of rutin equivalent per 1 mL of Paludose™ herbal mixture. The equation of standard curve was y = 0.0289x + 0.1722, where R^2 = 0.995.

Identification and quantification of chemical compounds in Paludose™ using High Performance Liquid Chromatography (HPLC) Techniques: The chromatographic analysis was done using Agilent Technologies® HPLC 1200 series, binary pump, micro-vacuum degasser, standard and preparative autosampler, thermostatic column compartment, diode array and multiple detector with ChemStation software (Stan *et al*., 2016). The column used was Agilent ® EclipseXDB-C18, 4.6 x 150 mm, 5 µm diameter particle size. The mobile phase was 0.1% formic acid and acetonitrile. The mobile phase was filtered through a 0.45 µm membrane filter, then de-aerated ultrasonically prior to use. The flow rate injection volume and wavelengths were 0.7.0 mL/minute and 280 nm respectively. The identification was done using the retention times obtained from the chromatograms of the standard compared with that obtained from the herbal extract. The quantification was determined from the regression equation from the standard used.

Ethical consideration: The standard guidelines and procedures for the care and use of laboratory animals in research and teaching prepared by National Institute of Health (NIH, 2011) were followed.

Experimental animals: Fifty (50) male Swiss mice (18-20 g) and 30 male Wistar rats (150-160 g) were purchased from the central animal house, University of Ibadan. The animals were acclimatized for 2 weeks and maintained under standard

laboratory conditions in the animal house of Department of Pharmacology and Therapeutics, University of Ibadan, Ibadan, Oyo State, where the experiments were conducted. Animals were placed in sufficiently sized polystyrene cages to allow for normal postural adjustments with freedom of movements. The animals had free access to standard pelletized animal feed (ACE®) and portable, clean tap water *ad libitum*.

Dose Determination of Paludose™ Herbal Mixture: The dose given to each experimental animal was extrapolated from the adult dose by correlating the volume given to an adult human (weight-70 kg) to the weight of the experimental animal. The dose of Paludose™ herbal mixture as stated is 2 teaspoon full twice daily. The extrapolated dose was 0.143 mL/kg (1X). The dose administered include 0.143 mL/kg (1X), 0.29 mL/kg (2X) and 0.57 mL/kg (4X) respectively.

Evaluation of the Anti-nociceptive activities of Paludose™ Herbal Mixture

Acetic acid-induced mouse writhing assay: The acetic acid-induced mouse writhing assay as described by Koster *et al.* (1959) and Igbe *et al.* (2012) was carried out to evaluate the possible peripheral anti-nociceptive effect of Paludose™. Twenty-five male Swiss mice were weighed and randomly assigned to five groups (n=5): Group 1 received normal saline (10 mL/kg p.o.), Group 2-4 received selected doses (0.143, 0.29 and 0.57 mL/kg, p.o.) of Paludose™ herbal mixture, and Group 5 received diclofenac (30 mg/kg p.o.). One-hour post-treatment, acetic-acid 0.06% v/v (10 mL/kg, i.p.) was administered and the number of cumulative writhing behaviour in 30 minutes was recorded. The percentage inhibition of writhing behaviour was calculated as follows;

$$\text{Percentage inhibition} = 100 - \left(\frac{test}{control}\right) \times 100$$

Hot Plate Analgesia Test: The test was carried out to investigate the central anti-nociceptive effect of Paludose™ herbal mixture in Swiss mice using Ugo Basile hot and cold plate (Woolf *et al.,* 1998; Igbe *et al.,* 2012). Twenty-five Swiss mice were weighed and randomly divided into 5 groups (n=5); Group 1 received normal saline (10 mL/kg, p.o.), Group 2-4 received selected doses (0.143, 0.29 and 0.57 mL/kg, p.o.) of Paludose™ herbal mixture, and Group 5 received tramadol (20 mg/kg p.o.). Pre-dosing base-line latencies were determined before the experiment was carried out. The reaction latency was recorded at 30, 60, 90, and 120 minutes post-treatment. A maximum of hot plate latency of 20 seconds was used to prevent tissue damage to the paws. Pre-dosing latencies were determined before the administration of drugs or vehicle. The maximum possible effect was evaluated using the formula below;

$$\text{Maximum Possible Effect} = \left(\frac{(post-drug\ reaction\ latency)-(pre-drug\ reation\ latency)}{(maximum\ latency)-(pre-drug\ latency)}\right) \times 100$$

Elucidation of possible mechanism of anti-nociception of Paludose™ in Swiss mice: To investigate the possible mechanism of Paludose™ herbal mixture-induced antinociception, mice (n=5) grouped were pre-treated with naloxone (5 mg/kg, s.c; non-selective opioid receptor antagonist) (Alchaider, 1991); glibenclamide (10 mg/kg, i.p; sensitive potassium channel blocker) (Ishola *et al*., 2014); L-Nitro arginine methyl ester, L- NAME (10 mg/kg, i.p; competitive inhibitor of nitric oxide synthase) or vehicle (normal saline). Fifteen minutes after pre-treatment, Paludose™ herbal mixture (1X, 0.143 mL/kg) or vehicle (10 mL/kg, p.o.) was administered. One-hour post-treatment, acetic-acid induced writhing assay was carried out. The cumulative number of writhing was recorded for each animal.

Evaluation of the Anti-inflammatory Activity of Paludose™ Herbal Mixture

Carrageenan-induced rats' paw edema: This test was carried out to evaluate the effect of Paludose™ herbal mixture on the acute inflammation induced by carrageenan (Winter *et al.,* 1962, Ishola *et al.,* 2011). Thirty male Wistar rats fasted overnight were randomly grouped into 6 (n=5). Group 1 and 6 received normal saline (10 mL/kg, p.o.), group 2, 3, and 4 received 1X, 2X and 4X of Paludose™ herbal mixture p.o. respectively while group 5 received diclofenac (30 mg/kg, p.o.). One hour after treatment, 100 µL of 1% carrageenan was injected into the right hind paw of animals in group 1-5 while group 6 was injected with 100 µL of normal saline. Changes in circumference of the hind paw was recorded for 1, 2, 3, 4, and 5 h post-carrageenan injection. The percentage inhibition of edema was expressed as follows;

$$\text{Percentage inhibition of oedema} = 100 - \left(\frac{(post-treatment\ circumference)-baseline\ (test)}{(post-treatment\ circumference)-baseline\ (control)}\right) \times 100$$

Biochemical assays

Sample preparation: Freshly excised inflamed paws of the carrageenan-induced rats were placed on ice-pack, minced and subsequently homogenized with a homogenizer in cold phosphate Buffer of pH 7.4. The homogenates were then centrifuged using a cold centrifuge at 10,000 rpm for 10 minutes at 4 ºC. The resulting supernatants were used for biochemical analysis using spectrophotometric techniques.

Biochemical evaluation of the effects of Paludose™ herbal mixture on rats' paw homogenate: The level of depletion of reduced glutathione, a vital antioxidant that prevents damages to important cellular components was determined using the method described by Anderson *et al.,* (1985). The amount of thiobarbituric acid reactive substances (product) (TBARS) in the rat paw homogenate from the reaction between the chromogenic reagent (2-thiobarbituric acid) and malondialdehyde (MDA), an end-product of lipid peroxidation was used as an index of lipid peroxidation as described by Vashney and Kale (1990). The nitric oxide level was estimated as nitrite using Griess reagent whose reaction relies on the simple colorimetric reaction between nitrite, sulfanilamide and N-(1-naphthyl) ethylenediamine (NNED) to produce a pink azo-product with maximum absorbance at 540 nm as previously described (Wang *et al.,* 2004). The concentration of pro-inflammatory cytokines; tumour necrosis factor-alpha (TNF-α) and interlukin-6 (IL-6) in the rats' paw homogenate were evaluated using enzyme-linked immunosorbent assay kits (ELISA MAX™ Deluxe Set) from Biolegend®, San Diego, USA, in accordance to the manufacturer's instruction.

Statistical Analysis: All values were expressed as mean ± S.E.M (standard error of mean) and analyzed using Graph Pad Prism version 5.00 (GraphPad Software Incorporated, La Jolla. USA). Comparison among the groups were done using

one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. P-values less than 0.05 ($p < 0.05$) were considered statistically significant

RESULTS

Phytochemical Composition of Paludose™ Herbal Mixture: The total phenolic content (TPC), total flavonoid content (TFC) and HPLC profiling of Paludose™ herbal mixture were determined. The total phenolic (TPC) and flavonoid contents (TFC) of the herbal mixture were found to be 2291.58 ± 0.01 mg/g of GEA and 131.36 ± 0.01 mg/g rutin equivalent respectively. In addition, the HPLC profiling of phenolic constituents found in Paludose™ herbal mixture revealed some phenolics as shown on the chromatogram (Figure 1). Out of the four compounds identified using appropriate standards, gallic acid is the most abundant (Table 1).

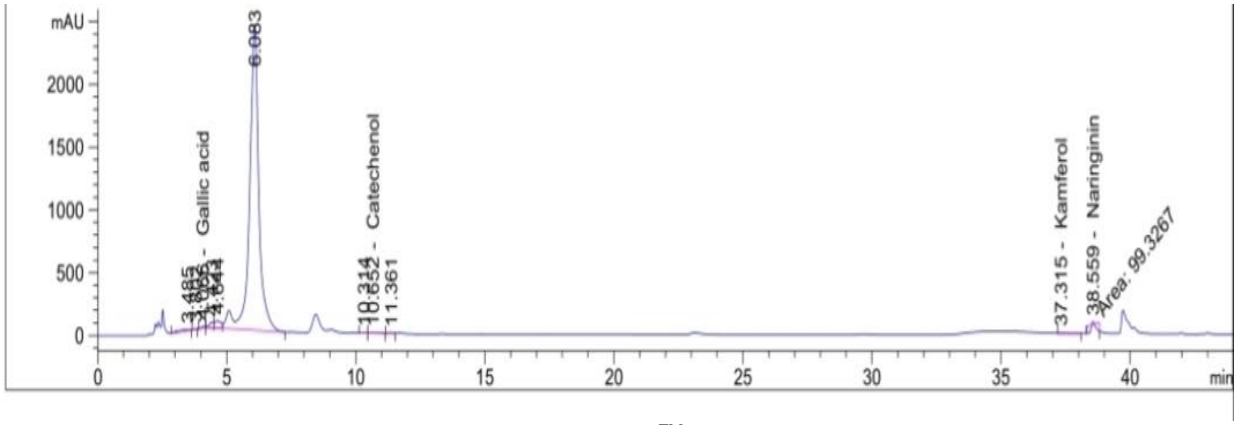

Figure 1: HPLC Chromatogram of bioactive components of Paludose™ herbal Mixture

Table 1:
Phenolic Constituents in Paludose™ Herbal Mixture as revealed by HPLC

S/N	Compound	Retention time (min)	Amount (mg/L)	Area
1	Gallic acid	4.07	5.87	77.34
2	Catechenol	10.65	1.08	99.23
3	Kaempferol	37.32	1.57	199.02
4	Naringenin	38.56	1.73	99.33

Table 2:
Effect of Paludose™ herbal mixture on the number of writhes induced by acetic acid in 30 minutes.

Treatment	Mean ± SEM	Percentage Inhibition (%)
PALUDOSE™ (1X)	8.75 ± 4.03*	83.58
PALUDOSE™ (2X)	15.00 ± 4.02*#	71.70
PALUDOSE™ (4X)	24.00 ± 2.48*#	54.72
Diclofenac (30 mg/kg)	4.05 ± 0.50*	92.36

N=5/group, *$p \leq 0.05$ vs negative control group, #$P \leq 0.05$ vs Diclofenac treated (positive control) group.

Anti-nociceptive Activity of Paludose™ Herbal Mixture

Effect of Paludose™ herbal mixture on acetic acid-induced mouse writhing assay: The anti-nociceptive activity of Paludose™ herbal mixture in acetic-acid induced mouse writhing assay using 0.6% acetic acid is presented on table 2. The intraperitoneal injection of 0.6% acetic acid in experimental mice produced mean abdominal writhes of 53.00 ± 5.69 in 30 minutes. However, pre-treatment with Paludose™ herbal mixture reduced the cumulative number of writhes, with peak effect obtained at 0.143 mL/kg (8.75 ± 4.03, 83.58% inhibition). Mice pretreated with diclofenac experienced a reduction in the mean number of writhes by 92.36% in comparison to vehicle-treated control group.

Effect of Paludose™ herbal mixture on nociceptive behaviour in hot plate analgesia test: Table 3 shows the time course reaction latencies of mice exposed to thermally induced nociception. The pre-treatment of mice with Paludose™ show various effect in the different concentrations. 1X showed the peak effect at 30 minutes' post-treatment (12.19 ± 2.00, 41.85% inhibition). Similarly, the standard drug, tramadol (20 mg/kg) increased the latency from 5.52 ± 0.77 to 15.44 ± 1.98 (68.51% maximum possible effect) at 30 minutes. The analgesic effects of the herbal mixture and tramadol gradually decreased from 30 minutes to 120 minutes. However, Paludose™ at 4X with low analgesic effect (15.20%) at 30 minutes had a better effect at 120 minutes (29.97%) than 1X Paludose™ (9.79%). In addition, tramadol had 31.35% analgesic effect at 120 minutes (Table 3).

Table 3.: Time course effect of Paludose™ herbal mixture on hot plate-induced nociception in mice

Treatment	Baseline	30 mins	60 mins	90 mins	120 mins
Vehicle	6.67 ± 0.056	6.66 ± 0.52	7.77 ± 0.87	6.4 ± 0.06	7.10 ± 0.07
1X	6.57 ± 0.97	12.19 ± 2.00* ***41.85 %***	9.39 ± 1.10 ***20.99 %***	8.89 ± 2.28# ***26.11 %***	7.28 ± 0.93 ***9.79 %***
2X	4.84 ± 0.50	10.12 ± 1.55# ***34.8 %***	9.12 ± 1.11 ***28.23 %***	8.32 ± 1.53 **22.95 %**	6.96 ± 2.34 ***13.98 %***
4X	5.92 ± 0.40	8.06 ± 1.28# ***15.20 %***	10.52 ± 2.33* ***32.67 %***	10.74 ± 1.25* **34.23 %**	10.74 ± 1.96 ***29.97 %***
Tramadol 20 mg/kg	5.52 ± 0.77	15.44 ± 1.98* ***68.51 %***	12.74 ± 0.63* ***49.86 %***	9.36 ± 1.13* ***26.52 %***	10.06 ± 0.77 ***31.35 %***

Values expressed as mean ± S.E.M (n=5). Percentage maximum possible effect in bold and italics. *P≤0.05 vs negative control, #P≤0.05 vs tramadol

Elucidation of possible mechanism of antinociceptive effect of Paludose™ herbal mixture: As shown in table 2, intraperitoneal injection of 0.6% v/v acetic-acid induced writhing reflex in vehicle-treated control group. However, pretreatment with Paludose™ herbal mixture at 0.143 mL/kg reduced the writhing behaviour in the experimental mice indicating anti-nociceptive activity. To investigate the involvement of opioidergic, ATP-sensitive potassium channel (KATP channel), and neuronal nitric synthase pathway in the antinociceptive effect of the Paludose™ herbal mixture, mice were pretreated with naloxone (5 mg/kg), glibenclamide (10 mg/kg), and L-nitro-arginine (10 mg/kg) respectively (Figure 2). The pretreatment of mice with naloxone, a non-selective opioid receptor antagonist at 5 mg/kg before oral administration of Paludose™ herbal mixture, significantly reduced the anti-nociceptive effect of Paludose™ herbal mixture in mice (Figure 2B). In contrast, L-nitro-arginine (10 mg/kg), a neuronal nitric synthase inhibitor, slightly reduced the anti-nociceptive effect of Paludose™ herbal mixture (Figure 2C), while glibenclamide (10 mg/kg), an ATP-sensitive k+ channel blocker, failed to antagonize the anti-nociceptive effect of the Paludose™ herbal mixture in mice (Figure 2A).

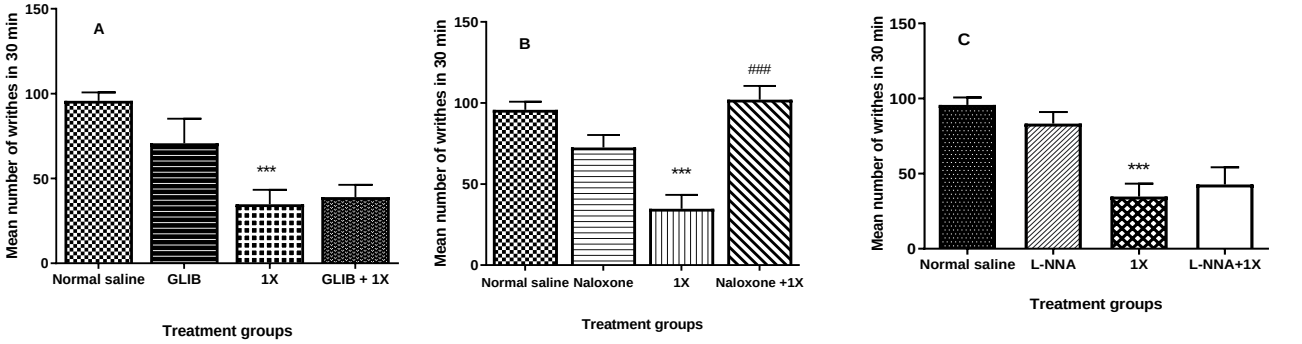

Figure 2.: Effect of **(A)** glibenclamide (GLIB), **(B)** naloxone, and **(C)** L-nitro-arginine (L-NNA) on the analgesic activity of Paludose™ herbal mixture in acetic acid-induced writhing assay in mice.
Values are expressed as mean ± S.E.M (n=5/group). ***P <0.05 versus negative control (normal saline). ###P ≤ 0.05 vs naloxone-treated group.

Anti-inflammatory Activity of Paludose™ Herbal Mixture
Effect of Paludose™ herbal mixture on carrageenan-induced paw oedema: Intra-plantar injection of carrageenan into the right hind paw of the rats induced a time course increase in paw circumference. Treatment with 0.143 mL/kg of Paludose™ herbal mixture (1X) produced a time course inhibition of oedema with peak effect (68.46 ± 8.46%) at 3 h post-carrageenan injection and produced effect similar to diclofenac at the 4th hour (p≤ 0.05; figure 3A) while treatment with 2X and 4X produced lesser time course inhibition of oedema with peak effects (53.50 ± 5.33, 64.45 ± 12.53%) at 3 hours and 4 hours post-carrageenan injection respectively (Figure 3B & 3C). Diclofenac reduced paw edema with its peak at the 5th hour.

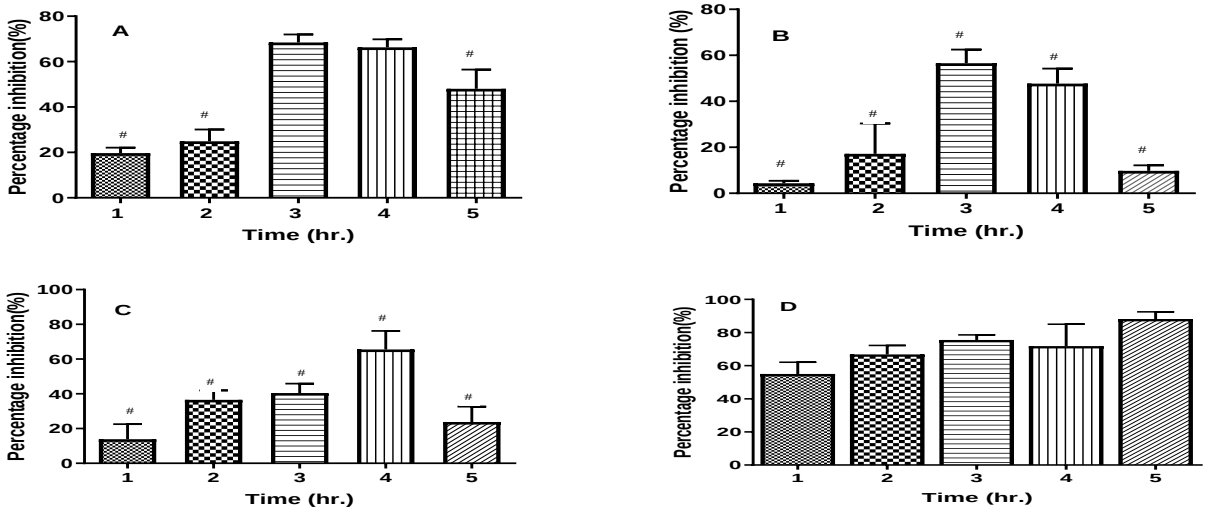

Figure 3: Percentage inhibition of carrageenan-induced paw edema in rats treated with **(A)** 1X, **(B)** 2X, **(C)** 4X, and **(D)** diclofenac
Values are expressed as mean ± S.E.M (n=6/group). #P ≤ 0.05 vs positive control, diclofenac (30 mg/kg).
1X; 0.143 mL/kg, 2X; 0.286 mL/kg, and 4X; 0.572 mL/kg of Paludose™ herbal mixture

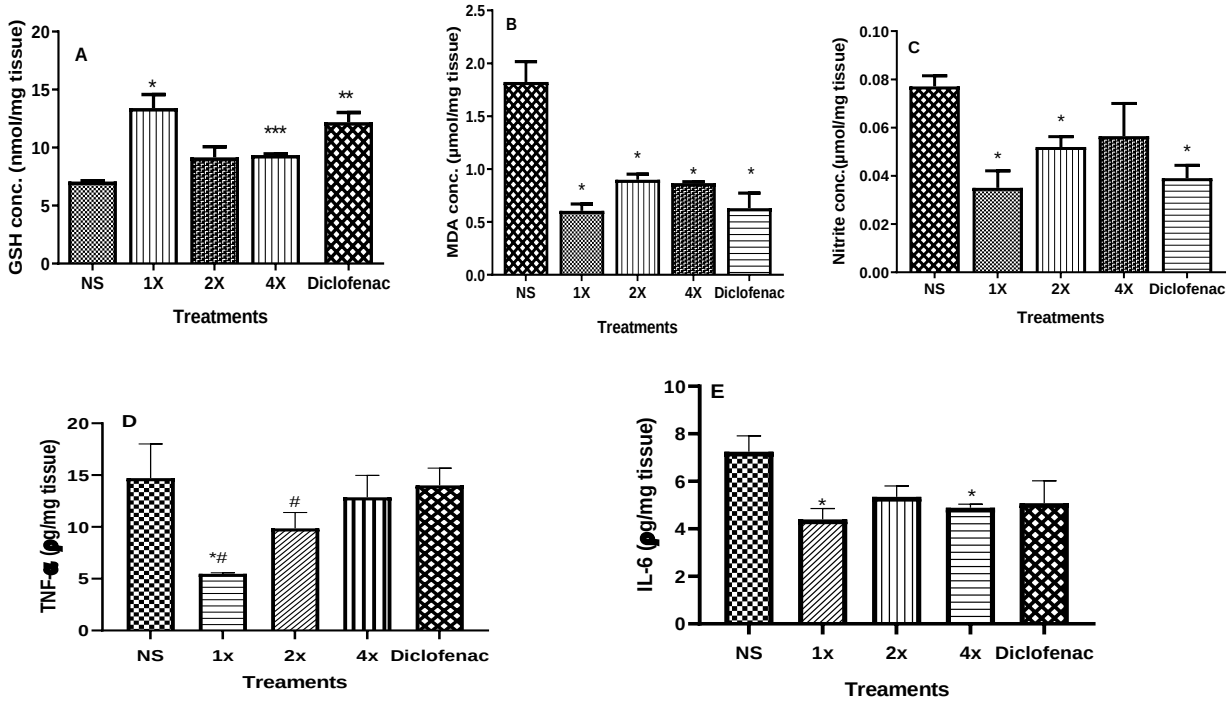

Figure 4.: Effect of Paludose™ herbal mixture on biochemical parameters in rats' paw edema induced by carrageenan. **(A)** GSH (reduced glutathione), **(B)** MDA (malondialdehyde), **(C)** Nitrite, **(D)** TNF-α (tumour necrosis factor alpha), and **(E)** IL-6 (interleukin-6) levels.

Values are expressed as mean ± SEM, (n=6/group). *P≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.0001 vs negative control group, #P ≤0.05 vs diclofenac-treated group.

Effects of Paludose™ herbal mixture on biochemical parameters in carrageenan-induced rats' paw edema

Effect of Paludose™ herbal mixture on reduced glutathione level: The effect of Paludose™ herbal mixture on reduced glutathione (GSH) level in carrageenan-induced rats' paw homogenate is presented in figure 4A. Carrageenan injection significantly decreased GSH level in the paw of rats administered with normal saline (7.08 ±0.07 nmol/mg tissue) when compared to diclofenac-treated group (12.20 ± 0.83 nmol/mg tissue). However, treatment with Paludose™ herbal mixture at 0.143 mL/kg (1X) and 0.572 mL/kg (4X) doses significantly increased GSH levels (13.41 ± 1.17; 9.35 ± 0.10 nmol/mg tissue) in comparison with the normal saline-treated group (7.08 ± 0.07 nmol/mg tissue).

Effect of Paludose™ herbal mixture on lipid peroxidation: Figure 4B presents the effect of Paludose™ herbal mixture on lipid peroxidation (MDA levels) in experimental animals.

There was a significant increase in the level of MDA in animals treated with normal saline (1.82 ± 0.36 µmol/mg tissue) following carrageenan-induced paw edema when compared with diclofenac-treated group (0.63 ± 0.08 µmol/mg tissue). However, animals treated with selected concentration of Paludose™ herbal mixture (1X, 2X, and 4X) decreased MDA level significantly when compared to normal saline-treated group. Interestingly, 0.143 mL/kg Paludose™ (1X) showed similar MDA level (0.61 ± 0.01 µmol/mg tissue) when compared with group treated with diclofenac.

Effect of Paludose™ herbal mixture on nitrite level: As presented in Figure 4C, carrageenan significantly increased the level of nitrite in the paw of rats administered with normal saline (0.08 ± 0.005 µmol) compared to paw of the rats treated with diclofenac (0.04 ± 0.005 µmol). Likewise, 0.143 mL/kg (1X) and 0.286 mL/kg (2X) of Paludose™ herbal mixture significantly reduced the level of nitrite (0.04 ± 0.00 and 0.05 ± 0.00 µmol). However, treatment with 0.572 mL/kg (4X) showed no significant difference (0.06 ± 0.01 µmol) when compared with the normal saline-treated group.

Effect of Paludose™ herbal mixture on pro-inflammatory cytokines (TNF-α and IL-6) level: The effect of Paludose™ herbal mixture on the level of proinflammatory mediators in rats' paw of carrageenan-induced animals are presented in figure 4D and figure 4E. Treatment with 0.143 mL/kg of Paludose™ herbal mixture significantly reduced the level of TNF-α when compared with the control group (normal saline) (Figure 4D). Similarly, there was a significant reduction in IL-6 levels in groups that received 0.143 (1X) and 0.286 mL/kg (2X) doses of Paludose™ herbal mixture in comparison with the normal saline-treated group (figure 4E)

DISCUSSION

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for the treatment of pain and inflammation (Doomra and Goyal, 2020). However, prolonged use of these agents leads to gastric ulcers, upper gastrointestinal bleeding, and renal disorders among others (Gunaydin and Bilge, 2018). Thus, there is a need for the discovery of new anti-inflammatory and analgesic drugs with minimal deleterious effects and cost effectiveness. Hence, the study investigated the phytochemical constituents of Paludose™ herbal mixture as well as its analgesic and anti-inflammatory activities in rodents.

The phytochemical investigation of the Paludose™ herbal mixture revealed significant level of phenolics and flavonoids. However, the HPLC analysis provided more precise information in identification and quantification of the bioactive constituents present in the Paludose™ herbal mixture based on the chemical nature. Among the polyphenols; gallic acid, coumaric acid, apigenin, and naringenin were found to be present while flavonoids present include; rutin, quercetin, and kaempferol. Reports from several studies have indicated that these phytochemicals constitute a major group of bioactive compounds that are known to possess anti-inflammatory, analgesic, antioxidant, anti-diabetic, anti-cancer amongst other biological properties of medicinal plants (Vendramini-Costa *et al*., 2012; Tatipamula and Kukavica, 2021).

Finding from this study showed that Paludose™ herbal mixture possesses anti-nociceptive activity in chemical and/or thermal-induced pain possible through opioidergic pathway, as well as the anti-inflammatory effect on carrageenan-induced acute inflammation. Consequently, it is vital to employ different test which differs in stimulus quality, intensity and duration to assess the antinociceptive effect of a new agent (Ishola *et al*., 2011). The peripheral anti-nociceptive effects of Paludose™ herbal mixture were assessed using the acetic acid-induced nociceptive assay. Contraction of the abdominal muscles and the extension of the forearm occur as a result of the injection of acetic acid. This response described is believed to be due to the liberation of endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinins, and substance-P, which stimulate nerve endings (Oliveira-Fusaro *et al*., 2012). The abdominal constriction response is also thought to be mediated by local peritoneal mast cells, acid-sensing ion channels, release of lipoxygenase products, and via the activation of mitogen- activated protein kinase (MAPK) and microglia in the spinal cord which modulates central pain through several complex processes (Mishra *et al*., 2011; Zhang *et al*., 2011). The study revealed that Paludose™ herbal mixture significantly reduced the number of writhing at all doses when compared with the negative control. The lowest dose (0.143 mL/kg) appears to be the most effective with comparable activity to that of the positive control, diclofenac (30 mg/kg). This result may suggest the role of Paludose™ herbal mixture in the inhibition of cyclooxygenase or lipoxygenase pathway which is the general pathway of common peripheral-acting analgesic agents (Shulan *et al*., 2011), but further work needs to be done on this.

The central anti-nociceptive effects of Paludose™ herbal mixture were assessed using the hot plate assay. The hot plate assay makes use of the latency measurement to assess acute cutaneous pain sensitivity. Oftentimes, an increase in reaction latency time of experimental animals exposed to thermally induced pain by the hot plate is used to assess substances or chemical compounds for possible central analgesic activity (Heidari *et al*., 2009). The animal is exposed to thermal pain on a larger surface area making it a sensitive test, consequently elevating the reaction latency time of experimental animals. From the study, treatment with Paludose™ herbal mixture produced a significant increase in the latency time as early as 30 minutes in animals treated with the lowest dose (0.143 mL/kg). The beneficial effect was comparable to tramadol (20 mg/kg).

To investigate the possible mechanism of antinociception of Paludose™ herbal mixture, acetic acid-induced writhing test was chosen. The result from this study provides evidence suggesting the involvement of opioidergic system in the antinociceptive activity of the herbal mixture as pre-treatment with naloxone, a competitive antagonist of opioid receptors, significantly reversed the antinociceptive effect of Paludose™ herbal mixture. The opioid receptors involved in pain modulation are situated in both the central nervous system and the peripheral nervous system. These receptors also bind to endogenous opioid peptides, which are involved in pain modulation and numerous functions in the body (Kuan and Shyu, 2016; Liu *et al*., 2020). Evidences from this study also showed failure in reversal of the anti-nociceptive effect of Paludose™ herbal mixture in animals pretreated with L- nitro-arginine and glibenclamide, hence the anti-nociceptive effect

is not related to nitregenic and K-ATP sensitive system pathways.

Paludose™ herbal mixture (0.143 mL/kg) administered orally, one hour prior to carrageenan injection caused an inhibition of rats' paw edema in the 3rd, 4th, and 5th hour which is comparable to the inhibition produced by standard anti-inflammatory agent, diclofenac (30 mg/kg). Carrageenan is a mucopolysaccharide derived from the Irish sea moss Chrondus, and also a potent phlogistic agent with neither antigenic nor apparent systemic effects (Chakraborty *et al*., 2004). It is used to induce acute inflammation in addition to being a suitable model for screening of non-steroidal anti-inflammatory drugs (Duarte *et al*., 2016). Carrageenan–induced paw edema is believed to be biphasic; the first phase occurs between 1-2 hours following carrageenan injection and is attributed to the release of mast cell autacoids like histamine, serotonin and bradykinin while the second phase occurs between 3-6 hours after carrageenan injection as a result of the release of prostaglandins, arachidonic acid byproducts, and the continuity between phases 1 and 2 which is due to the release of kinins (Vazquez *et al*., 2015). In addition, migration of neutrophils, release of oxygen free radicals and proteolytic enzymes are believed to contribute to the development of the second phase (Barth *et al*., 2016). Paludose™ herbal mixture at the dose of 0.143 mL/kg showed significant inhibition at the 3rd and 4th hour, where it may have acted by inhibiting prostaglandin, a by-product of arachidonic acid metabolism. Moreso, following carrageen-induced paw edema, hyperalgesia and redness occurred due to the action of pro-inflammatory agents released into the tissue. Mediators such as histamine, prostaglandins, bradykinin, tachykinin, cytokines and substance P are involved in this process (Merht, 2019). In this study, carrageenan in rats resulted in redness, swelling, and painful edematous paw tissue with a significant increase in edema volume till it reached the maximum after 4 hours post-injection as previously obtained by Abd-Allah *et al*., (2018). The percentage inhibition is a good index for assessing the anti-inflammatory activity. The results revealed that Paludose™ herbal mixture significantly inhibited the second phase of carrageenan-induced paw edema.

The level of reduced glutathione and MDA level (a measure of lipid peroxidation) were used to assess the oxidative status and the effect of Paludose™ herbal mixture in carrageenan-induced acute inflammation in the paw of experimental animals. The level of MDA increased following carrageenan injection as a result of released neutrophilic ROS/RNS, while the level of GSH, in response to carrageenan injection decreased as commonly reported in inflammatory conditions. A significant increase in MDA level and a decrease in the level of GSH in animals that received normal saline in this study are similar to that previously reported (Abbas *et al*., 2014). Interestingly, animals that received Paludose™ herbal mixture showed marked suppression of MDA level at doses of 0.143 mL/kg and 0.572 mL/kg, thus preventing the reduction of GSH and consequently improving the antioxidant status of the experimental animals. Glutathione, a non-enzymatic reducing molecule that traps free radical yielding thiol radicals and prevents oxidative damage is depleted in inflammatory condition (Smeyne and Smeyne, 2013). In this study, pretreatment of Paludose™ herbal mixture prevented this depletion which was significantly decreased in animals treated with normal saline.

Inflammatory responses stimulate inducible nitric oxide synthase (iNOS) which is responsible for the overproduction of nitric oxide (NO) at the site of inflammation (Ziskoven *et al*., 2011). Nitrite level was elevated in the paw of rats that were induced with carrageenan in the study. However, Paludose™ herbal mixture prevented the increased nitrite level. Nitric oxide combined with superoxide anion peroxynitrite are oxidizing agents capable of promoting lipid peroxidation while MDA causes oxidative deterioration of polyunsaturated lipids consequently producing radical products that induce cellular damage (Saeidnia *et al*., 2013).

Inflammation is mediated by cytokines such as TNF-α and IL-6 (Li and Jamdade, 2021). Currently, the choice of an anti-inflammatory agent such as NSAIDs are inhibitors of these cytokines. Tumor necrosis factor-alpha is a major mediator in inflammatory responses that activates T cells and macrophages thereby stimulating the secretion of other inflammatory cytokines (Wang *et al*., 2020). In a study conducted by Huang *et al*., TNF-α was shown to be one of the pro-inflammatory mediators in carrageenan–induced inflammatory reaction and induced a further release of kinins and leukotrienes, which may have contributed to the maintenance of a long-lasting nociceptive effect (Huang *et al*., 2011). The level of TNF-α and IL-6 significantly reduced in the paw of experimental animals treated with 0.143 mL/kg of Paludose™ herbal mixture when compared to those treated with normal saline. TNF-α and IL-6 are vital mediators known to cause damages in tissues when they are overexpressed in inflammatory conditions (Wang and He, 2018).

Paludose™ herbal mixture significantly ameliorated the deleterious effect of carrageenan in the rats' paw, a measure of its anti-inflammatory effects. Improvement in the antioxidant status was also observed as well as the reduction in mediators of inflammatory and nitrergic pathways. Furthermore, the anti-inflammatory and anti-nociceptive activities of Paludose™ herbal mixture as observed in this study could be linked to its richness in phenolics and flavonoids. Flavonoids have been known in previous studies to be potent inhibitors of pro-inflammatory cytokines and cyclooxygenase (Vidyalakshmi *et al*., 2010; Ribeiro *et al*., 2015). These compounds act by several mechanisms including inhibition of transcription of the nuclear factor (NF-κB) by inhibiting kinases involved in signal transduction (Gonzalez-Gollago *et al*., 2014). These bioactive compounds can inhibit and release arachidonic acid from membranes by inhibiting lipoxygenase, cyclooxygenase, and phospholipase A2 (Chen *et al*., 2018).

In conclusion, **t**his study demonstrated that Paludose™ herbal mixture possesses peripheral and centrally acting analgesic effect. There is possible involvement of the opioidergic pathway in its analgesic effect. It appeared to attenuate inflammation in carrageenan-induced paw edema by inhibiting cytokines and nitrite. Oxidative stress was inhibited as antioxidants status was boosted in the experimental animal. This study reveals that Paludose™ herbal mixture is also able to reduce paw edema induced by carrageenan and its peripheral mechanism of pain, suggesting their action may be related to the inhibition of the synthesis and release of the various inflammatory mediator. The activities observed in this study might be due to the presence of flavonoids including rutin, quercetin, and kaempferol, identified in the herbal product.

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