Original Paper   http://ajol.info/index.php/ijbcs   http://indexmedicus.afro.who.int

Anti-microbial, anti-inflammatory and anti-oxidant bioactivities of the methanolic extract of *Alafia zambesiaca* (stem-bark)

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ABSTRACT

Alafia zambesiaca is a medicinal plant used widely in Ghana for the treatment of gastric/peptic ulcers. The objective of this study was to comparatively assess the effect of the methanolic and the diethylether extracts of the plant phytochemicals on three bioactivities indicative of gastric/peptic ulcers etiology. Using standard laboratory assays involving broth dilution, carrageenan-induced foot swelling of 7-day old chicks and DPPH radical scavenging, this study compared and contrasted the antimicrobial, the antioxidant and the anti-inflammatory bioactivities of the methanolic extract with that of the diethyl ether extract of the stem-bark of *Alafia zambesiaca*. Phytochemical analyses proffered mechanistic explanations for the differing solvent-specific extract bioactivities by reporting the presence of tannins, flavonoids, saponins, glycosides and triterpenoids in the methanolic extract and by demonstrating that saponin is the only detectable phytochemical in the diethylether extract. The methanolic extract was comparatively the more potent anti-oxidant *in vitro* as demonstrated by its relatively higher anti-oxidant capacity, its comparatively higher total phenolic content and its disproportionately lower IC_{50}'s in the DPPH and H_2O_2 antioxidant assays. Consistently for each of the panel of four bacterial and two fungal pathogenic microbial cell lines, the methanolic extract showed higher anti-microbial activity recording, in each case, MICs that were quantitatively lower than that of the diethylether extract. *In vivo* anti-inflammatory activity using the carrageenan induced chick feet edema method indicated the methanolic extract's evocation of a dose-dependent reduction in foot edema and the diethyl ether extract's lack of display of measurable anti-inflammatory activity. Taken together, the more polar methanolic extract contains quantitatively more phytochemicals that have qualitatively more potent bioactivities and this observation gives credence to the use of aqueous stem-bark extracts of *Alafia zambesiaca* for the ethnomedicinal management of gastric ulcers. *Alafia zambesiaca* mediates its anti-ulcer effects in gastric mucosa possibly through polar phytochemical-triggered suppressive effects on microbial proliferation, via polar phytochemical-evoked mitigation of concurrent cellular oxidative stress and through polar phytochemical-modulation of inhibitory effects on cellular inflammatory events.

INTRODUCTION

The incidences of gastric ulcers are primarily linked to the pathogenic activities of *Helicobacter pylori* on the gastric, duodenal or stomach mucosa (Dovjak, 2017). The persistence of the *Helicobacter pylori* infection and its resultant virulent activities elicits inflammatory responses from gastric tissues

and generates Reactive Oxygen Species (ROS) as additional secondary responses (Dajani and Klamut, 2000). Gastric ulcer is a multifactorial process. Key etiologic mechanisms underlying the development and the progression of gastric ulcers therefore include the persistence of microbial infection, the elicitation of oxidative stress and the evocation of inflammation (Dajani and Klamut, 2000 ; Dovjak, 2017). The efficacious use of *Alafia zambesiaca* against gastric ulcers suggests that its phytochemical pool provide resilience against pathophysiological processes that confer susceptibility to gastric ulcers and that ensures its progression to disease of increasing severity such as microbial proliferative growth and microbial virulence, such as ROS generation and ROS-mediated oxidative processes and such as cellular inflammatory responses.

Although Ghanaian ethnomedicine has historically used the stem-bark of *Alafia zambesiaca* for the management of gastric and peptic ulcers that present with different clinical manifestations, a survey of the literature indicated that no systematic approach has been made to evaluate these anti-ulcer properties of the stem-bark of *Alafia zambesiaca.* The scientific literature reports on the phytochemistry of *Alafia zambesiaca* are meager and the anti-ulcer properties of *Alafia zambesiaca* are completely unexamined. Logically, the literature is also surprisingly silent on the defining biomedical properties that permit *Alafia zambesiaca* to evoke the collective potential bioactivities of anti-inflammatory, antioxidant and antimicrobial properties that are considered key underlying mechanisms of its net anti-ulcer effects (Charles-Dominique, 2016). Therefore, the central hypothesis of this study is that *Alafia zambesiaca* attenuates and/or manages gastric and peptic ulcers through a mechanism that involves the suppression of microbial proliferation, the mitigation of concurrent cellular oxidative stress in mucosa tissues and the inhibition of downstream cellular inflammatory events. The objective of this study was to comparatively assess the effects of the methanolic and the diethylether extracts of the plant phytochemicals on three bioactivities indicative of gastric/peptic ulcers etiology.

Using conventional *in vitro* and *in vivo* bioactivity-specific assays, this study reports that differential extraction-solvent dependent phytochemical pool bestows *Alafia zambesiaca* stem-bark extracts with unique biochemical properties that encompasses the endowment of its methanolic extract with: more potent anti-oxidant activities *in vitro*; with stronger anti-microbial activities *in vitro* and with a more efficacious *in vivo* anti-inflammatory activities. This study is instrumental in providing a deeper understanding of the anti-ulcer effects of *Alafia zambesiaca* and provides some mechanistic insights into this previously unexamined ethnomedicinal-based efficacy of *Alafia zambesiaca* as an anti-ulcer remedy.

MATERIALS AND METHODS
Chemicals

All reagents were of analytical grade. Nutrient broth and nutrient agar, DPPH (2, 2-Diphenyl-2-picrylhydrazyl) and Ascorbic Acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diethylether, Ethanol and Methanol were obtained from Merck Chemical Supplies (Damstadt, Germany).

Sample collection

The plant sample was purchased from central market in Kumasi, Ashanti Region, Ghana. Plants were identified based on exomorphic characters and a review of literature as *Alafia zambesiaca* by the Department of Herbal Medicine, herbarium at KNUST, Kumasi (Kupicha, 1981; Leeuwenberg, 1997). A voucher sample was deposited at the herbarium of KNUST.

Sample preparation and extraction

The plant sample was air dried continuously at room temperature (27°C) until successive weighing of the plant sample yielded no loss in weight. Plants were then pulverized and ground into powder and stored in air-tight containers.

Extraction of phytochemicals was carried out by the Soxhlet method using methanol and diethyl ether as extraction solvents. Extraction solvent was then evaporated on a rotary evaporator. Dried extracts were kept in air-tight glass containers and were kept frozen until further analyses were needed.

Phytochemical screening

Phytochemical screening was conducted on both the powdered sample and on the two extracts for the presence of flavonoids, coumarins, tannins, saponins, glycosides, steroids and triterpenes, anthraquinones and alkaloids as described by Trease and Evans (1984).

Diagnostic TLC

Silica gel coated plates Thin-Layer Chromatography (TLC) was utilized to show the approximate number of distinct chemical entities within each extract. Iodine vapor was the visualization agent for chromatographic bands. Retention factor (Rf) of resolved bands were computed as the ratio of the distance moved by the spot to the distance moved by the solvent.

Assessment of anti-microbial activity
Culture and maintenance of microorganisms

Microorganisms used were acquired from ATTC and were stored at the microbiology lab of the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi. Two gram negative (*Pseudomonas aeruginosa* and *Escherichia coli*), two gram positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and two fungal strains (*Candida albicans* and *Tinea corporis*) were used for the study. Earlier reports have described the genotype, storage, culture and maintenance of these microbes (Mensah et al., 2019).

Broth dilution assay

Broth dilution assay was performed using previously reported protocols (Mensah and Amarh, 2018). *Alafia zambesiaca* extract were diluted to different concentrations and

applied on the list of pathogenic microbial specimen as specified in Table 2. Ciprofloxacin and clotrimazole were used as positive controls. Un-inoculated sterile broth media with and without extracts were used as negative controls. Minimum inhibitory concentrations (MIC) of extracts were expressed in µg/mL and were taken as the lowest extract concentration that displayed complete microbial growth inhibition. MICs were visualized by the absence of violet coloration of the reaction mixture after the addition of 0.1 mL of MTT dye.

Agar diffusion assay

The protocol for the agar diffusion is a modified form of a previous report (Mensah and Golomeke, 2015). The size of the diameters of the zone of inhibition is indicative of the level of microbiocidal activity of the applied sample. A larger zone of inhibition around the wells denotes high susceptibility to the antimicrobial agent.

Assessment of anti-inflammatory activity
Animals

Prior report has described all the salient features involved in the housing, feeding and the preparation of the day-old chicks used for the assessment of the anti-inflammatory activity (Mensah and Armah, 2018). In all cases, 5 chicks were used per each extract concentration and per each control drug. Ten chicks were used as negative controls (Mensah et al., 2020).

Carrageenan-induced foot edema in 7-day old chicks

Detailed description of the carrageenan-induced foot edema model in 7-day old chick is reported elsewhere (Mensah and Amarh, 2018). Induction of inflammation in the sub-plantar of the right foot of the chicks and the subsequent treatment of chicks with extracts and with control drugs is similarly described in the previous literature (Mensah and Amarh, 2018). Serial monitoring of the swollen foot with and without extract treatment at hourly intervals over the 6 h post treatment (pt) time-course and over the 30-300 mg/kg extract dose-range were earlier described by Mensah and

Amarh (2018). The stem-bark extracts of *Alafia zambesiaca* at three different concentrations (30, 100, 300 mg/kg) were then administered to the chicks orally while the standards Diclofenac (10-100 mg/kg) and Dexamethasone (0.3-3.0 mg/kg) were administered as positive controls via injection. Negative control animals were given only normal saline.

Data analysis for the anti-inflammation assay

Data analysis for the carrageenan-induced inflammation employed a one-way analysis of variance (ANOVA) and differences between the groups of chicks were followed by Dunnett's post hoc test. Analysis of differences in AUCs expresses the entire foot volume for each treatment group. Equation (1) was then used to estimate the percentage inhibition of edema for each treatment group.

$$\% \ inhib. of \ edema = \frac{AUC_{control} - AUC_{treated}}{AUC_{control}} 100 \ (1)$$

Where, % inhib. of edema = % inhibition of edema.

The ED$_{50}$ (effective dose, 50%) and total foot volume for each treatment was calculated as the area under the curve (AUC) which were subsequently used to determine the percentage inhibition for each treatment. GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla, California, USA) was used for all statistical analyses and for the ED$_{50}$ calculations.

Assessment of the antioxidant activity
DPPH scavenging assay

Extracts' ability to scavenge free radicals was assessed with the 2, 2-diphenyl-1-picrylhydrazil (DPPH) assay as previously described (Mensah and Amarh, 2018). The percentage of DPPH radical scavenged was calculated using equation (2).

$$\% \ \textbf{Inhibition} = \ \textbf{Ao} - \frac{\textbf{Ai}}{\textbf{Ao}} \times \textbf{100} \qquad (2)$$

where *Ao* is the absorbance of the control and *Ai* is the absorbance of the sample. Ascorbic acid was the standard control.

Hydrogen peroxide scavenging assay

The protocol for the assessment of the H$_2$O$_2$ radical scavenging activity is described elsewhere (Mensah and Armah, 2018). Sample absorbances were taken at 510 nm on a UV-VIS spectrophotometer. Results were computed using equation (3).

$$\% \ \textbf{H}_2\textbf{O}_2 \ \textbf{Scavenging Activity} = \frac{A_{test}}{A_{control}} \times \textbf{100} \ (3)$$

Where A_{test} is the absorbance of the extract and $A_{control}$ is the absorbance of the control. Ascorbic acid was used as a standard.

Total antioxidant capacity (Phosphomolybdenum method)

The Total Antioxidant Capacity of the methanolic extract was assessed by the phosphomolybdenum method using a slight modification of protocols described by Mensah and Armah (2018). The blank solution was made by substituting the test sample with the solvent. The Total Antioxidant Capacity was expressed as Ascorbic Acid Equivalent (AAE) per 100 g of extract.

Total phenolic content (Folin-Ciocalteu method)

A modified procedure previously described by Mensah and Amarh (2018) was used for the assessment of the Total Phenolic Content of extracts. Gallic acid was used as a standard and the Total Phenolic Content of extracts were estimated by interpolation from a standard Gallic acid curve. The Total Phenolic Content of the extracts was expressed in Gallic Acid Equivalents (GAE) per 100 g of extract.

Statistical analysis

Statistical comparisons of data for each experimental point were made by analysis of variance (ANOVA), and statistical values were considered significant at p<0.05.

RESULTS
Extraction

Solvent strength-dependence of the yield of the total extractable phytochemicals from the Soxhlet extraction demonstrated that methanol yielded a total crude extract (12.8% dry weight) that was 4-fold higher in dry

weight when compared to that of the diethylether (2.9% dry weight) ($p<0.5$) (Table 1). Bioactive compound concentrations in the extracts are likely modest and may be present within the range of values deemed potentially pharmacological. *Alafia zambesiaca* secondary metabolites that suppress microbial growth, inhibit ROS and mitigate inflammation are therefore more likely present at bioactive concentrations in performed assays. Utilized concentrations of extracts in the bioassays are low and can be considered physiologically relevant.

Phytochemical screening

As shown in Table 1, the contrast in the scope of solvent-extracted phytochemical pool is sharp and highlights once again the differing strength of the two utilized solvents. While the methanolic extract yielded a wide range of phytochemicals that included Tannins, Flavonoids, Triterpenoids, Glycosides and Saponins, the diethylether extract demonstrated the presence of only Saponins. It is interesting to note that the powdered raw sample of the stem-bark of *Alafia zambesiaca* tested positive for coumarins as well and this observation suggests that none of the two utilized solvents was capable of coumarin extraction.

TLC

The three discernible TLC bands of the methanolic extract along with the two TLC bands for the diethyl ether extract comprise some of the discrete chemical entities detected chromatographically among the extracted phytochemical pool (Table 1). For both extracts, chromatographic bands were reasonably well-resolved as demonstrated by the wide variation in Rf values that had uniform spread (Table 1).

Anti-microbial assay

Microbial colonization, particularly *Helicobacter pylori* infection of the gastric mucosa, has been implicated in the development and the progression of gastric ulcers and in other forms of ulcerations. The efficacy of *Alafia zambesiaca* to evoke anti-microbial effects improves its clinical outcome as an anti-ulcer agent. To examine the anti-microbial effects of extracts of *Alafia zambesiaca*, the standard agar diffusion and the normalized broth dilution methods were used. Estimated zone of inhibition (agar diffusion) and assessed minimum inhibitory concentration or MIC (broth dilution) became relative quantitative measures of microbiocidal efficacies of extracts as described in Materials and Methods.

Agar diffusion assay
Methanolic extract

Pronounced dose-dependent zones of growth inhibition were evoked by the methanolic extract for all six microbial species (Table 2)**.** Maximal growth inhibition at all applied extract concentrations occurred with *Streptococcus pyogenes*. Individual growth inhibition of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* were comparable when paired against each other although they were manifestly weaker to that of *Streptococcus pyogenes* ($p<0.5$). Together, the methanolic extract triggered growth inhibitions of all pathogenic bacteria at a substantially lower potency (2-2.5-fold lower) than that of the standard drug (Ciprofloxacin) (Table 2) ($p<0.5$). The two fungal strains (*Candida albicans* and *Taenia corporis*) displayed growth inhibition at slightly reduced zones (1-5 mm units lower) when compared to that of all pathogenic bacteria species. Growth inhibition of *Taenia corporis* occurred at a marginally higher concentration than that of *Candida albicans*. The fungal control drug (Clotrimazole), used at a lower effective concentration, demonstrated a 1.5-2-fold difference higher growth inhibition on both fungi (Table 2) ($p<0.5$).

Diethylether extract

The diethylether extract failed to demonstrate an effective anti-microbial effect as was displayed by the methanolic extract on the test micro-organisms (Table 2) ($p<0.5$). Beside *Candida albicans* that evinced dose-dependent inhibition, all the other microbial

species (including all the bacteria specimen and *Taenia corporis*) showed minimal inhibition, even at the highest examined extract concentration (100 mg/mL). To re-state this observation, no observable zone of inhibition was displayed by all microbial species at tested concentrations that were below 100 mg/mL (Table 2) (p<0.5).

Broth dilution assay
Methanolic extract

Against all microbial panelists, the methanolic extract displayed broad potent anti-microbial effects on a scale comparable to that of the control drugs Ciprofloxacin and Clotrimazole (Tables 3-4). MICs were particularly low for bacteria [(0.19 mg/mL for *Pseudomonas aeruginosa* and *Escherichia coli*) and (0.39 mg/mL for *Streptococcus pyogenes*)]. Interestingly, growth of *Staphylococcus aureus* was inhibited at all tested extract concentrations]. MICs were similarly low for fungi [0.19 mg/mL for both *Candida albicans* and *Taenia corporis*]. Compared to the methanolic extract, the control drug showed little variability in growth inhibition potency (MICs) on the individual microbial and on the collective microbial samples (Tables 3-4) (p<0.5).

Diethylether extract

Besides *Staphylococcus aureus* whose MIC was quantitatively equal to that generated by the methanolic extract (0.19 mg/mL), all other MICs generated by the dietheylether extract were disproportionally higher in quantitative value and were demonstrably 3-fold differences higher than that of the methanolic extract [(32-fold difference higher for *Pseudomonas aeruginosa* and *Escherichia coli*) and (18-fold difference higher for *Streptococcus pyogenes*); (4-fold difference higher for the fungi *Candida albicans*) and (8-fold difference higher for the fungi *Taenia corporis*)] (Tables 3-4) (p<0.5).

In all cases and for all microbial species, a strong qualitative correlation was found between broth dilution MICs and agar diffusion zones of inhibition (Tables 2-4). The qualitative patterns of microbial growth

inhibition evoked by the two extracts were identical for both methods, with individual microbial species inhibited to the same relative extent when viewed in the prism of methodological differences. Nevertheless, higher relative sample (extract and control drugs) concentrations were generally utilized for the microbial growth inhibition in the agar diffusion assay due to the obvious resistance to sample diffusion and impaired contact with microbial specimen presented by the agar gel.

Anti-inflammatory assay

Given that inflammation has been etiologically linked to the development of and the increasing severity of gastric ulcers, targeting inflammation with extracts of *Alafia zambesiaca* may improve clinical outcomes. Using carrageenan-induced inflammation model in 7-day old chicks as described in Material and Methods, anti-inflammatory studies examined the time course induction of the loss of inflammation and studied the net dose-dependent effects of extracts on inflammation.

The 6 h pt time course measurement of loss of inflammation post extract treatment was a practicable experimental time course that corresponded to the early/acute inflammation phase and yielded baseline anti-inflammation time course profiles for extracts. Additionally, the three utilized *Alafia zambesiaca* extract concentrations were within physiological ranges and can be comparable to doses normally utilized by patients for treatment under clinical conditions.

Time-course studies

Time-course experiments were conducted to examine the windows of efficacy of anti-inflammatory action of extracts. For both methanolic and diethylether extracts, the onset of anti-inflammatory effects and its concomitant extent of maximum inhibition were undulating - portrayed as rising and falling phases of anti-inflammatory responses. Figures 1-2 compare the time course of presentation of inflammation and anti-

inflammation for the two extracts and for the two control drugs.

Methanolic extract

Time course changes in inflammation triggered by the different doses during the first hourly stage pt show highest recorded values per each sample concentration. This observation implies that initial anti-inflammatory responses for all sample concentrations were meagre (Figure 1a). Anti-inflammation persisted through the next hour as inflammation decreases of about 10% from its 1 h peak value were recorded after 2 h. Inflammation decreases were, however, not sustained as about 5% increases were observed after 3 h. After a temporary recovery of inflammation with an upward swing after 3 h, a second fall in inflammation was observed at 4 h pt where the displayed anti-inflammation persisted throughout the remaining 2 h study period. Specifically, decreases in inflammation were intensified into 5 h pt and finally into 6 h pt. Peak anti-inflammatory effect for all examined sample concentrations were recorded by the 300 mg/kg at 6 h pt (Figure 1a).

Diethylether extract

The 1 h pt phase of the anti-inflammatory response of the diethylether extract for the 3 doses showed a profile similar to that shown by the methanolic extract-treated chicks (Figure 1b). Each dose recorded the minimum observable anti-inflammation effect at the 1 h pt time-point. Inflammation progressively decreased for each applied dose of extract and yielded dose-specific profiles. However, the extract-mediated anti-inflammatory recovery after the 1 h pt in this cohort of chicks was slower and weaker and such weakness persisted up to the 6th hour. The recorded 1 h peak inflammation: decreased significantly for the 30 mg/kg sample with increased in pt time; maintained constant for a short period and decreased slightly for the 100 mg/kg; and increased for a short duration for the 300 mg/kg before decreasing progressively. The 30 mg/kg sample concentration saw progressive decreases in inflammation up until 6 h pt; the 100 mg/kg displayed progressive decreases between 4-6 h pt and so did the 300

mg/kg that recorded continuous progressive decrease between 4-6 h pt. Maximal anti-inflammation effect for all doses was recorded at 6 h pt (Figure 1b).

Diclofenac

In a pattern seen with the extracts, the 1 h pt time period recorded the minimal anti-inflammatory response as all sample concentrations of diclofenac displayed highest increases in foot volumes (Figure 2a). A similar undulating gradation in anti-inflammation response with increasing time was observed with diclofenac-injected chicks, although the steepness of the ebbs and flows were less deep. For all sample concentrations, foot volume decreases were recorded after 2 h pt but were not sustained for longer periods. Anti-inflammatory responses, therefore, decreased at 3 h but were then followed by progressive increases at 4-6 h pt. Peak anti-inflammatory response was recorded by the 100 mg/kg sample at 6 h pt (Figure 2a).

Dexamethasone

Maximum observable increases in foot volumes were recorded 1 h pt (Figure 2b). Quantitative estimations of anti-inflammatory responses were undulating - showing no distinct long-term pattern of sustained increase or sustained decrease with time. The highest dose of 3 mg/kg showed peak inhibition at 6 h after a series of ebbs and flows between 1-5 h pt. The 1 mg showed consistent progressive decreases 1-6 h after its minimum inhibition at 1 h pt. Similarly the 0.3 mg/kg dose recorded minimum inhibition at 1 h and maximum inhibition at 6 h pt while showing progressive decrease in foot volume from the peak increase in foot volume at 1 h pt to the least increase in foot volume at 6 h pt (Figure 2b).

Dose response

To examine the anti-inflammatory efficacy of extracts, the effect of increasing concentrations of extracts (30, 100, 300 mg/kg) and of control drugs [(Dexamethasone: 0.3, 1.0, 3.0 mg/kg); (Diclofenac: 10, 30, 100 mg/kg)] on the net loss of inflammation were examined relative to vehicle-administered controls. Chicks with induced inflammation of

the right foot were treated with the three sample doses for 6 h, and then the net loss of inflammation in the swollen foot were estimated and presented as bar graphs (Figures 3-4). For extracts, the doses used were 3-5-fold higher than doses typically used for ethnomedicinal remedy.

Methanolic extract

Anti-inflammatory responses induced by the methanolic extract were dose-dependent and were significant when evaluated against vehicle-administered controls. Maximal anti-inflammatory response of 64.1% was triggered by the highest applied dose of 300 mg/kg (Figure 3a). This anti-inflammatory response is quantitatively almost 2-fold stronger than that evoked by the 100 mg/kg sample dose (32.6%). The lowest dose (30 mg/kg) produced a 13.4% loss in inflammation, a response that constituted the minimum anti-inflammatory effect for the methanol extract (Figure 3a) ($p<0.5$).

Diethylether extract

Anti-inflammatory effects showed a clear non-linear dose response relationship for the 3 utilized extract concentrations. Both the minimum administered dose (30 mg/kg) and the larger dose (100 mg/kg) were ineffective, increasing the net inflammation to levels just beyond that induced by the vehicle-control anti-inflammatory response (Figure 3b). This observation suggests a null or inverse relationship between the applied extract dose and the anti-inflammatory response it evokes. Anti-inflammatory effects triggered by the highest dose (300 mg/kg) were, however, a paltry 9.6%, and was about 6-fold units lower than that induced by the same dose of the methanol-extract (Figure 3b) ($p<0.5$).

Diclofenac

Compared to the anti-inflammatory responses induced by the two extracts maintained at 10-fold higher concentrations, diclofenac, at 10-fold lower concentrations, triggered a comparatively steeper dose-response relationship. The three doses of 10, 30 and 100 mg/kg were sufficient to inhibit inflammation by 50.9%, 61.1% and 66.5% respectively when considered relative to vehicle-treated control chicks (Figure 4a) ($p<0.5$). This observation agrees with diclofenac's more potency as an anti-inflammatory agent than that either extract.

Dexamethasone

Although concentrations of dexamethasone (0.3, 1.0 and 3.0 mg/kg) were lowest in the series of utilized sample doses, it, nevertheless, evoked the strongest dose-response relationship when viewed by the proportional effects of the quantitative level of induction triggered by the individual applied sample doses. Doses of 0.3 mg/kg and 1.0 mg/kg significantly evoked substantial anti-inflammatory responses of 11.2% and 32.6% respectively (Figure 4b) ($p<0.5$). The highest dose of 3.0 mg/kg triggered a comparably high 60.7% anti-inflammation relative to that induced by the 30 mg/kg diclofenac dose (Figures 4a-4b) ($p<0.5$). In conclusion, dexamethasone causes the largest concentration-dependent suppression per dose of the carrageenan-induced inflammation of 7-day old chicks.

Comparative ED$_{50}$ values

ED$_{50}$ values show in both qualitative and quantitative terms, the relative ordering of the potency of anti-inflammatory potentials of extracts and control drugs. By far the control drugs were the most potent accounting for more than a 100-fold difference in anti-inflammatory efficacy relative to both extracts (Table 5) ($p<0.5$). The methanolic extract had a 2-fold difference lower ED$_{50}$ (higher anti-inflammatory effect) compared to the diethylether extract and this observation supports earlier dose-response data that showed higher anti-inflammation efficacy of the methanolic extract ($p<0.5$). Based on estimated ED$_{50}$ values, the order of decreasing anti-inflammation potency of the samples is: dexamethasone > diclofenac > methanolic extract > diethylether extract.

Anti-oxidant assays

ROS generation is etiologically linked to the development and the severity progression of gastric ulcers. Therefore, targeting ROS with extracts of *Alafia zambesiaca* may improve clinical outcomes. Using standardized bio-assays, extracts were therefore assessed for anti-oxidant bioactivities with *in vitro* models that included the estimation of the Total Phenolic Content (TPC), the assessment of Total Antioxidant Capacity (TAC) and the evaluation of the (2, 2-Diphenyl-1-Picrylhydrazyl) DPPH and H_2O_2 scavenging potentials.

Total Phenolic Content (TPC)

TPC of botanical extracts can serve as a reliable guide of potential antioxidant effects for extracts as a linear correlation is tentatively acknowledged to exist between TPC and induced antioxidant effects. To quantitatively estimate the TPC, extracts were subjected to the standard *in vitro* spectrophotometrical-based Folin-Ciocalteu colorimetric assay with Gallic acid as standard as described in Materials and Methods.

Solvent-specific extract differences in phenolic contents were substantial as the methanolic extract showed a 2-fold difference higher phenolic content compared to the diethylether extract (Table 6) ($p < 0.5$). This observation implies that methanol was a strong enough polar solvent to extract a phytochemical pool that has a larger proportion of polyphenols (for example tannins) than could diethylether.

Total Antioxidant Capacity (TAC)

TAC reflects the sum of the anti-oxidant potentials evoked by the total phytochemical constituents of an extract or a sample. As TAC broadly denotes the antioxidant activities of polyphenols, tannins and other unclassified antioxidants constituents, its quantitative level is reflective and predictive of the antioxidant status of an extract. TAC was assessed using the standard *in vitro* Phosphomolybdenum assay with Ascorbic acid as standard as described in Materials and Methods.

The 1.5-fold TAC increase of the methanolic extract over the TAC of the diethylether supports earlier assertions that the high solvent strength and high polarity of methanol enabled the extraction of a larger and broader antioxidant-capable phytochemical pool from *Alafia zambesiaca* (Table 6) ($p < 0.5$).

Anti-oxidant activities

As discussed above, quantitative trends in the TPC (Table 6) and in the TACs (Table 6) show direct parallels to observed anti-oxidant activities estimated as DPPH (Figure 5; Table 7) and as H_2O_2 (Figures 6 and 7) radical scavenging effects.

Comparative IC_{50}s show a more potent DPPH scavenging activity for the methanolic extract relative to the diethylether extract (the methanolic extract has a 1.3-fold lower IC50) (Table 7) ($p < 0.5$). Similarly, methanol extract demonstrated a stronger H_2O_2 scavenging activity (the methanolic extract possess a 1.8-fold lower IC_{50}) (Table 7) ($p < 0.5$).

Nevertheless, both the methanolic and the diethylether extracts were incapable of inducing IC_{50}s that were quantitatively comparable to that of the Ascorbic acid and to that of the Gallic acid controls (Table 7; Figures 5-6). In both assays, and for both extracts, at least a 10-fold differential IC_{50}s values were observed between the Ascorbic acid/Gallic acid controls and the individual extracts (Table 7) ($p < 0.5$). The implication of this observation is that both extracts were comparatively weaker antioxidants than the controls that are conventionally acceptable strong antioxidants.

For both extracts, DPPH and H_2O_2 radical scavenging activities increased with increasing extract concentrations. But increases in scavenging effects were only marginal at higher extract concentrations and the phenomenon yielded sigmoidal curves that shifted to higher percentage scavenging for the methanol extract (Figure 5 and Figure 6).

Table 1: Thin layer chromatography (TLC)-based estimation of total number of chemical entities and standard phytochemical analyses-based estimation of phytochemical composition of the methanolic and dietheylether extracts of the stem-bark of *Alafia zambesiaca.*

Sample	TLC Results		Phytochemicals Present
	Number of spots	**Rf values**	
Methanolic Extract	3	0.125, 0.235, 0.931	Tannins, Flavonoids, Triterpenoids, Glycosides, Saponins
Diethyl ether Extract	2	0.353, 0.588	Saponins

Table 2: Estimated zones of inhibitions (mm) for the methanol and the diethylether extracts of the stem-bark of *Alafia zambesiaca* in the agar diffusion assay. Zones of inhibitions (mm) estimated for the standard control drugs (Ciprofloxacin and Clotrimazole) in the agar diffusion assay are included for comparison.

|	Organisms	Concentration (mg/ml)/Zones of inhibition(mm)				Ciprofloxacin	Clotrimazole
		100	**50**	**25**	**12.5**		
Methanolic Extract	*Pseudomonas aeruginosa*	16	11	9	8	42	
	Escherichia coli	18	10	9	9	23	
	Staphylococcus aureus	18	10	9	9	41	
	Streptococcus pyogenes	17	15	14	13	33	
	Candida albicans	11	9	8	7		16
	Tinea corporis	16	11	10	9		33
Diethyl Ether Extract	*Pseudomonas aeruginosa*	15	No zone	No zone	No zone	30	
	Escherichia coli	12	No zone	No zone	No zone	20	
	Staphylococcus aureus	16	15	No zone	No zone	39	
	Streptococcus pyogenes	15	No zone	No zone	No zone	34	
	Candida albicans	18	8	9	No zone		10
	Tinea corporis	13	No zone	No zone	No zone		33

"No zone" denotes lack of microbiocidal effect.

Table 3: Growth inhibitory effects of extracts of the stem-bark of *Alafia zambesiaca* as estimated by MICs in the Broth Dilution assay. Estimated MICs for the standard control drugs (Ciprofloxacin and Clotrimazole) are included for comparison.

| Sample	Test Organisms	Concentration (mg/ml)							
		12.5	6.25	3.12	1.56	0.78	0.39	0.19	0.09
Methanolic Extract	*P. aeruginosa*	-	-	-	-	-	-	+	+
	E. coli	-	-	-	-	-	-	+	+
	S. aureus	-	-	-	-	-	-	-	-
	S. pyogenes	-	-	-	-	-	+	+	+
	C. albicans	-	-	-	-	-	-	+	+
	T. corporis	-	-	-	-	-	-	+	+
		12.5	6.25	3.12	1.56	0.78	0.39	0.19	0.09
Diethyl Ether Extract	*P. aeruginosa*	-	-	-	-	+	+	+	+
	E. coli	-	-	-	-	+	+	+	+
	S. aureus	-	-	-	-	-	-	-	+
	S. pyogenes	-	-	-	+	+	+	+	+
	C. albicans	-	-	-	-	+	+	+	+
	T. corporis	-	-	-	+	+	+	+	+
		Concentration (µg/ml)							
		12.5	6.25	3.12	1.56	0.78	0.39	0.19	0.09
Ciprofloxacin	*P. aeruginosa*	-	-	-	-	-	-	+	+
	E. coli	-	-	-	-	-	-	+	+
	S. aureus	-	-	-	-	-	-	-	+
	S. pyogenes	-	-	-	-	-	-	-	+
		12.5	6.25	3.12	1.56	0.78	0.39	0.19	0.09
Clotrimazole	*C. albicans*	-	-	-	+	+	+	+	+
	T. corporis	-	-	-	+	+	+	+	+

+ indicates microbial growth; - indicates no microbial growth.

Table 4: MICs of all samples [(methanolic extract and diethylether extract of the stem-bark of *Alafia zambesiaca*; control drugs (Ciprofloxacin and Clotrimazole)] in the broth dilution assay summarized against all utilized test organisms for comparison.

| ORGANISM	MIC(mg/ml)		MIC(µg/ml)	
	Methanol Extract	Diethyl Ether Extract	Ciprofloxacin	Clotrimazole
Pseudomonas aeruginosa	0.39	12.5	0.391	
Escherichia coli	0.39	12.5	0.391	
Staphylococcus aureus	0.19	0.19	0.195	
Streptococcus pyogenes	0.78	3.12	0.195	
Candida albicans	0.39	1.56		1.56
Tinea corporis	0.39	3.12		1.56

(a) **(b)**
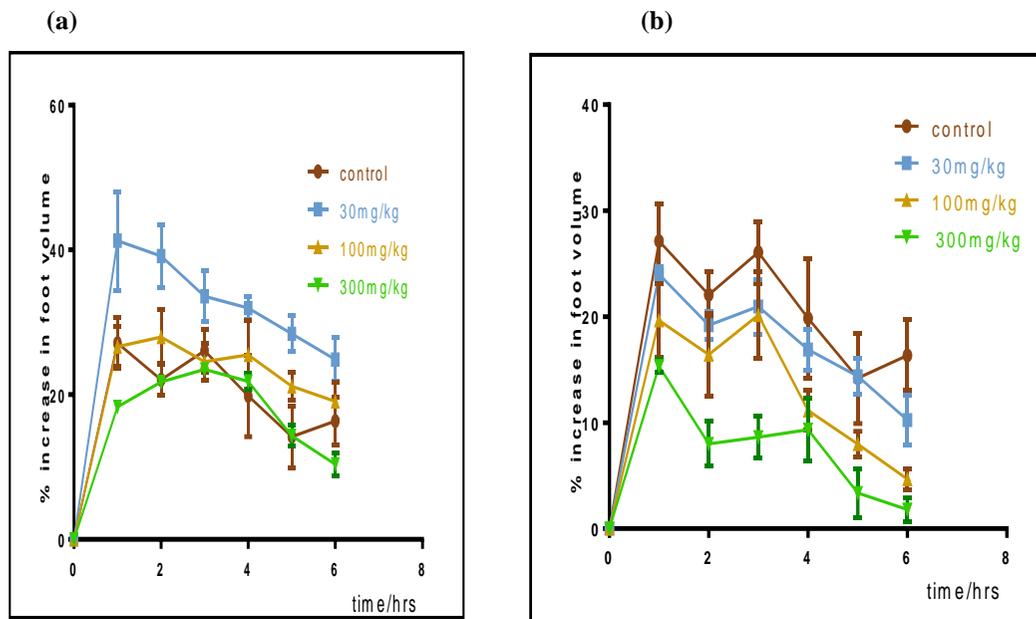
Figure 1: Extract-mediated time course of inhibitory effects on the carrageenan-induced inflammation of the 7-day old chick foot. Anti-inflammatory inhibitions in oedema volumes (%) per post treatment (pt) time (h) were estimated for: (a) the methanolic extract of the stem-bark of *Alafia zambesiaca*; (b) the diethylether extract of the stem-bark of *Alafia zambesiaca*. Three different doses (30, 100, 300 mg/kg) of samples were orally administered and each dose-mediated anti-inflammatory effect on oedematous volume is depicted by a different color.

(a) **(b)**
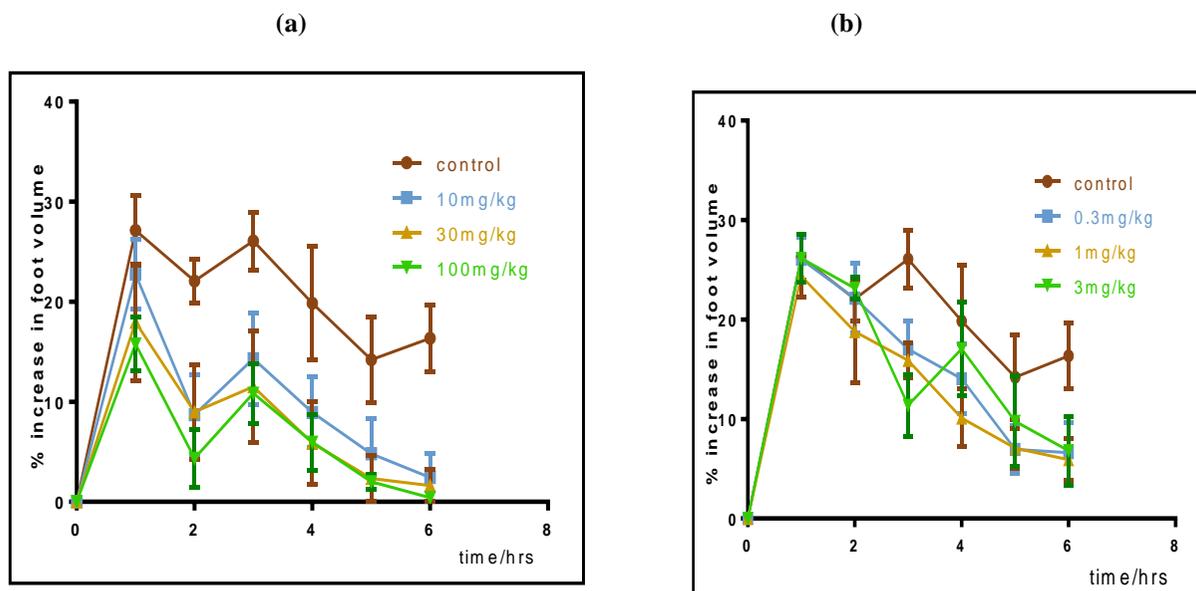
Figure 2: Time course of anti-inflammatory effects evoked by control drugs on oedema volumes in the carrageenan-induced inflammation assay. Inhibition of oedema volumes induced by the three different dose injections of: (a) Diclofenac: 10, 30, 100 mg/kg) and (b). Dexamethasone: 0.3, 1.0, 3.0 mg/kg pt are depicted by curves colored differently.

(a)　　　　　　　　　　　　　　　　(b)
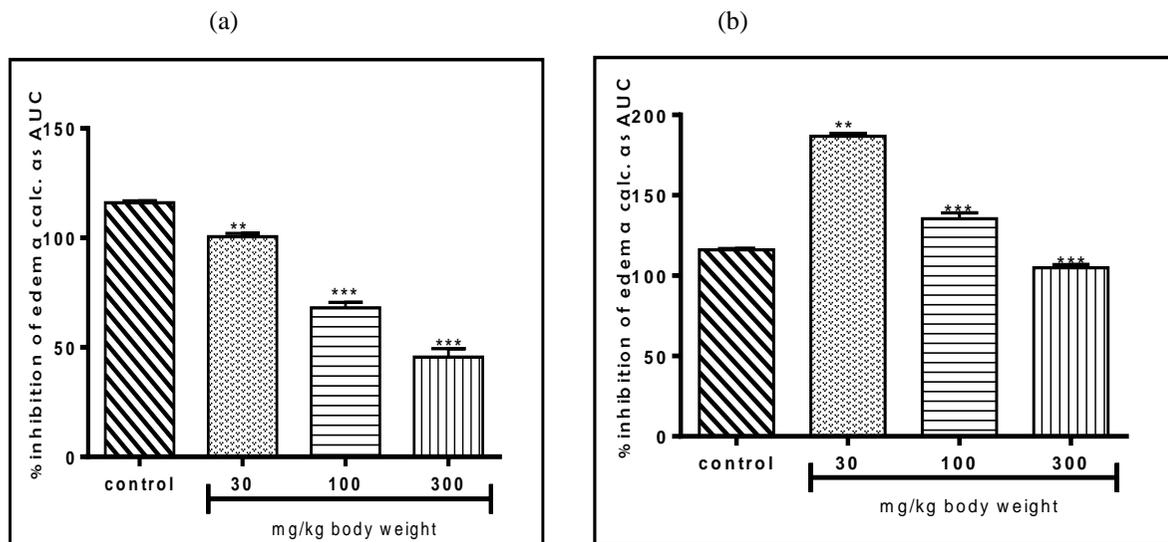
Figure 3: The dose-dependent anti-inflammatory responses of swollen chick foot to increasing concentrations (30, 100, 300 mg/kg) of: (a). methanolic extract and (b) diethylether extract. Estimated anti-oedematous responses to extract doses for 6 h pt were graded and are presented as area under the curve (AUC). (***P <0.001, **P < 0.01 and *P < 0.05 compared to the saline-treated control group).

(a)　　　　　　　　　　　　　　　**(b)**
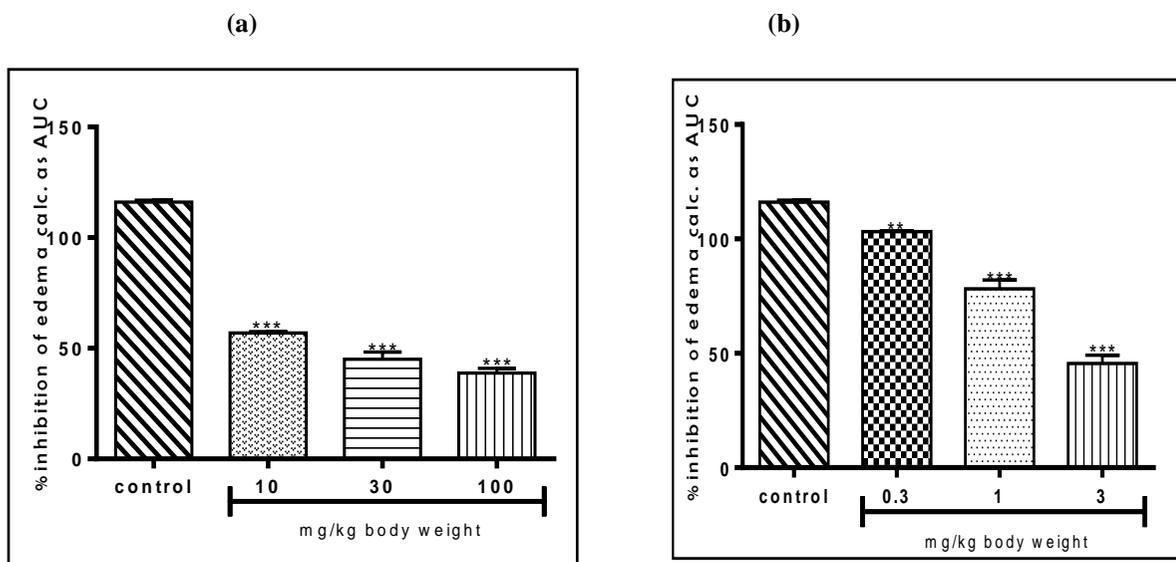
Figure 4: Control drug-induced graded anti-inflammatory responses (%) to graded doses of: (a). Diclofenac: 10, 30, 100 mg/kg) and (b). Dexamethasone: 0.3, 1.0, 3.0 mg/kg. A 6 h pt inflammation suppression by the three doses is presented as bar graphs. AUC in the bar graphs refer to area under the curve. (***P <0.001, **P < 0.01 and *P < 0.05 compared to the saline-treated group).

Table 5: Estimated anti-inflammatory ED_{50}s triggered by the methanolic and the diethylether extracts and by the standard control drugs (Dexamethasone and Diclofenac) in the carrageenan-induced oedema of 7-day chick foot.

Sample	ED_{50} (mg/kg)
Methanolic Extract	198.7
Diethyl Ether Extract	363.7
Dexamethasone	1.982
Diclofenac	5.850

Table 6: Total phenolic content (gGAE/100 g) and total antioxidant capacity (gAAE/100 g) of the methanolic and diethylether extracts of the stem-bark of *Alafia zambesiaca*.

| Sample	Total antioxidant capacity (gAAE/100 g)	Total phenolic content (gGAE/100 g)
Methanolic extract	15.820	13.588
Diethyl ether extract	10.4747	6.7686
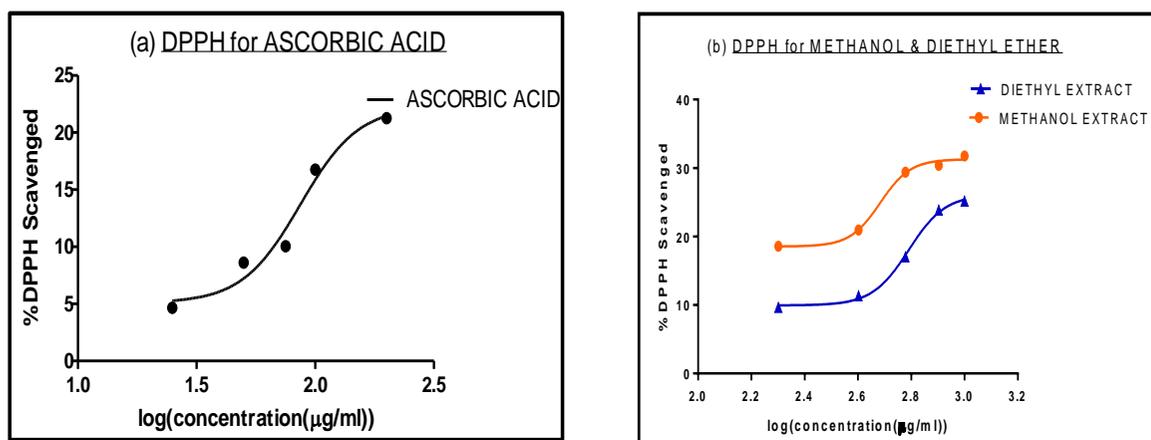
Figure 5: Radical scavenging efficacies of the methanolic extract and of the diethylether extract of *Alafia zambesiaca* graphically depicted in the DPPH assay for (a) Ascorbic acid control aligned with that of (b) both the methanol and diethylether extracts.

Table 7: Estimated DPPH and H_2O_2 anti-oxidant IC_{50}s triggered by the methanolic and the diethylether extracts and by their respective Ascorbic acid and Gallic acid controls.

Sample	DPPH scavenging (IC_{50}) (μg/mL)	H_2O_2 scavenging (IC_{50}) (μg/mL)
Methanolic Extract	482.4	388.3
Diethyl Ether Extract	617.4	688.4
Standard (Ascorbic acid [a], Gallic acid [b])	42.47[a]	64.16[b]

Triplicate repeats were used in each assay.
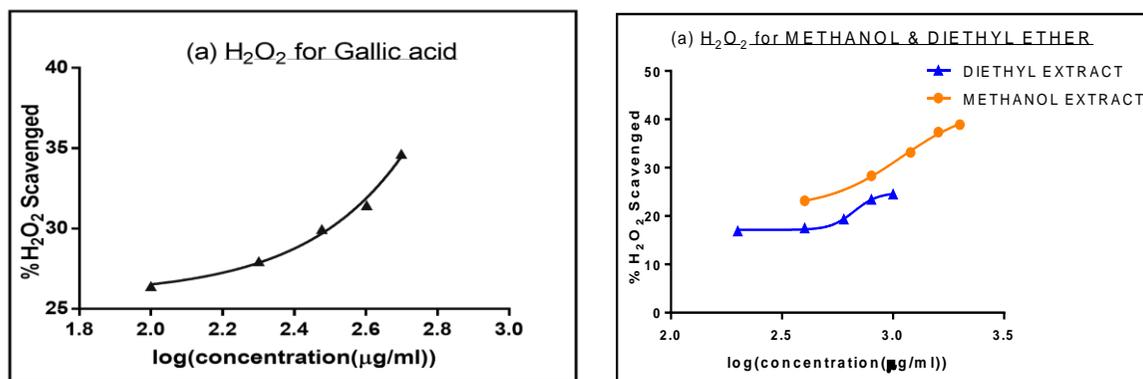
Figure 6: Radical scavenging efficacies of the methanolic extract and of the diethylether extract of *Alafia zambesiaca* graphically depicted in the H_2O_2 assay for: (a) Gallic acid control aligned with that of (b) both both the methanol and diethylether extracts.

DISCUSSION

This study described the strong dependence of solvent-specific phytochemical compositions on the net bioactivities of *Alafia zambesiaca* extracts and highlighted the three interesting extract-specific bioactivity differences that encompass the anti-microbial, anti-oxidant and anti-inflammation effects. Although the breadth of health disorders that employ *Alafia zambesiaca* for prophylaxis and for therapy is broad, key use prevalence in the Ghanaian ethnomedicinal context is for the management of peptic and gastric ulcers and for the treatment of ulcerated wounds on skin and muscles (Charles-Dominique, 2016). In gastric ulcers and in different pathological ulceration conditions, disease etiology derives from multiple sources but invariably encompass these three key elements of microbial infection, of ROS generation and of inflammatory responses (Dajani and Klamut, 2000; Dovjak, 2017). The natural product chemistry of *Alafia zambesiaca* is therefore of public health interest as its polar phytochemical pool is ethnomedicinally known to modulate cellular and tissue function/dysfunction within an ulcerated environment.

How does the polar extract of *Alafia zambesiaca* confer its anti-ulcer effects to cells and tissues of the gastric mucosa? Details of how *Alafia zambesiaca* mediates anti-microbial and antioxidant and anti-inflammatory responses to elicit its anti-ulcer effects are issues of current interest. The experimental data is scant but the evidence it presents of stronger anti-microbial, more potent anti-oxidant and greater anti-inflammatory bioactivities of the methanolic extract of *Alafia zambesiaca* is compelling and provides hints at the operative anti-ulcer mechanism. The presence in sufficient clinical quantities of polar phytochemicals such as Tannins, Flavonoids, Triterpenoids, Glycosides and Saponins with proven efficacy against different diseases mechanistically mediates studied biochemical functions. Ultimately, the observed efficacy of the polar constituents of *Alafia zambesiaca* depends on the strength of its mechanistic interaction with genetic and with epigenetic targets as well as on the potency of triggered downstream molecular events.

This observation supports the hypothesis that constituents of the polar extracts of *Alafia zambesiaca* are more

effective clinically in neutralizing ulcerated environment likely through the evocation of differential gene expression and the modulation of epigenetic activities of key genes that regulate and promote the formation and the progression of stomach ulcers. This hypothesis may be further supported once compelling additional evidence on the mechanistic action of specific identified phytoconstituents are provided.

Do specific phytochemicals of *Alafia zambesiaca* elicit specific functional roles or evoke broad and complementary functional roles? What are the identity of these bioactive phytochemicals and the identity of their genomic and epigenomic molecular targets? Definitive resolution of these pertinent issues requires cutting-edge research work. Based on its historical ethnomedicine use and on the strength of the data from this report, the polar methanolic extract of *Alafia zambesiaca* may now be regarded not only as repository of suitable phytochemical pool with which to manage such ulcers but also to elucidate some of the primary molecular events that occur mechanistically in its mitigation. *Alafia zambesiaca* phytochemical pool likely accomplishes this medicinal objective through an effective anti-inflammatory response that potentially targets NF-κB pathway genes (Borthakur et al., 2012); through an excellent anti-oxidative response that is mediated by COX-2 regulated genes (Murakami et al., 2009); and through an anti-microbial regime modulated by a hierarchical genetic program that ultimately evokes microbicidal and microbiostatic effects in resident microbial colonies (Chaparian et al., 2020). Polar phytochemicals of *Alafia zambesiaca* likely activate an antioxidant response through oxidative pathways that synchronizes with anti-inflammatory-enhancing effects and simultaneously contributes a microbiocidal and microbiostatic properties. Polar phytochemicals of *Alafia zambesiaca* can thus target three signaling pathways of antioxidant,

of anti-microbial and of anti-inflammatory properties that underlie the etiological development and severity progression of ulceration.

As a first step towards the assessment of how the phytochemical constituents of *Alafia zambesiaca* regulate both genetic and epigenetic events on targets, single active phytochemicals will have to be isolated on a platform of bioassays of clinical relevance to gastric ulcers and the phytochemical isolates structurally characterized. Biological characterization of the isolated phytoconstituents with regard to their safety and efficacy can then be undertaken through the monitoring of specific bio-marker activation/deactivation. Subsequently, the biochemical mechanism underlying its anti-ulcer activities can then be elucidated through an initial *in vitro* interaction with tissue specific receptors and later through *in vivo* regulation of specific molecular targets in an animal model. Extract molecular-based bioactivity is partly dependent on phytochemical structural binding interaction with cognate receptors and possibly on their pharmacokinetic interaction with the gastric microbiome. Such a proposed study will address critical gaps in our understanding of the mechanism of action of specific bioactive phytoconstituents of *Alafia zambesiaca* and, more broadly, elucidate how the triggered anti-microbial, anti-inflammatory and antioxidant pathways observed in this study interact to evoke beneficial anti-ulcer effects.

The rich availability of varied phytochemical classes with proven utility against diseases renders *Alafia zambesiaca* a botanical with high translational potential; particularly as a plant that is critical for the preservation and the maintenance of normal cellular function and for withstanding tissue dysfunction when exposed to an ulceration environment. Clearly, based on ethnomedicinal usage and on the strength of the presented evidence, *Alafia zambesiaca* has potential to reduce ulcers and an in-depth study of its

phytoconstituents will thereby pave the way for drug discovery leading eventually to better treatment modality against gastric ulcers and ulcer-related diseases. Since the molecular mechanisms through which *Alafia zambesiaca* mitigates ulcers remain unknown, it can be proposed that its future elucidation will be key to the development of a broad understanding of phytochemical regulation of molecular processes underlying both normal and disease gastric mucosa. Proposed study results should drive the future goal of moving *Alafia zambesiaca* towards a safe and effective botanical-inspired drug for the management of ulcers.

Towards this end, bioactive secondary metabolite isolation and structural characterization can be followed by organic synthetic-based structural elaboration to generate potential novel chemical entities. Pharmacological efficacy of individual phytochemicals can form the basis of a drug design program in which organic synthetic-based structural elaboration or derivatization can yield compounds with superior potency and improved specificity. Isolated and chemically identified bioactive phytochemicals derived from this study can be used alone or together with other drugs to boost the efficacy of ulcer pharmaceutical therapies through biochemical synergy (Caesar and Cech, 2019). Isolation of a single bioactive compound or utilization of multiple bioactive compounds in synergy in *in vitro* cell-culture models or in *in vivo* animal models may reveal mechanisms by which *Alafia zambesiaca* exhibit its anti-ulcer effects, and may elucidate the importance of specific mechanistic pathway(s) in mediating such anti-ulcer activities. More specifically, singly isolated and purified compounds can be serially tested (individually or in combination) for anti-microbial proliferation and anti-virulence activities against a panel of Helicobacter pylori clinical isolates.

The acceptance and widespread use of *Alafia zambesiaca* by the general public makes this report particularly appropriate as it sheds light on the molecular scientific underpinnings of gastric ulcer mitigation. The key observation from this study is that polar *Alafia zambesiaca* phytochemicals enhance the regulatory capability of gastric cells and tissues: to respond to acute inflammation; to react to microbial infiltration, specifically to Helicobacter pylori infection and to counter ROS-generative processes.

Conclusion

Alafia zambesiaca is a medicinal plant used in Ghana for the treatment of gastric/peptic ulcers. This study compared and contrasted the antimicrobial, the antioxidant and the anti-inflammatory bioactivities of the stem-bark methanol extract with that of the diethylether extract of *Alafia zambesiaca*. Different phytochemical compositions were proposed to underlie the mechanistic basis for the differing solvent-specific extract bioactivities. Tannins, flavonoids, saponins, glycosides and triterpenoids were observed in the methanolic extract while saponin was the only detectable phytochemical in the diethyl ether extract. Based on the strength of its varied phytochemical composition, the methanolic extract was the more potent anti-oxidant *in vitro* as demonstrated by its relatively higher total anti-oxidant capacity, its comparatively higher total phenolic content and its disproportionately lower IC_{50}'s in both the DPPH and H_2O_2 antioxidant assays. *In vivo* anti-inflammatory activity using the carrageenan induced chick feet oedema method indicated a dose-dependent reduction in foot edema by the methanol extract but displayed a lack of anti-inflammatory activity by the diethyl ether extract. Consistently for each pathogenic microbial cell type, the methanol extract showed higher anti-microbial activities against the panel of four bacterial and two fungal strains with MICs that were

quantitatively lower than that of the diethylether extract. By far the more polar methanol extract contains the more potent bioactive phytochemicals and this observation gives credence to the use of aqueous stem bark extracts of *Alafia zambesiaca* for the ethnomedicinal management of ulcers. *Alafia zambesiaca* likely mediates its anti-ulcer effects through polar phytochemical-modulation of suppressive effects on microbial proliferation, mitigation of concurrent cellular oxidative stress and inhibitory effects of cellular inflammatory events. This study does not only shed much-needed light on the molecular basis of its anti-gastric ulcers effects but may also have therapeutic potential in future drug development effort.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

JKM: Conceived the research idea, guided and instructed RT on the bench work and edited the final manuscript. RT: Performed most of the experimental work that generated the data for this manuscript and wrote the draft of the manuscript. YJ: Supervised the work on the anti-inflammatory section.

ETHICAL STATEMENT

The school of Pharmacy of KNUST adheres to international protocols on the ethical treatment of animals.

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