Anti-inflammatory, Analgesic, and Cytotoxic Effects of The Phytexponent: A Polyherbal Formulation

Halvince O. Odira¹, Simon O. Mitema¹, Isaac M. Mapenay¹, and Gervason A. Moriasi²

Abstract
The Phytexponent is used to treat pain and inflammation in complementary and alternative medicine practices; however, empirical data supporting its pharmacological efficacy and safety is scanty, hence the present study. We used the carrageenan-induced paw oedema and the acetic acid-induced writhing techniques to determine the anti-inflammatory and analgesic efficacies, respectively, of the Phytexponent in Swiss albino mice models. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay technique was used to investigate the in vitro cytotoxic effects of the Phytexponent in the Vero E6 cell line. The Phytexponent exerted significant ($P < .05$) anti-inflammatory effects in the carrageenan-induced paw oedema mouse model in a dose- and time-dependent manner, with significantly higher efficacy at 250 mg/Kg BW, than indomethacin (4 mg/Kg BW), in the first, second, and third hour ($P < .05$). Besides, the Phytexponent significantly reduced the acetic acid-induced writhing frequency in mice ($P < .05$), in a dose-dependent manner, depicting its analgesic efficacy. Notably, the Phytexponent (at doses: 125 mg/Kg BW and 250 mg/Kg BW) exhibited significantly higher analgesic efficacy than the Indomethacin ($P<.05$). Moreover, the Phytexponent was not cytotoxic to Vero E6 cells (CC50 >1000 µg/ml) compared to cyclophosphamide (CC50 = 2.48 µg/ml). Thus, the Phytexponent has significant in vivo anti-inflammatory and analgesic efficacy in mice models and is not cytotoxic to Vero E6 cell line, depicting its therapeutic potential upon further empirical investigation.

Keywords
carrageenan-induced paw oedema, acetic acid-induced writhing, Vero E6 cell line

Received August 3, 2021. Received revised January 1, 2022. Accepted for publication February 6, 2022.

Introduction
Inflammation is a response of a tissue to a noxious stimuli, such as physical injury, irritant agents, and pathogens, which is characterized by increased vascular permeability, changes in blood flow, and migration of leucocytes to the affected sites.¹ Pain refers to an unpleasant emotional and sensory experience that results from tissue damage and acts as a signal to warn against further insults.² Pain, fever, and inflammation are associated with a myriad of pathological processes in the body.³ There are various anti-inflammatory and analgesic drugs for the treatment of inflammation and pain;⁴⁵ however, they are inaccessible and unaffordable especially in low income and remote settings, they are of low efficacy and cause adverse effects with life-threatening consequences.⁶⁷ In this regard, focus has shifted to investigating natural products, especially medicinal plants, as one of the most promising therapeutic agents for inflammatory diseases.⁸ Research data shows that plant-derived natural products are a bulwark of future drug discovery, especially for the treatment of inflammation and pain.⁹¹¹ This is encouraging, considering

¹ Department of Public Health, Pharmacology, and Toxicology, College of Veterinary and Agricultural Sciences, University of Nairobi, Nairobi, Kenya
² Department of Medical Biochemistry, School of Medicine, College of Health Sciences, Mount Kenya University, Thika, Kenya

Corresponding Author:
Halvince Odira, Department of Public Health, Pharmacology, and Toxicology, College of Veterinary and Agricultural Sciences, University of Nairobi, P.O. Box 29053-00625, Nairobi, Kenya. Email: halvincedera@yahoo.com

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access page (https://us.sagepub.com/en-us/nam/open-access-at-sage).
that more than 80% of the human population in third world countries, especially in Africa, depend on traditional medicine for healthcare needs.\textsuperscript{12} In Kenya, various communities manage pain, fever, and inflammation using plant-derived remedies, as part of their traditional and complementary medicine practices\textsuperscript{13–19}.

Even though medicinal plants have extensive and longstanding utilisation in alternative and complementary therapy, various concerns regarding their safety have been raised.\textsuperscript{20} For instance, most countries lack legislative guidelines for the practice of traditional medicine, thus allowing unscrupulous practitioners to thrive.\textsuperscript{21} Additionally, there is scanty data on dosage regimens, herb-herb and herb-drug interactions, and associated effects to effectively guide prescriptions.\textsuperscript{22} Furthermore, the lack empirical data on safety and toxicity profiles of many medicinal plants further cripples the confidence accorded to herbal medicine; hence, it is imperative to evaluate toxicity and safety of herbal preparations used to manage various diseases to avert the development of undesirable effects and fatalities.\textsuperscript{20,22}

The Phytexpenton is used in complementary and alternative medicine, in Kenya, and in many other countries, to manage inflammation and pain, and associated syndromes, with an appreciable level of efficacy. Some of the plants used to formulate the Phytexpenton are used in traditional medicine since they possess various pharmacologic activities against a variety of disease conditions. For instance, extracts of \textit{Viola tricolor} have been traditionally used to treat inflammatory lung disease and skin ailments, such as ulcers, itching, scabs, psoriasis, and eczema.\textsuperscript{23} Besides, \textit{Echinacea purpurea} is commonly used to alleviate common cold symptoms, and has been shown to possess anti-inflammatory and immunostimulatory properties.\textsuperscript{23} Additionally, \textit{Allium sativum} (Garlic) is widely used as a food ingredient, and its over 200 phytochemicals are used to treat various conditions that are associated with inflammation, including some types of cancer, and as an aphrodisiac.\textsuperscript{23}

Recently, Moriasi \textit{et al.}\textsuperscript{24} demonstrated significant \textit{in vitro} anti-inflammatory and antioxidant activities, and the presence of bioactive phytochemicals with diverse pharmacologic effects, including anti-inflammation and antioxidation. However, there is a scarcity of documented studies on the \textit{in vivo} efficacy of this polyherbal product, its mode(s) of action in various disease states, its toxicity, and safety.

Therefore, this study was designed to investigate the \textit{in vivo} anti-inflammatory, analgesic, and cytotoxic effects of the Phytexpenton formulation of \textit{Viola tricolor}, \textit{Echinacea purpurea}, \textit{Allium sativum}, \textit{Matricaria chamomilla}, and \textit{Triticum repens}, as a potential alternative source of affordable, accessible, potent, and safe analgesic and anti-inflammatory lead compounds for drug discovery and development.

\section*{Materials and Methods}
\subsection*{The Source of the Phytexpenton Polyherbal Formulation}

The Phytexpenton formulation (Pharmapath 27, Belgium; LOT NO:17E19) was purchased from a local vendor and stored at room temperature according to the manufacturer’s guidelines, and retrieved only when required.

\subsection*{Experimental Animals}

Swiss-albino mice weighing 24 ± 1 g, and aged between four and five weeks old were used in this study. The experimental animals were housed under standard conditions (Temperature: 25 ± 2 °C; Relative humidity: 55%-61%; 12 hours of dark and 12 hrs of light cycle), in polypropylene rectangular cages measuring 30 cm × 20 cm × 13 cm, in which soft wood shavings were added as bedding material. The mice were fed on standard laboratory rodent food (pellets) and clean water \textit{ad-libitum}, and maintained at natural day-night cycle. The experimental mice were acclimatised to the laboratory settings for 72 hours prior to experimentation.

\subsection*{Determination of \textit{in Vivo} Anti-Inflammatory Activity of the Phytexpenton Prepartion}

The Carrageenan-induced paw oedema technique of Winter \textit{et al.}\textsuperscript{25} was adopted to investigate the antiinflammoty effects of the Phytexpenton with minor modifications. Briefly, experimental mice were randomised into 8 groups comprising of 5 mice per group. The normal control group mice [A] were orally administered with 10 ml/Kg BW of normal saline. The negative control group mice [B] were given normal saline (10 ml/Kg BW) orally, and after 30 minutes, they were injected with 100 µl of 1% Carrageenan (Sigma-Aldrich, Germany) via the subplantar region of the right hind paw (s.p). The positive control group mice [C] received 10 mg/Kg BW of indomethacin orally and 100 µl of 1% carrageenan through the subplantar region of the right hind paw after 30 minutes. Mice in other experimental groups (D-H) were orally treated with the Phytexpenton at dose levels of 15.625 mg/Kg BW, 31.25 mg/Kg BW, 62.5 mg/Kg BW, 125 mg/Kg BW, 250 mg/Kg BW and 500 mg/Kg BW, respectively, and injected with 100 µl of 1% Carrageenan via the subplantar route after 30 minutes. The changes in paw diameter sizes were measured before induction of inflammation, and after 1 hour, 2 hours, 3 hours, and 4 hours, respectively, following the induction of inflammation, using a plethysmographic technique, and the percentage inhibitions of inflammation were calculated and tabulated.

\subsection*{Determination of the Analgesic Effects of the Phytexpenton}

The analgesic activity of the Phytexpenton was evaluated according to the method described by Koster \textit{et al.}\textsuperscript{26} with slight modifications. Briefly, the normal control group mice [A] received normal saline (10 mg/Kg BW; \textit{p.o}) only. The negative control group [B] mice were orally administered with normal saline at a dose of 10 mg/Kg BW (\textit{p.o}) and 100 µl of acetic acid (0.6% w/v; \textit{i.p}) (Lot##L148661503; Loba Chemie) after 30 minutes. On the other hand, the positive
control group [C] mice received indomethacin (4 mg/Kg BW; 
po) and 100 μl of acetic acid (0.6% v/v; ip) after 30 minutes. Besides, the mice in other experimental groups 
[D-H] were orally administered with the Phytexponent at 
dose levels of 15.625 mg/Kg BW, 31.25 mg/Kg BW, 62.5 mg/Kg BW, 125 mg/Kg BW, 250 mg/Kg BW, and 500 mg/Kg BW, respectively, 30 minutes before the intraperitoneal injection of 100 μl of 0.6% v/v of acetic acid. The total number of writhes was recorded for each experimental mouse after 5 minutes of writhing induction for 15 minutes, and expressed as the percentage inhibition of writhing.

**In Vitro Cytotoxicity Assay**

**Vero E6 cell line culture.** The normal kidney epithelial cell line derived from the African green monkey (Vero E6) was obtained from the American Type Culture Collection (ATCC) (Rockville, USA). The Vero cell line (Vero E6) was cultured in T75 culture flasks containing Eagle’s Minimum Essential Medium (EMEM)(ATCC® 30-2003™, Sigma-Aldrich, Chem, St. Louis, MO), in an aseptic environment to avoid contamination, and supplemented with penicillin (100 units/ml)-streptomycin(100 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) to reduce extraneous bacterial contamination, and 10% foetal bovine serum (10% FBS) (Bio Whittaker®, Verviers, Belgium). The culture was incubated at 37 °C in an incubator (SHEL LAB™, Sheldon Mfg., Inc., OR, USA) with 5% CO₂ in air and 65% humidity. The growth of cells was controlled thrice a week, on Monday, Wednesday, and Friday, respectively, trypsinised, and passaged following the modified procedure of Bibi et al. 27

**Determination of the effects of the Phytexponent on cell viability.** The standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay technique 27,28 was used to determine the viability of Vero E6 cells in the presence and absence of the Phytexponent. In this assay, 100 μl of the growth medium was transferred into each well of the 96-multiwell plate and then seeded with 20,000 Vero E6 cells and allowed to attach overnight. Various serial concentrations of the Phytexponent and Cyclophosphamide (Sigma-Aldrich, St. Louis, MO, USA) (positive control) were added to respective wells in triplicate according to the assay protocol. After that, the multiwell plates were incubated for 48 hours at 37 °C, 5% CO₂ and 95% relative humidity in an incubator.

Following culturing, 10 μl of freshly prepared MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the plates were further incubated for 4 hours. With the help of a micropipette, there spective supernatants were aspirated followed by addition of 100 μl of dimethyl sulphoxide (DMSO) (Lot#A218101702, Loba Chemie) to solubilise the MTT crystals. The plates were then agitated and optical densities of each well were measured using an enzyme-linked immunosorbent assay (ELISA) scanning multiwell spectrophotometer (Multiskan Ex lab-systems) at 562 nm. The percentage inhibitions of cell proliferation (percentage cytotoxicity) was calculated using the following formula (Eq. 1) described by Fatemeh and Khosro.29

\[
\% \text{ Cytotoxicity} = 1 - \left( \frac{\text{Optical density of treated cells}}{\text{Optical density of control}} \right) \times 100
\]

(1)

**Data Management and Statistical Analysis**

The obtained data were first tabulated on Excel (Microsoft 365) spreadsheet and then exported to GraphPad prism version 9.2 for analysis. The data were subjected to descriptive statistics and the results were expressed as mean ± standard error of the mean (±SEM) of independent replicate experiments. Then, One-Way analysis of variance (ANOVA), or Two-Way ANOVA were performed, as appropiate, to determine significant differences among means of independent treatment groups followed by Tukey’s post hoc test for pairwise comparisons and separations of means at α = 0.05. Unpaired student t-test statistic was performed to compare between the cytotoxic effects of the Phytexponent and cyclophosphamide at 95% confidence level.

**Results**

**Anti-Inflammatory Activity of the Phytexponent in Swiss Albino Mice**

The Phytexponent exerted significant (P<.05) anti-inflammatory effects in carrageenan-induced paw oedema mouse model in a time-dependent manner (Table 1). At a dose of 31.25 mg/Kg BW, the percentage inhibition of inflammation by the Phytexponent was 1.117 ± 0.193% at the first hour, while at the fourth hour it was 11.162 ± 0.091% (P<.05; Table 1). At a dose of 62.50 mg/Kg BW, the percentage inhibitions of inflammation ranged from 6.240 ± 0.242% at the first hour to 17.407 ± 0.186% at the fourth hour (P<.05; Table 1). The percentage inhibition of inflammation caused by the Phytexponent at a dose of 125 mg/Kg BW ranged from 9.645 ± 0.020% at the first hour to 31.795 ± 0.090% at the fourth hour (P<.05; Table 1). At a dose of 250 mg/Kg BW, the percentage inhibition of inflammation by the Phytexponent ranged from 14.000 ± 0.102% at the first hour to 37.931 ± 0.133% in the fourth hour (P<.05; Table 1). Notably, the Phytexponent formulation significantly inhibited inflammation in dose- and time-dependent manner (P<.05; Table 1).

**Analgesic Activity of the Phytexponent**

The findings revealed a positive dose-dependent significant increase in the percentage inhibition of acet criticism-induced writhing in mice (P<.05; Figure 1). Notably, at doses of 125 mg/Kg BW and 250 mg/Kg BW of the Phytexponent, the percentage
inhibitions of acetic acid-induced writhing were significantly higher than the percentage inhibitions caused by indomethacin (reference drug) \( (P < .05; \text{Figure 1}) \). However, indomethacin exhibited a significantly higher inhibition of acetic acid-induced writhing in mice compared with the inhibitions caused by the Phytexponent at dose levels of 31.25 mg/Kg BW and 52.50 mg/Kg BW \( (P < .05; \text{Figure 1}) \).

Bars with dissimilar letters are significantly different (One-Way ANOVA followed by Tukey’s test; \( P > .05 \)).

**Table 1.** Anti-Inflammatory Effects of the Phytexponent in Swiss Albino Mice.

| Status                  | Treatment                                | %Inhibition of Carrageenan-induced paw oedema (\( \bar{x} \pm \text{SEM} \))               |
|-------------------------|------------------------------------------|-----------------------------------------------------------------------------------------|
|                         |                                          | 1stHr 2ndHr 3rdHr 4thHr                                                              |
| Normal control          | Normal saline only                       | -0.040 ± 0.018e 0.039 ± 0.014f 0.020 ± 0.007e 0.020 ± 0.007e                         |
| Negative control        | Carrageenan + Normal saline              | -23.70 ± 0.103f  -25.623 ± 0.170f -26.221 ± 0.117f -27.679 ± 0.173f                |
| Positive control        | Carrageenan + Indomethacin (4 mg/Kg BW)  | 9.580 ± 0.199f 17.000 ± 0.058e 24.989 ± 0.057e 37.250 ± 0.341e                     |
| Experimental Group A    | Carrageenan + Phytexponent (31.25 mg/Kg BW) | 1.117 ± 0.193f 3.088 ± 0.140f 5.192 ± 0.180f 11.162 ± 0.091f                       |
| Experimental Group B    | Carrageenan + Phytexponent (62.50 mg/Kg BW) | 6.240 ± 0.242d 8.368 ± 0.216d 10.768 ± 0.080d 17.407 ± 0.186d                    |
| Experimental Group C    | Carrageenan + Phytexponent (125 mg/Kg BW) | 9.645 ± 0.020c 12.645 ± 0.031c 24.851 ± 0.010c 31.795 ± 0.090c                   |
| Experimental Group D    | Carrageenan + Phytexponent (250 mg/Kg BW) | 14.000 ± 0.102a 18.097 ± 0.043a 29.946 ± 0.128a 37.931 ± 0.133a                   |

Values are expressed as \( \bar{x} \pm \text{SEM} \); Means with similar superscript letters within the same column and similar subscript letters within the same row are not significantly different (One-Way ANOVA followed by Tukey’s test; \( P > .05 \)).

**Figure 1.** Analgesic effects of the phytexponent in acetic acid-induced writhing in mice.

Inhibitions of acetic acid-induced writhing were significantly higher than the percentage inhibitions caused by indomethacin (reference drug) \( (P < .05; \text{Figure 1}) \). However, indomethacin exhibited a significantly higher inhibition of acetic acid-induced writhing in mice compared with the inhibitions caused by the Phytexponent at dose levels of 31.25 mg/Kg BW and 52.50 mg/Kg BW \( (P < .05; \text{Figure 1}) \).

Bars with dissimilar letters are significantly different (One-Way ANOVA followed by Tukey’s test; \( P < .05 \)).

**In Vitro Cytotoxic Effects of the Phytexponent**

In this study, the results depicted a significantly positive dose-dependent increase percentage cytotoxicity of the Phytexponent on Vero cell line (normal cell line) \( (P < .05; \text{Table 2}) \). Similarly, the reference drug (cyclophosphamide) caused a dose-dependent increase in cytotoxicity to Vero cell *in vitro* \( (P < .05; \text{Table 2}) \). A comparison between the cytotoxic effects of the Phytexponent and cyclophosphamide revealed that, at all the tested concentrations, the cytotoxicity of cyclophosphamide was significantly higher than that of the Phytexponent in Vero cells \( (P < .05; \text{Table 2}) \). Furthermore, the median cytotoxic concentrations (CC\(_{50}\)) were >1000 µg/ml (1137.83 µg/ml) for the Phytexponent and 2.48 µg/ml for cyclophosphamide (Table 2).

**Discussion**

Currently, the management of inflammation mostly utilises the non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, indomethacin, naproxen, ketoprofen, and ibuprofen,
which inhibit the activity of the cyclooxygenase-2 (COX-2) enzyme, thereby interfering with the synthesis of prostaglandins like prostaglandin E-2 (PGE2). However, NSAID therapy causes dependence, is arguably unaffordable, inaccessible, and is often associated with adverse effects such as nephrotoxicity, cardiotoxicity, hepatotoxicity, intestinal bleeding, gastric ulcers, among other effects. In light of this, we investigated the anti-inflammatory, analgesic, and cytotoxic effects of the Phytexponent of selected medicinal plants as a potential source of safer, efficacious, accessible, and affordable anti-inflammatory and analgesic molecules.

In this study, inflammation was induced in mice using carrageenan, a natural carbohydrate derived from edible red seaweed, widely used to screen plant extracts and molecules for anti-inflammatory efficacy. Carrageenan induces a biphasic inflammatory response, whereby distinct modulators are produced. In the early phase of carrageenan-induced inflammation, cyclooxygenase, histamine, and serotonin are produced, whereas in the late phase, which occurs after one hour, is characterized by PGE2 synthesis, mediated by bradykinin and leukotrienes. In this case, the early and late phases are characteristic of acute and chronic inflammation, respectively.

The upregulated synthesis of inflammatory mediators is due to the activation and enhanced activity of the inducible nitric oxide synthase (iNOS), COX-2 enzymes, pyrogenic cytokines, including the tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin (IL-6), among others. Therefore, for an agent to be considered as having anti-inflammatory activity, it ought to alter the consequences of carrageenan-induced inflammation, culminating in the amelioration of its typical features such as oedema, pyrexia, redness, algesia, and tissue dysfunction.

Previous studies have established that a solution of 1% carrageenan (prepared in physiologic saline), when injected at a volume of 50-150 µl into the subplantar region, is sufficient to cause inflammation, which manifests in oedema. In this study, a subplantar injection of 100 µl of 1% carrageenan into the right hind paw of experimental mice effectively induced inflammation, as evidenced by well-pronounced swelling around the injected site. The negative inhibitions of oedema are indicative of progressive increase in oedema size, due to the inflammatory response to Carrageenan. The findings revealed a progressive increase in oedematous paw size of the negative control mice throughout the treatment period, which indicates a successful induction of inflammation. Conversely, the reference drug (indomethacin) and the Phytexponent effectively reduced oedema in a dose- and time-dependent manner in mice as depicted by the percentage inhibitions of paw oedema. Moreover, the Phytexponent successfully inhibited both the early and late phases of inflammation as depicted by the progressive increase in the percentage inhibitions of oedema. The time-dependent increase in percentage inhibition of oedema may be attributed to a higher bioavailability of the Phytexponent’s active molecules, following metabolism and distribution to target sites.

Indomethacin is a non-steroidal anti-inflammatory drug that interferes with the synthesis of prostaglandins from arachidonic acid by inhibiting the cyclooxygenase (COX) enzyme. The COX enzyme exists in two isoforms: COX-1 and COX-2, respectively. Scientific evidence shows that COX-1 mainly catalyses the synthesis of prostaglandins, which are essential for maintaining the health and proper functioning of the gastrointestinal tract, platelet activity, renal functioning, and other vital physiological functions in the body. On the other hand, COX-2 facilitates the synthesis of prostaglandins, which mediate pain, fever, and inflammation. However, studies have demonstrated the existence, in some instances, of a crossover of the biological effects between COX-1 and COX-2 in the body. Just like other NSAIDs, indomethacin nonselectively inhibits both COX-1 and COX-2 to confer anti-inflammatory activity. Even though the specific mode of action of the Phytexponent has not been established, the observations made herein partly suggest that its anti-inflammatory effects could be via the inhibition of the COX enzyme.

Pain is an unpleasant emotional and sensory experience resulting from tissue damage. It acts as a warning signal to protect the body from actual or potential injury; however, it is associated with a disabling accompaniment of discomfort and adverse effects, characterising various medical conditions. As a result, pain forms a critical component of disease diagnosis, and its management is among the most important therapeutic priorities in medical practice. Various analgesic agents are used manage acute and chronic pain in patients. Currently, the most typical group of analgesic drugs used to manage pain comprises the NSAIDs, whose efficacy is based on the central and peripheral inhibition of prostaglandin synthesis.

Although NSAIDs interfere with prostaglandins’ normal synthesis and functioning, their side effects are predictable and include decreased homeostasis, renal dysfunction, hepatic dysfunction, peptic ulceration, intestinal bleeding, among others. Empirical evidence shows that over 20% of patients under long-term NSAID therapy develop duodenal and gastric ulcers with

**Table 2. In Vitro Cytotoxic Effects of the Phytexponent on Vero Cell Line.**

| Well | Concentration (µg/ml) | % Cytotoxicity on Vero cell line |
|------|----------------------|-------------------------------|
|      | Phytexponent | Cyclophosphamide |
| A    | 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| B    | 1.37 | 2.82 ± 1.41 | 64.56 ± 0.34 |
| C    | 4.12 | 5.57 ± 1.52 | 55.52 ± 1.27 |
| D    | 12.35 | 5.34 ± 1.47 | 64.05 ± 1.55 |
| E    | 37.04 | 7.23 ± 2.02 | 68.46 ± 2.51 |
| F    | 111.1 | 8.66 ± 1.93 | 73.73 ± 3.13 |
| G    | 333.3 | 18.88 ± 2.00 | 85.73 ± 0.99 |
| H    | 1000 | 43.69 ± 0.61 | 96.03 ± 0.47 |
| CC50 (µg/ml) | >1000 | 2.48 |

Values are expressed as x ± SEM; Means with similar superscript letter along the columns are not significantly different (One-Way ANOVA followed by Tukey’s test; P < 0.05); Means with different subscripts letters across rows are significantly different (unpaired student t-test; P < 0.05).
The acetic acid-induced writhing is an experimental reflex model of visceral pain that has been extensively utilised to screen drugs and chemicals for analgesic efficacy in laboratory animals.26 In the present study, 0.6% of acetic acid was intraperitoneally administered into experimental mice to induce pain by activating chemosensitive nociceptors, which manifests in writhing.52 Writhing is described as the arching of the back, extension of limbs, and the abdominal musculature contraction.53 In this experiment, the level of analgesia is indicated by the percentage reduction in abdominal writhing frequency.

This study showed a dose-dependent increase in the percentage inhibition of acetic acid-induced writhing by the Phytoexponent in mice, indicating its potential analgesic efficacy. Similarly, indomethacin, the positive control drug, successfully inhibited the acetic acid-induced writhing in mice resulting in high percentage inhibitions. Moreover, the results showed that the Phytoexponent at dose levels of 125 mg/Kg BW and 250 mg/Kg BW had significantly higher percentage inhibitions of writhing compared to indomethacin. These observations suggest that the Phytoexponent is more potent at these doses than indomethacin. Partly, this observation could be attributable to the various phytactive principles present in the Phytoexponent,24 which may have acted at different sites in a multitarget fashion to thwart pain as opposed to a single target effect (inhibition of the COX enzyme) of indomethacin.

Preliminary studies have demonstrated that each medicinal plant, which comprises the Phytoexponent, has anti-inflammatory and analgesic properties54–59. Additionally, a recent study demonstrates significant in vitro anti-inflammatory and antioxidant efficacy of the Phytoexponent.24 Therefore, a combination of the analgesic- and anti-inflammatory-associated phytocompounds of individual plants in the Phytoexponent may have synergistically conferred the bioactivities reported in the present study.8 Moreover, studies have shown that chronic pain can be successfully managed by agents which modify the neurochemistry of the spinal cord dorsal horn, like anticonvulsants, local anaesthetic analogues, tricyclic antidepressants, γ-aminobutyric acid (GABA) agonists, and N-methyl-D-aspartate (NMDA) antagonists60–62. Opiates are useful in managing chronic pain; however, tolerance, dependence, and loss of efficacy limit their usefulness.63,64

To this end, only NMDA antagonists and epidural morphine have consistently demonstrated preemptive analgesic efficacies65–67. Therefore, to adequately manage pain and inflammation, a multifaceted approach using multitarget agents is the most viable strategy to alleviating pain in affected patients.58 The results of this study, therefore, posit that the Phytoexponent, by virtue of its analgesic and anti-inflammatory efficacy, could be a promising candidate for further development.

Even though the specific mode of action and the specific analgesic and anti-inflammatory bioactive molecules of the Phytoexponent have not been elucidated, its is suggestive that it targets various pathways associated with immunity, inflammation, and pain.69 Possibly, the phytocompounds present in this formulation could be maintaining the redox homeostasis, thereby preventing cell damage, and modulating immunity, modifying the inflammatory and pain transduction pathways, which together ensure the proper functioning of cellular molecules and avert cellular damage18,70–72.

Medicinal plants have long standing usage in managing various diseases and play an integral role in meeting primary healthcare needs, especially in Sub-Saharan Africa.73 Indeed the world health organisation estimates that over 80% of the global population depends on herbal medicine for their healthcare needs.74 Despite the extensive utilisation of herbal products to manage various diseases, serious concerns regarding their efficacy and safety have been raised.20

In the present study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay technique27,28 was employed to assess in vitro cytotoxicity and safety effects of the Phytoexponent to assess its safety. This has was first described by Mosmann,25 and has been extensively applied in the screening of anticancer potential of chemicals and plant extracts. The MTT assay measures the activity of mitochondrial enzymes, especially the succinate dehydrogenase (SDH), whose function is impaired by toxic agents leading to mitochondrial collapse and cell death.26 During the assay, the mitochondrial nicotinamide adenine dinucleotide (NAD) reduced the MTT to a purple formazan product, which is determined colorimetrically at a specific wavelength (520 nm). The amount of formazan produced is directly proportional to the number of cells in a particular cell line.28 This technique was selected due to its high reproducibility, safety, sensitivity, and robustness in determining cell viability and cytotoxicity.76

According to the National Cancer Institute (NCI) criteria, plant extracts with CC50 < 30 µg/ml are considered to be cytotoxic after 48–72-hour exposure to cells.77 The observation from this study revealed that the reference drug (cyclophosphamide) was a potent cytotoxic agent by its low CC50 value (CC50 = 2.48 µg/ml) as consistently demonstrated in other studies.78,79 On the other hand, the Phytoexponent demonstrated low cytotoxic effects, as witnessed by its high CC50 (CC50 > 1000 µg/ml). These results indicate that this polyherbal formulation might be safe and may be used to treat pain and inflammation without eliciting cytotoxic effects. However, extensive toxicity studies should be conducted to establish their safety profile.

In current medical practice, pharmaceutical drugs are designed to confer specific biological effects that are accompanied by specific side effects.41,80 However, medicinal plants demonstrate a broad spectrum of bioactivities; thus, there are no defined toxic profiles.81 This is attributable to the enormous phytoconstituents that act synergistically to affect various physiological functions in a non-specific manner71,82–85. If a medicinal plant contains toxic compounds, the toxic effects elicted could be fatal; therefore, it is critical to validate medicinal plants’ safety to avert potential fatalities. This study’s findings demonstrate that the Phytoexponent is non-toxic to Vero cell-lines-normal cells and is a potential source of safe analgesic and anti-inflammatory agents.
Conclusions and Recommendations

Our study findings showed that the Phytexponent possess in vivo anti-inflammatory and remarkable analgesic efficacy in experimental mice, and is non-toxic to Vero E6 cell line. Therefore, the Phytexponent is a promising source of efficacious anti-inflammatory and analgesic compounds, against various maladies, especially those it is used to manage in complementary and alternative medicine. Further studies aimed at establishing the specific mechanism(s) through which the Phytexponent confers the anti-inflammatory and analgesic effects should be done. Extensive toxicity and safety evaluation of this polyherbal product should be performed to give way for its further development.

Acknowledgments

The authors acknowledge the Laboratory staff of the Department of Public health, Pharmacology and Toxicology of the University of Nairobi, and the Kenya Medical Research Institute for their technical support. We also thank Prof. Epaphrodite Twahirwa of the Department of Pharmaceutics and Pharmaceutical Chemistry of Mount Kenya University for his mentorship.

Authors’ Contributions

Halvince Odira, performed the experiments, and drafted the manuscript under the supervision of Simon Mitema and Isaac Mapenay. Gervason Moriasi designed the experiments, donated research materials, performed data analysis, and interpreted the results. All authors reviewed the draft manuscript and approved the final version for publication.

Data Availability

All data is included in the manuscript, and any additional information is available from the authors upon request.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The experimental mice were used and disposed of as per the guidelines set out by the Faculty of veterinary Medicine (FVM) of the University of Nairobi biosafety, animal use, and ethics committee (BAUEC) (FVM BAUER/2020/265). The cell line (Vero E6) was used and disposed of according to the protocols set out by the Scientific Ethical Review Unit (SERU) of the Kenya Medical Research Institute (KEMRI/RES/7/5/2). Permission to conduct this study was also obtained from the Kenya National commission for science, technology, and innovation (NACOSTI) (BAHAMAS ABS/P/20/7744).

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iDs

Halvince O. Odira https://orcid.org/0000-0002-0436-8641
Gervason A. Moriasi https://orcid.org/0000-0001-5604-9987

Supplemental Material

Supplemental material for this article is available online.

References

1. Lordan R, Tsoupras A, Zabetakis I. Inflammation. In: The Impact of Nutrition and Statsins on Cardiovascular Diseases. Elsevier; 2019:23-51. doi:10.1016/B978-0-12-813792-5.00002-1
2. Raja SN, Carr DB, Cohen M, et al. The revised international association for the study of pain definition of pain: concepts, challenges, and compromises. Pain. 2020;161(9):1976-1982. doi:10.1097/j.pain.0000000000001939
3. Walter EJ, Hanna-Jumma S, Carraretto M, Forni L. The pathophysiological basis and consequences of fever. Crit Care. 2016;20(1):200. doi:10.1186/s13054-016-1375-5
4. Yaksh TL, Dirig DM, Malmbeg AB. Mechanism of action of nonsteroidal anti-inflammatory drugs. Cancer Invest. 1998;16(7):509-527. doi:10.3109/07357969809011705
5. Giorno TBS, dos Santos CHC, de Carvalho MG, et al. Study on the antinociceptive activity and mechanism of action of isolated saponins from Scolmatura brasiliensis (Cogn.) Baill. Molecules. 2019;24(24):1-12. doi:10.3390/334244584
6. Herrero JF, Romero-Sandoval EA, Gaitan G, Mazzario J. Antinociception and the new COX inhibitors: research approaches and clinical perspectives. CNS Drug Rev. 2003;9(3):227-252. doi:10.1111/j.1527-3458.2003.tb00251.x
7. Olela B, Mbia J, Wachira T, Moriasi G. Acute oral toxicity and anti-inflammatory and analgesic effects of aqueous and methanolic stem bark extracts of Piliostigma thomningii (Schumach). Evidence-Based Complement Altern Med. 2020;2020(2020):1-10. doi:10.1155/2020/5651390
8. Moriasi GA, Ireri AM, Nelson EM, Ngugi MP. In vivo anti-inflammatory, anti-nociceptive, and in vitro antioxidant efficacy, and acute oral toxicity effects of the aqueous and methanolic stem bark extracts of Lonchocarpus eriocalyx (Harms.). Helioy. 2021;7(5):e07145. doi:10.1016/j.helioy.2021.e07145
9. Calixto JB, Scheidt C, Otuki M, Santos ARS. Biological activity of plant extracts: novel analgesic drugs. Expert Opin Emerg Drugs. 2001;6(2):261-279. doi:10.1517/14728214.6.2.261
10. Fürst R, Zündorf I. Plant-Derived anti-inflammatory compounds: hopes and disappointments regarding the translation of preclinical knowledge into clinical progress. Mediators Inflamm. 2014;2014(March 2015):1-9. doi:10.1155/2014/146832
11. Nunes C, dos R, Barreto Arantes M, et al. Plants as Sources of Anti-Inflammatory Agents. Molecules. 2020;25(16):3726. doi:10.3390/molecules25163726
12. World Health Organization (WHO). WHO Traditional Medicine Strategy 2014-2023. 2013. doi:2013.
13. Mukungu N, Abuga K, Okalebo F, Ingwela R, Mwangi J. Medicinal plants used for management of malaria among the luhyua community of kakamega east sub-county, Kenya. J Ethnopharmacol. 2016;194(Dec 2016):98-107. doi:10.1016/j.jep.2016.08.050
14. Karera PG, Kenji GM, Gachanja AN, Keriko JM, Mungai G. Traditional medicines among the Embu and Mbeere peoples of
Kenya. *African J Tradit Complement Alter Med.* 2007;4(1):75-86.

15. Mutie FM, Gao LL, Kathambi V, et al. An ethnobotanical survey of a dryland botanical garden and its environs in Kenya: The Mutomo hill plant sanctuary. *Evidence-based Complement Alter Med.* 2020;2020(2020):1-22. doi:10.1155/2020/1543831

16. Nankaya J, Nampushi J, Petenya S, Balslev H. Ethnomedicinal plants of the loita maasai of Kenya. *Environ Dev Sustain.* 2020;22(3):2569-2589. doi:10.1007/s10668-019-00311-w

17. Odongo E, Mungai N, Mutai P, Karumi E, Mwangi J, Omale J. Ethnomedicinal survey of medicinal plants used in kakamega county, western Kenya. *Appl Med Res.* 2018;4(1):22. doi:10.5455/amr.20180315095706

18. Ochwang’i DO, Kimwele CN, Oduma JA, Gathumbi PK, Mbaria JM, Kiama SG. Medicinal plants used in treatment and management of cancer in kakamega county, Kenya. *J Ethnopharmacol.* 2014;151(3):1040-1055. doi:10.1016/j.ejphar.2013.11.051

19. Keter LK, Mutiso PC. Ethnobotanical studies of medicinal plants used by traditional health practitioners in the management of diabetes in lower Eastern province, Kenya. *J Ethnopharmacol.* 2012;139(1):74-80. doi:10.1016/j.jep.2011.10.014

20. George P. Concerns regarding the safety and toxicity of medicinal plants - An overview. *J Appl Pharm Sci.* 2011;1(6):40-44.

21. Arora M. *Pharmacovigilance & Clinical Trials.* 2015;3(4):6887.

22. Kaur J, Kaur S, Mahajan A. Herbal medicines: possible risks and benefits. *Am J Phytomedicine Clin Ther.* 2013;2(2013):226-239.

23. Hellinger R, Koehbach J, Feduch H, et al. Immunosuppressive activity of an aqueous *Viola tricolor* herbal extract. *J Ethnopharmacol.* 2014;151(1):299-306. doi:10.1016/j.jep.2013.10.044

24. Moriasi G, Nelson E, Twahirwa E. *In vitro* anti-inflammatory, antioxidiant, and qualitative phytochemical evaluation of the phytoexponent preparation of selected plants advanced techniques in biology & medicine. *Adv Tech Biol Med.* 2021;9(1 [277]):1-9. doi:10.4172/2379-1764.1000277

25. Winter CA, Risley EA, Nuss GW. Carrageenin-induced edema in hind paw. *Exp Biol Med.* 1962;3(111):544-547.

26. Koster R, Anderson, M, De Beer EJ. Acetic Acid for Analgesic activity of *Aesculus indica* herbal extract. *J Ethnopharmacol.* 2014;151(1):299-306. doi:10.1016/j.jep.2013.10.044

27. Bibi Y, Nisa S, Zia M, Waheed A, Ahmed S, Chaudhry MF. *In vitro* cytotoxic activity of *Aesculus indica* against breast adenocarcinoma cell line (MCF-7) and phytochemical analysis. *Pak J Pharm Sci.* 2012;25(1):183-187.

28. Van Meerloo J, Kaspers GJL, Cloos J. Cell sensitivity assays: the MTT assay. *Methods Mol Biol.* 2011;731(March):237-245. doi:10.1007/978-1-61779-80-5_20

29. Fatemeh K, Khosro P. *In vitro* cytotoxic activity of aqueous root extract of *Althea kurdica* against endothelial human bone marrow cells (line K562) and human lymphocytes. *Bull Env Pharmacol Life Sci.* 2013;2(26):22-29.

30. Felson DT. Safety of nonsteroidal antiinflammatory drugs. *N Engl J Med.* 2016;375(26):2595-2596. doi:10.1056/NEJMe1614257

31. Harirforoosh S, Asghar W, Jamali F. Adverse effects of nonsteroidal antiinflammatory drugs: an update of gastrointestinal, cardiovascular and renal complications. *J Pharm Sci.* 2013;16(5):821-847. doi:10.18433/j3vvw2f

32. Fokunang C. Overview of non-steroidal anti-inflammatory drugs (nsaids) in resource limited countries. *MOJ Toxicol.* 2018;4(1):5-13. doi:10.15406/moj.toxicol.2018.04.00081

33. Gan TJ. Diclofenac: an update on its mechanism of action and safety profile. *Curr Med Res Opin.* 2010;26(7):1715-1731. doi:10.1185/03007995.2010.486301

34. Necas J, Bartosikova L. Carrageenan: a review. *Vet Med (Praha).* 2013;58(4):187-205. doi:10.17221/6758-VETMED

35. Mansouri MT, Hennmati AA, Naghizadeh B, Mard SA, Rezaie A, Ghorbanzadeh B. A study of the mechanisms underlying the anti-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. *Indian J Pharmacol.* 2015;47(3):292-298. doi:10.4103/0253-7613.157127

36. Bukhari IA, Gilani AH, Moe SA, Saeed A. Analgesic, anti-inflammatory and anti-platelet activities of *Buddleja crispa*. *BMC Complement Altern Med.* 2016;16(1):1-7. doi:10.1186/s12906-016-1021-4

37. Coura CO, Souza RB, Rodrigues JAG, et al. Mechanisms involved in the anti-inflammatory action of a polysulfated fraction from *Gracilaria cornea* in rats. *Hazard A, ed. PLoS One.* 2015;10(3):e0119319. doi:10.1371/journal.pone.0119319

38. Amri O, Zekhnini A, Bouhaimi A, Tahrousch S, Hatimi A. Anti-inflammatory activity of methanolic extract from *Pistacia atlantica* desf. *Leaves. Pharmacogn J.* 2017;10(1):71-76. doi:10.5530/pj.2018.1.14

39. Cai C, Chen Y, Zhong S, et al. Anti-Inflammatory activity of N-butanol extract from *Ipomoea stolonifera* in vivo and *In vitro*. *Song L, ed. PLoS One.* 2014;9(4):e95931. doi:10.1371/journal.pone.0095931

40. Capasso R, Di Cesare Mannelli L. Special issue “plant extracts: biological and pharmacological activity.”. *Molecules.* 2020;25(21):5131. doi:10.3390/molecules25215131

41. Lucas S. The pharmacology of indomethacin. *Headache.* 2016;56(2):436-446. doi:10.1111/head.12769

42. Fitzpatrick F. Cyclooxygenase enzymes: regulation and function. *Curr Pharm Des.* 2004;10(6):577-588. doi:10.2174/138161204453144

43. Stolfi C, De Simone V, Pallone F, Monteleone G. Mechanisms of action of non-steroidal anti-inflammatory drugs (NSAIDs) and mesalazine in the chemoprevention of colorectal cancer. *Int J Mol Sci.* 2013;14(9):17972-17985. doi:10.3390/ijms140917972

44. Attiq A, Jalil J, Husain K, Ahmad W. Raging the war against inflammation with natural products. *Front Pharmacol.* 2018;9(SEP):1-27. doi:10.3389/fphar.2018.00976

45. Treede R-D, Rief W, Barke A, et al. Chronic pain as a symptom or a disease: the IASP classification of chronic pain for the international classification of diseases (ICD-11). *Pain.* 2019;160(1):19-27. doi:10.1097/j.pain.0000000000001384

46. Young Blood MR, Ferro MM, Munhoz RP, Teive HAG, Camargo CHF. Classification and characteristics of pain associated with Parkinson’s disease. *Parkinson Dis.* 2016;2016(2016):1-8. doi:10.1155/2016/6067132

47. Cox F. Basic principles of pain management: assessment and intervention. *Nurs Stand.* 2010;25(1):36-39. doi:10.7748/nst2010.09.25.1.36.c7983
78. Ogbole OO, Segun PA, Adeniji AJ. *In vitro* cytotoxic activity of medicinal plants from Nigeria ethnomedicine on rhabdomyosarcoma cancer cell line and HPLC analysis of active extracts. *BMC Complement Altern Med.* 2017;17(1):1-10. doi:10.1186/s12906-017-2005-8

79. Baharum Z, Akim AM, Taufiq-Yap YH, Hamid RA, Kasran R. *In vitro* antioxidant and antiproliferative activities of methanolic plant part extracts of *Theobroma cacao*. *Molecules*. 2014;19(11):18317-18331. doi:10.3390/molecules191118317

80. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. *Immunity*. 2013;39(1):74-88. doi:10.1016/j.immuni.2013.06.014

81. Singh S, Sedha S. Medicinal plants and their pharmacological aspects. *Fpi*. 2018;1(4):156-170.

82. Hussein R A, El-Anssary A A. Plants secondary metabolites: the key drivers of the pharmacological actions of medicinal plants. * Herb Med*. 2018;2018(2018):1–21. doi:10.5772/intechopen.76139

83. Moriasi G, Ireri AM, Ngugi MP. *In vivo* cognitive-enhancing, *Ex vivo* malondialdehyde-lowering activities and phytochemical profiles of aqueous and methanolic stem bark extracts of *Piliostigma thonningii* (Schum.). *Int J Alzheimer’s Dis*. 2020;2020(March 2020):1–15. doi:10.1155/2020/1367075

84. Jared MO, Bibiane AW, Gervason AM, Lameck NA, Japhet KN. The antibacterial, antioxidant and phytochemical composition of *Combretum tanaense* (J. Clark). *Root Extracts*. 2018;23(4):1-8. doi:10.9734/EJMP/2018/40956

85. Moriasi G, Ireri A, Ngugi MP. *In vitro* antioxidant activities of the aqueous and methanolic stem bark extracts of *Piliostigma thonningii* (schum.). *J evidence-based Integr Med*. 2020;25(July 2020):1–9. doi:10.1177/2515690X20937988