Ethanolic leaf extract from Strophanthus gratus (Hook.) Franch. (Apocynaceae) exhibits anti-inflammatory and antioxidant activities

Samuel Ofori – Baah and Lawrence Sheringham Borquaye

Abstract: Chronic inflammation is associated with many diseased conditions. In particular, free radicals and oxidative stress play a major role in the development of tissue damage and some pathological events which result in inflammation. Plant extracts have widespread folkloric use in the management of various diseased conditions. One of such plants used in Ghana for managing inflammation-related conditions is Strophantus gratus. This study evaluated the anti-inflammatory and antioxidant activities of the ethanolic extract of the leaves of Strophantus gratus. Crude ethanolic leaf extract was obtained by Soxhlet extraction. The crude extract was then fractionated to obtain hexane, ethyl acetate, and butanol fractions. Anti-inflammatory activity of the extract and fractions were evaluated using carrageenan-induced paw edema model in 7-day old chicks. Phosphomolybdenum assay was used to assess the total antioxidant activity of the extracts. The concentration of the crude extract required to reduce the induced inflammation by 50% (ED$_{50}$) was determined to be $129.7 \pm 10.5$ mg/kg. The ethyl acetate fraction produced a similar ED$_{50}$ value ($133.5 \pm 14.7$ mg/kg). The ED$_{50}$ values of the hexane and butanol fractions were however greater than 1000 mg/kg, indicating that the activity of the crude extract was concentrated in the ethyl acetate fraction. The total antioxidant capacity of the crude extract and the ethyl acetate fraction was determined to be $6.7 \pm 1.0$ g/100g and $8.3 \pm 1.4$ g/100g ascorbic acid equivalent respectively. Taken together, the results

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PUBLIC INTEREST STATEMENT
Many plants are used in herbal formulations to treat various diseases. In Ghana, Strophantus gratus is used to treat diseases which has inflammation as one of the symptoms. However, there is no scientific proof that validates the use of this plant in managing inflammation related conditions. This research work showed that the leaf extracts of Strophantus gratus do indeed possess anti-inflammatory capabilities and can also neutralize reactive oxidant species.
provide scientific validation for the use of *Strophantus gratus* in managing inflammation associated ailments in traditional herbal medicine.

**Subjects:** Pharmacology; Medicinal & Pharmaceutical Chemistry; Organic Chemistry

**Keywords:** total antioxidant capacity; carrageenan-induced paw edema; phosphomolybdenum assay; solvent fractionation; inflammation

1. **Introduction**

Certain processes in the body such as wound healing require a multifactorial network of chemical signals to initiate and maintain a host of responses designed to heal the afflicted tissue. Inflammation is a part of the body’s defensive mechanism that is invoked when the body is exposed to irritants and pathogens or some cells become damaged (Eming, Krieg, & Davidson, 2007; Koh & DiPietro, 2011; Vane & Botting, 1987). Inflammation is characterized by five main cardinal symptoms termed as PRISH (pain, redness, immobility or loss of function, swelling, and heat). Inflammation associated diseases are usually treated with steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs mainly act by inhibiting cyclooxygenase (COX) enzymes and as such interfere with the production of inflammatory prostaglandins. Prostaglandins (PG) and other metabolites derived from PG are involved in a number of different physiological and pathophysiological processes (Vane & Botting, 1987). Undoubtedly, NSAIDs have proven efficacy in managing inflammation, pain, and fever.

Prolonged use of NSAIDs, however, has been associated with a number of adverse side effects (Bjarnason & Hayllar, 1993; Rainsford, 1999). NSAIDs are associated with cardiovascular, renal and gastrointestinal complications. The most common side effects include gastritis, ulcers, perforation, and enteropathy (Laine, 2003; Whelton, 2000). In Denmark, there has been a rise in the prevalence of NSAID-related peptic ulcers from 39% in 1993 to 53% in 2002 (Lassen, Hallas, & De Muckadell, 2006). In 2014, it was reported that the prevalence of gastrointestinal complications related to exposure to NSAIDs in the Indian sub-continent was about 40% (Chatterjee et al., 2015). Again, reports of mortality in patients suffering from upper gastrointestinal bleeding or perforations due to the use of NSAIDs has increased dramatically (Straube, Tramer, Moore, Derry, & McQuay, 2009). The numerous adverse side effects of anti-inflammatory agents on the market has necessitated efforts towards the search for and the development of new anti-inflammatory agents with improved efficacy and lesser side effects.

Free radicals are known to play a significant role in the development of tissue damage and some pathological events (Kehrer & Klotz, 2015; Kowluru & Mishra, 2015). The relationship between oxidative stress and inflammation is well documented in literature (Mangge, 2014). Chronic diseases linked with higher production of reactive oxygen species (ROS) result in oxidative stress and a variety of protein oxidations (Berlett & Stadtman, 1997; Liguori et al., 2018). Protein oxidations tend to release inflammatory signal molecules and peroxiredoxin 2 (PRDX 2) which has been recognized as an inflammatory signal (Hussain et al., 2016; Knoops, Argyropoulou, Becker, Ferté, & Kuznetsova, 2016; Salzano et al., 2014). ROS generated in the brain can modulate synaptic and non-synaptic communications between neurons that result in neuroinflammation (Popa-Wagner, Mitran, Sivanesan, Chang, & Buga, 2013). Glutathione (GSH) is a natural antioxidant produced in the body. Lower levels of GSH causes an increase in the production of ROS due to the imbalance in redox conditions in the body. This results in an imbalanced immune response, inflammation, and susceptibility to infection. Although the body possesses an endogenous antioxidant defense system, antioxidants from natural products can retard tissue damage caused by free radicals hence enhancing the endogenous antioxidant defense system and reducing inflammation (Ghezzi, 2011).

In recent times, focus on medicinal plant research has increased all over the world and there is a widespread belief that plant-derived drugs are safer, cost-effective and possess fewer side effects than conventional drugs (Nasri, 2013). The use of medicinal plants in health care delivery has existed since ancient times and still plays an integral role in modern health care especially in developing
countries. Many important drugs such as artemisinin (antimalarial), morphine (pain medication) and taxol (antitumor) are natural products derived from plants. Ghana is home to a rich biodiversity of flora and most of these plants are frequently used in traditional herbal remedies. One of such plants is *Strophanthus gratus* (Hook.) Franch. Belonging to the Apocynaceae family, *Strophanthus gratus* (S. gratus) is a vigorous evergreen clambering shrub and also glabrous with leaves oblong that can be up to 6 inches in length. The plant is mainly located in tropical Africa and southern Africa with other varieties found in parts of Asia (Endress & Bruyns, 2000). *S. gratus* is used traditionally to treat snake bites and also in making arrow poisons. The leaves are crushed and applied to guinea-worm sores for healing (Irvine, 1961). The significant traditional use of the plant in herbal medicine indicates a potential anti-inflammatory and antioxidant action and could, therefore, provide a remedy for the challenges associated with NSAIDs.

Despite the widespread use of the plant in traditional herbal medicine, very little scientific validation of its use exists in literature. The antimicrobial and antioxidant activities of the aqueous and alcoholic extracts of the stem bark have been reported in Ghana (Henneh, 2013). The absence of scientific validation of the biological activities of this important Ghanaian herbal plant motivated us to carry out this study. This work aimed at investigating the anti-inflammatory and antioxidant activities of ethanolic extract of the leaves of *S. gratus*. The phosphomolybdenum assay was used to determine the total antioxidant capacity of the extracts whereas the carrageenan-induced foot edema model of inflammation in chicks was used to assess anti-inflammatory activity.

2. Materials and methods

2.1. Reagents

Analytical grade chemicals were used throughout the study. Standard drugs—dexamethasone, diclofenac sodium and ascorbic acid—were obtained from Sigma-Aldrich (St. Louis, USA). Carrageenan, sodium phosphate dibasic, sodium phosphate monobasic, sulfuric acid, ammonium molybdate tetrahydrate, ethanol, ethyl acetate, butanol and hexane were also obtained from Sigma-Aldrich (St. Louis, USA). Extracts and fractions were filtered through sterile 0.2–0.6 µm polypropylene filters before use.

2.2. Plant material and extraction

2.2.1. Sample collection

The leaves of the plant, *S. gratus*, were collected from the Botanical Gardens, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi—Ghana, in September 2018. The plant was authenticated by Mr. Clifford Asare at the Pharmacy Herbarium, Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Science, KNUST. Voucher specimens were deposited at the herbarium.

2.2.2. Sample preparation and extraction

The sample was air-dried for 7 days at room temperature. Air-dried leaves were milled into fine powder. Approximately 1 kg of the powdered leaf material was Soxhlet extracted with 2 L of 90% ethanol. The extract was concentrated under vacuum on a Cole Parmer rotary evaporator (N-1110, China) and dried under nitrogen gas to remove any residual solvent. The concentrated extract was stored in a refrigerator below 4 °C until used for analysis.

2.2.3. Fractionation

A sample of the crude extract was then sequentially fractionated using hexane, ethyl acetate and butanol. The fractionation process involved dissolving a mass of 1 g of the crude extract in 70 mL distilled water and performing a liquid-liquid extraction using 70 mL of each solvent (hexane, ethyl acetate, and butanol). The solvents were used in order of increasing polarity. The extraction with each solvent was performed thrice and combined. Fractions were then concentrated, dried under nitrogen gas to remove any residual solvent and stored below 4 °C until used for analysis.
2.3. Anti-inflammatory assay

2.3.1. Chicks
One-day post-hatch chicks (Gallus gallus) were gotten from Akate Farms (Kumasi, Ghana) and main-
tained in the Animal House of the Department of Pharmacology, KNUST. Chicks were housed in stainless
steel cages (34 × 57 × 18 cm$^3$) with about 15 chicks per cage. The cages were kept at room temperature,
with an alternating 12-hour light-dark cycle. Food and water were available ad libitum through gravity-
fed feeders and water troughs. The cages were maintained daily during the first quarter of the light cycle.
Seven-day old chicks were used for the assay. A group sample size of 5 chicks (randomly selected) was
employed for the study. The National Institute of Health guidelines for the care and use of laboratory
animals was adhered to in the handling of the chicks and all experimental protocols conformed to this
standard.

2.3.2. Extracts and standard drug preparation
Extracts, which have been dried under nitrogen gas, were reconstituted in normal saline (0.9% w/
v sodium chloride). Standard dexamethasone and diclofenac were prepared similarly, by dissol-
ving in normal saline to appropriate concentration. Carrageenan was prepared as a 2% suspen-
sion in normal saline. Normal saline only, which served as drug vehicle, was used as the negative
control.

2.3.3. Carrageenan-induced paw edema
The carrageenan-induced paw edema model of inflammation in 7-day-old chicks was used to
assess the anti-inflammatory activity of the extracts from S. gratus. The methods of Roach and
Sufka (2003) and Borquaye et al. (2017) were followed with some modifications (Borquaye et al.,
2017; Roach & Sufka, 2003). Dexamethasone (0.3, 1 and 3 mg/kg) and diclofenac (10, 30 and
100 mg/kg) were used as positive controls. Inflammation was induced by injecting sub-plantar
10 µL of carrageenan (2% suspension in saline) into the right footpads of the chicks (time, t = 0).
After an hour and 30 minutes (when inflammation was maximum), extracts (at concentrations of
30, 100 and 300 mg/kg) and standard drugs were administered to the chicks orally and intraper-
itoneally respectively. A digital Vernier Caliper was used to measure the foot volume before
injection and at various time points after injection. A control group received normal saline and
served as a negative control.

2.3.4. Analysis of data
For each foot volume, the raw scores were individually normalized as the percentage difference
from the initial foot volume at time zero, then averaged for each treatment group. The change in
foot volume was calculated with equation (1)

\[
\% \text{ Change in foot volume} = \frac{\text{Foot volume at time, } t = 0 - \text{Foot volume at time, } t = 0}{\text{Foot volume at time, } t = 0} \times 100
\]  

(1)

The total foot volume for each treatment group was calculated in arbitrary units as the Area Under
the Curve (AUC). Percentage inhibition of edema for each treatment group was then determined
with equation (2) as follows:

\[
\% \text{ Inhibition of edema} = \frac{\text{AUCcontrol} - \text{AUCtreatment}}{\text{AUCcontrol}} \times 100
\]  

(2)

One-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test was used to analyze
differences in AUCs. The ED$_{50}$ values (dose responsible for 50% of maximal effect) for each extract
was determined using an iterative computer least square method with the following nonlinear
regression (three-parameter logistics) equation.

\[
Y = \frac{a + (b - a) \times 10^{\log ED_{50} - x}}{1 + 10^{\log ED_{50} - x}}
\]
where $X$ is the logarithm of dose and $Y$ is the response. $Y$ starts at $a$ (the bottom) and goes to $b$ (the top) with a sigmoid shape (Borquaye et al., 2017). GraphPad Prism Version 6.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and $ED_{50}$ determinations.

### 2.4. Antioxidant assay

#### 2.4.1. Phosphomolybdenum (PM) assay

The PM assay used was the same as that previously employed in our lab (Gyesi, Opoku, & Borquaye, 2019). The assay principle is based on the reduction of Mo(VI) to Mo(V) by the analyte. This is followed by the formation of the characteristic green phosphate/Mo(V) complex at acidic pH (Prieto, Pineda, & Aguilar, 1999). To 5 mL of the PM reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a test tube was added 0.5 mL of each test extract or standard (ascorbic acid was used as standard) at varying concentrations. Capped test tubes were shaken and incubated for one hour 30 minutes at 95 °C. After samples had cooled to room temperature, absorbance at 695 nm were taken against a blank solution. The blank solution was made by replacing test sample with solvent in the mixture and incubated under similar conditions. Ascorbic acid was used as the standard. Antioxidant capacity was expressed as equivalents of ascorbic acid with equation 3

$$TAC = \frac{C \times V}{M} \times 100$$  \hspace{1cm} (3)

where TAC is the total antioxidant capacity in gAAE/100g of the test sample, $C$ is the concentration of ascorbic acid (µg/mL), $V$ is the volume of the reaction mixture and $M$ is the mass of the extract in the reaction mixture.

### 3. Results

Soxhlet extraction of powdered $S$. gratus leaf sample with ethanol produced extracts with a yield of 5.7% (Table 1). Further sequential fractionation of this ethanolic extract with hexane, ethyl acetate, and butanol afforded fractions with yields ranging from 12–46%. The ethyl acetate fraction had the highest yield of 45.5%, followed by butanol, then hexane, as indicated in Table 1.

The in vivo anti-inflammatory assay was carried out using the carrageenan-induced paw edema in chick model. Upon induction of inflammation, the increase in paw size of chicks was observed after 90 minutes. Upon intervention (through the introduction of drug or extract), a noticeable decrease in paw size is observed (Figure 1, A, B, C). This was also observed even in the case of the negative control (where saline, instead of drug or extract was administered). A sharp decrease in paw size was observed when either extract or drug was administered but a much gentle decrease was observed in the control group. The trend in the time course curve was similar for extracts and standard drugs (Figure 1 A, B, C) and fractions (Figure 2 A, B, C). The total edema, expressed as AUCs depicted a dose-dependent relationship between extract, fraction or drug administration and total edema (Figures 1 and 2, A', B', C'). The dose of extract or drug required to achieve half-maximal effect ($ED_{50}$) is represented in Table 2. Whereas the standard drugs diclofenac and

<table>
<thead>
<tr>
<th>Extract</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>Crude Ethanolic Extract (SGCEE)</td>
<td>5.7\textsuperscript{a}</td>
</tr>
<tr>
<td>Butanol Fraction (SG-B)</td>
<td>27.0\textsuperscript{b}</td>
</tr>
<tr>
<td>Ethyl Acetate Fraction (SG-EA)</td>
<td>45.5\textsuperscript{b}</td>
</tr>
<tr>
<td>Hexane Fraction (SG-H)</td>
<td>12.8\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Yield calculated based on the amount of powdered leaf sample used in Soxhlet extraction. \text{Yield} = \frac{\text{mass of crude extract}}{\text{mass of leaf sample}} \times 100

\textsuperscript{b}Yield calculated based on the amount of crude extract used for fractionation. \text{Yield} = \frac{\text{mass of fraction}}{\text{mass of crude extract}} \times 100
dexamethasone required less than 20 mg/kg to achieve the half-maximal effect, the crude ethanolic extract required 129.7 ± 10.5 mg/kg to achieve the same effect. For the fractions, the ethyl acetate fraction had an ED\textsubscript{50} value similar to the crude ethanolic extract whereas both butanol and hexane fractions had ED\textsubscript{50} values of 1312 and 1611 mg/kg respectively.

The total antioxidant capacity, as determined from the phosphomolybdenum assay, was 6.7 gAAE/100g and 8.3 gAAE/100g for crude ethanolic extract and ethyl acetate fraction respectively (Table 3). There was no significant difference (P < 0.05) between the antioxidant capacities of both extract and fraction.

4. Discussions
Over the years, plants have served as an important source of compounds for managing inflammation-associated ailments and other diseases. Salicin, a natural product derived from the willow tree, has been used to treat inflammatory conditions since time immemorial (Vane & Botting, 1987). Many other plants are widely distributed in folkloric medicine for the treatment of inflammatory-related conditions. However, scientific validation for most of these plants are absent and represents a major challenge for the industry (Angell & Kassirer, 1998). In this study, the anti-inflammatory and antioxidant activities of the ethanolic leaf extract of \textit{S. gratus} was evaluated. The ethanolic extract...
was then fractionated sequentially with different solvents to identify the fraction where the anti-inflammatory principles were localized. The antioxidant activity of the most potent fraction was also evaluated.
The extraction of leaf samples was achieved using ethanol as solvent. The choice of ethanol as solvent was to mimic the traditional method of plant preparation, where the plant sample is extracted with ethanol. Extraction via Soxhlet afforded a yield of about 6%. This yield is similar to that usually obtained for solvent extraction of plant materials (Laryea & Borquaye, 2019). Fractionation of the ethanolic extract with solvents of increasing polarity revealed that most of the constituents of the ethanolic extract were of medium polarity. Ethyl acetate fraction was the most abundant, with a yield of ~50%. Unsurprisingly, the hexane fraction gave the least yield. The ethanolic extract was initially evaluated for its ability to reduce edema induced in the foot paw of chicks. An ED$_{50}$ of about 129.7 mg/kg was recorded (Table 2). The anti-inflammatory activity of the crude ethanolic extract is good, considering the fact that this is only a crude extract. To localize the anti-inflammatory principles, the ethanolic extract was fractionated with solvents of varying polarities. Interestingly, the ethyl acetate fraction recorded a similar ED$_{50}$ (133.5 mg/kg) as the crude ethanolic extract. The other fractions (hexane and butanol) recorded ED$_{50}$’s greater than 1000 mg/kg. Thus, the anti-inflammatory principles were localized in the ethyl acetate fraction. There are two distinct phases associated with inflammation induced by carrageenan—an initial phase mediated by mast cell degranulation with histamine and serotonin releases and final stage characterized by inflammatory mediators such as prostaglandins, proteases, and lysosomes (Eddouks, Chattopadhyay, & Zeggwagh, 2012). These two stages can be clearly identified on the time course curves (Figures 1 and 2). It is possible the extracts interacted with inflammatory processes in both stages, as has been speculated for other plant extracts (Mensah, Donkor, & Fleischer, 2011; Mensah & Amoh—Barimah, 2009).

There is a strong link between oxidative stress and inflammation. It has been reported that patients who suffer from inflammatory diseases are often present with diminished levels of antioxidants either as a result of inadequate dietary intake or, most likely, due to increased demand of antioxidants in situations of overwhelming production of reactive oxygen species by activated immune effector cells such as macrophages (Mangge, 2014). Medicinal plants with anti-inflammatory properties have been shown to possess antioxidant properties (Mensah et al., 2011; Gonçalves, Dinis, & Batista, 2005; Schinella, Tournier, Prieto, De Buschiazzo, & Ríos, 2002). We therefore investigated the antioxidant property of the crude ethanolic extract of the leaves of S. gratus. Since the ethyl acetate fraction possessed the greatest anti-inflammatory activity amongst the fractions, we evaluated its antioxidant activity as well. No significant difference (p > 0.05) existed between the total antioxidant capacities of the two analytes, using the phosphomolybdenum assay. Since the anti-inflammatory activities of the two extracts were similar, it is possible both contain comparable active principles.

Although information on the biological activity of S. gratus is scanty in the literature, other species in the genus Strophanthus have been shown to possess various biological activities. Iheanacho and coworkers evaluated the phytochemicals and toxicity of the root extracts of Strophanthus hispidus (2016). Extracts from Strophanthus hispidus were shown to have antimicrobial, antioxidant and wound healing properties (Agyare et al., 2013) whereas aqueous root extract of Strophanthus hispidus was also shown to

### Table 3. Total antioxidant capacity of crude extract and ethyl acetate fractions of the leaf of *Strophantus gratus*

<table>
<thead>
<tr>
<th>Concentrations (ppm)</th>
<th>Crude Ethanolic Extract (g/100g)</th>
<th>Ethyl Acetate Fraction (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>8.2 ± 2.5</td>
<td>10.4 ± 2.1</td>
</tr>
<tr>
<td>400</td>
<td>7.1 ± 3.0</td>
<td>8.8 ± 1.5</td>
</tr>
<tr>
<td>600</td>
<td>6.0 ± 1.3</td>
<td>7.5 ± 0.9</td>
</tr>
<tr>
<td>800</td>
<td>5.4 ± 2.6</td>
<td>6.5 ± 3.1</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>6.7 ± 1.0$^a$</td>
<td>8.3 ± 1.4$^a$</td>
</tr>
</tbody>
</table>

SD: Standard Deviation

$^a$No significant difference exists between mean AAE of crude extract and ethyl acetate fraction since p > 0.05
exhibit anti-inflammatory activity (Agbaje & Fageyinbo, 2012; Ishola, Awodele, Oreaqba, Murtala, & Chijioke, 2013). Other extracts of the same plant have been shown to possess cardiac protective and anti-hypertensive activities (Gundamaraju et al., 2014) and hypoglycemic activity (Ojiako & Igwe, 2009). Since most of the species in the genus are usually used for similar indications in traditional herbal remedies, it is possible that S. gratus will possess similar biological activities as that of S. hispidus.

5. Conclusion
The results of this study indicate that the ethanolic crude extract of the leaves from Strophanthus gratus reduces carrageenan-induced paw edema in chicks in a dose-dependent manner. The use of the plant to treat inflammation resulting from wounds and snake bites, thus, has scientific justification as the plant has been shown to possess anti-inflammatory and antioxidant activities.

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Competing interests
The authors declare that there were no competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

Author contributions
LSB conceived the study. All experiments were designed by LSB and SOB. Sample collection and all experimental procedures were carried out by SOB. Data analysis was by SOB and LSB. The initial manuscript was drafted by SOB and edited by LSB. All authors read and approved the final manuscript.

Data availability
All data generated or analyzed during this study are included in this published article.

Ethics
The project proposal and procedures were reviewed and approved by the Institution Ethics Review Board for Animal Use at the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

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