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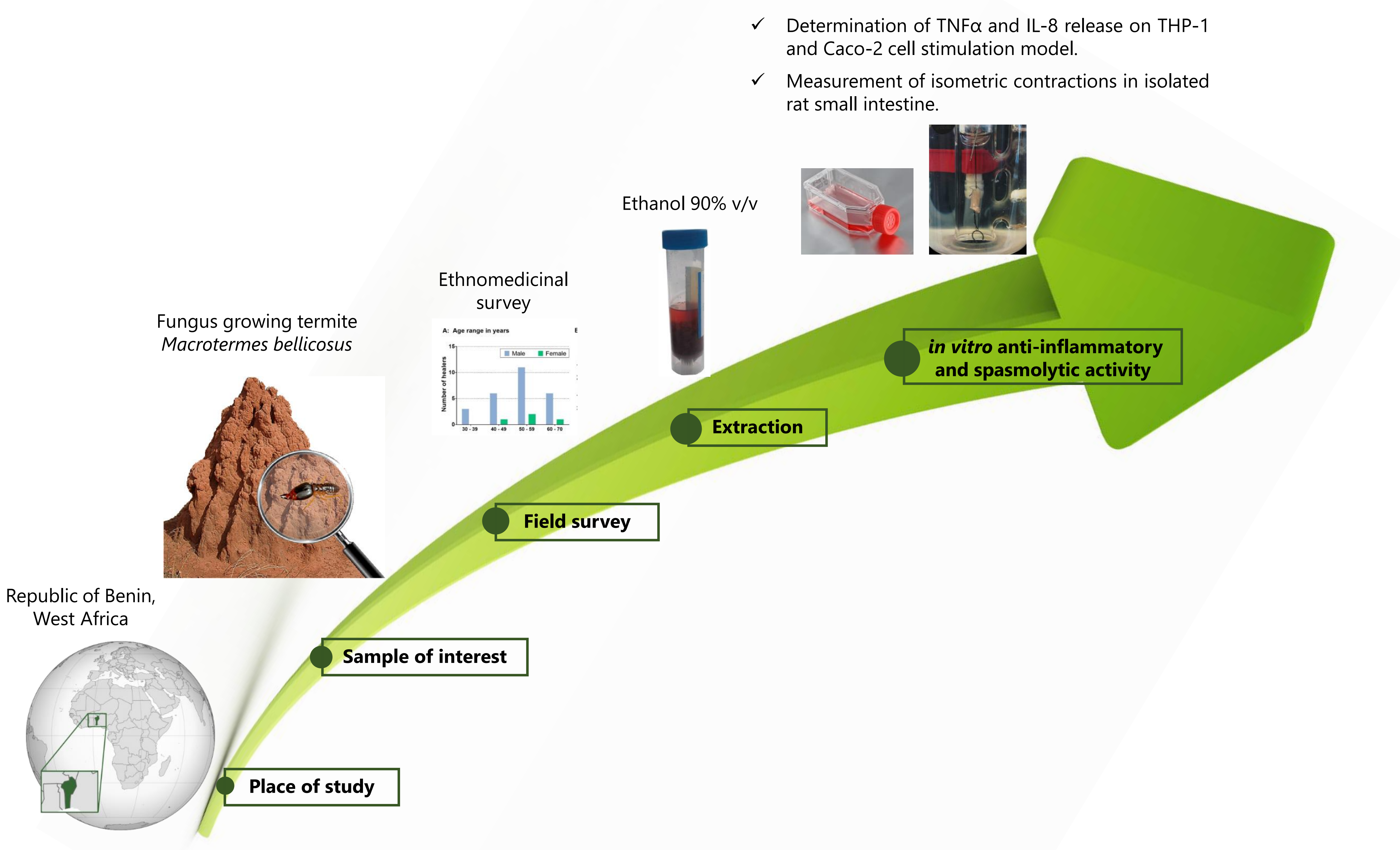
✓ Determination of TNFα and IL-8 release on THP-1 and Caco-2 cell stimulation model.
✓ Measurement of isometric contractions in isolated rat small intestine.
Ethanol 90% v/v
Ethnomedicinal survey
Fungus growing termite
Macrotermes bellicosus
Republic of Benin, West Africa
in vitro anti-inflammatory and spasmolytic activity
Extraction
Field survey
Sample of interest
Place of study

Ethnomedicinal survey and *in vitro* confirmation of anti-inflammatory and antispasmodic properties of the termite strain *Macrotermes bellicosus* used in traditional medicine in the Republic of Benin.

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Abstract

Ethnopharmacological relevance: Insects and insect-derived products play a vital role in traditional medicine in many parts of the world since ancient times. Among these insects, fungus-growing termites like *Macrotermes bellicosus* (*M. bellicosus*) are widely used in nutrition and traditional medicine in various societies of sub-Saharan Africa. **Aim of the study:** Aim of the present study was to explore the traditional applications of *M. bellicosus* and subsequently investigate the anti-inflammatory and spasmolytic activity of samples collected in Benin. **Material and methods:** An ethnomedicinal survey with thirty active healers in Benin was conducted and the anti-inflammatory activity of an ethanolic extract of *M. bellicosus* was investigated on LPS-induced TNFα release from differentiated human macrophages (THP-1) and IL-8 release from cytokine (IL-1β/TNFα/IFNγ)-challenged human intestinal epithelial cells (Caco-2) was measured by enzyme-linked immunosorbent assay. Furthermore, the influence of *M. bellicosus* extract on basal tone and induced contractions in isolated rat small intestinal preparations was determined to examine the influence on intestinal motility. **Results:** The survey of 30 active healers demonstrated that *M. bellicosus* and its products (termites' mound and fungus comb) are used in Benin for therapeutic purposes mainly to treat infectious and inflammatory diseases including digestive disorders, snake bites and diarrhea. It was found that *M. bellicosus* extract inhibited both LPS-induced TNFα release from human macrophages and cytokine-induced IL-8 release of intestinal epithelial cells comparable to budesonide. In addition, isometric contraction measurement with isolated rat small intestinal preparations demonstrated a mild spasmolytic effect of the termite extract in higher concentrations with a suppression of induced contractions and relaxation of basal tone. **Conclusion:** *M. bellicosus* which is used in traditional medicine in Benin to treat infectious and inflammatory diseases showed anti-inflammatory activity by inhibiting pro-inflammatory cytokine release and a moderate influence on intestinal motility.

Keywords: Termite, *Macrotermes bellicosus*, traditional medicine, ethnopharmacology, anti-inflammatory activity, spasmolytic activity, Benin.

List of abbreviations: ACh – acetylcholine; MBE – *Macrotermes bellicosus* extract

1. Introduction

Due to the prevalence of many diseases without suitable medical treatments available, the need for the discovery and development of new effective drug therapy is constantly high. Avenues of research are numerous, but the exploration of natural resources appears to be particularly promising as they are, by their biodiversity, the largest pool of active substances playing an important role in drug discovery (Harvey, 2008; Newman and Cragg, 2012). Unlike medicinal plants that are prominent in pharmaceutical research, the use of animals in traditional medicine and more specifically invertebrate animals such as insects has long been neglected. Among these insects, fungus-growing termites and their products (termite mound and fungus comb) play an important role in nutrition and traditional medicine in various societies of sub-Saharan Africa (Figueirêdo et al., 2015). Wildlife in Benin is very rich and varied, however, it is still not completely exploited scientifically. Benin is located in the tropics where most fungus-growing termites' subfamily of *Macrotermitidae* live (Lee and Wood, 1971). *Macrotermes bellicosus (M. bellicosus)*, a species belonging to the *Macrotermitidae* is native to Benin and traditionally used in nutrition and healing practice (Smeathman, 1781). So far, it has been reported mainly as a food source (Figueirêdo et al., 2015) presumably due to high levels of proteins and lipids found in this genus (Ntukuyoh et al., 2012). Furthermore, it was mentioned to be widely known in the agricultural context among traditional farmers as an important termite pest, causing considerable damage to agricultural crops (Yêyinou Loko et al., 2017). Reports on its medicinal use however are scarce and focus on the application of the mandibles to suture wounds (Marie, 1955).

The present study aims to explore the ethnomedicinal value of *M. bellicosus* in different regions of northern Benin and investigate their pharmacological properties. Based on the field data obtained, the research focus of the pharmacological studies was laid on the anti-inflammatory and intestinal motility-modulating potential of the soldier caste of this termite species. Thus, in the first instance, an ethnopharmacological survey was conducted with active healers from different regions of northern Benin. Secondly, the anti-inflammatory activity of an ethanolic *M. bellicosus* extract was tested using activated human monocyte-derived macrophages (THP-1) and intestinal epithelial cells (Caco-2) on which inflammation was induced *in vitro* and the release of pro-inflammatory mediators was determined. Furthermore, isometric contraction measurement was performed with isolated rat ileum/jejunum preparations to examine the influence of *M. bellicosus* on intestinal motility.

2. Materials and methods

2.1. Study area and ethnomedicinal survey

Traditional medicine is an integral part of the health care system in the Republic of Benin and eighty percent of the population relies on traditional medicine not least due to the high cost of allopathic pharmaceuticals (Massougbodji and Tevoedjre, 1997). In order to obtain and document information regarding the traditional therapeutic uses of *Macrotermes bellicosus (M. bellicosus)*, in northern Benin, an ethnomedicinal survey was conducted among traditional practitioners between January and February 2015. The survey was conducted in Kérou, Matéri, Tanguiéta and Natitingou (Fig. 1), four of the nine districts in the Atacora department located in northwestern Benin. Thirty (30) indigenous practitioners of traditional medicine were identified based on the recommendation of the oldest residents in the community (leaders, community agents, and representatives of rural associations) and interviewed using a previously prepared questionnaire. All participants were informed about the survey and written consent was obtained before personal visits were made to their facilities, centers and homes. Questionnaires were designed in English, translated and addressed to the traditional healers in their local dialect whereby a picture of *M. bellicosus* (soldier and worker caste) and their termite mound was used for visualization. The main questions focused on general knowledge and therapeutic use of *M. bellicosus*, its termite mound and fungus comb, including vernacular names as well as the mode of application. The full survey can be found in the supplement (Appendix A).

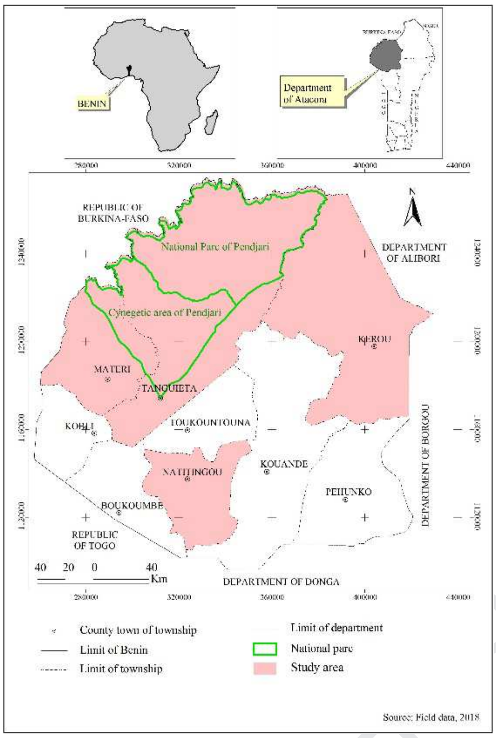

Figure 1: Geographical description of study area. Map of Atacora department showing the location of Kérou, Matéri, Tanguiéta and Natitingou districts.

2.2. Termite material

Termites belonging to the soldier caste of *M. bellicosus,* were collected in Abomey Calavi (6◦26'N, 2◦21'E) in the country of Benin during February 2016. The samples were authenticated by Dr. Laura E. Yêyinou Loko at the Faculty des Sciences et Techniques (FAST), Abomey Calavi University (UAC), Benin. Voucher specimen (ID-number AB-02-2016) have been deposited at the Inter-Regional University of Industrial Engineering Biotechnologies and Applied Sciences, IRGIB, Cotonou, Benin. A certificate for exportation was obtained from the Ministry of Agriculture and Husbandry.

2.3. *Macrotermes bellicosus* extract preparation

Termite samples were extracted according to a method adapted from Solavan et al. (2007). Thus, five grams of non-treated raw alive termites were collected from the field and extraction was initiated within one hour from time of collection. The material was stirred vigorously in 10 mL of ethanol HPLC grade 90% v/v (Alfa Aesar^{TM}) and macerated for one week at room temperature. The samples were subjected to sonication (15 min) followed by centrifugation (10 min, 6000 rcf) and the hydro-alcoholic phase was isolated from the residual deposit, filtered (Whatman^{TM} Grade 1 qualitative filter paper; pore size 11 µm) and the solvent evaporated. As a result, an extraction yield of 200 µg was obtained. The prepared extract was stored at -20ºC and used for the experiments. Analytical characterization of the extract used for the pharmacological investigations was performed using untargeted GC-MS analysis to putatively identify major peaks. Data evaluation and putative compound identification was performed by Chemstation (Agilent Waldbronn, Germany) and NIST 2011 (National Institute of Standards and Technology, USA) reference spectra database electron ionization (EI) spectra and kovats index information. Details can be found in Appendix B - supplementary data. For the pharmacological investigations, stock solutions of the dried extracts were prepared using DMSO whereby the final DMSO concentration did not exceed 0.01%.

2.4. Anti-inflammatory activity

2.4.1. Chemicals and reagents

RPMI medium 1640, DMEM high glucose medium, fetal bovine serum (FBS) and non-essential amino acids were purchased from Biowest, Nuaille, France. Penicillin/streptomycin (P/S) was procured from Biochrom AG, Berlin, Germany. Phorphol-12-myristate-13-acetate, lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4; impurities < 1% protein, Lowry), budesonide, triton-X, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO; ≥ 99.7%) and dimethylformamide (DMF) were obtained from Sigma-Aldrich, Steinheim, Germany. TNFα, IL-1β, IFNγ were obtained from Biomol, Hamburg, Germany. TNFα and IL-8 ELISA kits were purchased from BD OptEIA, BD Biosciences, Franklin Lakes, NJ, USA.

2.4.2. Cell culture and inflammatory stimulation

The human leukemic cell line THP-1 (ATCC, TIB-202) (Tsuchiya et al., 1980) which is commonly used to model macrophage function (Chanput et al., 2014) was cultured in RPMI 1640 supplemented with FBS (10%) and penicillin/streptomycin (P/S, 1%) at standard cell culture conditions. Differentiation to macrophage-like cells was induced with phorbol-12-myristate-13-acetate (100 ng/ml) for 48 hours. Differentiated THP-1 cells were then stimulated with LPS (100 ng/ml, LPS from *E. coli* 0111:B4) for 4 hours to induce a pro-inflammatory response as it has been demonstrated before (Chanput et al., 2013). Caco-2 is a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma (ATCC, Rockville, MD) commonly used to model the human intestinal epithelium (Sambuy et al., 2005). Caco-2 cells were cultured in DMEM containing 10% FBS, 1% P/S and non-essential amino acids (1%) at standard cell culture conditions. Inflammation on differentiated Caco-2 cells was induced by stimulation with a cytokine mix of TNFα (10 ng/mL), IL-1β (5 ng/mL) and IFNγ (10 ng/mL) for 24 hours.

2.4.3. Quantification of protein release

The influence of the *M. bellicosus* extract (1 – 200 µg/mL) on cytokine release was tested in double determination on LPS-stimulated THP-1 cells and cytokine-challenged Caco-2 cells. Untreated cells, cells only stimulated with LPS or cytokine mix and budesonide treated cells served as control. After incubation, TNFα and IL-8 were quantified in the cell free supernatants and remaining cells were immediately subjected to cell viability testing (MTT). Quantification of TNFα and IL-8 by enzyme-linked immunosorbent assay, ELISA (BD OptEIA™ Human ELISA kits, BD Biosciences, Franklin Lakes, NJ, USA) was performed according to manufacturer's instructions.

2.5. Cell viability assay (MTT assay)

Concomitant MTT assays were performed with the treated cells to ensure a stable cell viability throughout the assays. Thus, an MTT assay was used, to determine the metabolic activity of viable cells which are able to convert yellow soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) to purple formazan. Briefly, after cells (THP-1/Caco-2) were stimulated with LPS or cytokine mix, the supernatant has been removed and remaining cells were treated with 100 µL MTT (0.3 mg/ml in PBS) per well for two hours. Untreated cells and cells treated with Triton X (0.1%) served as control. After complete cell lysis using SDS lysis buffer (20% SDS, 30% DMF, pH = 4.7), the amount of resulting purple formazan was detected spectrophotometrically at 570 nm.

2.6. Influence on intestinal motility

2.6.1. Animal and tissue preparation

Adult male and female Wistar rats (13 - 17 weeks old, 300 – 500 g body weight) were obtained from the animal care facility of the Medical Faculty, University of Leipzig (Germany). Rats were housed in cages of five at room temperature in a 12-h light/dark cycle. Tab water and standard food pellets were available *ad libitum*. The rats were anaesthetized with CO_2 and sacrificed by decapitation. The abdomen was immediately opened by midline incision and the distal segment of the small intestine (approx. 15 cm) was excised and stored in aerated modified Krebs solution (118 mM NaCl, 25 mM Na_2HCO_3, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 11 mM glucose; pH = 7.4) at 37°C. All experiments were performed according to the German Animal Welfare Act and approved by the Institutional Review Board of Animal Care Committee (reference No. T 05/16 file reference: DD24-5131/347/7).

2.6.2. Isometric contraction measurement

Isometric contraction measurement was performed like described before (Vissiennon et al., 2015). Briefly, an ileum/jejunum segment of approximately 1.5 cm was prepared, cleaned and suspended in 20 mL organ baths (TSE Systems, Bad Homburg, Germany) containing aerated (95% O_2, 5% CO_2) modified Krebs solution maintained at 37°C. The preparations were allowed to equilibrate for 20 minutes with a preloaded tension of 10 mN. Tonic contraction was induced by application of acetylcholine (ACh, 1 mM) at minute 20 for conditioning and to test the sensitivity of the preparations used. Further, single ACh applications (minute: 40, 100 and 160) were used as internal standards. Between these controls, the *M. bellicosus* extract in ascending concentrations was applied two minutes prior induction of contraction (minute: 60, 80, 120 and 140) and the influence on basal tone directly after extract application as well as the intensity of ACh-induced contractions in relation to control applications was assessed.

2.7. Data evaluation

Data evaluation was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, California, USA). Data is expressed as mean±SEM; n represents the number of independent experiments/different animals used. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Concentration-response-curves and IC_{50}/IC_{25} values were obtained by non-linear least-square fit analysis, whereby IC_x describes the concentration of extract that induced x% inhibition.

3. Results

3.1. Ethnopharmacological survey

3.1.1. Sociodemographic characteristics of surveyed traditional healers

The interviewed traditional healers ranging in age from 30 to 70 years were mainly older than 50 years (66.6%) with the majority being men (86.7%) and very few being women (13.3%). Table 1 gives an overview of the sociodemographic characteristics of the study group. The medium age of the interviewed practitioners was 53.6±10.1 years and time of practice averaged on 28.6±11.3 years. Only one of the practitioners held a university degree while the majority has no formal education (n = 17; 56.7%); 30% have at most primary school education and a small proportion (10%) has a secondary school education. A majority of the healers have been in practice for 20 years and longer. Six ethnic groups (Bariba, Yoruba, Berba, Fon, Waama and Ditamari) were almost equally represented in the study group.

Demographic characteristics	Number of healers	Percentage
Age (years)		
30 - 39	3	10.0
40 - 49	7	23.3
50 - 59	13	43.3
60 - 70	7	23.3
Level of education		
No formal education	17	56.7
Primary	9	30.0
Secondary	3	10.0
University	1	3.3
Gender		
Female	4	13.3
Male	26	86.7
Experience (years)		
5 -10	2	6.7
11 - 20	6	20.0
21 - 30	9	30.0
31- 40	8	26.7
> 40	5	16.7
Ethnic group		
Bariba	5	16.7
Yoruba	5	16.7
Berba	6	20.0
Fon	4	13.3
Waama	6	20.0
Ditamari	4	13.3

Table 1: Sociodemographic characteristics of the interviewed traditional practitioners (n = 30) in the study area.

3.1.2. Vernacular names of *Macrotermes bellicosus* and criteria of recognition

The vernacular names of *Macrotermes bellicosus (M. bellicosus)* varied through the ethnic groups of the study area and are listed in Table 2. Reported vernacular names include *Toubanga*, *Kokoro*, *Tourou*, *Kossou kossou*, *Touman* and *Ditour*. Among healers of the same ethnic group, the reported vernacular names were consistent. The healers reported that criteria of recognition of the termite itself include mainly *size* (n = 29), *shape of mandibles* (n = 24) and *body color of soldier and worker termites* (n = 29) in connection with the nature of the termite mound where recognition criteria include *size* (n = 30), and *shape* (n = 24) of the mound as well as the *presence of the symbiotic fungi* (n = 18). Some healers also consider characteristics of the nest such as the presence of a *straw roof* (n = 22) or *wood* (n = 2) to identify the termite species.

Ethnic group (No.)	Vernacular name	No. of responses
Bariba (5)	Toubanga	5
Yoruba (5)	Kokoro	5
Berba (6)	Tourou	6
Fon (4)	Kossou kossou	4
Waama (6)	Touman	6
Ditamari (4)	Ditour	4

Table 2: Vernacular names of *Macrotermes bellicosus* per ethnic group.

3.1.3. Cultural, nutritional and medical uses of *Macrotermes bellicosus* and its products

Among the interviewed practitioners, the majority (n = 19, 63.33%) indicated to eat *M. bellicosus* (10 stated to not eat *M. bellicosus*; 1 answered with “no, but I know people who eat them”). Main consumed castes are the winged and soldier termites (each n = 19). Some respondents (n = 8) additionally reported to consume the queen (for cultural events or rituals for example before marriage to acquire virility, strength, courage and to become more respected by the people), whereby the animals are eaten fried, dried, roasted or raw (queen caste). Generally, reasons for consumption were medicinal purposes (19), nutrition (19), usage in cultural events/rituals (16) and spiritual protection (11). Collection of winged termites is realized during the rainy season by placing a lamp above a bowl of water to attract them by the light and scoop them from the water where they had fallen. The trapping of soldier termites is done by braking a small part of the termite mound and introducing a grass stem into a hole of the termite mound. The soldiers will bite in the stem and will be transferred into a container. The queen is collected from the royal chamber and often a whole termite mound has to be demolished in order to reach the queen chamber. With regards to its medicinal use, two thirds of the traditional practitioners indicated to use *M. bellicosus* and its products (termites’ mound and fungus comb) in a medical context (Table 3). Main indication for the medicinal use include infectious and inflammatory diseases like digestive disorders; mumps; snake bites; cough; diarrhea; dysentery; and pulmonary infection. Depending on the treated disease, oral and topical administration are the main reported modes of application described by the healers, whereby mostly powdered termites of the soldier, worker and alate caste are utilized and only in one case, the use of the termite queen (*in toto*, taken orally) was reported to treat pulmonary infections. In addition to the termites itself, the fungus comb as well as the termite mound were reported to be applied orally as powdered substance to treat diarrhea and digestive disorders or topically to treat mumps.

Medicinal use of *Macrotermes bellicosus*		**Number of responses**	**[%]**
	No	10	33.3
	Yes	20	66.7
Treated disease	**Mode of application**		
Cough	Dried powdered termites (soldiers and workers and/or alates) eaten with corn or cassava porridge	13	13.0
Digestive disorders	Dried powdered termites (soldiers and workers and/or alates) and powdered parts of the termite mound taken orally	20	20.0
Diarrhea	Powdered parts of the fungus comb and dried powdered termites (soldiers and workers and/or alates) eaten with corn porridge	13	13.0
Dysentery	Infusion of termite mound and dried powdered termites (soldiers and workers and/or alates) taken orally	13	13.0

Mumps	Dried powdered termites (soldiers and workers and/or alates) and powdered parts of the termite mound suspended in water and applied topically	17	17.0
Pulmonary infection	Dried powdered termites (soldiers and workers and/or alates) and raw queen termite (*in toto*) taken orally	7	7.0
Snake bites	Dried powdered termites (soldiers and workers and/or alates) suspended in water used as a poultice	17	17.0

Table 3: Medicinal use of *Macrotermes bellicosus* and modes of application.

3.2. Anti-inflammatory activity of a *Macrotermes bellicosus* extract

Based on the ethnomedicinal uses of *M. bellicosus* in inflammatory diseases registered throughout the survey, investigation of the anti-inflammatory activity of a *M. bellicosus* extract was performed. Thus, its effect on TNFα-release from activated differentiated THP-1 cells and IL-8 release from cytokine-challenged Caco-2 cells was monitored to evaluate the inflammatory response.

LPS (100 ng/mL, 4 hours) significantly increased TNFα-release from differentiated THP-1 cells (170.85±101.16 pg/mL vs. 2124.20±220.91 pg/mL; $p < 0.0001$, Fig. 2). This effect was inhibited after simultaneous application of the glucocorticoid budesonide (1nM) down to 688.69±92.63 pg/mL ($p < 0.0001$). Comparably, simultaneous treatment with *M. bellicosus* extract (1 – 200 µg/mL) led to an inhibition of the LPS-induced TNFα release to 15.45±17.27 pg/mL in the highest concentration with an IC_{50} of 41.99 µg/mL. Similarly, cytokine (TNFα, IL-1β, IFNγ)-stimulation of differentiated Caco-2 cells led to a significant increase of pro-inflammatory IL-8 release (6.79±1.14 pg/mL vs. 378.05±54.87 pg/mL; $p < 0.0001$, Fig. 2), which was inhibited after simultaneous budesonide application (274.01±68.97 pg/mL; $p < 0.01$). Treatment with *M. bellicosus* extract (10 – 100 µg/ mL) likewise inhibited the cytokine-challenged IL-8 release down to 56.53±18.28 pg/mL; $p < 0.0001$ (IC_{50} = 56.93 µg/mL). Overall metabolic activity of the cell population which was monitored throughout the assays using the MTT-test was not altered after treatment with LPS and cytokine mix, budesonide or *M. bellicosus* extract in the applied concentrations.

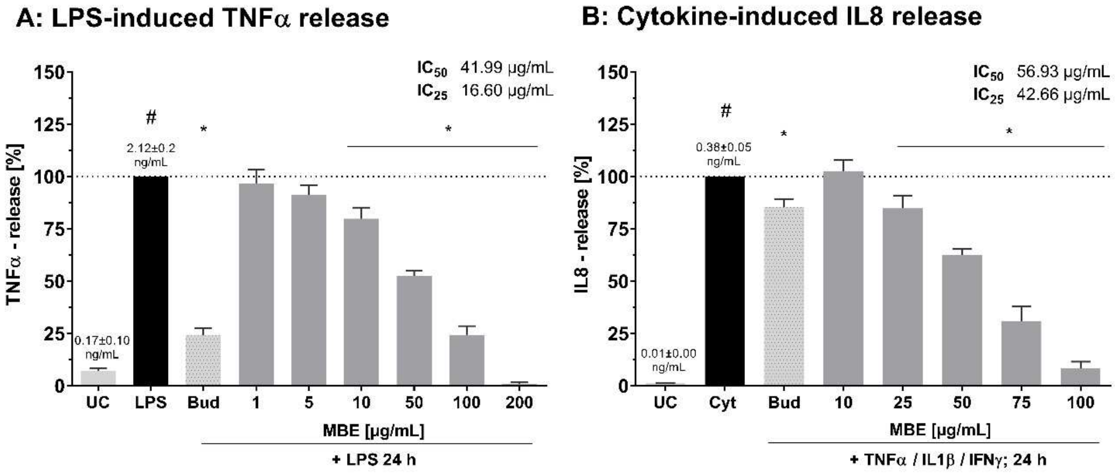

Figure 2: Effect of *Macrotermes bellicosus* extract (MBE) on LPS-induced TNFα release from THP-1 cells and cytokine induced IL8 release from Caco2 cells. UC: Untreated control, LPS: Lipopolysaccharide 100 ng/mL, Cyt: TNFα 10 ng/mL, IL-1β 5 ng/mL, IFNγ 10 ng/mL. Bud: Budesonide 1 nM (THP-1) / 0.1 µM (Caco2). Data are presented as mean±SEM and non-linear regression curve; * $p < 0.01$ vs. LPS or Cytokines mix, # $p < 0.01$ vs. UC, n = 12.

3.3. Influence of a *Macrotermes bellicosus* extract on intestinal motility

The influence of *M. bellicosus* extract on intestinal motility was characterized using isometric contraction measurement with isolated rat small intestinal preparations in an organ bath equipment. Thus, acetylcholine (ACh, 1 mM) was used to induce control contractions which were set 100%. Application of *M. bellicosus* extract (0.05 – 2.0 mg/mL) into the organ bath two minutes prior induction of contraction resulted in a concentration-dependent decrease of the induced contractions down to 41.85%±6.5 (% control) in the highest concentration (Fig. 3; IC_{50} = 1.54 mg/mL; IC_{25} = 0.81 mg/mL). Additionally, basal tone was assessed for two minutes after application of *M. bellicosus extract.* Application of 1 – 2 mg/mL induced a significant decrease in basal tone after 110 seconds down to -1.15±0.7 mN (1 mg/mL) and -2.07±2.4 mN (2 mg/mL). The effect was most pronounced in the highest concentration after 120 s (2 mg/mL, 120 s: -6.37 mN±5.5; Fig. 3).

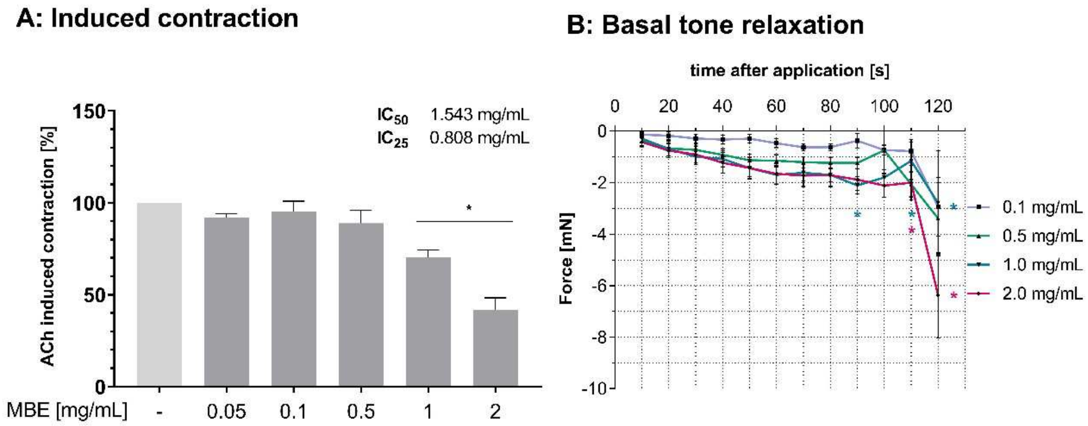

Figure 3: Influence of *Macrotermes bellicosus* extract (MBE) on (A) acetylcholine (ACh)-induced contractions and (B) basal tone in rat ileum/jejunum preparations. Data is presented as mean±SEM; * $p < 0.01$ vs. control; n = 6.

4. Discussion

The relevance of insects as a natural resource for food and medicinal purposes in various cultures around the world is well regarded. Of these, termites are commonly used insects in traditional popular medicine in the tropics and the African continent in particular (Figueirêdo et al., 2015). The present study provides an overview on the traditional medicinal use of the fungus growing termite species *Macrotermes bellicosus (M. bellicosus)* in the Republic of Benin and reports on pharmacological activity reinforcing its traditional medicinal application.

The sociodemographic characteristics of the surveyed traditional healers registered in this study were age distribution, gender, education, period of practice and ethnicity. It was observed from the collected information that most of the interviewed traditional healers were older than 50 years with long time of experience and a dominance of male gender while the majority has no formal education. This observation is in line with other ethnomedicinal studies, whereby it is well regarded that the level of education of traditional healers is not necessarily related to their potential and knowledge which depends mostly on their cultural heritage, intuition and experience achieved during practical work over the years (Agyare et al., 2009; Mathibela et al., 2015).The ethnicity of the interviewed healers was balanced and represents around 42.1% of the ethnic distribution of the Republic of Benin considering the Fon (24.2%) , the Yoruba (8%), the Bariba (7.9%) and the Ditamari (2%) ethnic groups in the study area (Kpeki, 2008).

The fungus growing termite species *M. bellicosus* was designated, with varying vernacular names which differed but were consistent throughout the ethnic groups. Parts of these observations were in line with findings from Yêyinou Loko et al. (2017), who has focused on the agricultural relevance of termites in northern Benin and interviewed farmers about their familiarity with termites in general and their utilization. Identical results were obtained with regards to the vernacular names such as *Toubanga* (Bariba) and *Ditour* (Ditamari). Additional vernacular names could be assigned for further ethnic groups like Yoruba (*Kokoro*), Fon (*Kossou kossou*), Berba (*Tourou*) and Waama (*Touman*). With regards to the therapeutic application, the use of termite mound soil of *M. bellicosus* for the treatment of mumps was also mentioned in this study. However, no medicinal use of the termites itself was reported by Yeyinou Loko et al. which is most likely due to the different target audience.

According to the interviewed traditional practitioners *M. bellicosus* and its products (termites' mound and fungus comb), is used by oral and topical administration for the treatment of infectious and inflammatory diseases. Main forms of application include the dried and powdered termites which are mixed with food (e.g. corn porridge) or infused for internal use or suspended in water for external application. The indicated medicinal use and application forms were found equally distributed between the ethnic groups and no correlation between cultural background and traditional medicinal use was detected. In line with this observation, it was demonstrated, that, since traditional knowledge is influenced by ancestry, inter-cultural diffusion and interaction with the natural environment, ethnic groups resemble each other in traditional healing approaches because they exist in similar environments, regardless of whether they are geographically proximate or share common ancestors (Saslis-Lagoudakis et al., 2014). Thus, the observed similarities in the ethnomedicinal usage might be explained by the fact that the ethnic groups under study are geographically close, exposed to similar environments and able to exchange knowledge readily. The reported ethnomedicinal uses are in line with the general perception of termites in traditional medicine as their use for medicinal purposes is not uncommon in the folk medicine in different parts of the world like South America (Brazil) or India. Human diseases which have been reported to be treated by termites include influenza, asthma, bronchitis, whooping cough, sinusitis, tonsillitis and hoarseness (Alves et al., 2011) as well as rheumatic diseases, body pain, better health and anemia (Wilsanand et al., 2007). Therapeutic application is usually realized internally whereby termites like *Nasutitermes macrocephalus* (Silvestri, 1903) are for example "mixed with sugar and taken as syrup" to treat Bronchitis, catarrh in the chest, coughs, influenza, sore throat, sinusitis, tonsillitis and hoarseness (Alves et al., 2011).

The pharmacological properties assessed in this study focused on inflammation (immune activation) and gastrointestinal motility based on the outcome of the ethnomedicinal survey. In the traditional medicinal context, the termites are often applied *in toto* or as a powder after drying. However, in order to subject the termites to bioactivity testing it was necessary to prepare an extract of the ingredients with ethanolic extraction.

Untargeted GC-MS analysis was performed prior to pharmacological testing, to proof the extraction process and preliminary check for the presence of bioactive compounds. Putative compound identification revealed the presence of quinones, sugars derivatives, fatty acid and steroid like compounds. The results were in accordance to literature data which reported the presence of vitamin E and linoleic acid in *Macrotermes spp* (Adepoju et al., 2014; Igwe et al., 2011). Other studies also mentioned the presence of hydroquinone in the labial gland of some termite species (Moore, 1968; Maschwitz and Tho, 1974; Olagbemiro et al., 1988). Presumably, due to the extraction process some ethylated compounds were found. Based on the preliminary GC-MS experiments the presence of bioactive compounds in the extract can be assumed, thus further and more detailed chemical characterization using other hyphenated techniques and NMR are currently performed.

The anti-inflammatory action of *M. bellicosus* extract observed in this study was realized via inhibition of the immune response/activation by reducing inflammatory TNFα response from macrophages and chemotactic (IL8-) signaling from intestinal epithelial cells. This inhibitory effect was comparable to the effect of the glucocorticoid budesonide used as a positive control and in higher concentrations even more pronounced. So far, anti-inflammatory effects have only been observed for the cultured microbial symbionts of termites like the fungus Xylaria or the fungus-associated bacteria *Actinomadura* sp. RB99 not for ingredients of the termite itself (Chang et al., 2017; Chen et al., 2019; Lee et al., 2018).

With regards to intestinal motility, the extract of *M. bellicosus* showed only weak effects in lower concentrations. Higher concentrations however, indicated an inhibiting effect on induced contractions as well as basal tone relaxing properties. Since these effects were only observed in mg/mL-range of the dried extract a pharmacological relevance cannot be concluded with certainty. However, considering the fact that a full inhibition of bowel movement is not necessarily beneficial in the treatment of gastrointestinal disorders, a moderate alleviation of intestinal motility could still mediate therapeutic relevance. Further it should be considered, that a correlation between the concentration of the applied dried ethanolic extract of *M. bellicosus* and the content in the whole animals is not possible. Bioactive components which might be responsible for a spasmolytic effect like for example smooth muscle relaxing polyphenols could be present in the ethanolic extract only in low concentrations due to unexhaustive extraction but might mediate a symptom relieving effect when consumed with the whole powdered animal to treat digestive disorders as indicated by the healers.

Hitherto, no reports have been made about antispasmodic or motility reducing properties of termites. However, antispasmodic properties have been described for the aerial parts, leaf and root of trees of *Combretum spp.* and fruits of *Tamarindus indica* (Ali and Shah, 2010; de Morais Lima et al., 2012) which are native plants to the savannah ecosystem that occur on termite mounds and could explain the occurrence of antispasmodic effects in higher concentration of the *M. bellicosus* exerted by residues of digested active plant metabolites (Dossou-Yovo et al., 2014; Sinsin et al., 2008). These hypotheses however remain subject to further investigation. So far, the reported traditional indications with regard to intestinal disorders might only partially be related to a potential influence on intestinal motility and are more supported by the apparent anti-inflammatory properties.

The presented data adds relevant knowledge about the medicinal tradition on *M. bellicosus* in the study region. In relation to already existing information on the use of termites in nutrition and traditional medicine the present study showed great similarities in relation to mode and form of application as well as addressed disease-fields. Similarities in the indication fields cover mainly infectious diseases like mumps, cough and pulmonary infections. In contrast to the reports on medicinal use of other termite species however, *M. bellicosus* is at the same time used in the context of intestinal disorders including digestive disorders, diarrhea, and dysentery. The observed inhibiting effect on cytokine release as well as indices for motility-modulating activity in higher concentrations are supporting pharmacological features for their therapeutic application in these diseases.

5. Conclusion

The study provides for the first time broader information on the medicinal use of *M. bellicosus* and substantiating pharmacological evidence on its anti-inflammatory effects *in vitro*. These observations can reinforce the utilization of the termite species as a traditional medicine for inflammatory and infectious diseases as reported by the traditional practitioners in the study area.

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Conflict of Interests

The authors declare that there is no conflict of interest.

References

Adepoju, O. T.; Omotayo, O. A., 2014. Nutrient composition and potential contribution of winged termites (*Marcrotermes bellicosus* Smeathman) to micronutrient intake of consumers in nigeria. *Br. J. Appl. Sci. Technol. 4* (7), 1149.

Agyare, C; Asase, Alex; Lechtenberg, Matthias; Niehues, Michael; Deters, Alexandra; Hensel, Andreas, 2009. An ethnopharmacological survey and in vitro confirmation of ethnopharmacological use of medicinal plants used for wound healing in Bosomtwi-Atwima-Kwanwoma area, Ghana. Journal of ethnopharmacology 125 (3), 393–403.

Ali, N; Shah, Swa, 2010. Spasmolytic Activity of Fruits of *Tamarindus indica* L. Journal of young pharmacists: JYP 2 (3), 261–264.

Alves, RRN; Barbosa, José A. A; Santos, Silene L. D. X; Souto, Wedson M. S; Barboza, Raynner R. D., 2011. Animal-based remedies as complementary medicines in the semi-arid region of northeastern Brazil. Evidence-based complementary and alternative medicine: eCAM 2011, 179876.

Chang, J-C; Hsiao, George; Lin, Ruo-Kai; Kuo, Yueh-Hsiung; Ju, Yu-Min; Lee, Tzong-Huei, 2017. Bioactive Constituents from the Termite Nest-Derived Medicinal Fungus Xylaria nigripes. Journal of natural products 80 (1), 38–44.

Chanput, W; Mes, Jurriaan J; Savelkoul, Huub F. J; Wichers, Harry J., 2013. Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds. Food & function 4 (2), 266–276.

Chanput, W; Mes, Jurriaan J; Wichers, Harry J., 2014. THP-1 cell line: an in vitro cell model for immune modulation approach. International immunopharmacology 23 (1), 37–45.

Chen, M-C; Wang, Guei-Jane; Kuo, Yueh-Hsiung; Chiang, Yin-Ru; Cho, Ting-Yu; Ju, Yu-Ming; Lee, Tzong-Huei, 2019. Isoprenyl phenolic ethers from the termite nest-derived medicinal fungus Xylaria fimbriata. Journal of food and drug analysis 27 (1), 111–117.

de Morais Lima, GR de; Sales, Igor Rafael Praxedes de; Caldas Filho, Marcelo Ricardo Dutra; Jesus, Neyres Zínia Taveira de; Sousa Falcão, Heloina de; Barbosa-Filho, José Maria; Cabral, Analúcia Guedes Silveira; Souto, Augusto Lopes; Tavares, Josean Fechine; Batista, Leônia Maria, 2012. Bioactivities of the genus Combretum (Combretaceae): a review. Molecules (Basel, Switzerland) 17 (8), 9142–9206.

Dossou-Yovo, HO; Vodouhe, G F; Sinsin, B, 2014. Assessment of the medicinal uses of plant species found on termitaria in the Pendjari biosphere reserve in Benin. Journal of Medicinal Plants Research 8 (8), 368–377.

Figueirêdo, RECR de; Vasconcellos, Alexandre; Policarpo, Iamara Silva; Alves, Rômulo Romeu Nóbrega, 2015. Edible and medicinal termites: a global overview. Journal of ethnobiology and ethnomedicine 11, 29.

Harvey, AL, 2008. Natural products in drug discovery. Drug discovery today 13 (19-20), 894–901.

Igwe, C.U.; Ujowundu, C.O.; Nwaogu, L.A. and Okwu, G, N., 2011. Chemical analysis of an edible african termite, *Macrotermes nigeriensis*; a potential antidote to food security problem. Biochem & anal biochem; 1:105. doi:10.4172/2161-1009.1000105.

Kpeki, S.B., 2008. Ethnicité, taxonomie locale et distribution géographique de quatre espèces de légumes-feuilles traditionnels au Benin: *Acmella uliginosa*, *Ceratotheca sesamoides*, *Justicia tenella* et *Sesamum radiatum*. Thèse d'ingénieur agronome, Cotonou, Benin, 76 pp.

Lee, K.E., Wood, T.G., 1971. Termites and soils. Academic Press, London, New York, 251 pp.

Lee, SR; Lee, Dahae; Yu, Jae Sik; Benndorf, René; Lee, Sullim; Lee, Dong-Soo; Huh, Jungmoo; Beer, Z. Wilhelm de; Kim, Yong Ho; Beemelmanns, Christine; Kang, Ki Sung; Kim, Ki Hyun, 2018. Natalenamides A¯C, Cyclic Tripeptides from the Termite-Associated Actinomadura sp. RB99. Molecules 23 (11).

Marie, R., 1955. Contribution a l'histoire des insectes en thérapeutique. Ph.D. Dissertation (Pharmacy), Strasbourg.

Maschwitz, U. and Tho, Y. P., 1974. Chinone alsWehrsubstanzen bei einigen orientalischen Macrotermitinen. Insectes Soc. 21:231–234.

Massougbodji, M; Tevoedjre, A, 1997. Communication with WHO. Cotonou, Ministère de la Santé, de la Protection Sociale, et de la Condition Féminine and Ministère du Plan, de la Restructuration Economique, et de la Promotion de l'Emploi, République du Bénin 27 October 1997.

Mathibela, MK; Egan, Bronwyn A; Du Plessis, Helena J; Potgieter, Martin J., 2015. Socio-cultural profile of Bapedi traditional healers as indigenous knowledge custodians and conservation partners in the Blouberg area, Limpopo Province, South Africa. Journal of ethnobiology and ethnomedicine 11, 49.

Moore, B. P., 1968. Studies on the chemical composition and function of the cephalic gland secretion in Australian termites. J. Insect Physiol. 14:33–39.

Newman, DJ; Cragg, Gordon M., 2012. Natural products as sources of new drugs over the 30 years from 1981 to 2010. Journal of natural products 75 (3), 311–335.

Ntukuyoh, AI; Udiong, D. S; Ikpe, Edidiong; Akpakpan, Aniekan, 2012. Evaluation of nutritional value of termites (*macrotermes bellicosus*): Soldiers, workers, and queen in the niger delta region of nigeria. International Journal of Food Nutrition and Safety 1, 60–65.

Olagbemiro, T. O., Lajide, L., Sani, K. M., and Staddon, B. W. 1988. 2-Hydroxy-5-methyl-1,4-benzoquinone from the salivary gland of the soldier termites *Odontotermes magdalenae*. Experientia 44:1022–1024.

Sambuy, Y; Angelis, I. de; Ranaldi, G; Scarino, M. L; Stammati, A; Zucco, F., 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell biology and toxicology 21 (1), 1–26.

Saslis-Lagoudakis, CH; Hawkins, Julie A; Greenhill, Simon J; Pendry, Colin A; Watson, Mark F; Tuladhar-Douglas, Will; Baral, Sushim R; Savolainen, Vincent, 2014. The evolution of traditional knowledge: environment shapes medicinal plant use in Nepal. Proceedings. Biological sciences 281 (1780), 20132768.

Sinsin, B; Sogbohossou, E. A; Nobime, G; Adi, M., 2008. Dénombrement aérien de la faune dans la Réserve de Biosphère de la Pendjari. GTZ, Pendjari project.

Smeathman, H, 1781. Some account of the termites, which are found in Africa and other hot climates. In a letter from Mr. Henry Smeathman, of Clement's Inn, to Sir Joseph Banks, Bart. P. R. S. Philosophical Transactions of the Royal Society of London 71, 139–192.

Solavan, A; Paulmurugan, R; Wilsanand, V., 2007. Antibacterial activity of subterranean termites used in south Indian folk medicine. 0972-5938.

Tsuchiya, S; Yamabe, M; Yamaguchi, Y; Kobayashi, Y; Konno, T; Tada, K., 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). International journal of cancer 26 (2), 171–176.

Vissiennon, C; Goos, Karl-Heinz; Goos, Ole; Nieber, Karen, 2015. Antispasmodic effects of myrrh due to calcium antagonistic effects in inflamed rat small intestinal preparations. Planta medica 81 (2), 116–122.

Wilsanand, V; Varghese, Preema; Rajitha, P., 2007. Therapeutics of insects and insect products in South Indian traditional medicine. Indian Journal of Traditional Knowledge 6, 563–568.

Yêyinou Loko, LE; Orobiyi, Azize; Agre, Paterne; Dansi, Alexandre; Tamò, Manuele; Roisin, Yves, 2017. Farmers' perception of termites in agriculture production and their indigenous utilization in Northwest Benin. Journal of ethnobiology and ethnomedicine 13 (1), 64.

Supplementary data to the manuscript *"Ethnomedicinal survey and in vitro confirmation of anti-inflammatory and antispasmodic properties of the termite strain Macrotermes bellicosus used in traditional medicine in the Republic of Benin"* by Hammoud Mahdi et al.

Appendix B. Untargeted GC-MS analysis of an ethanolic (90% v/v) extract of *Macrotermes bellicosus*

Analytical parameters

Detection	GC-single quad, EI (-70eV), fullscan m/z = 50-600 da
Column	Optima 5MS 30 m x 250 µm x 0.25 µm
Carrier gas	Helium
Injection mode	splittless
Sample characteristic	0.1 µg/µL (dry extract in MeOH)
Injection volume	1 µL
Flow rate	1 mL/min
Heater temperature	280 °C
Oven program	1 min 80°C; 20 °C/min to 310 °C for 8 min
Run time	20. 5 min

Total Ion Chromatogram (TIC)

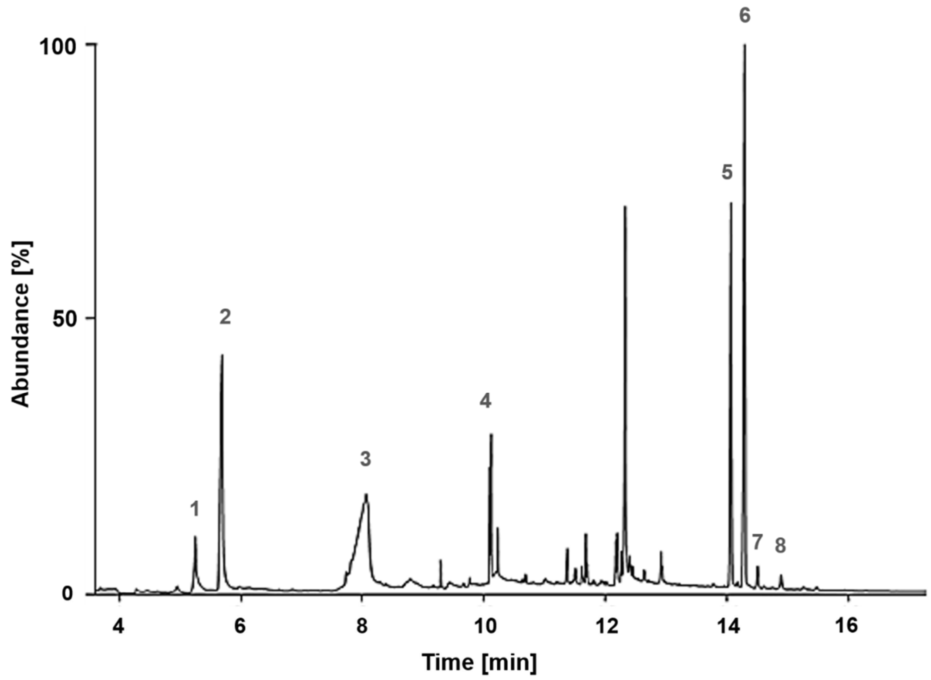
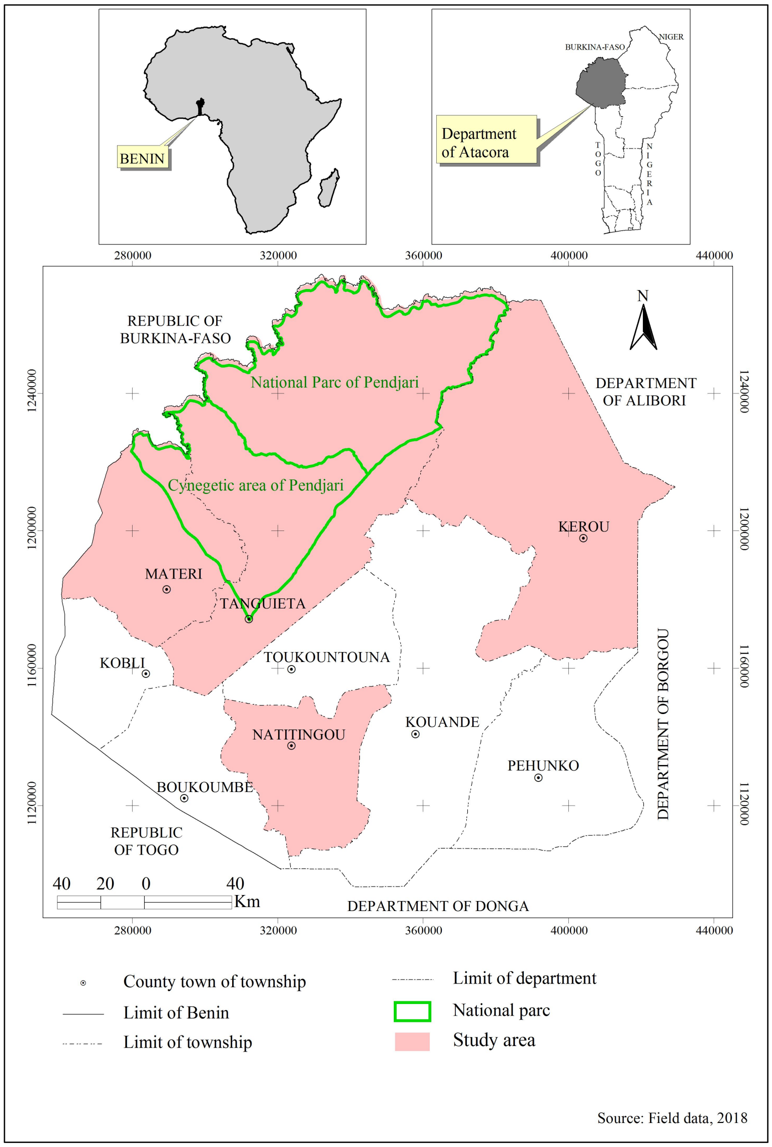


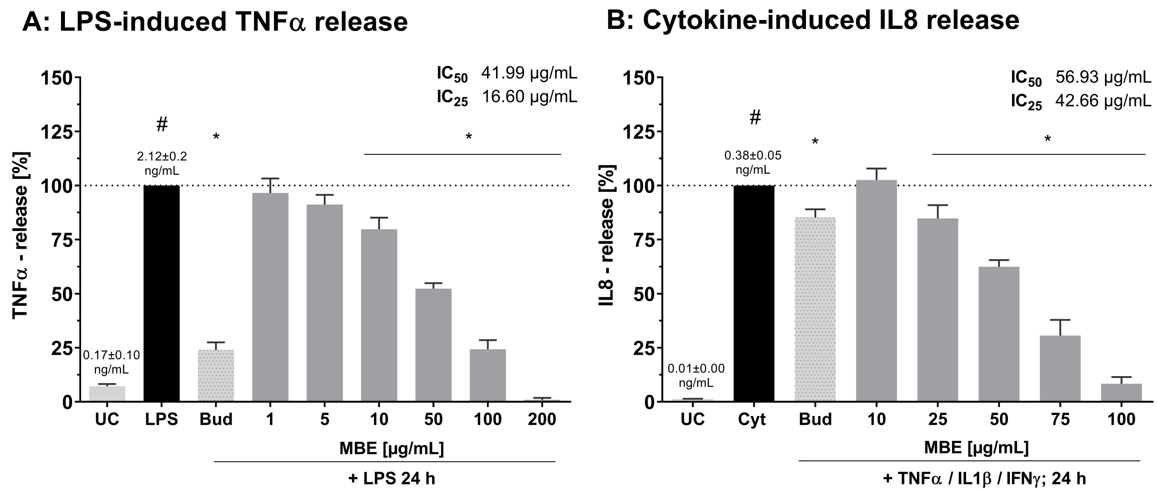
Fig S2. Total Ion Chromatogram (TIC) of the *Macrotermes bellicosus* extract (EtOH 90 % v/v) GC-MS analysis.

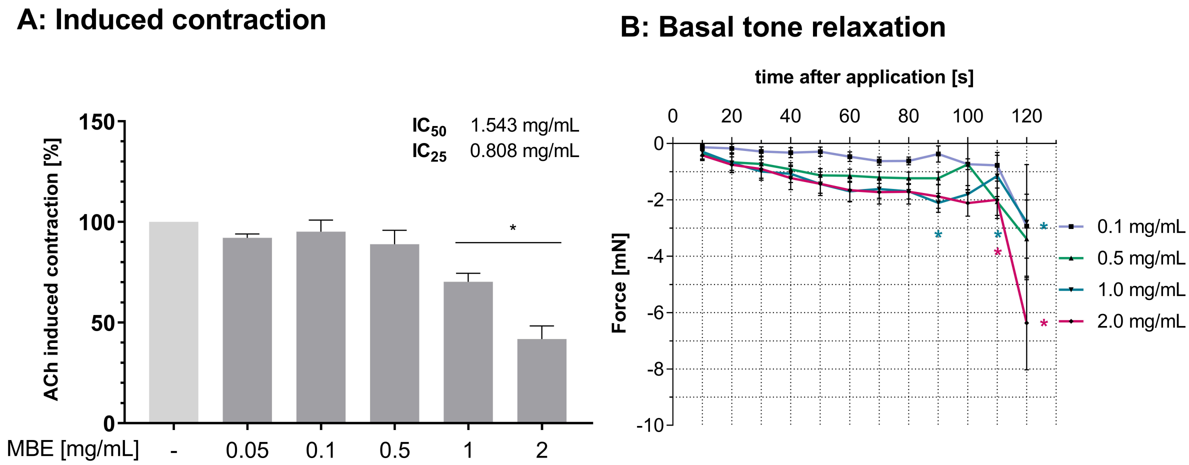
Putatively detected compounds: putative identification by NIST11 library after manual subtraction. Retention indices were calculated using benzoic acid methyl ester, caffeine and permethrin.

Putative Compound	Rt (min)	Library RI (median)		Calculated RI		m/z
		Semi-standard non-polar	Standard non-polar	Semi-standard non-polar	Standard non-polar	
1 - Benzohydroquinone	5.2	1241	1334	1289	1273	110.1
2 - Methylhydroquinone	5.6	1225	-	1363	1347	124.1
3 - Ethyl-hexopyranoside	8.1	-	-	1826	1810	208.1
4 - Linoleic acid ethyl ester	10.0	2162	2141	2178	2161	308.5
5 - Vitamin E	14.1	3138		2937	2921	430.4
6 - Cholesterol	14.2	3087	3052	2955	2939	386.7
7 - Ergosta-5,22-dien-3-ol	14.5		3079	3011	2995	398.7
8 - Campesterol	14.8	3131	3117	3066	3050	400.7

Rt: retention time; RI: retention index


BENIN
BURKINA-FASO
NIGER
Department
of Atacora
TOGO
NIGERIA
REPUBLIC OF
BURKINA-FASO
National Parc of Pendjari
Cynegetic area of Pendjari
DEPARTMENT
OF ALIBORI
KEROU
MATERI
TANGUIETA
KOBLI
TOUKOUNTOUNA
NATITINGOU
KOUANDE
PEHUNKO
BOUKOUMBE
REPUBLIC
OF TOGO
DEPARTMENT OF BORGOU
DEPARTMENT OF DONGA
N
40 20 0 40
Km
280000
320000
360000
400000
440000
1240000
1200000
1160000
1120000
County town of township
Limit of Benin
Limit of township
Limit of department
National parc
Study area
Source: Field data, 2018


A: LPS-induced TNFα release
IC50 41.99 µg/mL
IC25 16.60 µg/mL
TNFα - release [%]
150
125
100
75
50
25
0
#
*
*
2.12±0.2 ng/mL
0.17±0.10 ng/mL
UC
LPS
Bud
1
5
10
50
100
200
MBE [µg/mL]
+ LPS 24 h
B: Cytokine-induced IL8 release
IC50 56.93 µg/mL
IC25 42.66 µg/mL
IL8 - release [%]
0.38±0.05 ng/mL
0.01±0.00 ng/mL
UC
Cyt
Bud
10
25
50
75
100
MBE [µg/mL]
+ TNFα / IL1β / IFNγ; 24 h


A: Induced contraction
IC50 1.543 mg/mL
IC25 0.808 mg/mL
ACh induced contraction [%]
150
100
50
0
*
MBE [mg/mL]
-
0.05
0.1
0.5
1
2
B: Basal tone relaxation
time after application [s]
0
20
40
60
80
100
120
Force [mN]
0
-2
-4
-6
-8
-10
0.1 mg/mL
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL