Trop J Nat Prod Res, October 2023; 7(10):5005-5013 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Anti-inflammatory, Analgesic and GCMS Analysis of Ethanol Extract of a Polyherbal Phytomedicine Used in Nigerian Ethnomedicine

Alfred F. Attah[1], Rashidat O. Ayanniyi[2], Fatimoh I. Ojuade[2], Franklyn C. Opara[2], Hammed Faleke[3], Kolawole J. Adedoyin[4], Samuel O. Adeyeye[4]

[1]*Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, University of Ilorin, Ilorin, Nigeria.*
[2]*Department of Pharmacology and Therapeutics, University of Ilorin, Ilorin, Nigeria*
[3]*Department of Biochemistry, University of Ibadan, Ibadan, Nigeria*
[4]*Tropical Design Nutritional Products, Georgia, USA*

ARTICLE INFO

Article history:
Received 01 May 2023
Revised 14 June 2023
Accepted 14 October 2023
Published online 01 November 2023

ABSTRACT

While access to orthodox medicine is limited in rural areas of most developing countries, access to traditional herbal remedies are easy and cheap. However, several of these remedies lack scientific evidence to support their use. Herein, we investigated the anti-inflammatory, analgesic, phytochemical and GCMS analysis of the aqueous ethanol extract of a polyherbal preparation containing *Zingiber officinale*, *Citrus limon*, *Aloe vera*, *Allium sativum* and honey used in South-west Nigeria for the management of pain and inflammatory related conditions. The extract was subjected to acute (OECD) and sub-acute toxicity studies, in-vivo anti-inflammatory (Carrageenan-induced model) and analgesic studies (acetic acid-induced). The extract was further analyzed phytochemically by Gas Chromatography-Mass spectrometry for characterization of its metabolites. This study showed that polyherbal extract is not toxic in vivo, at both acute and sub-acute levels, and exhibits analgesic and anti-inflammatory effects at various doses of 200 mg/kg and 400 mg/kg. The GC-MS revealed the presence of important constituents including gingerol, limonene, vitamin E, retinoic acid, palmitic acid, and beta-sitosterol. Findings from this work indicated that the investigated polyherbal extract is safe, produced anti-inflammatory and analgesic activities which may be due to the presence of identified anti-inflammatory compounds. Our findings provide some evidence supporting the use of this anti-inflammatory polyherbal preparation in Nigerian South-west ethnomedicine. Further extensive in-vitro, in-vivo and mechanistic studies are warranted to fully unveil the therapeutic potential of the extract, its lead bioactive compounds, and their possible development into effective anti-inflammatory products for future clinical use.

Keywords: Ethnopharmacology, *Zingiber officinale*, Citrus limon, *Aloe vera*, *Allium sativum*, honey

Introduction

Inflammatory responses are series of stimuli that leads to the recruitment of molecules including cytokines, chemokines, growth factors, proteases, oxidative stress products, and lipid mediators after system assault by a viral infection, bacterial infection or physical injuries.[1,2] However, players of inflammation differ depending on the duration and nature of systemic abuse. In an acute inflammatory response, the time range of development is usually within minutes or hours and involves the migration of leukocytes, particularly neutrophils, and exudate of fluids and plasma protein towards the affected area.[3] Chronic inflammation takes a longer time to develop and it is characterized by the recruitment of lymphocytes and macrophages in the histology, leading to fibrosis and tissue necrosis.[4]

*Corresponding author. E mail: attah.fau@unilorin.edu.ng
Tel: 23408030915635

Citation: Attah AF, Ayanniyi RO, Ojuade FI, Opara FC, Kolawole J. Adedoyin KJ, Adeyeye SO. Anti-inflammatory, Analgesic and GCMS Analysis of Ethanol Extract of a Polyherbal phytomedicine used in Nigerian Ethnomedicine. Trop J Nat Prod Res. 2023; 7(10):5005-5013. http://www.doi.org/10.26538/tjnpr/v7i10.41.

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The use of non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, and corticosteroids had been the treatment approach for inflammation via the inhibition of cyclooxygenase 2 (COX2). However, long-term usage threatens the human organs such as the liver, kidneys and the gastrointestinal tract due to their side effects.[3] Hence, the need for an alternative with lesser or no adverse effect while proffering a comparable therapeutic effect for the treatment of inflammation cannot be overemphasized. Traditional remedies have recently gained increased acceptance and application in Africa as inflammatory-related diseases, epidemics, and pandemics appear to be on the increase.[4]

Medicinal plants have proved to be an excellent alternative to conventional medicines due to their richness in diverse types of phytochemicals and micronutrients such as alkaloids, flavonoids, terpenes, tannins, saponins, zinc, iron etc. These phytochemicals are responsible for the therapeutic activities of medicinal plants such as antioxidant, antimicrobial, antidiabetic, anticancer, neuroprotective effects.[3,4]

Ginger - *Zingiber officinale* Roscoe (family Zingiberaceae) is one of the plants prominently used for several disease management. It is abundant in different bioactive compounds and minerals. Its fresh form contains mainly gingerols (6-gingerol, 8-gingerol, and 10-gingerol), which are converted to shogaols after heating,[5] and finally to paradols upon hydrogenation. In addition to the gingerols and its derivatives, there are also several other phenols in ginger such as zingerone, gingerenone-A, quercetin and 6-dehydrogingerdione terpenes, polysaccharides, lipids, organic acids, and raw fibers. Antioxidant, antiviral, anti-inflammatory, anti-tumor, neuroprotective, and

antifungal are among the medicinal properties of the *Z. officinale* due to the accumulation of many of these nature-derived bioactive compounds.[5]

The lemon, or *Citrus limon* (L.) Burm., is a member of the Rutaceae family and is indigenous to Asia. Lemon fruit is an important component of a healthy diet, a rich source of nutrients, and offers health advantages. Lemons are an intriguing source of dietary fibre and health-promoting bioactive compounds including carotenoids, essential oils, vitamins, minerals, and anti-inflammatory flavonoids. Macroscopically, Lemon, is a yellow or pale-yellow fruit with up to ten seeds that is used in many different food cultures around the world. The fruit's juice, which is praised for its tart, acidic, and fresh flavor, is frequently used as an ingredient in both homemade and commercial cuisines. Lemon is rich in flavonoids, especially flavanones and flavone glycosides. Flavonols, flavone aglycones, and polymethoxyflavones have also been reported in lower concentrations than those of flavanones and flavones.[6]

Garlic - *Allium sativum* L. (family Amaryllidaceae) is the second most utilized species of the genus Allium after onions (*Allium cepa* L.). Garlic is an organosulphur-containing herbaceous annual spice with an aromatic pungent flavor. Its main bioactive compound is allicin [S-(2-propenyl)-2-propene-1-sulfinothioate], which is responsible for the characteristic smell and taste.[6] In addition to allicin, other sulphur and cysteine-containing compounds (alliin, allyl mercaptan) also contribute to garlic's aromatic features. Bioactive compounds present in garlic have been reported to be responsible for its different medicinal properties such as anti-inflammatory, antibacterial, antidiabetic, antioxidant, antifungal, cardioprotective, anticancer, and antihypertensive.[7]

***Aloe vera** (L.) Burm.f.* (family Asphodelaceae) is an herbaceous, perennial succulent xerophyte which is a water-storing vasculature due to its native habitat in the desert. *Aloe vera* plant is one of the most utilized plants globally as its medicinal uses can be dated as far back as 1500 BC across ancient countries like China, Greece, Egypt, and Mexico.[8] It is morphologically featured with a water-storing mucilaginous tissue which is reported to contain about 75 compounds including vitamins, minerals, enzymes, sugars, anthraquinones or phenolic compounds, lignin, saponins, sterols, amino acids, and salicylic acid. Several reports had described the anti-inflammatory efficiency of *Aloe vera* particularly in wound healing process.[9]

Honey, produced by the Apis spp (Family Apidae) contains macro and micronutrients that are influenced by a number of parameters, including bee type, floral supply, and ambient and processing conditions. Sugar, protein, enzymes, minerals, vitamins, amino acids, and a wide spectrum of polyphenols are among the approximately 200 components found in honey. Each honey has a varied color, flavor, viscosity, and medicinal activity due to the various ratio of these chemicals. As a result, the combination of all of these chemicals works synergistically in a variety of applications making honey one of the most powerful natural wound healing agents.[10] Traditional uses and clinical applications of honey include antimicrobial, antioxidant, anti-inflammatory, anticancer, antihyperlipidemic, and cardioprotective properties, as well as the treatment of eye, gastrointestinal tract, neurological fertility disorders and wound healing activity.[11] Traditional medications are frequently made as polyherbal formulations and have often been observed to have improved therapeutic effects and reduced toxicity. This is simply because of the reduced dose of each component. Each of these plants have been reported to have some beneficial effect but until this time of reporting, no scientific report is available on the beneficial effect of this indigenous polyherbal formulation under investigation. Our study is therefore focused on establishing the general safety and possible beneficial effect of this polyherbal phytomedicine in experimental animals.

Materials and Methodology

Plant collection and Extraction

These commonly sold plant parts used during this experiment were purchased in August, 2022 from Bodija market (7° 26' 14" north and 3° 54' 57" east), Ibadan, Oyo state, Nigeria and identified by Dr F. A Attah and Mr Bolu of the Herbarium unit, Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, University of Ilorin, Nigeria using the Flora of West Tropical Africa as main reference and by comparing with herbarium specimens. The following collection numbers were respectively assigned to *Zingiber officinale, Citrus limon, Aloe vera*, and *Allium sativum* – FPS-PDDHI: 028, 029, 030 and 031. Samples were washed and dried before size reduction. Powdered plant samples (500 g) from each plant source were macerated and extracted separately and independently using 5 L of 70% ethanol in distilled water for 72 h. The content was intermittently shaken for thorough extraction, the menstruum containing the extracted metabolites was decanted and filtered. These were further concentrated under reduced pressure using rotary evaporator and the concentrated sample was lyophilized and kept in the refrigerator at (-4ºC) and used when needed.

Experimental Animals

Wistar rats (150–160 g) both male female were obtained from the Department of Biochemistry, University of Ilorin, the animals were housed and maintained in standard plastic cages in the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences with light and darkness (12/12 h) and free access to food and water ad libitum.

Experimental procedure

The experiment was performed in accordance with the procedures laid down by the University of Ilorin Ethics Committee for care and use of laboratory animals and in accordance with the principles of laboratory animal care by the National Institute of Health (NIH publication No. 85-23, which was revised in 1985). The rats were acclimatized for one week before the start of the experiment and Ethical clearance was gotten from the Ethical Review Committee of the University of Ilorin, Ilorin, Nigeria. The experiment was given an approval no: UERC/ASN/2022/2353

Acute Oral Toxicity

The acute oral toxicity study was performed as per the guidelines of Organization for Economic Cooperation and Development as described by Obohon and colleagues.[12] Healthy female albino rats were used for this study. The rats were divided into 2 groups, with 3 animals in each group. From 12 hours before until 3 hours after the oral administration, the animals were kept without access to food. The control group received normal saline at 1 mL/kg by oral gavage while the treatment group received 2,000 mg/kg of the extract. The safety of the 2,000 mg/kg dose was subsequently confirmed in another 3 animals as recommended in the OECD guidelines. Immediately after administration, all of the animals were observed for signs of toxicity and mortality, with special attention given during the first 6 to 24 hours.

Sub-acute toxicity

Four experimental groups of six female wistar rats each were established as control group (treated orally with normal saline I mL/kg) and different doses of the polyherbal extract (125 mg/kg, 250 mg/kg, and 500 mg/kg) for 28 days. During the duration of the treatment, signs of toxicity, including body weight changes were recorded on days 0, 7, 14, 21, and 28 of the experiment. After the completion of the study, all animals were fasted overnight before blood sampling and the rats were euthanized in an airtight glass chamber saturated with diethyl ether.

Hematological assays

Blood samples were collected by cardiac puncture into ethylene diamine tetra acetic acid (EDTA) bottles. Hematological examination was performed as previously described by Ebohon *et al*., [13]using an automated hematological analyzer (Beckman Coulter JT series Hematological Analyser). The hematological parameters of interest that were estimated include; white blood cell (WBC) count, Red blood cells (RBC) count, hemoglobin (HGB), mean corpuscular hemoglobin concentration, mean corpuscular volume, monocyte, neutrophil, lymphocyte and platelet count.

Histopathological Examination of the Organ.

Liver and kidney removed and fixed in 10% neutral buffered formalin and then dehydrated by successively passing through a gradient of

mixtures of ethyl alcohol and water. The samples were rinsed with xylene and embedded in paraffin. Organs sections (5 µm thickness) were cut, stained (hematoxylin and eosin dye) and examined under light microscope.

Relative Organ Weight.
The relative organ weight (ROW) of each animal was calculated as follows:
Relative organ weight (%) = $Organ\ weight/Body\ weight \times 100$

Serum Biochemistry.
Biochemical (albumin, alkaline phosphatase, alanine aminotransferase, aspartate transaminase, bilirubin, cholesterol, triglyceride creatinine and urea) analysis was facilitated by assay kits used for liver and kidney function tests which were products of Randox Laboratories (Antrum, UK).[14] AST and ALT were measured at 405 nm while that of ALP was taken at 405 nm. Absorbance of urea was determined at 560 nm and creatinine at 492 nm. Reading of the absorbance was done with nano-UV/vis spectrophotometer (Optima, USA)

Evaluation of Anti-Inflammatory Activity.
Carrageenan-Induced Hind Paw Edema
Female albino rats were divided into four groups of 6 animals. Group A (control) received 0.2 mL of normal saline; Group B and Group C will receive aqueous doses of 200 mg/kg and 400 mg/kg of extract respectively. Group D (positive control) animals were administered with diclofenac sodium (10 mg/Kg body weight.). Inflammation was induced by subplantar administration of freshly prepared 0.9% salt solution of carrageenan (100µL of 1% w/v) into the hind paws of the animal. Paw thickness were measured just before the carrageenan administration and at 30 minutes interval for 3 hours after carrageenan injection.[14,15] The percentage inhibition of edema in extract-treated group was calculated using the formula below.

$$\text{Percent Inhibition} = \frac{\text{Difference in paw size (control)} - \text{Difference in paw size (agent)}}{\text{Difference in paw size(control)}} \times 100$$

In-vivo analgesic activity
Acetic Acid Writhing Method
The writhing test was carried out as described by Ajayi et al.,[14] with some modification (Spindola et al. 2010). Female albino mice were divided into 5 groups of 6 mice each. The animals were treated orally with diclofenac (20 mg/kg), distilled water (10 mL/kg), and 200 mg/kg and 400 mg/kg of extract. Writhing was induced with intraperitoneal injection of 0.6% w/v acetic acid solution (20 mL/kg) 30 minutes after pre-treatment. Five minutes after injection of the acetic acid solution, the writhes (abdominal constrictions and hind limbs stretching) were counted for 10 minutes. The percentage of analgesic activity (writhes reduction) was calculated as follows:

$$\frac{\text{number of writhes (control)} - \text{Number of writhes (agent)}}{\text{Number of writhes (control)}}$$

Mechanistic study
The Mice were randomly selected in eight groups (n= 8). The first phase involved the pre-treating mice the selected antogonist: Groups I and II received Normal saline (0.2 mL) and extract (400 mg/kg) respectively. Group III- naloxone (2 mg/kg), Group IV- prazosin (1 mg/kg), Group V-yohimbine (1 mg/kg), Group VI-propranolol (20 mg/kg), Group-VII metergoline (2 mg/kg) and Group VIII- l-arginine (50 mg/kg).
Thirty (30) minutes after the pre-treatment with antagonists, 400 mg/kg of the extract was administered to all the animals in groups III-VIII. 0.6% v/v of Acetic acid (10ml/kg) was administered intraperitoneally to induce pain. Sixty (60) minutes after the above treatments, the numbers of writhes were counted, and percentage inhibition calculated and compared to that of the control group.

$$\text{Percent Inhibition} = \frac{\text{Number of writhes (control) - Number}}{\text{Number of writhes (control)}}$$

GC-MS analysis
The major phytochemical components of the polyherbal phytomedicine were profiled using the gas chromatography-mass spectrometry (GC-MS, model-GCMS-QP2010SE Shimadzu, Japan) technique carried out at Shimadzu Training Centre for Analytical Instruments (STC) Lagos, Nigeria. The Restek Rtx-5MS fused silica capillary column (5%-diphenyl-95%-dimethylpolysiloxane) of 30 m×0.25 mm internal diameter (di) and 0.25 mm in film thickness was used to separate the metabolites. The mass spectrometer was operated in electron ionization mode of 70 eV and an acquisition mass ranged of 45-700 amu. To identify separated metabolites, mass spectra were matched with libray using the NIST computer data bank, as well as by comparing the fragmentation pattern with those reported in the literature.[16]

Statistical analysis
Data were collected from the different methods used above and analyzed using GraphPad Prism version 9.05 for Windows (GraphPadSoftware, San Diego, CA, USA). The results were expressed as mean ± SEM and a comparison of mean values between different groups was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests.

Results and Discussion

Acute toxicity and sub- acute toxicity
In the first and second phases of the acute toxicity study, no signs of toxicity, abnormalities, or mortality were seen in the animals after an oral dose of 2000 mg/kg of extract was given to three animals. As a result, the median lethal dose (LD_{50}) of the extract in rats was calculated to be higher than or equal to 2000 mg/kg. During the 14-day observation period, all of the animals were active and healthy.
Effect of polyherbal extract on body weight and relative organ body weight
No significant difference ($p < 0.05$) in the weight of the rats at Day 0 and Day 28, weight gain and percentage weight gain as depicted in Table 1. When comparing groups treated with (125, 250, and 500 mg/kg) of the extract to the control group, there was no significant difference ($p>0.05$) in the relative liver to body weight ratio as depicted in Table 2.

Effect of polyherbal extract on hematological parameters
All measured hematological parameters in groups treated with the extract were not significantly different ($p < 0.05$) when compared with those obtained from control group (Table 3).

Effect of Polyherbal extract on Serum biochemical parameters
A significant decrease ($p < 0.05$) of 31.68% in the levels of alkaline phosphatase in the group administered with 125 mg/kg of the extract as compared with the control group (Table 4). A progressive increase of 6.06%, 8.96% and 15.47% respectively was observed in the high density lipoprotein cholesterol levels (HDL-C) for group treated with doses of 125 mg/kg, 250 mg/ and 500 mg/kg of extract when compared to the values of the control group after 28 days of administration. All other parameters in groups treated with extract were not significant different from those of control.

Effect of Extract on histological Examination of Liver
Histopathological examination of the liver sections of the rats treated with various doses of the extract for 28 days are consistent with the normal liver histology of rats with no variations with the liver histology results observed in rats in the control group (Figure 1A-D).
Effect of Extract on histological Examination of kidney
Histopathological examination of the kidney sections of the rats treated with various doses of the extract for 28 days are consistent with the normal kidney histology of rats with no variations with the kidney histology results observed in rats in the control group (Figure 1E-H).
Polyherbal formulations are commonly used in traditional medicine and have been found to often offer better therapeutic benefits with lower toxicity compared to single herbal formulations. This is believed to be due to the synergistic effects of pharmacodynamics and pharmacokinetics present in polyherbal phytomedicines, making them a preferred choice.[17] The principal aim of evaluating the safety of any

medicinal plant is to identify the nature and significance of adverse effect and to establish the exposure level at which this effect is observed.[18] The results of the acute toxicity study indicate that the polyherbal extract at 2000 mg/kg dose did not pose any toxic threat or death to the test animals at both the first and second phases, thus, the LD_{50} of the extract is larger than or equivalent to 2000 mg/kg body weight. According to OECD criteria under its Globally Harmonised Classification System (GHS) for chemical substances and mixtures, substances with LD_{50} > 2000–5000 mg/kg are categorised as unclassified or category 5.[11] The approximate lethal dose of the extract was determined to be higher than 2000 mg/kg and the polyherbal extract is likely to be safe. This is not surprising, as existing data showed that many constituents of the extract are not prominently toxic.[19,22]

Substances administered in chronic disease conditions often require repeated dosing toxicological evaluation (sub-acute toxicity study) since daily use may result in accumulation in the body with gradual effects on tissues and organs.[23,24]The sub-acute toxicity study is effective in evaluating target organ and hematological or biochemical effects of extracts that are not observable in acute toxicity testing. It is also important in determining human safety, particularly in the development of pharmaceuticals. [24]Therefore, this study evaluated the sub-acute toxicity profile of the extract in rats by measuring body weight, hematological, biochemical, and histological parameters.

Alterations in body weight and body weight gain have been employed as a measure of the general health status of experimental animals.[24]Oral administration of the extract daily over the period of 28 days exposure to extract showed no significant changes in the body weight gain of rats across all groups. Again, in toxicity studies, changes in the weight of organs are valuable indicators of toxicity, as well as the effects on enzymes, physiological disruptions, and damage to target organs. An increase in the weight of an organ indicates hypertrophy, while a decrease indicates necrosis in the targeted organ. However, organ weight measurements must be interpreted in conjunction with other findings such as gross pathology, clinical pathology, and histopathology to gain a complete understanding of the effects of the substance being tested.[24,25] Oral administration of the extract daily over the period of 28 days exposure test showed no significant changes in the relative liver and kidney weight of rats across all groups. The relative and kidney weights of rats obtained lie within normal ranges. This is consistent with findings from the histopathological examination of the liver and kidney of rats across all groups which were found to be consistent with normal histology.

Analysis of haematological parameters are used to study the extent of toxicity of drug substances including plant extracts.[18] Alterations in the haematopoietic system have a higher predictive value for human toxicity when data are translated from animal studies.[24-25]

Table 1: Effect of polyherbal extract on body weight of rats

Groups	Day 0(g)	Day28 (g)	Weight gain	Percentage weight gain
Control	116.0	137.1	21.1	15.52%
125 mg/kg of extract	116.7	149.3	32.6	20.46%
250 mg/kg of extract	128.8	145.4	16.6	11.42%
500 mg/kg of extract	117.4	130.4	12.9	17.96%

n=5, Data are expressed as mean ± S.E.M

Table 2: Effect of polyherbal extract on relative organ weight (ROW) in rats

Organ	Groups			
	Control	MLRE (125 mg/kg)	MLRE (250 mg/kg)	MLRE (500 mg/kg)
Kidney ($\times 10^{-3}$)	3.36 ± 0.171	3.19 ± 0.150	4.90 ± 0.694	3.08 ± 0.084
Liver ($\times 10^{-3}$)	38.90 ± 2.158	36.65 ± 1.738	40.56 ± 1.410	35.89 ± 2.396

n=5, Data are expressed as mean ± S.E.M

Table 3: Effect of polyherbal extract on haematological parameters in rats

Parameter	Groups			
	Control	MLRE (125 mg/kg)	MLRE (250 mg/kg)	MLRE (500 mg/kg)
WBC (X10^3/microliter)	14.74 ± 1.001	14.62 ± 1.260	12.68 ± 2.209	9.96 ± 0.457
%RBC (X10^6/microliter)	7.29 ± 0.203	7.62 ± 0.125	6.846 ± 0.054	7.28 ± 0.314
HGB (g/dL)	10.96 ± 0.092	11.32 ± 0.115	10.94 ± 0.107	11.12 ± 0.305
HCT (%)	37.78 ± 0.414	39.60 ± 2.200	36.40 ± 0.446	38.60 ± 1.274
MCV (fL)	53.42 ± 0.715	51.94 ± 10.280	52.58 ± 0.597	53.10 ± 0.880
MCH (pg)	15.86 ± 0.483	14.88 ± 0.618	16.16 ± 0.344	15.34 ± 0.463
MCHC (g/dL)	29.00 ± 0.192	28.30 ± 0.354	30.06 ± 0.204	28.84 ± 0.526
PLT (X10^3/microliter)	67.00 ± 58.990	874.40 ± 0.583	676.4 ± 47.220	597.20 ± 48.590
LYM (%)	90.66 ± 1.486	85.12 ± 0.301	84.66 ± 2.829	81.06 ± 4.329

n=5, Data are expressed as mean ± S.E.M

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

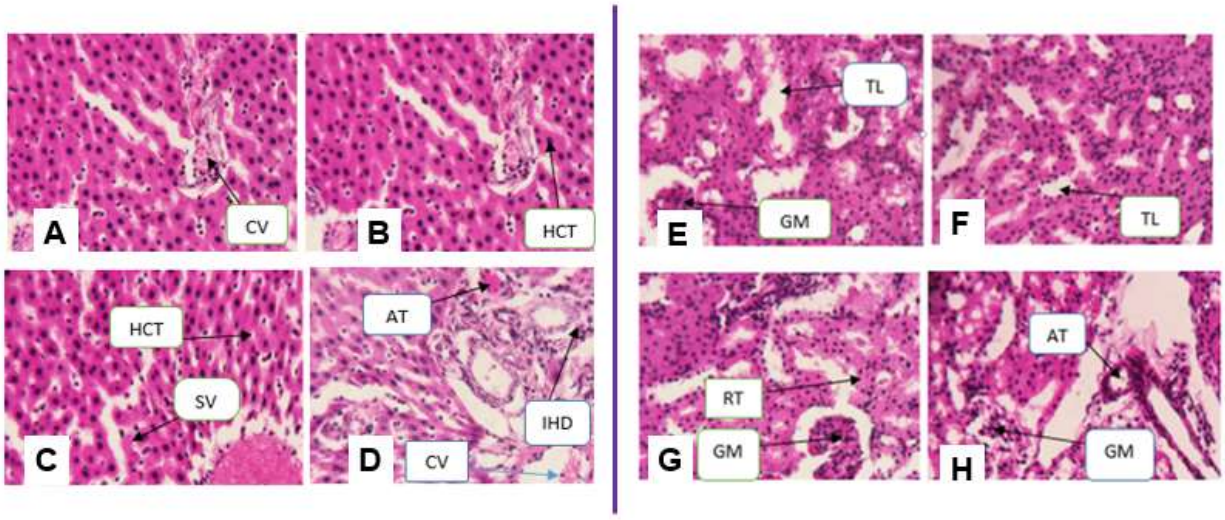

Figure 1: Micrographs of Liver sections ((A-D) and kidney sections (E-H) from rats treated with different doses of MLRE A) Control, B) 125 mg/kg, C) 250 mg/kg, D) 500 mg/kg respectively. 40×Magnification., Central vein (CV), Hepatocyte (HCT), Sinusoid (SV), Intrahepatic bile duct (IHD), Arteriole (AT), Tubular lumen (TL), Glomerulus (GM), Renal tubules (RT), Arteriole (AT)

Table 4: Effect on polyherbal extract on serum biochemical parameters

Parameter	Groups (mg/kg of extract)			
	Control	**125**	**250**	**500**
Urea (mg/dl)	180.60 ± 13.06	174.9 ± 11.67	183.90 ± 18.02	181.10 ± 24.08
Creatinine (mg/dl)	2.13 ± 0.20	1.63 ± 0.64	1.73 ± 0.43	2.32 ± 0.63
Sodium (mg/dl)	57.56 ± 1.09	63.60 ± 2.77	59.44 ± 2.48	60.44 ± 3.71
Potassium (mg/dl)	1.32 ± 0.08	1.02 ± 0.13	1.34 ± 0.05	1.46 ± 0.07
Chloride (mg/dl)	26.97 ± 0.62	25.04 ± 0.62	24.54 ± 0.41	25.88 ± 1.12
HCO_3 (mg/dl)	14.41 ± 1.44	8.80 ± 0.84	15.90 ± 1.33	15.02 ± 2.00
Protein (mg/dl)	31.08 ± 0.41	31.54 ± 0.42	32.75 ± 0.62	29.93 ± 1.86
Albumin(mg/dl)	9.62 ± 0.37	9.38 ± 0.18	10.26 ± 0.30	10.18 ± 0.73
AST (U/L/mg protein)	35.35 ± 4.90	28.43 ± 3.03	33.63 ± 3.71	38.26 ± 3.70
ALT (U/L/mg protein)	17.52 ± 0.46	15.56 ± 0.29	15.67 ± 1.15	15.87 ± 1.16
ALP (U/L/mg protein)	67.30 ± 0.76	45.98 ± 3.02*	63.02 ± 2.44	70.39 ± 3.23
Total bil (mmol/L)	41.14 ± 2.14	39.98 ± 2.71	42.22 ± 1.39	43.47 ± 3.49
Dir bil (mmol/L)	23.77 ± 1.90	19.95 ± 1.88	19.60 ± 1.02	22.21 ± 1.89
Triacylglycerol (mmol/L)	4.69 ± 0.28	4.38 ± 0.19	5.29 ± 0.44	4.61 ± 0.26
HDL cholesterol (mmol/L)	17.98 ± 2.41	19.14 ± 0.74	19.75 ± 1.31	21.27 ± 2.74
Total cholesterol((mmol/L)	31.97 ± 1.86	34.29 ± 0.99	39.28 ± 1.89	36.84 ± 1.61

Table 5. Effect of polyherbal extract on acetic acid-induced writhing response in mice

Groups/Treatment	Number of writhes (per 10 mins)	Percentage of inhibition
Control	60.90 ± 1.887	_
Diclofenac sodium 20 mg/kg	25.40 ± 0.678	58.29
200 mg/kg of extract	43.40 ± 0.871	28.74
40 mg/kg of extract	30.25 ± 1.648	50.32

Values were expressed as mean ± SEM of 5 determinations; * p<0.001, ** p<0.05 versus Control

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

After 28 days of treatment with the extract, the hematological parameters showed non-significance (P > 0.05) when compared to control group. However, there was a reduction of 6.11%, 6.62% and 10.6% in the lymphocyte levels and WBC counts, for group treated with doses of 125 mg/kg, 250 mg/kg and 500 mg/kg of extract respectively after 28 days of administration (Table 3).

Table 6: Effect of polyherbal extract on carrageenan-induced paw oedema in mice

Treatments	% Inhibition					
	at 30 mins	at 60 mins	at 90 mins	at 120 mins	at 150 mins	at 180 mins	at 210 mins
Diclofenac	10.21±1.302	28.98±3.050	48.54±3.426	63.62±2.936	67.49±2.031	69.60± 2.151	74.73±4.368
400 mg/kg	4.74±0.691	16.56±2.578	31.62±2.250	42.64±3.566	34.42± 2.806*	30.07±2.932*	24.92±1.766*
200 mg/kg	2.04±0.881*	12.56±0.387*	23.50±2.378*	37.69±0.475*	36.91±2.308	33.15±3.245*	24.61±0.980*

n=5, Data are expressed as mean ± S.E.M * p<0.05 versus Diclofenac.

Table 7. Effect of different receptor blockers on analgesic activity of polyherbal extract on acetic acid-induced writhing test in Mice

Groups/Treatment	Number of writhes (per 10 mins)	Percentage of inhibition
Control	56.92 ± 1.313	_
400 mg/kg of extract	28.28 ± 0.667*	50.32
Naloxone + extract	31.70 ± 1.271**	44.31
Prazosin + extract	32.30 ± 1.562**	43.25
Yohimbine + extract	33.40 ± 2.835**	41.32
Propanolol + extract	37.70 ± 0.916	33.77
Metergoline + extract	40.90 ± 1.134	28.14
L-Arginine+ extract	47.60 ± 2.141***	16.37

Values were expressed as mean ± SEM of 5 determinations; * p<0.001, ** p<0.05 versus Control, *** p<0.05 versus 400 mg/kg of extract.

The study found no significant changes in liver-related biochemical markers, including AST, ALT, ALP, bilirubin, albumin, and protein, when compared to the control group (Table 4). These markers are essential in assessing the cellular integrity and functionality of the liver.[26] Elevated liver enzymes, ALP, ALT and AST, are sensitive indicators of hepatocellular damage caused by liver injury induced by hepatotoxins. Conversely, decreased serum levels of total proteins, bilirubin, and albumin indicate impaired synthetic liver function and suggest liver damage as indicated by previous studies.[2427] However, the extract did not affect the serum levels of transaminase activities, ALP, bilirubin, albumin, and protein, indicating that its sub-acute administration did not induce hepatotoxicity. These results suggest that the plant extract may be safe for traditional medicinal use, although the ALP levels increased significantly at the 125 mg/kg dose level, which was considered unlikely related to treatment given the small magnitude of change and lack of dose-response or histological changes in the liver. Similarly, no significant changes were observed in kidney-related biochemical markers, including creatinine, urea, and electrolyte levels, when compared to the control group, indicating that the sub-acute administration of the extract was not associated with any kidney toxicity. Creatinine levels, which are crucial indicators of the glomerular filtration rate,[24] urea and electrolyte levels are common parameters used to evaluate the concentrating and diluting capacity of tubular functions of the kidneys and the kidneys' overall function.[26]
The histological analysis of the cellular structure of the liver and idney (as shown in Fig. 1) did not reveal any abnormalities in the extract-treated rats compared to the control rats. This finding suggests that the extract did not adversely affect the morphology of these vital organs. These results are consistent with the biomarkers of liver and kidney damage, which were not significant different in the groups that received the extract when compared to the control group.

Effect of Extract on Acetic Acid-Induced Writhing Response in Mice.
The intraperitoneal injection of acetic acid (0.6%) caused strong nociceptive response in the control group, with (60.90±1.8887) abdominal contortions.Treated animals with 400 and 200 mg/kg of the extract inhibited writhings caused by acetic acid by 50.32% and 28.74%, respectively. Diclofenac sodium (20 mg/kg), the standard for this experiment, reduced writhing by 58.29% (Table 5).

Anti-inflammatory, Analgesic activity of extract
Effect of Extract on Carrageenan Induced Paw Oedema
Significant differences in anti-inflammatory activity were observed for all time intervals for rats administered with 400 mg/kg of the extract and Diclofenac versus those that were administered with normal saline as seen in Table 6. No Significant differences in anti-inflammatory activity were observed for time intervals of 30 minutes for rats administered with 200 mg/kg of the extract versus the rats in control, however significant differences were observed at all other measured

time intervals for rats administered with 200 mg/kg of the extract versus the rats in control.

Mechanistic study

The pretreatment of animals with l-arginine significantly increased the number of writhes produced by the extract (47.60±2.141) when compared with the number of writhing produced by the extract alone (56.92±1.313). However, the pretreatment of animals with naloxone, prazosin, yohimbine, propranolol or metergoline each did not significantly decrease or increase the number of writhes produced by the extract (Table 7).

In the anti-inflammatory assay of the extract via the Carragenan Induced left Paw Oedema method, significant inhibition of the oedema/increased rat paw size induced by carrageenan were recorded in all the groups of rats given the 400 mg/kg of the extract and Diclofenac at all-time intervals measured in the study. This suggests that the extract posseses some anti-inflammatory activities, especially at the 400 mg/kg dose. The observed anti-inflammatory and analgesic effects of the extract may be attributed to the presence of various bioactive compounds in the individual plant sources. Earlier studies on some African medicinal plants particularly the individual components of the extract reported that they contain flavonoids and phenolic compounds which are known antioxidants and possess anti-inflammatory properties as well.[28-30] Garlic bulb contains allicin, which has been shown to have anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines.[31] Honey contains flavonoids and phenolic acids, which have been reported to possess anti-inflammatory and analgesic effects.[32] *Aloe vera* gel contains aloin, which has been shown to have anti-inflammatory effects by inhibiting the activity of cyclooxygenase (COX) and lipoxygenase (LOX).[33] The juice of *citrus limon* contains limonene, which has been reported to possess anti-inflammatory and analgesic effects by inhibiting the production of pro-inflammatory cytokines and increasing the levels of anti-inflammatory cytokines.[34] Ginger rhizomes have been reported to contain gingerols and shogaols, which exhibit anti-inflammatory and analgesic effects by inhibiting prostaglandin synthesis and COX-2 activity.[35]

The central and peripheral pathways have been observed to be involved in analgesia. Plants having anti-inflammatory properties have also frequently been observed to have potent analgesic effects.[36] The acetic acid writhing reflex test is used to assess for compounds that mediate their anti-nociceptive effect via peripherally related mechanisms. When acetic acid is injected into the peritoneal cavity, endogenous mediators such as tumour necrosis factor (TNF-), interleukins, and prostaglandins are produced.[36]

The peripheral mediated analgesic activity of acetic acid is associated with the stimulation of peripheral receptors especially the local peritoneal receptors at the surface of cells lining the peritoneal cavity, and may be due to either its action on visceral receptors sensitive to acetic acid or the inhibition of the synthesis of allogenic substances.[37] In this study, the extract at 400 mg/kg dose significantly ($P < 0.05$) reduced the number of acetic acid-induced writhes in the mice. However, the reduction in number of writhes produced by the extract at 400 mg/kg was lesser than the reduction produced by the standard drug, Diclofenac.

Popular mechanisms for peripherally mediated analgesic activity include inhibition of prostaglandin synthesis, GMP-ATP-sensitive K+ channel cascade, serotonergic pathway, adrenergic pathway, inhibition of cytokine production, L-Arginine-Nitric Oxide pathway amongst others.[38,39]

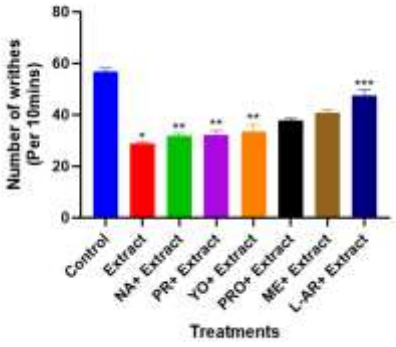

Figure 2: Effect of different receptor blockers on analgesic activity of extract on acetic acid-induced writhing test in mice. Values presented as Mean ± SEM; Values were expressed as mean ± SEM of 5 determinations; * p<0.001, ** p<0.05 versus Control, *** p<0.05 versus 400 mg/kg of extract. NAL=naloxone, PRA= prazosin, YOH=yohimbine, PRO=propranolol, MET= metergoline, L-ARG=l-arginine

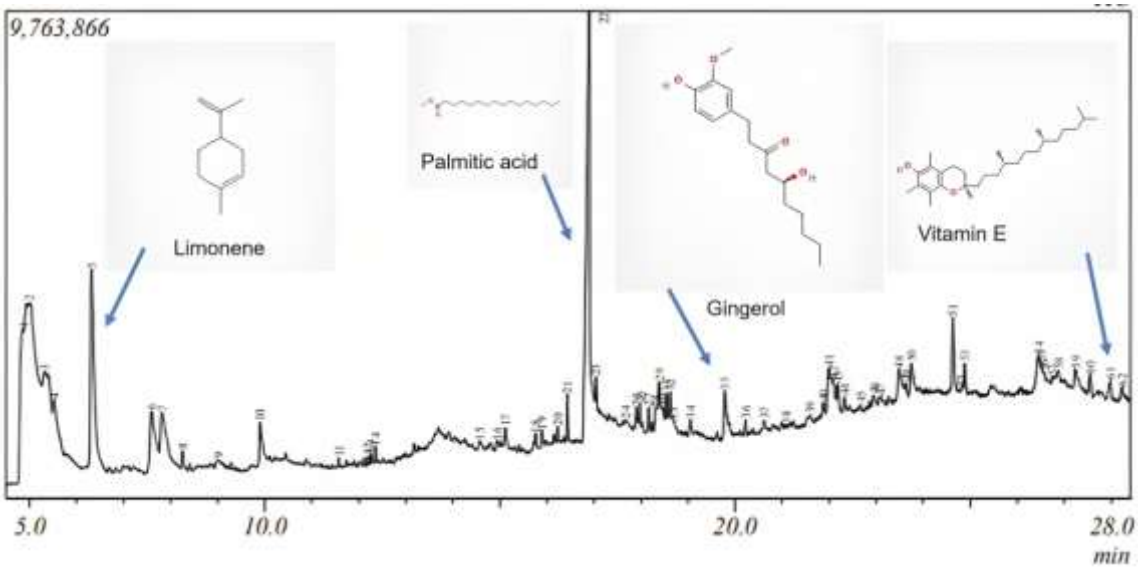

Figure 3: GCMS chromatogram of the 70% ethanol extracts of RA polyherbal phytomedicine with clear peaks showing some marker compounds including gingerol (peak 35 from *Zingiber officinale*), Limonene (peak 5 from *Citrus limon*), Vitamin E, (peak 61 - *Aloe vera*), Retinoic acid (peak 33 - *Aloe vera*), Palmitic acid – peak 22 and beta sitosterol – peak 42.

A mechanistic study on the extract was further conducted to determine more specific pathways implicated in the analgesic activity of the extract. The extract at dose of 500 mg/kg was used for this mechanistic study because it gave the peak response in the writhing test.
Further studies on the analgesic properties of the extract were conducted by using six different receptor blockers; naloxone, prazosin, yohimbine, propranolol, metergoline and L-Arginine. The significant difference in analgesic activity between mice that received the extract after being pretreated with l-Arginine and mice who were administered with 400 mg/kg and no prior pretreatment with any receptor blockers demonstrate that the L-arginine-NO-cGMP pathway is involved in the extract's analgesic effects. This conclusion derives from the fact that that the analgesic effect caused by the extract was reversed by pretreating mice with l-arginine, the substrate of nitric oxide synthase and suggests that the L-arginine-NO-cGMP may play a crucial role in analgesic activity of the extract. In living systems, NO is produced from l-arginine by the action of the enzyme iNOS, which is induced by bacterial lipopolysaccharide (LPS) stimulation in many cells including macrophages. This pathway has been linked to thermal inflammatory hyperalgesia and neuropathic pain, and overproduction of NO has been associated with a range of clinical disorders such as convulsions, pain, and schizophrenia.[40] The analgesic activity of the extract may not involve the opioidergic, adrenergic and serotonergic pathway. These notions are because the pretreatment of mice with naloxone (a nonselective opioid receptor antagonist), prazosin (a α1-adrenoceptor antagonist), yohimbine (a α2-adrenoceptor antagonist), propranolol (a β-adrenoceptor antagonist) and metergoline (a serotonin antagonist) each failed to reverse the analgesic activity of the extract.

GC-MS analysis of the polyherbal extract
Polyherbal extract was formulated from an equal amount of each of the following crude drugs; *Zingiber officinale*, *Allium sativum*, *Aloe vera*, *Citrus limon* and honey. The chromatogram from the GC-MS analysis (Figure 1) revealed that the extract possesses a wide range of plant metabolites, a majority of which have been reported for each of the individual plants extract making up the polyherbal extract.[5,6] Some peaks which correspond to known compounds present in the constituent of the polyherbal extract (Figure 1). These compounds include gingerol (from *Zingiber officinale*), Limonene (from *Citrus limon*), Vitamin E, (*Aloe vera*), Retinoic acid (*Aloe vera*), Palmitic acid, beta sitosterol, etc. Some other marker compounds detected using GCMS include Palmitic acid (N-hexadecanoic acid - peak 22), D-Limonene (peak 5), Butanedioic acid, monomethyl ester (or Succinic acid_peak 6,7), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-m, 4 Terpinen-4-ol, 2,3-dihydrobenzofuran, 2-Ethylbutyric acid and tridecyl ester.
The GC-MS analysis was used for the identification and quantification of the secondary metabolites of the polyherbal extract. Several known[41] anti-inflammatory compounds have been identified in the GC-MS analysis which may be responsible for the documented activity and thus chemically validating the bioactivity of the extract. Furthermore, the GC-MS fingerprint of this polyherbal extract could help in the quality control of the standardized phytomedicine.

Conclusion

This study showed that the polyherbal extract is not toxic at both acute and sub-acute stages as there was no mortality through the duration of the study which was also corroborated with the findings from the liver and kidney function biomarkers which did not show toxicity of the polyherbal phytomedicine at the doses used. This study also demonstrates the anti-inflammatory and analgesic potentials of the polyherbal extract particularly at 400 mg/kg body weight in rodents. The L-arginine-NO-cGMP pathway is specifically involved in the analgesic effects of the extract. Hence, the polyherbal extract demonstrates to be a promising regimen for the treatment of disease conditions associated with inflammation and analgesia with a little to no adverse effect. The GCMS data revealed marker compounds in the investigated phytomedicine which will be helpful in the proper identification and quality control of the anti-inflammatory phytomedicine. The polyherbal phytomedicine requires further elaborate standardization[42] to make it suitable for a possible for application as a complimentary medicine.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

References

1. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, Ferrucci L, Gilroy DW, Fasano A, Miller GW, Miller AH. Chronic inflammation in the etiology of disease across the life span. Nat. Med. 2019 Dec;25(12):1822-32.. doi:10.1038/s41591-019-0675-0.
2. Lordan, R., Ronan Lordan, Alexandros Tsoupras, Ioannis Zabetakis, Chapter 2 – Inflammation. In: The Impact of Nutrition and Statins on Cardiovascular Diseases; Academic Press, 2019, (Pp 23-51); ISBN 9780128137925. https://doi.org/10.1016/B978-0-12-813792-5.00002-1
3. Gakhar A. Anti-inflammatory perspectives of diverse natural resources. Journal of Medical Pharmaceutical and Allied Sciences (Jmpas). 2021;10(4):1379.. https://doi.org/10.22270/jmpas.v10i4.1379
4. Weavers H, Martin P. The cell biology of inflammation: From common traits to remarkable immunological adaptations. Journal of Cell Biology (JCB). 2020 Jul6;219(7). e202004003. doi:10.1083/jcb.202004003.
5. Mao QQ, Xu XY, Cao SY, Gan RY, Corke H, Beta T, Li HB. Bioactive compounds and bioactivities of ginger (Zingiber officinale Roscoe). Foods. 2019 May 30;8(6):185.. doi:10.3390/foods8060185.
6. Ballistreri G, Fabroni S, Romeo FV, Timpanaro N, Amenta M, Rapisarda P. Anthocyanins and other polyphenols in citrus genus: Biosynthesis, chemical profile, and biological activity. InPolyphenols in plants 2019 Jan 1 (pp. 191-215). Academic Press.
7. El-Saber Batiha G, Magdy Beshbishy A, G. Wasef L, Elewa YH, A. Al-Sagan A, Abd El-Hack ME, Taha AE, M. Abd-Elhakim Y, Prasad Devkota H. Chemical constituents and pharmacological activities of garlic (Allium sativum L.): A review. Nutrients. 2020 Mar 24;12(3):872.
8. Rozani M, Kusbaryanto A. Efficacy Aloe vera in treatment: A literature review. J Complement Med Alt Healthcare. 2019;10(1):555776. doi:10.19080/JCMAH.2019.09.555776.
9. Sajjad A, Subhani Sajjad S. Aloe vera: An ancient herb for modern dentistry—A literature review. Journal of Dental Surgery (J Dent). 2014;2014., 210463. doi:10.1155/2014/210463
10. Rao PV, Krishnan KT, Salleh N, Gan SH. Biological and therapeutic effects of honey produced by honey bees and stingless bees: a comparative review. Revista Brasileira de Farmacognosia. 2016 Sep;26:657-64. doi:https://doi.org/10.1016/j.bjp.2016.01.012.
11. Ranneh Y, Akim AM, Hamid HA, Khazaai H, Fadel A, Zakaria ZA, Albujja M, Bakar MF. Honey and its nutritional and anti-inflammatory value. BMC Complement. Med. Ther.. 2021 Dec;21(1):1-7.. doi:10.1186/s12906-020-03170-5.
12. Ebohon O, Irabor F, Omoregie ES. Sub-acute toxicity study of methanol extract of Tetrorchidium didymostemon leaves using biochemical analyses and gene expression in Wistar rats. Heliyon. 2020 Jun 1;6(6):e04313.
13. Hogan IA, Kuo YC, Abubakar AN, Lawal B, Agboola AR, Lukman HY, Onikanni SA, Olawale F, Fadaka AO, Ibrahim

YO, Babalola SB. Attenuation of hyperglycemia-associated dyslipidemic, oxidative, cognitive, and inflammatory crises via modulation of neuronal ChEs/NF-κB/COX-2/NOx, and hepatorenal functional deficits by the Tridax procumbens extract. Biomedicine & Pharmacotherapy. 2023 Feb 1;158:114114.
14. Ajayi AM, Ola CB, Ezeagu MB, Adeleke PA, John KA, Ologe MO, Ben-Azu B, Umukoro S. Chemical characterization, anti-nociceptive and anti-inflammatory activities of Plukenetia conophora seed oil in experimental rodent models. Journal of Ethnopharmacology. 2023 Apr 6;305:116017.
15. Falodun A, Okunrobo LO, Uzoamaka N. Phytochemical screening and anti-inflammatory evaluation of methanolic and aqueous extracts of Euphorbia heterophylla Linn (Euphorbiaceae). African Journal of Biotechnology (AJB). 2006;5(6):529-31.s
16. Grace OM, Kolawole IA, Cajethan OE. GC-MS analysis of bioactive compounds and evaluation of antimicrobial activity of the extracts of Daedalea elegans: A Nigerian mushroom. African Journal of Microbiology Research. 2020 Jun 30;14(6):204-10.
17. Parasuraman S, Thing GS, Dhanaraj SA. Polyherbal formulation: Concept of ayurveda. Pharmacogn Rev . 2014 Jul;8(16):73.. https://doi.org/10.4103/0973-7847.134229
18. Ibrahim MB, Sowemimo AA, Sofidiya MO, Badmos KB, Fageyinbo MS, Abdulkareem FB, Odukoya OA. Sub-acute and chronic toxicity profiles of Markhamia tomentosa ethanolic leaf extract in rats. J. Ethnopharmacol.. 2016 Dec 4;193:68-75.. https://doi.org/10.1016/j.jep.2016.07.036
19. Ahd K, Dhibi S, Akermi S, Bouzenna H, Samout N, Elfeki A, Hfaiedh N. Protective effect of ginger (Zingiber officinale) against PCB-induced acute hepatotoxicity in male rats. RSC advances. 2019;9(50):29120-30.
20. Wu J, Zhang Y, Lv Z, Yu P, Shi W. Safety evaluation of Aloe vera soft capsule in acute, subacute toxicity and genotoxicity study. PloS one. 2021 Mar 26;16(3):e0249356. https://doi.org/10.1371/journal.pone.0249356.
21. Dahiya A, Prakash A, Agrawala PK, Dutta A. Investigation on Oral Toxicity of Diallyl Sulfide: A Principle Organosulfur Compound Derived from Allium Sativum (Garlic) in mice. Defence Life Science Journal. 2022 Jan 21;7(1):3-10.
22. Yamprasert R, Chanvimalueng W, Mukkasombut N, Itharat A. Ginger extract versus Loratadine in the treatment of allergic rhinitis: a randomized controlled trial. BMC complementary medicine and therapies. 2020 Dec;20:1-1.
23. Bariweni MW, Yibala OI, Ozolua RI. Toxicological studies on the aqueous leaf extract of *Pavetta crassipes* (K. Schum) in rodents. J. Pharm. Pharmacogn. Res. 2018;6(1):1-6. https://www.redalyc.org/journal/4960/496055725001/496055725001.pdf
24. Unuofin JO, Otunola GA, Afolayan AJ. Evaluation of acute and subacute toxicity of whole-plant aqueous extract of *Vernonia mespilifolia* Less. in Wistar rats. J. Integr. Med. 2018 Sep 1;16(5):335-41.. https://doi.org/10.1016/j.joim.2018.07.003
25. Ugwah-Oguejiofor CJ, Okoli CO, Ugwah MO, Umaru ML, Ogbulie CS, Mshelia HE, Umar M, Njan AA. Acute and sub-acute toxicity of aqueous extract of aerial parts of Caralluma dalzielii NE Brown in mice and rats. Heliyon. 2019 5(1):e01179.. https://doi.org/10.1016/j.heliyon.2019.e01179
26. Thangavelu L, Balusamy SR, Shanmugam R, Sivanesan S, Devaraj E, Rajagopalan V, Veeraiyan DN, Chellappan DK, Dua K, Kim YJ, Perumalsamy H. Evaluation of the sub-acute toxicity of Acacia catechu Willd seed extract in a Wistar albino rat model. Regulatory Toxicology and Pharmacology RTP. 2020;113:104640. https://doi.org/10.1016/j.yrtph.2020.104640
27. Kifayatullah M, Mustafa MS, Sengupta P, Sarker MM, Das A, Das SK. Evaluation of the acute and sub-acute toxicity of the ethanolic extract of Pericampylus glaucus (Lam.) Merr. in BALB/c mice. J. Acute Dis. 2015 4(4):309-15.. https://doi.org/10.1016/j.joad.2015.06.010
28. Falodun A, Poh CF, Adelusi SA, Emmanuel O. Phytochemical and anti-inflammatory evaluation of Khaya grandifoliola stem bark extract. Int J PharmTech Res. 2009;1(4):1061-4.
29. Dzoyem JP, Eloff JN. Anti-inflammatory, anticholinesterase and antioxidant activity of leaf extracts of twelve plants used traditionally to alleviate pain and inflammation in South Africa. Journal of Ethnopharmacology. 2015;160:194-201.
30. Bozin B, Mimica-Dukic N, Samojlik I, Goran A, Igic R. Phenolics as antioxidants in garlic (Allium sativum L., Alliaceae). Food Chem. 2008 111(4):925-9.
31. Bayan L, Koulivand PH, Gorji A. Garlic: a review of potential therapeutic effects. Avicenna J phytomedicine. 2014; 4(1):1.
32. Yu W, Sun F, Xu R, Cui M, Liu Y, Xie Q, Guo L, Kong C, Li X, Guo X, Luo L. Chemical composition and anti-inflammatory activities of Castanopsis honey. Food & Function. 2023.
33. Xiao J, Chen S, Chen Y, Su J. The potential health benefits of aloin from genus Aloe. Phytotherapy Research. 2022;36(2):873-90.
34. Li C, Zhu H, Zhao K, Li X, Tan Z, Zhang W, Cai Q, Wu X, Mo J, Zhang L. Chemical constituents, biological activities and anti-rheumatoid arthritic properties of four citrus essential oils. Phytotherapy Research. 2022 Jul;36(7):2908-20.
35. Grzanna R, Lindmark L, Frondoza CG. Ginger—an herbal medicinal product with broad anti-inflammatory actions. J Medicinal food. 2005;8(2):125-32.
36. Elkhateeb A, El-Shabrawy M, Abdel-Rahman RF, Marzouk MM, El-Desoky AH, Abdel-Hameed ES, Hussein SR. LC-MS-based metabolomic profiling of Lepidium coronopus water extract, anti-inflammatory and analgesic activities, and chemosystematic significance. Medicinal Chem Res. 2019 28:505-14.
37. Bhuiyan MMR, Bhuiya NMA, Hasan MN, Nahar UJ. *In vivo* and in silico evaluation of antinociceptive activities of seed extract from the *Holarrhena antidysenterica* plant. Heliyon, 2020; 6(5), e03962.
38. Ping X, Xie J, Yuan C, Jin X. Electroacupuncture Induces Bilateral S1 and ACC Epigenetic Regulation of Genes in a Mouse Model of Neuropathic Pain. Biomedicines. 2023;11(4):1030.
39. Odoma S, Zezi AU, Danjuma NM, Ahmed A, Magaji MG. Elucidation of the possible mechanism of analgesic actions of butanol leaf fraction of Olax subscorpioidea Oliv. Journal of ethnopharmacology. 2017;199:323-7.
40. Sheikholeslami MA, Ghafghazi S, Parvardeh S, Koohsari S, Aghajani SH, Pouriran R, Vaezi LA. Analgesic effects of cuminic alcohol (4-isopropylbenzyl alcohol), a monocyclic terpenoid, in animal models of nociceptive and neuropathic pain: Role of opioid receptors, L-arginine/NO/cGMP pathway, and inflammatory cytokines. European Journal of Pharmacology. 2021;900:174075.
41. Nyaitondi, o. D., Wanjau, R. N., Nyambaka, h., & Hassanali, A. (2018). Anti-bacterial properties and GC-MS analysis of extracts and essential oils of selected plant product. Asian JNat Prod Biochem, 16(1), 44-58.
42. Falodun A. Herbal medicine in Africa-distribution, standardization and prospects. Res. J. Phytochem. 2010;4(3):154-61