In Vivo Analgesic and Anti-Inflammatory Effects of Some Ethanol Leaf Extracts of Antisickling Remedy and its Polyherbal Combination in Experimental Animals.

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Abstract: Resulting from antisickling properties of the three medicinal plants and their polyherbal extracts, this study therefore assessed their analgesic and anti-inflammatory activities in mice and rats since sickle cell disorder symptoms are characterized by painful crises with remarkable inflammatory reactions. In this present study, the analgesic potential of ethanol leaf extracts of Piper guineense (P), Gongronema latifolium (G), Cymbopogon citratus (C) and their polyherbal combination (PGC) was centered on the hot plate; tail immersion, analgesiometer, and acetic acid induced writhing assays. Carrageenan-induced paw oedema in animal models was used for the anti-inflammatory assay. The extracts exerted central and peripheral acting mode of action with highest protective potentials at 250 mg/kg (hot plate), 500 mg/kg (both tail immersion and analgesiometer) and dose-dependent action (hot plate and acetic acid induced writhing). Both Gongronema latifolium ethanol leaf extract (GLLET) and PGC exhibited dose-dependent activity in inhibiting inflammation while Piper guineense ethanol leaf extract (PGLET) and Cymbopogon citratus ethanol leaf extract (CYCLET) exhibited inhibition of inflammation at all doses with some discrepancy at certain hours in 250, 500 and 1000 mg/kg of the rat body weight. This present study revealed that the PGLET, GLLET, CYCLET and PGC possess potential analgesic and anti-inflammatory activities. The antisickling polyherbal PGC extract regulate pain and inflammation for the first time confirming folkloric used in management of sickle cell disease. Thus, this study demonstrates that PGC can be used as rich source of analgesic and anti-inflammatory drugs.

Keywords: antisickling, polyherbal, analgesic, anti-inflammatory, carrageenan, Indomethacin

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I. Introduction

Pain is the hallmark of Sickle cell disorder (SCD) with the acute sickle cell painful episode (painful crisis) causing more than 90% of hospital admissions among adult patients leaving with SCD [1]. It is a long – lasting problem for people leaving with SCD, which may be precipitated by other components of symptomatic effects. Major fundamentals for the effective management of sickle cell pain relates to the patient, the pathophysiology of the disease and the pharmacology of analgesics. Sickle cell disease (SCD) is also a chronic inflammatory state in which leucocyte-endothelium elicit interactions play a role in vaso-occlusion. Anti-inflammatory agents possess the ability to inhibit the cyclooxygenase COX-1and COX-2 pathway of arachidonic acid metabolism which produces prostaglandins. Analgesic and anti-inflammatory agents are therefore used to counter pain and inflammation [2].

Many of African medicinal plants have demonstrated analgesic and anti-inflammatory activities in animals’ studies. Both pain and inflammation constitute major crisis in patients living with sickle cell disease, thus it’s desirable that antisickling agents should not only serve as agents to manage the disorder but deal with pain and inflammation resulting from the crisis. However, previous studies revealed that the three plants (Piper guineense, Gongronema latifolium and Cymbopogon citratus) used for polyherbal combination serves as analgesic and anti-inflammatory agents but there is no report on analgesic and anti-inflammatory of the antisickling polyherbal product. The leaves of Piper guineense are used as a spice to flavor meat preparation, fresh pepper soup and consumed as vegetables in meals. They are considered as aperitif, antibacterial activity, antimicrobial, carminative and euphelic [3]. The leaves are also used to treat respiratory infections, female
inertility and low sperm count in male, relieve flatulence, rheumatism, and syphilis [4]. In Nigeria, the leaves of *G. latifolium* are used as vegetables in the preparation of soups to which they add a bitter-sweet flavor [5]. *G. latifolium* also possess moderate to promising antioxidant, anti-inflammatory, hepatoprotective, antiplasmodial, antiasthmatic, anti-sickling, anti-ulcer, analgesic, antipyretic, gastrointestinal relaxing, laxative and stomachic activities [6,7]. Different studies of *Cymbopogon citratus* have also shown that the leaves demonstrated antioxidant, anti-inflammatory, cytoprotective, anti-microbial, anti-plasmodial, anti-fungal, anti-diarrhoeal, anti-mutagenic, anti-malaria, antinoceceptive, anti-hepatotoxic and anti-obesity effects [8-10]. This present study therefore for the first time ascertained scientific evidence to the folklore uses of polyherbal combination in the treatment of pain and inflammation in sickle cell patient using various animal models.

II. Materials And Methods

2.1 Plant Materials

The plant materials were collected on the basis of information obtained from ethnomedicinal survey. Fresh leaves of *Piper guineense* Schumach & Thonn and *Gongronema latifolium* were collected in June, 2016 from medicinal garden of Department of Pharmacognosy, Faculty of Pharmacy in the Teaching and Research farm while *Cymbopogon citratus* was collected along Road 18 from Obafemi Awolowo University Senior Staff quarters, Ile-Ife, Osun State, Nigeria. Identification and authentication of the plants were carried out by Prof H. C. Illoh and voucher specimens were deposited at the Herbarium unit of Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Edo State, Nigeria for reference purposes. The herbarium voucher specimen numbers include UBHdt/SN/095, UBHdt/SN/154 and UBHdt/SN/129 for *Piper guineense* Schumach. & Thonn, *Gongronema latifolium* Benth and *Cymbopogon citratus* (DC.) Stapf respectively.

2.2 Extraction of plant samples

Fresh dried powdered leaves (500 g) each were separately extracted by cold maceration in 70% (v/v) ethanol for 48 h on a mechanical shaker. Also, fresh dried powdered leaves of *Piper guineense*, (P) *Gongronema latifolium* (G) and *Cymbopogon citratus* (C) were mixed together in same proportion forming polyherbal combination PGC. The powdered PGC (1.5 kg) were extracted using 70% ethanol separately. The extracts were then filtered and evaporated to dryness in vacuo at 50°C. The percentage yields of each extracts were determined. The ethanol extracts were stored in sample bottle until use.

2.3 Drugs and chemicals

All the drugs and chemicals used in this present study were of international standards. Normal saline, Aspirin*®* and indomethacin® were purchased at Obafemi Awolowo University campus pharmacy in Ile-Ife while ethanol and acetic acid were purchased from Sigma-Aldrich (USA). Normal saline is used for negative control while Aspirin and indomethacin are used as positive control.

2.4 Animals

Swiss albino mice of both sex weighing 18-30g were used for analgesic test and Wistar rats of both sex weighing 150-260g were used in the anti-inflammatory study. Animals were kept and maintained under adequate laboratory condition with 12h/12h light and dark cycles and were fed with commercial pelleted rats and water ad libitum as a standard diet. Furthermore, university animal ethical committee approved the experimental protocol according to standard principles for laboratory animal use and care (UBER – 18-102).

2.5 Acute Toxicity Test (LD<sub>50</sub>)

The acute toxicity test of the extracts was determined in mice with little modifications [11,12]. A single oral dose of 2000 mg/kg extract was administered to a group of six healthy mice. The animals were observed for any gross impact for the first 4h and mortality after 48h. The mortality or lethality was counted after 48h and the Lethal Dose (LD<sub>50</sub>) was determined.

2.6 Assessment of Analgesic Activity

This was carried out using hot plate, tail immersion, analgesiometer and Acetic Acid Induced Writhing tests with little modification of previous method [13].

2.6.1 Hot Plate Test

White mice were divided into groups of five, three groups received 250, 500 and 1000 mg/kg doses of each extract, a fourth group was given Aspirin (100mg/kg) and one other group served for the negative control (Normal saline 0.9%). After one hour, the animals in all groups were individually exposed to the hot plate. The time taken in seconds for fore paw licking or jumping was taken as reaction time.
2.6.2 Tail Immersion Test
The test agents were administered as in hot plate assay above. One hour after administration of the plant extracts or the standard drugs, the tail (up to 5 cm) of each mice was immersed in hot water at 50 ± 1°C (in a 1000 ml beaker). The reaction time (seconds) for both the treated animals and the control was measured by stop watch as the time when the animal withdrew their tails completely from the hot water in the beaker. The mean reaction time was calculated for each plant extract dose, aspirin and normal saline.

2.6.3 Analgesiometer Test
Analgesic activity was determined by placing the left hind paw of the rat on a plinth under a cone-shaped pusher of the Ugo Basile analgesiometer. It generates a linearly increasing mechanical force or pressure on hind paw. As the applied pressure increases, it gets to a point where the animal struggles to free its paw. The strength at which each rat withdrew its paw was recorded and considered as indicative of pain. The stimulus was terminated and force threshold was read in seconds. The groups administered with tested extracts were compared to control and standard drug groups.

2.6.4 Acetic Acid Induced Writhing Test
All the three plant and its Polyherbal combination were administered intraperitoneally using different doses as defined in design study, one hour prior to administration of 0.1 ml acetic acid (1%). Animals were observed individually and the number of writhes was counted for 20 min commencing 5 min after injection of acetic acid. The significant reduction in a number of writhes of treated groups was compared to that of the control and standard groups. The percentage inhibition of abdominal constrictions was calculated using the following formula.

\[
\text{Inhibition} (%) = \frac{\text{Mean No. of writhes (Control)} - \text{Mean No. of writhes (Test)}}{\text{Mean No. of writhes (Control)}} \times 100
\]

2.7 Evaluation of Anti-inflammatory Activity

2.7.1 Carrageenan – Induced Rat Paw Oedema
The anti-inflammatory screening was evaluated using the rat paw oedema method [14, 15]. Acute inflammation is measured in terms of change in volume of the rat hind paw induced by sub plantar injection of carrageenan. Animals (n = 5 per group) received the graded doses of the extracts; the standard was indomethacin 100mg/kg. The negative control animals received 0.2ml of the vehicle. Oedema was induced one hour later with carrageenan (0.2ml) injected into the supplanter region of the right-hand paw of the animals. The volume of distilled water displaced by the treated paw was measured before and 1, 2, 3, 4 and 5 hours after the induction of oedema. Inflammation was assessed as the difference between the zero-time volume of the treated paw (\(V_0\)) and the volume at the various time (\(V_t\)) after the administration of the phlogistic agent.

\[
\% \text{ Inhibition} = 100 \left(1 - \frac{(a-x)}{b-y}\right)
\]

Where
\[
\begin{align*}
& a = \text{Mean paw volume of treated rats at various time after carrageenan injection.} \\
& x = \text{Mean paw volume of treated rats before carrageenan injection.} \\
& b = \text{Mean paw volume of control rats at various time after carrageenan injection.} \\
& y = \text{Mean paw volume of control before carrageenan injection.}
\end{align*}
\]

2.8 Statistical Analysis
All values are expressed as mean ± standard error of the mean (SEM) and data was analyzed by one-way analysis of variance (ANOVA) using statistical package for social science (SPSS 20) followed by Turkey post-hoc test. The analysis was performed with 95% confidence interval and the value, p<0.05, is considered as statistically significant.

III. Results

3.1 Acute Toxicity Test
Neither mortality nor signs and symptoms of toxicity were observed in all the extracts and their polyherbal combination at the limit dose of 5000mg/kg. They can then be considered safe according to Locke’s guidelines. The acute toxicity study of ethanol leaf extracts of all the three antisickling plants and their polyherbal combination PGC up to the limit dose of 2000 mg/kg body weight of the animals used did not produce any signs and symptoms of toxic effects or mortality after 48hrs of oral administration. The result of the acute toxicity test showed that all the three plants and the combined extract PGC appeared to be safe.
3.2 Analgesics Assay of Anti-sickling Polyherbal Formulation

3.2.1 Hot Plate Test

The result of the hot plate thermal induced pain carried out on the three individual plants and their polyherbal combination are shown in figures 1. Fig. 1 showed the analgesic activity of ethanol leaf extract of *Piper guineense* (PGLET), *Gongronema latifolium* (GLLET) *Cymbopogon citratus* (CYCLET) and polyherbal PGC on the hot plate test. PGLET exhibited a dose-dependent analgesic activity (13.8 to 28.6 secs) and also compared favourably with standard drug Aspirin (10.21 secs), *Cymbopogon citratus* (CYCLET) showed analgesic though not dose-dependently (21.6, 13.2, 19 secs for the respective doses) but compared favourably with the standard drug while *Gongronema latifolium* (GLLET) was active at 500mg/kg and 1000mg/kg but not at 250mg/kg. The mean latency time of pain responses to thermal stimuli in the hot plate for the polyherbal PGC was 35.4, 23.2 and 25.2 secs for respective doses. The polyherbal PGC extract exerts good analgesic effect at 250 and 1000 mg/kg with the highest activity at the lowest dose of 250 mg/kg when compared with control and standard drug. There was no significant difference for all the treated doses and the control.

![Hot Plate test](image)

Fig. 1: Graphs showing the effects of *Piper guineense*, *Gongronema latifolium*, *Cycmbopogon citratus* and polyherbal PGC extracts on hot plate thermal induced pain in mice. PGLET= *Piper guineense* leaf ethanol, GLLET= *Gongronema latifolium* leaf ethanol, CYCLET= *Cymbopogon citratus* leaf ethanol and PGC= combination of *Piper guineense*, *Gongronema latifolium* and *Cymcbopegon citratus* in ratio 1:1:1. Values are expressed as mean ± SEM (n=5). No significant effect (p < 0.001)

3.2.2 Tail Immersion test

The result of the analgesic effect of all the three antisickling plants and polyherbal extract (PGLET, GLLET CYCLET and PGC) to thermal stimuli by tail immersion are represented in Fig. 2. PGLET exhibited low (2.0 secs) and non-dose-dependent analgesic activity against thermally induced response in tail withdrawal latency as compared with Aspirin (5.11 secs). Both GLLET and CYCLET demonstrated dose-dependent analgesic effect with the highest analgesic potential of 4.2 secs (GLLET) and 2.6 secs at 1000 mg/kg body weight of animals. The PGC extract exhibited analgesic activity at all doses with highest analgesic effect at 500 mg/kg (2.2 secs) while at 250 mg/kg (1.6 secs) the extract demonstrated poor analgesic effect as compared with the standard drug (5.11 sec). The analgesic activity was very minimal with the tail immersion method (2.2 secs as against 1.77 secs for negative control). However, Aspirin the positive control produces significant analgesic effect in tail withdrawal from hot water than all the extracts and the normal saline.
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Fig. 2. Graphs showing the effects of *Piper guineense*, *Gongronema latifolium*, *Cymbopogon citratus* and polyherbal PGC extracts against thermally induced pain by tail immersion test in mice.

PGLET = *Piper guineense* leaf ethanol, GLLET = *Gongronema latifolium* leaf ethanol, CYCLET = *Cymbopogon citratus* leaf ethanol and PGC = combination of *Piper guineense*, *Gongronema latifolium* and *Cymbopogon citratus* in ratio 1:1:1.

Values are expressed as mean ± SEM (n=5). No significant difference (p < 0.001)

3.2.3 Analgesiometer Test

The result of the effect of the extracts PGLET, GLLET, CYCLET and PGC on mechanically induces pain test using analgesiometer is given in figure 3. In this model, all the extracts used exhibited analgesic potentials though not dose-dependently. PGLET demonstrated good analgesic potential at both 250 mg/kg (8.74 ± 1.89 secs) and 1000 mg/kg (12.2 ± 2.84 secs) as compared to aspirin (7.7 ± 2.22 secs) and normal saline (5.7 ± 2.27 secs) with the highest analgesic activity at 1000 mg/kg of body weight of the animal. GLLET indicated maximum analgesic ability at 500 mg/kg (11.1 ± 2.12 secs). CYCLET also possess a non-significant analgesic effect with the maximum effect observed at all doses in comparison with aspirin. PGC demonstrated an analgesic potential to reduce pain exerted on the tail of the mice at all doses when compared with aspirin and normal saline. The extract indicated maximum activity at 500 mg/kg (15.4 sec).

Fig 3: Graphs showing the effects of *Piper guineense*, *Gongronema latifolium*, *Cymbopogon citratus* and polyherbal PGC extracts against mechanically induced pain by analgesiometer on tail of mice

PGLET = *Piper guineense* leaf ethanol, GLLET = *Gongronema latifolium* leaf ethanol, CYCLET = *Cymbopogon citratus* leaf ethanol and PGC = combination of *Piper guineense*, *Gongronema latifolium* and *Cymbopogon citratus* in ratio 1:1:1. Values are expressed as mean ± SEM (n=5). No significant effect (p < 0.001)
3.2.4 Acetic Acid Induced Writhing Test

The result of the analgesic ability of the PGLET, GLLET, CYCLET and antisickling polyherbal PGC extracts by the acetic acid writhing method is shown in Figure 4. All the extracts showed dose dependent activities with the highest activity observed at 1000 mg/kg. The anti-sickling polyherbal combination PGC extract was highly significant at all doses when compared with that of negative control (Normal saline).

Fig. 4: Graphs showing the effects of Piper guineense, Gongronema latifolium, Cycmbopogon citratus and polyherbal PGC extract on acetic acid induced writhing in mice.
PGLET= Piper guineense leaf ethanol, GLLET= Gongronema latifolium leaf ethanol, CYCLET= Cymbopogon citratus leaf ethanol and PGC= combination of Piper guineense, Gongronema latifolium and Cymbopogon citratus in ratio 1:1:1. Values are expressed as means ± SEM (n = 5). Non significant at p < 0.0001.

3.3 Evaluation of anti-inflammatory assay

The result of anti-inflammatory assays of individual extracts and their polyherbal formulation were shown in Table 1. The PGLET extract exhibited anti-inflammatory activity in all the doses, though with some discrepancy at the third hour in 1000mg/kg significantly at p≤ 0.0001. The highest inhibitory activity was observed in 1000 mg/kg (98.44±0.02 %). GLLET inhibited inflammation remarkably and dose-dependently. The activity of GLLET was compared favorably with the indomethacin with the highest inhibitory activity of 89.06 ± 0.08%). Anti – inflammatory activity of Cymbopogon citratus (CYCLET) exhibited increasing inhibitory activity against paw oedema at 250 and 500mg/kg, the highest activity being at the latter dose but did not compare favorably with standard drug Indomethacin. The combined extract PGC exhibited dose-dependent activity in inhibiting the paw oedema, though could not compare favourably with standard drug Indomethacin. The activity of antisickling polyherbal formulation increases as the dose increased with the highest percentage inhibition at 1000 mg/kg body weight of rats of 95.31% while indomethacin had 90%.
Table 1: Anti-inflammatory activities of PGLET, GLLET, CYCLET and PGC by carrageenan induced paw edema in Wistar rats.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg)</th>
<th>Change in paw volume ± SEM (mm)</th>
<th>Inhibition %</th>
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<td>Normal values</td>
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<td>1 h</td>
<td>1.2 ± 0.09</td>
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<td>1 h</td>
<td>1.6 ± 0.04</td>
<td>0.8 ± 0.05</td>
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<td>3 h</td>
<td>1.2 ± 0.05</td>
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<td>4 h</td>
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<td>Indomethacin</td>
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<td>4 h</td>
<td>1.3 ± 0.02</td>
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<td>5 h</td>
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<td>0.8 ± 0.03*</td>
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<tr>
<td>PGLET</td>
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<td>0.6 ± 0.02</td>
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<td>1 h</td>
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<td>GLLET</td>
<td>500</td>
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<td>CYCLET</td>
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<td>PGC</td>
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Values of the results are reported as mean ± SEM for group of five animals (n=5). The data was analyzed by one-way repeated measures Anova analysis of variance using Turkey’s and Dunnett’s multiple comparison tests. 

* = *** p < 0.0001, ** = ** p < 0.001 and * = *p < 0.05 as significant values from control.

IV. Discussion

In addition to anaemia, the leading symptom of sickle cell disease is pain which occurs as result of deformation of the shape of red blood cell leading to blockage in vessels and bones. This acute sickle cell pain crisis causes more than 90% of hospital administrations among patients who have sickle cell disease. Analgesics such as aspirin and paracetamol and sometimes, the narcotics like morphine, are used to reduce or remove this pain. The various classes of conventional analgesics vary from gastrointestinal disturbances to central nervous system reactions. Efforts are therefore on to find alternative drugs for this attendant conditions involved in sickle cell disease. This present study evaluated the analgesic effects of the ethanol extracts of P. guineense, G. latifolium, C. citratus and their polyherbal combination (PGC) using four analgesics tests such as hot plate, tail flick, analgesiometer and acetic acid writhing. All the extracts gave a analgesic effects centrally and peripherally. This result is in line with previous studies which proved that analgesic mechanism of action of the extracts of Scaphium lychnophorum demonstrated is peripherally and centrally mediated [16]. The tail flick test demonstrated minimal analgesic activity. The tail flick method of analgesia is very active in estimating the efficacy and potency of centrally acting analgesic drugs and is minimal in all the extracts evaluated. Centrally acting anti-nociceptive drugs are known to elevate the pain threshold of rodents to pressure and heat [17,18]. Tail immersion method which was used for evaluating centrally acting analgesic effects of drugs showed no increase in latency. Earlier studies indicated that analgesic effect against thermal noxious stimuli may be elicited through opioid receptors or through modulation of several neurotransmitters involved in relevant phenomena [19]. Pain induced by the analgesiometer provides a model for the study of non-inflammatory pain and centrally stimulated [20]. The extracts demonstrated the ability to reduce pain induce by pressure using analgesiometer which was in line with result previously obtained [21].

The analgesic activity evaluated by acetic acid writhing method had been reported to be effective for investigation of peripheral analgesic activity and acted as a chemical pain model [22]. The induction of pain occurs by the release of endogenous substances as well as other pain mediators such as arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis [23]. The dose-dependent inhibition of writhing exhibited by the extracts in this present study indicates a peripherally mediated analgesic activity based on the association of the model with stimulation of peripheral receptors. It was discovered that the pain activity of acetic acid may be due to the release of cytokines, such as TNF-C, interleukin-1N and interleukin-8, by resident peritoneal macrophages and mast cells [24]. Therefore, the analgesia action of the ethanol extracts of P. guineense, G. latifolium, C. citratus and their polyherbal combination (PGC) in the acetic acid writhing test may be due to inhibition of the release of these endogenous mediators by resident peritoneal cells. It was found that all the extracts significantly inhibited the acetic acid writhing test in a pronounced manner and compared favourably
with aspirin. This result is similar to previously obtained where plant extracts significantly inhibit the mouse acetic acid writhing action [25].

Although the ethanol extracts demonstrated analgesic activities in hot plate, tail immersion and analgesiometer but not as was pronounced in the acetic acid-induced method. This may suggest that the extracts act peripherally. Earlier report confirmed that Citral obtained from lemongrass, has been reported to possess anti-nociception properties by using acetic acid induced writhing and nociception induced by formalin. It was concluded that citral is capable of exhibiting peripheral antinociceptive property by inhibiting writhing and nociception [26].

**Anti-inflammatory Assay**

The carrageenan induced paw edema model has been reported as the most widely used method for anti-inflammatory effects of natural products which subsequently involves several mediators [27]. Edema formation in the rat paw has been postulated to be of three phases and these include; the initial phase (between 0 and 1.5 h) which is attributed to the action mediators such as histamine and serotonin; a second phase (1.5-2.5 h) mediated by bradykinin and a third phase (2.5-6 h) mediated by prostaglandins [28]. In this present study, the anti-inflammatory effect of the extracts showed a significant activity up to 5h. All the extracts demonstrated a pronounced reduction in the rat paw edema which could be due to reduction in the histamine and serotonin and reduces vascular permeability in carrageenan induced peritonitis [29]. Meanwhile, histamine as mediators of inflammation causes vasodilation and increase the vascular permeability [30]. The result obtained could also be as result of the effect of non-steroidal anti-inflammatory agents which primarily inhibit the cyclo-oxygenase involved in prostaglandin synthesis [31]. The result obtained in this study gives scientific basis for using ethanol extracts of *P. guineense*, *G. latifolium*, *C. citratus* and their combined extracts (PGC) as anti-inflammatory agents. This corroborates the reports of other authors [32].

**V. Conclusion**

This results of the ethanol leaf extracts of *Piper guineense*, *Gongronema latifolium*, *Cymbopogon citratus* and Polyeherbal PGC combination possess analgesic and anti-inflammatory properties in mice and rats. Furthermore, the preliminary study of polyherbal PGC establishes the scientific evidence on the rationale use as analgesics and anti-inflammatory agents for the first time. However, further study could be investigated for possible mechanism of actions of the extracts and isolation of active constituents responsible for such activities.

**References**


In Vivo Analgesic and Anti-Inflammatory Effects of Some Ethanol Leaf Extracts of Antisickling Remedy and Its Polyherbal Combination In Experimental Animals.


