Mahamat et al. Clinical Phytoscience (2018) 4:28
https://doi.org/10.1186/s40816-018-0089-4

Clinical Phytoscience

ORIGINAL CONTRIBUTION Open Access

Assessment of antimicrobial and immunomodulatory activities of termite associated fungi, *Termitomyces clypeatus* R. Heim (Lyophyllaceae, Basidiomycota)

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Abstract

Background: *Termitomyces clypeatus* (*T. clypeatus*) is an edible mushroom specie which grows in various areas in Cameroon. It is indicated by several healers in treatment of several infections*and* is frequently used for this purpose. However, no study has been reported on its antibacterial and immunomodulatory activities which were the aim of this study.

Methods: Disc diffusion method was used to appreciate the bactericidal activity against 4 bacteria and 2 yeast species. The immunomodulatory activities were assessed in mice, where the extract was administered by gavage and as supplement in the feed. The treatment was done for 10 days. Delayed type hypersensitivity test was carried out to assess the cell mediated immune response while the effect on humoral immunity was evaluated using hemagglutination assay and mice lethality test.. The body weight was also recorded in mice used for delayed hypersensitivity.

Results: It was found that extract of *T. clypeatus* highly inhibited the growth of bacteria and yeast at different ratios compared to the medium ($P < 0.05$). The extract of *T. clypeatus* has reversed the immunosuppressed effects of dexamethasone on antibody formation. In addition, it significantly decreased of the mice lethality rate in mice infected by *Pasteurella multocida* ($P < 0.05$). Administration of *T. clypeatus* also significantly increased the delayed type hypersensitivity response in healthy and dexamethasone immunosuppressed mice ($P < 0.05$), but it significantly reduced the body weight of mice after 10 days.

Conclusion: The results provided basic information demonstrating the antibacterial activity and immunostimulatory activity of *T. clypeatus* on both cell-mediated and humoral immunity.

Keywords: Cell-mediated immunity, Humoral immunity, Immunomodulation, Antibacterial, *Termitomyces clypeatus*

Background

Use of natural products as an alternative to conventional treatment for various diseases has been on the rise in the last few decades [1]. In public concern about dietary and health issues, the increase in the consumption of fungal food have been reported on a global basis [2]. Besides nutritional value which have industrial applications, many species of *Termitomyces* are cited to possess medicinal properties [2, 3]. In the last few decades, research has been focused on antibacterial, antifungal effects of mushroom. Nosocomial agents such as; *Escherichia coli, Pseudomonas aeruginosa, Enterococcus spp., Streptococcus spp., Candida albicans, and Enterobacter cloacae* have long been designated as the most frequent microorganisms causing bacteremia and fungemia [4]. Thus, various studies are focused to the use of plants or mushrooms as therapeutic agents against these agents. In addition, the effects on the immune system as the most therapeutic effects of

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medicinal plants in the treatment of infectious diseases have been demonstrated [5, 6].

Mushrooms have been used extensively in traditional medicine throughout the world. Several species, have been identified as potent immunomodulators or bactericide and they are used to treat infections [6, 7]. In several studies it was demonstrated that mushrooms of the genus *Termitomyces* possess the immunomodulatory or antibacterial activities [7]. *Termitomyces clypeatus* R. Heim is among the most common popular edible and medicinal mushrooms in many tropical countries including Brazil, India, Philippine and Cameroon [8–11]. It is recommended by the traditional healers to treat some gastrointestinal infectious diseases. However, no study was done to exhibit its pharmacological properties.

In the present study, *Termitomyces clypeatus* was tested for its immunomodulatory and antimicrobial activities.

Methods

Materials

Collection of specimen and preparation of extract

Fresh samples of *Termitomyces clypeatus* were bought in local markets of the North-West and West regions of Cameroon. The specimen was authenticated thanks to the laboratory investigation of their macroscopical and microscopical characteristics in the Laboratory of Biological Sciences of The University of Bamenda. The samples were washed and cut into small pieces. Then, they were dried at the oven for 3 days at 35 °C. The dried samples were ground to obtain a fine powder of the mushroom. The powder, 100 g was dissolved in 500 ml distilled water and boiled for 20 min. The solution was filtered using Watman paper N°1. The filtrate was evaporated in the oven at 35 °C and the dried product constituting the aqueous extract (AEM) used for the research work.

Animals

Adult healthy Swiss albino mice (*Mus musculus*) of both sex and having 10–12 weeks old and 17–19 g body weight were used in his study. They were raised at the animal house of the department of Biological Sciences, University of Bamenda, where the study was conducted. The colony was obtained from the National Veterinary Laboratory (LANAVET), Garoua, Cameroon. The experiments were conducted under conditions of temperature and light (Light:dark, 12 h:12 h, T°C, 25 ± 2 °C). The animals were fed with a standard mouse feed procured from LANAVET and water ad libitum.

Red blood cells

Fresh cow red blood cells (CRBC) were obtained from cow's blood as described by Heden (1946) [12] and used as antigen [13]. Briefly, veinous blood was collected from cow in EDTA-tubes and kept in the laboratory for 15 min. The plasma was therefore discarded and pellet (red blood cells) was washed three times in a large volume of pyrogen-free sterile normal saline by repeated centrifugation at 2500 rev/s for 10 min on each occasion. The washed CRBC was adjusted to a concentration of approximately 1×10^9 cells/ml and used for both immunization and challenge.

Test microorganisms

Five (5) strains of 5 bacteria (*Pasteurella multocida* NCTC 12178, *methicillin-sensitive Staphylococcus aureus MSSA, Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048 and *Salmonella typhi Ty2* ATCC 700931) and 2 yeast (*Candida albicans* ATCC10231 and *Candida glabrata* ATCC 66032 were used in this study. Microorganisms were provided by the Biochemistry Research Laboratory, Department of Biochemistry, Faculty of Science, University of Dschang-Cameroon.

Experimental design

Evaluation of antimicrobial activity

The AEM was subjected to antibacterial assay using the agar diffusion plate method as described by Alves and colleagues (2000), with some changes and guided by NCCLS/CLSI (2005) [6, 7]. Tests were carried out using 100 μl of suspension containing 10^8 per/ml of bacteria (*S. aureus, E. coli, E. aerogenes* and *S. typhi*) and 10^4 per/ml yeast (*C. albicans* and *C. glabrata*). Bacteria were inoculated into veal Broth (Difco) containing agar (1%) while yeasts were inoculated into malt extract gar (Difco). Petri dishes were prepared at 4 °C for 10 to 20 min. Then, four wells (6 mm diameter) were made and aseptically filled up with 100 μl of the extract at different concentrations (125, 250 and 500 μg/ml). Nystatin and Streptomysin sulfate (100 μg / ml) were used as positive control whereas Mueller-Hinton agar only was used as negative control for microbial growth. Then, the inoculated plates were incubated at 25 °C for 48 h for yeast and 37 ± °C for 24 h for bacterial strains. At the end of the incubation period, the diameter (mm) of clear zone considered as growth inhibition was measured. [14, 15]

Animal grouping

To assess the humoral and delayed type hypersensitivity (DTH) response, white mice weighing 15–19 g were divided into 2 groups: group 1 (animal not treated) and group 2 (treated with dexamethasone). Each group of mice was randomly shared into 4 subgroups of 5 animals each. Dexamethasone sodium, an immunosuppressing agent was intramuscularly administered at the rate of 5 mg/kg every day for 7 days. The first subgroup (subgroup 1) of each group or control subgroup was fed with

distilled water. The second subgroup (subgroup 2) of each group taken as positive control was received levamisole hydrochloride subcutaneously at the rate of 2.5 mg/kg thrice a week. The other subgroups (3 and 4) of each category were taken as subgroups tests and were daily fed with the extract (AEM) at the rate of 500 mg/kg and 1000 mg/kg by gavage. The test groups were also fed with feed supplemented with the powder of mushroom at 25 and 50% respectively for animal receiving 500 and animal receiving 1000 mg/kg. The Table 1 shows the animal grouping. This study was approved by Institutional Animal Ethical Committee.

Delayed type hypersensitivity response

Twenty mice were grouped as described above. The two test groups were daily fed with extract and powder of the mushroom for 10 days. Delayed type hypersensitivity (DTH) was assessed by evaluating the sensitivity to CRBC. On day 5 of the treatment, 0.2 ml of 1×10^9 cells/ml CRBC was administered subcutaneously in the plantar region of the right hind foot paw. Then, animals were challenged on day 10 by a second subcutaneous injection of the same amount of antigen into the left hind paw. The edema produced by antigenic challenge in the left hind paw was taken as the difference in the paw thickness before and 30 min, 24 h and 48 h after the challenge. The paw thickness was measured by volume displacement. Body weight of the animals was recorded before and on day of the challenge.

Humoral antibody response

Twenty mice were randomized and treated for 10 days as above. Then, they were immunized by an intraperitoneal injection (*i.p.*) of 0.2 ml of 1×10^9 CRBC/ml at the first day. In a late 5 days of treatment, the mice were challenged by injecting the same of CRBC. On day 11, animals were slaughtered and the blood samples were collected by cardiac puncture for analysis of serum. Antibody titer was determined by the haemagglutination technique [13]. Briefly, 25 μl of serum was put in 96 U-bottom microtitre plates and serially diluted two-fold using pyrogen free sterile normal saline. The last well on each row contained sterile normal saline was considered as control. The diluted sera were challenged with 25 μl of 1% (*v*/v) CRBC and incubated at 37 °C for 1 h. The highest dilution giving rise to visible haemagglutination was taken as antibody titer. The experiment was done in three replicates.

Table 1 Animal grouping following the treatment

Groups	Subgroups	Treatments
Group I	1	Control
	2	Levamisole
	3	AEM 500 mg/kg
	4	AEM 1000 mg/kg
Group II	1	Dexamethasone
	2	Dexamethasone + Levamisole
	3	Dexamethasone + AEM 500 mg/kg
	4	Dexamethasone + AEM 1000 mg/kg

Group I is constituted of normal mice
Group II is constituted of mice treated with dexamethasone

Mice lethality test

Thirty mice were divided into 5 groups of 6 animals. The first group was taken as control and was kept uninfected. Group 2 was taken as negative control and was infected and received distilled water. Group 3, taken as positive control, was infected and treated by levamisole. Groups 4 and 5 were taken as test groups and they were infected and fed with the extract at the dose of 500 and 1000 mg/kg respectively as described above. The extract was daily administered for 15 days starting from day 1. All the animals were immunized through intraperitoneal route with hemorrhagic septicemia vaccine, except negative control on 5th and 10th day of the treatment., Mice were subcutaneously challenged with *P. multocida* on 15 days of treatment and examined for about 72 h. *P. multocida* were injected at the LD_{50} of 10^5 cells in 0.5 ml of normal saline.

Statistical analysis

Values are expressed as Mean ± SD. Results obtained were statistically analyzed by using one-way ANOVA followed by tukey-krammer multiple comparison test. $P < 0.05$ was considered a significant value.

Results

Antimicrobial activity

The results on the antimicrobial activities of *T. clypeatus* tested in vitro against some bacteria and fungi are presented in Table 2 below. The extract of *T. clypeatus* showed a significant activity against *E. coli*, *E. aerogenes*, *S. aureus*, *S. typhi*, *C. albican* and *C. glabrata* compared to the control or simple medium. The inhibition zone was different for the various microbial strains. It was as follow: *E. coli* (2.6 to 6.6 mm), *E. aerogenes* (1.8 to 2.8 mm), *S. aureus* (55 to 10.5 mm), *S. typhi* (0 to 5.5 mm), *C. albicans* (3.9 to 8.5 mm) and *C. glabrata* (2.9 to 10.0 mm).

Effect on humoral response

Agglutination test was used to measure the level of antibodies to particulate CRBC. Serum from normal mice receiving the different doses of AEM has not shown significant haemagglutination titer compared to the control. While, the potentiation of humoral response by standard immunomodulatory drug levamisole has resulted in higher

Table 2 Antimicrobial activity of *T. clypeatus* against some bacteria and yeast species

Microbes Extract Conc. (μg/ml)	*E. coli*	*E. aerogenes*	*S. aureus*	*S. typhi*	*C. albicans*	*C. glabrata*
-ve control	-[a]	-[a]	-[a]	-[a]	-[a]	-[a]
125	2.6 ± 0.7[b]	1.8 ± 0.3[b]	5.5 ± 0.8[b]	-[a]	3.9 ± 0.7[b]	2.9 ± 1.8[b]
250	4.8 ± 1.7b[c]	2.5 ± 0.3b[c]	8.5 ± 1.1[cd]	2.7 ± 0.8[b]	6.5 ± 0.8[c]	6.9 ± 0.7[c]
500	6.6 ± 1.2[c]	2.8 ± 0.9[c]	10.5 ± 0.7[d]	5.5 ± 1.2[c]	8.5 ± 0.7[d]	10.0 ± 1.4[d]
+ve Control	13.0 ± 0.1[°°]	14.0 ± 0.0[°°]	17.0 ± 0.1[°°]	16.0 ± 0.1[°°]	16.5 ± 0.5[°]	13.2 ± 0.5[°]

-ve control indicates medium only while +ve control indicate the antibiotics which were Nystatin and Streptomysin sulfate: 100 μg. Comparison antibiotic: °Nystatin, °° Streptomysin sulfate. The symbol (–) indicate that no inhibition was detected. Each value is the mean ± SD of three replicates. Values with different small letters in the same column are significantly different at the level of 0.05 ($P < 0.05$)

antibody titer. In the category II of dexamethasone-treated groups, serum from mice receiving the doses of AEM showed a high haemagglutination titer as compared to dexamethasone group (Table 3).

Effect on the mortality rate in mice lethality test

Administration of *P. multocida* caused 100% mortality in 48 h in the negative control group, whereas 33.33% mortality was observed in positive control group within 72 h. Treatment with AEM500 and AEM1000 mg/kg caused the mortality rate of 50 and 33.33%, respectively as compared with positive control group. We observed the death of one mouse after 24 h and two mice later after 72 h in low dose treated group. In AEM1000 mg/kg treated group, we found one mouse dead with 48 h. Subsequently, one mouse died after 72 h. Treatment with 2.5 mg/kg of levamisole caused 40% mortality after 24 h (Table 4).

Effect on cell mediated immunity in normal mice

Cow red blood cells (CRBC) induced DTH reaction was used to study the effect of the extract on cell mediated immunity in normal mice. Pretreatment of the AEM and consumption of the mushroom powder has shown significant increase in paw thickness ($p < 0.01$) after 30 min, 24 and 48 h of challenge at the doses of 500 and 1000 mg/kg of extract (Fig. 1). The increase delayed type hypersensitivity response has observed at 30 min, 24 and 48 h after the antigenic challenge. Standard immunostimulant, levamisole, has also induced a significant increase in DTH response compared to control after 30 min and 24 h.

Table 3 Effect of the treatment by *T. clypeatus* on haemagglutination titer level in mice

Groups	Subgroups	Treatment		Titer level
Group I	1	–	Distilled water	36 ± 20,13[a]
	2	–	Levamisole	224 ± 192[c]
	3	–	AEM500 mg/kg	44 ± 24[a]
	4	–	AEM1000 mg/kg	48 ± 18,47[a]
Group II	1	Dexamethasone	Distilled water	6 ± 2,3[a]
	2	Dexamethasone	Levamisole	40 ± 16[b]
	3	Dexamethasone	AEM500 mg/kg	32 ± 22,62[b]
	4	Dexamethasone	AEM1000 mg/kg	37,33 ± 24,44[b]

Data represent the Means ± SD ($n = 5$). Values with different small letters in the same category are significantly different at the level of 0.05 ($P < 0.05$)

Effect on cell mediated immunity in dexamethasone treated mice

Potentiation of DTH response was also observed in Dexamethasone treated mice compared to untreated Dexamethasone treated mice. Increase in paw edema after challenge was observed in all extract treated groups similar to the standard, levamisole, when compared to negative control (dexamethasone treated group) (Fig. 2). Levamisole and mushroom treatment (AEM 500 and 1000 mg/kg) have shown mild increase in DTH response compared to control (dexamethasone untreated group). The increase in DTH response has observed in extract treated mice after 30 min and response has subsided up to 48 h.

Effect of T. clypeatus extract on mice's body weight

Body weights of mice in different groups showed a decrease in body's weight of mice after the period of the study in treated mice (Table 5). The capacity of *T. clypeatus* to reduce the weight of mice has observed in normal mice and dexamethasone treated mice. In contrast, mice in the control groups have significant increase in body's weight ($P < 0.05$).

Table 4 Mice lethality rate at 24, 48 and 72 h

Groups	After 24 h	After 48 h	After 72 h	Total lethality (%)
Untreated control	–	–	–	–
Negative control	33.33	66.66	–	100
Positive control	–	–	33.33	33.33
AEM 500 mg/kg	16.66	–	33.33	50.00
AEM 1000 mg/kg	–	16.66	16.66	33.33

Untreated control indicates the group of animals receiving distilled water, and non-infected by *P. multocida*. Negative control represents the group of animals receiving distilled water but infected by *P. multocida*. Positive control indicates the group of animals infected by *P. multocida* and receives levamisole. Each group has 6 mice and the data represent the percentage of dead animal

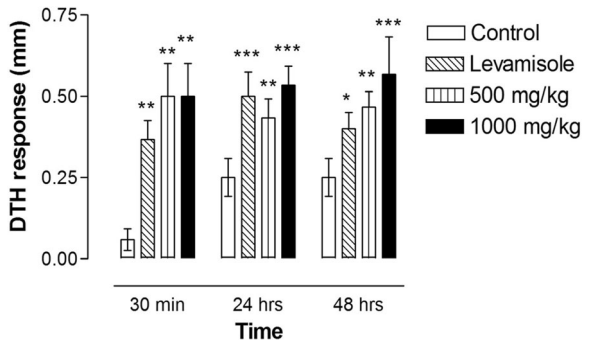

Fig. 1 Effect of *T. clypeatus* on DTH response using CRBC as an antigen in mice. Histogram represent Means ± SD ($n = 5$). Means with signs*, ** and ***superscripts are significantly different as compared to dexamethasone control at the level of 0.05 ($p < 0.05$)

Discussion

Many medicinal mushrooms are used to treat various specific health problems [13, 16]. As can be seen in Table 2, the extract of *T. clypeatus* showed activity on the other test microorganisms (2.5–10.5 mm). The result of this study on the antimicrobial activity of *T. clypeatus* showed activity against *S. aureus*, *E. coli* and S. thyphimurium, *E. aerogenes*, *C. albicans* and *C. glabrata* at different ratios. This may be indicative of the use of *T. clypeatus* in treatment of several infections. The significant difference in the size of inhibition between the microbial species could have arisen from the dissimilar biochemical pathways utilized by the pathogen [17].

Modulation of immune responses to alleviate infectious diseases has been of interest for many years. [18] One of the major immune response associated with the elimination of bacteria and fungi is the humoral response. Antibodies function as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells [19, 20]. The results of the study of the effect of *T. clypeatus* on antibody production are depicted in Table 3. The results showed that the extract increased dose-dependently the antibody titer in treated mice groups, but up 1000 mg/kg no significant difference has been observed when compared to untreated control (distilled water). In contrast, the extract has significantly reversed the inhibitory effect of dexamethasone. Despite the fact that the increase in production of antibody is not significant, the results indicate that the extract may stimulate the secretion of antibodies. This can be a result of the stimulatory activity of *T. clypeatus* on the secretion of antibodies by activated plasma cells or on the secretion of cytokines such as IL-4 from activated T lymphocyte cells [21]. Thus, this effect may be justify the reversion of the inhibitory effect of dexamethasone by the extract. Dexamethasone + levamisole group showed significant increase in HA titer as compared to dexamethasone + distilled water control group. This is evident since levamisole is known to restore corticosteroid induced depletion of immune response. Therefore, the extract may be had similar activity. An increase in production of antibodies may be an evidence of the importance of this mushroom demonstrated by the low mortality rate seen in *T. clypeatus* treated mice infected by *P. multocida* compared to control untreated.

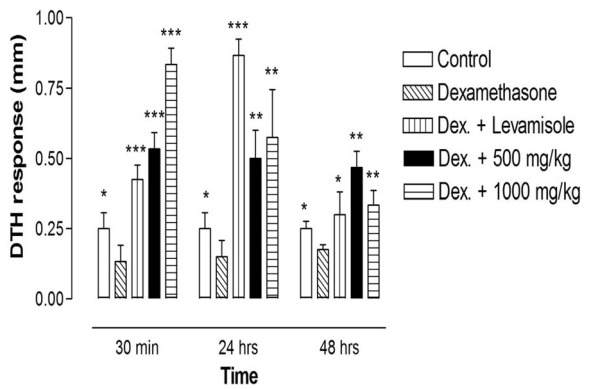

Fig. 2 Effect of *T. clypeatus* extract on DTH response using CRBC in Dexamethasone treated mice. Histogram represent Means ± SD ($n = 5$). Means with signs*, ** and ***superscripts are significantly different as compared to dexamethasone control at the level of 0.05 ($p < 0.05$)

The skin thickness increase was lower in the dexamethasone group when compared to the control. When compared to control, *T. clypeaus* and levamisole showed significant increase in skin thickness. Also, when compared to dexamethasone, Dexamethasone + *T. clypeatus*, Dexamethasone + levamisole showed significant increase in skin thickness. It has been demonstrated that DTH response results from the action of sensitized T lymphocytes, when challenged by antigen and were secrete a variety of molecules including proinflammatory lymphokines attracting more scavenger cells to the site of reaction [22]. Increased DTH response in treated animal showed that *T. clypeatus* might has a stimulatory effect on the lymphocytes and accessory cell types required for the expression of this reaction. A stimulatory activity on lymphocytes can boost phagocytic activity and increase concentration of lytic enzymes for more effective killing, which ultimately results in increase footpad thickness in immunized animals [23]. This suggests that *T. clypeatus* could enhance T cells proliferation and expression of the adhesion molecules, thus increasing migration of T cells to the site of inflammation.

Body weights of mice in treated groups decreased from the control or the dexamethasone groups indicating that the consumption of *T. clypeatus* has changed body weight of animals during the period of the study.

Table 5 Effect of extract and levamisole on body weight in mice

a. Effect on body weight in normal mice				
Periods	Control	Levamisole	AEM 500 mg/kg	AEM 1000 mg/kg
T0	18,65 ± 1,51[a]	17,42±2,46[a]	19,6 ± 2,80[a]	20,62 ± 2,68[a]
T1	21,35 ± 1,56[a]	20,72±2,60[a]	16,22 ± 1,94[b]	17,12 ± 2,19[b]
RWG (%)	14.47[#]	18.94[&]	-17.24[ξ]	-16.97[δ]
b. Effect on body weight in mice with dexamethasone induced immunosuppression				
	Dexam.	Dexam. + Lev.	Dex.+ 500 mg/kg	Dex. + 1000 mg/kg
T0	18,6±4,22[a]	18,87±1,84[a]	18,97 ± 1,32[a]	17,07±2,50[a]
T1	19,95±4,33[a]	21,57±1,87[a]	16,52 ± 1,23[a]	14,37±2,25[b]
RWG (%)	7.25[#]	14.30[&]	-18.91[ξ]	-15.81[ξ]

T0 before treatment, *T1* after treatment, *RWG* relative weight gain at the end of period of the treatment. Data represent Means ± SD (n= 5). Values with different letter (a, b) superscripts, along column, are significantly different at the level of 0.05 (p< 0.05). Values with different symbol (#, &, ξ) superscripts, along row, are significantly different at the level of 0.05 (p< 0.05)

Mushrooms and mushroom-derived polysaccharides, have been shown to have therapeutic properties against metabolic syndrome, which is characterized by obesity, hyperglycemia associated with diabetes, hypercholesterolemia, and hypertension [24–26]. This was attributed to their anti-hypercholesteremic effects which result in body weight. Hence the decreased in body weight by the result may be a result of the effect of *T. clypeatus* on lipid mass. As low lipid mass could not be affect significantly the production of antibodies or cell proliferation, this could be justify the improvement of immune response by the extract while it lowers the body weight. *T. clypeatus* is an edible mushroom, largely consume without adverse effects. Hence, the decreased in the body weight observed could not be compromised its use for its immunomodulatory. Otherwise, *T. clypeatus* was administered through oral route in two form as additive aliment (powder) and dissolved in water (extract). As the powder was used in crude form this could be lowered the animal's appetite which can also result in loss of body weight.

Conclusion

The data suggest that *T. clypeatus* possesses immunostimulantory activity which is evident by increasing the cell-mediated and humoral immunity. This is demonstrated by stimulation of DTH response and haemagglutination (HA) titer in mice. *T. clypeatus* also showed antimicrobial activity that in addition to its immunostimulatory activity can be justified its exploitation in treatment of the infectious diseases. However, there is need to carry further study to establish the bioactives compounds of *T. clypeatus* as antimicrobials and immunostimulants.

Acknowledgments
The Authors are grateful to Dr. Salah Marin, Head of Department of Animal Production, University of Bamenda, Cameroon for providing the necessary laboratory facilities.

Ethical approval and consent to participate
Not applicable

Authors' contributions
OM: Concepts, Design, Experimental Studies, Data acquisition and Analysis, Manuscript preparation, Statistical Analysis. NAL: Collection and identification of species. AAM: Data acquisition. KA: Design and Analysis. All authors read and approved the final manuscript.

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Consent for publication
All authors read and approved the final manuscript.

Competing interests
The authors wish to state that there are no competing interests associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Received: 21 September 2017 Accepted: 1 October 2018
Published online: 27 November 2018

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