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PHYSICOCHEMICAL, ANTIOXIDANT AND ANTI-INFLAMMATORY PROPERTIES OF A MULTIPURPOSE HERBAL FORMULATION (HB-01)

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Anti-inflammatory property, Herbal formulation, DPPH, Antioxidant, Acetylsalicylic acid

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ABSTRACT: An herbal formulation composed of *Allium cepa* (Onion), *Zingiber officinale* (Ginger), *Allium sativum* (Garlic), *Aloe vera*, *Andrographis paniculata* (King of bitters), *Allium ascalonicum* (Shallot), reportedly used as anti–inflammatory agent was investigated. This study evaluated the physicochemical, phytochemical properties and *in vivo* anti–inflammatory effect of the herbal formulation. The herbal formulation (Hp-01) was subjected to physicochemical analysis; visual and organoleptic test, thin layer chromatography (TLC), pH, trace elements, phytochemical screening, antioxidant activity using DPPH inhibition and total phenolic acid (TPA) content determination. The biological effect at three doses (half, normal and double) on acute inflammation using carrageenan-induced paw oedema, weight, haematological parameters and liver functions were investigated in Wistar rats. The herbal formulation (Hp-01) was an acidic (pH 3.18), bitter preparation with pungent odour and sediments. The TLC analysis showed a maximum of three spots. Trace elements content of Pb, Cd, Fe, and Zn were within acceptable limits at 0.053, 0.003, 1.190 and 4.190 mg/l respectively. Phytochemical screening showed the presence of anthraquinones, tannins, terpenoids, saponins (2.12%w/w), alkaloids (2.45%w/w) and flavonoids (49.45%w/w). The IC_{50} for DPPH radical scavenging activity was 2646.82±58.24 µg/ml indicating low activity, while TPA content was 0.0184±0.0005 mgGAE/g. The anti-inflammatory activity of the herbal formulation was comparable acetylsalicylic acid (reference) at the three doses tested ($p>0.05$). Also, there was no significant effect on the weight, liver function, some haematological parameters; PCV, RBC, Hb. The herbal formulation has comparable anti-inflammatory activity with acetylsalicylic acid and may be explored for new anti-inflammatory agents.

INTRODUCTION: The high cost of orthodox drugs, inadequate supplies, side effects and the belief that plants hold cure to many disease conditions have led to the reawakening of interest in the utilization of plants and plant products in recent years.

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Hence, majority of the people living in developing countries rely on medicinal plants for their primary health care needs [1]. With the ever-increasing use of herbal medicines worldwide and the rapid expansion of the global market for these products, the safety and quality of medicinal plant materials and finished herbal medicinal products have become a major concern for health authorities, pharmaceutical industries and the public [2].

Inflammation is the response of living tissues to injury and it begins when a stimulus such as infection, physical or chemical insult produces cellular damage, with classic signs such as local

redness, swelling, pain, heat and loss of function [3]. Inflammatory response changes with time and can be divided into acute and chronic phases. Chronic inflammation which occurs over months to years is marked by drastic increased production of inflammatory mediators [4]. The secondary chronic phase of inflammation may occurs after years of oxidative damage of degraded blood vessels and tissues, and have been associated with many diseased states such as arteriosclerosis, cardiovascular and cancer as well as inflammatory disorders [5].

Anti-inflammatory drugs act by inhibiting the enzyme cyclooxygenase (COX) activities thereby blocking the production of prostaglandins which are implicated in various inflammation reactions. Thus most anti-inflammatory drugs exhibit serious side effects and toxicity such as gastric intestinal ulceration and bleeding, renal damage, hypertension, hyperglyceamia, which tend to discourage their use. This has led to the renewed interest in alternative treatment sources of which herbal products are highly favoured.

In Nigeria, some reported plants with confirmed anti-inflammatory activity include *Aloe vera* [6], *Bryophyllum pinnatum*[7], *Securidaca longipe-dunculata* [8], *Alafia barteri, Combretum mucronatum* and *Capparis thonningi* [9], *Schwenkia Americana, Asparagus africanus, Dichrostachys cinerea, Ficus iteophylla, Indigofera pulchra* [10], *Acacia karoo and Margaritaria discordea* [4], *Andrographis paniculata* [11]. Some of the activities have been attributed to phytochemicals such as flavonoids [12] and saponins [10].

Folkloric use of medicinal plants is usually as combination of at least two plant/plant parts. WHO described herbal preparation as preparations comprising herbs, herbal materials and finished herbal products that may include comminuted or powdered herbal materials, or extracts, tinctures and fatty oils of herbal materials. They are available as solutions, tablets, capsules and powdered tea extracts [2].

Most herbal preparations are made from one or more herbs; if more than one herb is used, the term mixed herbal product can also be used to describe the preparation which may contain excipients in addition to the active ingredients [2].

Alternative medical practitioners always claim that their herbal preparations can cure all manners of ailments. There is the need to evaluate such herbal preparations; hence this study is aimed at verifying the claim of an herbal preparation (Hb-01) obtained from a practitioner.

The liquid herbal preparation (Hb-01) was reportedly used as anti- inflammatory, anti-diabetic, anti-ulcer, anti-malaria and anti-microbial, etc. The liquid herbal preparation that comprises of six major plants which are; *Allium cepa* (Onion), *Zingiber officinale* (Garlic), *Allium sativum* (Ginger), *Aloe vera*, *Andrographis paniculata* (King of bitters) and *Allium ascalonicum* (Shallot). Most of the component herbs are widely consumed as food condiments and have been reported to possess antioxidant, anti-inflammatory, immune-modulatory and anti-inflammatory activities. This study thus evaluated the antioxidant and anti-inflammatory properties of the herbal preparation (Hb-01).

MATERIALS AND METHODS:

Chemicals and Reagents: Folin–Ciocalteau reagent was purchased from Loba Chemie (India), while gallic acid, DPPH, carrageenan and Silica gel GF_{254}, was obtained from Sigma Aldrich (USA), vanillin from BDH Chemicals (Poole, England), alanine aminotransferase and aspartate amino-transferase kit (Randox Laboratories Ltd, UK). Sodium carbonate, ascorbic acid, methanol, ethyl acetate, hexane, n-butanol, ethanol, acetic acid, chloroform, ammonia, distilled water, sulphuric acid, acetylsalicylic acid (All reagents were of analytical grade).

Collection of Component Plants for Hp – 01: *Andrographis paniculata* and *Aloe vera* were collected from an herbal farm in Mokola Area of Ibadan, Oyo State, while *Allium sativum, Allium cepa, Allium ascalonicum* and *Zingiber officinale* were purchased from local herbal vendor at Oje market, Ibadan, Oyo State. *Andrographis paniculata* was identified and authenticated at Forestry Research Institute of Nigeria (FRIN) with voucher number FHI 110140.

Preparation of the Herbal Formulation (Hp - 01): The oral herbal liquid formulation comprises of *Allium cepa* (18.76%w/v), *Zingiber officinale* (10.44%w/v), *Allium sativum* (6.08%w/v), *Allium ascalonicum* (1.94%w/v), *Aloe vera* (22.6%w/v) and *Andrographis paniculata* (0.22%w/v). The plant components were washed thoroughly, blended together and sieved, after which honey (10%v/v) and ethyl alcohol (4%v/v) were added as sweetener and preservative respectively.

Physicochemical Evaluation:

Organoleptic Properties and pH Determination: The clarity and colour were determined by transferring the sample (10ml) into a clean test-tube. The taste of the Hb-01 was determined by pouring a little quantity on the tongue, while the pH was determined using pH meter.

Thin Layer Chromatographic Analysis (TLC): was carried out using Silica gel GF_{254} as stationary phase and three mobile phases [toluene: ethyl acetate (9.3: 0.7); acetone: water: toluene (5:2:3) and acetone: water: toluene (5:1:4). Visualisation was done using daylight, ultraviolet light (254 nm and 365 nm), and vanillin as spray reagents.

Determination of Trace Elements: The levels of trace elements such as cadmium, iron, zinc, lead in the herbal formulation was determined using atomic absorption spectroscopic analysis based on an earlier described procedure The trace metals were analysed at various wavelengths as follows; cadmium (228.8nm), lead (283.3nm), zinc (213.9nm) and iron (248.3nm) [13].

Phytochemical Screening: The qualitative screening was carried out in accordance with the standard protocol as described by Sofowora [14]. Also, the quantitative evaluation for flavonoids, saponins and alkaloids was based on earlier reported method [15].

Determination of Antioxidant Capacity: The antioxidant capacity and free radical scavenging activity were estimated using DPPH radical scavenging method [16].

The herbal product (Hb-01) (200mL) was lyophilized using a freeze drier; the yield was used to compute the concentrations. Methanolic solution of DPPH (0.3mM) (1mL) was added to 2.5ml of different concentrations of the sample solution, mixed properly and incubated room temperature for 30mins. The procedure was repeated with ascorbic acid at similar concentrations and methanol (2.5ml) which served as standard and negative control respectively. The absorbance was determined at 517nm. The determinations were done in triplicate for each sample. The DPPH radical scavenging activity was calculated according to the equation:

%DPPH scavenging = Abs of control- Abs of sample /Abs of control × 100

Determination of Total Phenolic Acid Content (TPA): Folin–Ciocalteau method was used to estimate total phenolic acid content of the Hb-01 sample [17]. Herbal preparation (Hb-01) (0.1mL) was mixed properly with 2ml of Na_2CO_3 solution (0.2%w/v) and incubated at room temperature for 2mins. Folin Ciocalteau reagent (0.1mL) was added and allowed to stand for 30mins at 25 ^{o}C. The absorbance was determined at 750nm. The total phenolic acid (TPA) content was determined using the Gallic acid calibration curve and the result expresses as gallic acid equivalents (mgGAE)/ml. The blank contains all the reagents and solvents except the sample. The procedure was done in triplicate.

Biological Evaluation:

Experimental Animals: Thirty Wistar rats of both sexes (120-180g) obtained from the Central Animal House University of Ibadan were used for this study. They were kept in rat cages at ambient temperature and humidity with 12 h light and dark cycle, fed with rat pellets (Ladokun feeds), and water was given *ad libitum*. The rats were allowed to acclimatize for two weeks. The animal study was done in accordance with the National Institute of Health Guidelines for Care of Laboratory animals of 1985. The rats were distributed into five groups (n=5; 3 males and 2 females) comprising three experimental test groups [half dose (HD), normal dose (ND), and double dose (DD)] and control groups comprising healthy control (HC) and positive control (acetylsalicylic acid) (PC).

Anti-Inflammatory Evaluation: The method described by Adedapo *et al*., in 2015 [18] using carrageenan induced hind paw oedema was used. The herbal preparation (Hb-01) based on the labelled dose at 1.67mL/kg, 3.33mL/kg and 6.66mL/kg (half, normal and double dose

respectively) and acetylsalicylic acid (reference drug) at 10mg/kg were administered orally using oral cannula. The doses were administered 1hour before the induction of oedema using right hind paw sub-plantar injection of carrageenan (0.1mL, 1%w/v) in saline. The negative control group was administered distilled water at the same volume. Linear paw circumference were measured using a micrometer screw gauge at 1, 2, 3, 4 and 5 h with increase in paw circumference as a measure of oedema, while reduction is a measure of anti inflammatory activity. The % inhibitions of the inflammation were determined.

Determination of the Body Weights: The weights of the rats were determined on the first day before treatment commenced, and at 7, 14, 21 and 28 days using animal balance.

Evaluation of the Biochemical and Haematological Parameters: At the end of the study period; 28days of Hb-01 administration (24hrs after the last dose), blood (3ml) was collected through the retro-ocular vein into labelled heparinised bottles, while another 5ml was collected into labelled non-heparinised bottles.

The heparinised blood samples were used to determine; haemoglobin concentration (Hb), packed cell volume (PCV), white blood cell count (WBC), red blood cell count (RBC), platelets levels and differential white blood cell count(neutrophils, lymphocytes, monocyte and eosinophils) [19].

On the other hand, non-heparinised bottles were kept in a slanting position to allow clotting before centrifuging at 3000 rpm for 10mins. The serum obtained was transferred into clean labelled bottles and stored at - 4 ^{o}C prior to analysis. The obtained sera were analysed for Alanine amino-transferase (ALT) and Aspartate amino-transferase (AST) using RANDOX enzyme kits.

Statistical Analysis: Data were expressed as mean ± SEM. Statistical analysis was carried out using one way analysis of variance (ANOVA) where necessary, $p< 0.05$ was considered as significant.

RESULTS: The herbal preparation was a bitter, golden brown liquid with pH 3.18. Thin layer chromatography using the three mobile phases gave varied number of spots with varied R_f values.

The levels of lead cadmium, iron and zinc were (0.053, 0.003, 1.190 and 4.190mg/L respectively. Phytochemical screening revealed the presence of anthraquinones, terpenoids, tannins, flavonoids, saponins, alkaloids and absence of cardiac glycosides. The total alkaloids, saponins and flavonoids contents were 2.45%w/w, 2.12%w/w and 49.45%w/w respectively.

Antioxidant evaluation using the DPPH inhibitory values (IC_{50}) obtained by linear regression analysis gave 2646.82±58.24µg/ml and 5.33±0.12µg/ml for the herbal formulation (Hb-01) and ascorbic acid respectively. Total phenolic acid content of the herbal preparation as determined from the gallic acid calibration curve ($y=0.009x–0.039$, $r^2=0.980$), was 0.0184 ± 0.0005 mgGAE/g. The herbal preparation (Hp–01) exhibited good anti-inflammatory effect at all doses tested on carrageenan induced-rat paw oedema which is comparable to acetylsalicylic acid ($p = 0.0536$) (**Fig. 1**).

A significant increase in the body weights was observed in all the treated groups except the negative control by the end of the study ($p<0.05$) (**Fig. 2**).

There was no significant difference in the haematological parameters; packed cell volume (PCV), erythrocyte counts, (RBC), leukocyte counts (WBC), haemoglobin concentration of the treatment groups given Hb–01 and healthy control (HN)($p>0.05$). For the differential leukocyte counts however, a significant decrease was observed in the lymphocytes and increase in neutrophils at normal dose (ND) when compared with healthy control group (HN) ($p< 0.05$) (**Table 1**).

A significant dose-dependent decrease in the AST and ALT was observed within the herbal product (Hb-01) treated groups which was higher than the negative control ($p=0.006$). However, the obtained results were much lower than the aspirin positive control ($p=0.007$) (**Table 2**). No significant difference was observed with the AST of herb treated group at normal dose and the healthy group. Also, the ALT values were significantly higher for the herb treated groups when compared with the healthy control ($p=0.0188$), while significantly lower than that of aspirin ($p=0.0139$) (**Table 2**).

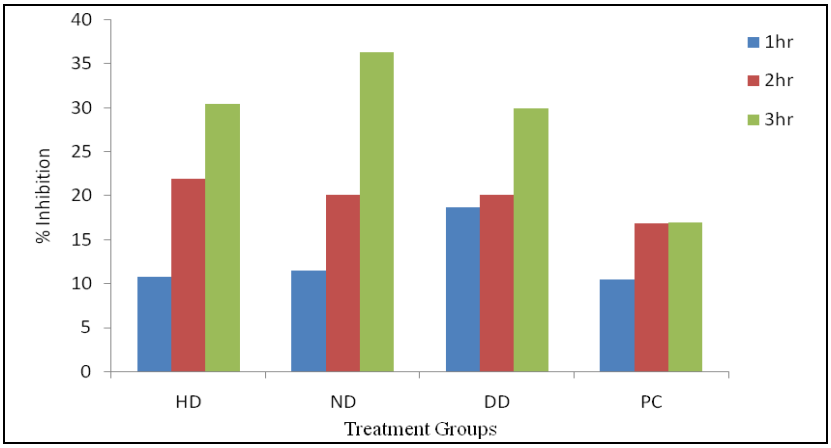

FIG. 1: PERCENTAGE INHIBITION OF CARRAGEENAN INDUCED-RAT PAW OEDEMA BY HB–01 AT 1H, 2H AND 3HR AFTER 2H MAXIMAL INFLAMMATION

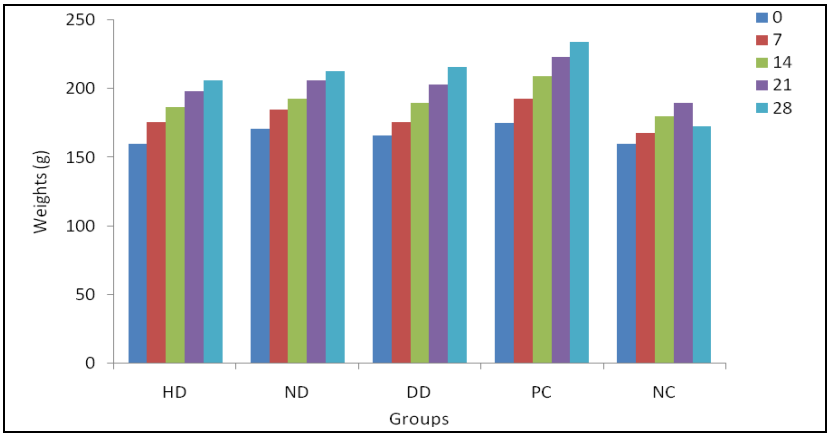

FIG. 2: BODY WEIGHT OF THE RATS AT SELECTED DAYS DURING HB-01 ADMINISTRATION TO WISTAR RATS FOR 28DAYS DURING ANTI-INFLAMMATORY EVALUATION

TABLE 1: HAEMATOLOGICAL PARAMETERS OBTAINED AFTER ADMINISTRATION OF DIFFERENT DOSES OF HB-01 TO WISTAR RATS FOR 28 DAYS

Treatment Group	PCV (%)	Hb(g/dl)	RBC ($\times 10^{12}$L)	WBC (/UI)	Platelet (/Ui)	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)
HN	44 ± 3.17	14.4 ±1.07	7.29 ±0.54	8830 ± 599	185600 ± 14854	7 1± 1.79	24 ± 1.90	3 ± 0.4
NC	43 ± 1.58	14.2 ±0.53	7.07 ±0.24	10420 ± 1890	210600 ± 27303	65 ± 3.01	30 ± 3.00	3 ± 0.51
PC	40 ±3.50	13.0 ±1.30	6.33 ±0.63	8140 ± 603	174400 ± 16064	64 ± 3.06	30 ± 3.37	2 ± 0.58
HD	46 ±2.08	15.1 ±0.73	7.83 ±0.38	8446 ± 1156	155200 ± 18271	63 ± 3.02	32 ± 2.94	2 ± 0.4
ND	52 ±0.81	14.4±0.26	7.26 ±0.14	9350 ± 1499	179200 ± 26490	59 ± 3.31	35 ±3.70	3 ± 0.68
DD	44 ± 1.88	14.8±1.07	7.51 ±0.54	7260 ± 599	144800 ± 14854	66 ± 1.79	29 ± 1.90	3 ± 0.4

Values expressed as Mean ±SEM

TABLE 2: LIVER ENZYMES (AST AND ALT) ACTIVITIES (U/L) OBTAINED AFTER ADMINISTRATION OF THREE DOSES OF HERBAL PREPARATION TO RATS FOR 28 DAYS

Group	AST Activities (U/L ± SEM)	ALT Activities (U/L ± SEM)
HN	2.37 ±1.41	23.08±4.10
PC	34.50 ±8.29	44.6±6.13
HD	7.00 ±1.15	37.3±1.02
ND	4.60 ±1.52	29.4±3.56
DD	3.60 ±1.55	27.0±1.35

Values expressed as Mean ±SEM

DISCUSSION: Inflammatory responses which can be divided into acute and chronic phases have been implicated in cardiovascular, rheumatic, arterio-sclerosis and cancer diseases [5]. Most anti-inflammatory drugs exhibit serious side effects and toxicities such as gastric intestinal ulceration and bleeding, renal damage, hypertension, hyper-glycaemia, which tend to discourage their use. The high cost of orthodox drugs, inadequate supplies, side effects and the belief that plants hold cure to many disease conditions have led to the reawakening of interest in the utilization of plants and plant products in recent years [20]. The use of herbal preparations is widespread in both developed and developing countries for varied reasons.

It is expedient for validation and provision of standardisation protocol for medicinal herbal preparations which use is on the increase as a result of poor economic strength of the population as done by many developed countries such as USA, China and India where there is quality control of such herbal products through provision of protocol for standardisation. In developing communities traditional medicine is of great importance to the healthcare needs of individuals and their communities hence, the need to verify some of the claims of such preparations.

This liquid herbal preparation formulary obtained from a practitioner is a multi-component aqueous extracts of six plants; *Allium cepa* (Onion), *Zingiber officinale* (Garlic), *Allium sativum* (Ginger), *Aloe vera*, *Andrographis paniculata* (King of bitters) and *Allium ascalonicum* (Shallot) leaves was prepared according to the instruction of the practitioner.

The herbal preparation (Hb-01) is an acidic golden brown liquid (pH 3.17) with pungent odour and slightly bitter taste which is as a result of the various plants composition. This shows the need for caution by ulcer patients; the herbal preparation should not be taken on an empty stomach.

The herbal preparation levels of lead, cadmium, iron, and zinc at 0.053mg/L,0.003mg/L, 1.190mg/L and 4.190mg/L respectively were within the acceptable limit for herbal product [21]. The presence of Zn at the highest concentration in the herbal preparation made it a good source of dietary minerals, since zinc is an essential trace mineral.

Phytochemical screening showed the presence of some important bioactive constituents such as anthraquinones, terpenoids, tannins, flavonoids, saponins and alkaloids with the absence of cardiac glycosides. Furthermore quantitative estimation of the total flavonoids, alkaloid, and saponins contents were found to be 49.45% w/w, 2.45%w/w and 2.12%w/w respectively. The presence of the above constituents might be responsible for the observed anti-inflammatory activity [15].

Inflammatory processes have been reported to induce oxidative stress with reduction in antioxidant capacity [5], thus, antioxidant capacity is associated with an anti-inflammatory potential which are desirable features for a bioactive compound. According to Sachan and Kumar in 2015 [22], therapeutic and benefits of traditional remedies are often attributed to a combination of active constituents. The DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging capacity is widely used as an indicator to determine the antioxidant potential of herbal preparations with percentage inhibition of DPPH and IC_{50} widely used to measure antioxidant and free radical scavenging power [23]. The DPPH inhibition for Hp – 01 at IC_{50} of 2646.82±58.23 µg/ml was low compared with the ascorbic acid (standard) of 5.33±0.115µg/ml indicating weak free radical scavenging activity; an indication of low antioxidant capacity, which is in agreement with the low total phenolic acid content (0.0184±0.0005 mgGAE/g).

Carrageenan-induced rat paw oedema is a form of acute inflammation as a result of the synthesis of mediators at the injured site. These mediators such as prostaglandins, histamine, bradykinins, leukotrienes and serotonin cause pain, fever and inflammation which are believe to be biphasic [24]. The first phase (2hour after carrageenan administration) involves the release of serotonin and histamine while the second phase (3 - 5h after carrageenan administration) is mediated by prostaglandins which are cyclooxygenase products [25]. Inhibition of these mediators from reaching the injured sites or exercising their pharmacological effects prevents inflammation [26].

The carrageenan – induced rat paw oedema showed a significant inflammation of the rat paw in the early hours of the study as expected while inhibitory effect was observed from 3, 4 and 5 hour by the Hb–01 (at all doses tested) and acetylsalicylic acid. The anti-inflammatory effect of Hb-01 was not dose dependent (**Fig. 1**), even at normal dose (ND) the formulation was able to suppress inflammation effectively in comparison to inhibitory effect of double dose (DD) of the formulation. The anti-inflammatory activities of Hb-01 at the three doses used in this study though higher than acetylsalicylic acid were significant ($p > 0.05$).

Flavonoids which have been associated with anti-inflammatory activities are known to target prostaglandins involved in late phase of acute inflammation [27]. Hence the high flavonoids content (49.45%w/w) might have contributed to the anti–inflammatory activity of Hp-01 formulation.

Although, an overall increase in weights was observed with the herbal formulation and acetylsalicylic acid compared with the healthy group; the increase in weight was not significant ($p > 0.05$) (**Fig. 2**). However, no significant effect was observed in haematological parameters (PCV, RBC, WBC, Hb and platelets) of the Hb-01 treated rats when compared with the healthy control (**Table 1**).

Serum marker enzymes are biochemical parameters associated with health indices and are of diagnostic significance in routine clinical evaluation of the state of health [28]. Alanine amino-transaminase (ALT) and Aspartate amino-transaminase (AST) are largely used in the assessment of liver damage by xenobiotics or any other hepatotoxin [29]. The increased release of ALT and AST into the blood by the liver and heart causing elevation in their plasma concentrations is an indicator of liver and heart damage [30].

The non-significant reduction in AST with increase in dose observed in the Hb-01 treated group ($p=0.2598$) were significantly higher than the healthy control group ($p=0.006$) and lower than the positive control group ($p=0.007$) is quite interesting. Ironically, the increased AST by the herbal product at half dose (HD) was not significant ($p=0.0635$) when compared with healthy group. Similar, the ALT values were significantly higher for the herb treated groups when compared with the healthy control ($p=0.0188$), while significantly lower than that of aspirin ($p=0.0139$) (**Table 2**). The AST and ALT levels for the normal dose were not significantly different from that of the healthy group ($p > 0.05$).

However, elevation of liver enzymes observed with the acetylsalicylic acid group (PC) observed in this study indicating a predisposition to liver damage is in agreement with some earlier reports on elevation of AST and ALT with modest dose of acetylsalicylic acid [31]. On the other hand, the reduction in the liver enzyme levels in the inflammatory condition at double dose is a pointer to possible hepatoprotective effect of the herbal preparation at high dose. Similar study on an herbal mixture containing three plants used as anti-inflammatory agents among Bedouin people of Saudi Arabia was reported to possess significant anti-inflammatory activity [32]. Therapeutic benefits of traditional remedies are often attributed to a combination of active constituents [25].

CONCLUSION: The outcome of this study showed that the combination of plants extracts corroborates the folkloric combination of the anti-inflammatory plants. The herbal preparation was found to be safe for consumption as there was no reported deleterious effect on the biochemical and haematological indices of the animal model used. Furthermore, the comparative anti-inflammatory activity of the herbal formulation (Hp - 01) with acetylsalicylic acid reported in this study justifies the use of the herbal formulation (Hb–01) in inflammatory disorders. This also showed that Hb-01 has potential for development as anti-inflammatory agent which could be exploited to isolate potential lead compounds for new drug entities.

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CONFLICT OF INTEREST: The authors hereby declare no conflict of interest in the publication of this paper.

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