Research Paper

CHANGES IN CYTOKINE EXPRESSION IN RATS ADMINISTERED EXTRACTS OF TERMITOMYCES ROBUSTUS AND OCIMUM GRATISSIMUM

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The study evaluated the immunomodulatory activities of extracts of Termitomyces robostus and Ocimum gratissimum on Wistar albino rats. A total of twenty Wistar albino rats were randomly grouped into five groups. The positive control was administered normal saline while the negative control received pyrogallol to suppress the immune system. Group one and two received 100 and 300 mg/kg body weight of methanol extract of Ocimum gratissimum and 10% pyrogallol while group three received 300 mg/kg body weight of Termitomyces robostus and 10% pyrogallol. The immunological activity was evaluated on cytokine expression and haemaglutination titre. The study lasted for 21 days. Significant (p<0.05) increases were induced by the 300 mg/kg body weight administration of both extracts on serum interleukin-2, interferon-γ, and tumor necrosis factor-α. The two doses administration of Termitomyces robostus resulted in significant (p<0.05) decrease in delayed type hypersensitivity while the administration of Ocimum gratissimum increased (p>0.05) the delayed type hypersensitivity. The primary and secondary antibody titres showed significant (p<0.05) increase. These comparisons were made against the negative control. The results from the study demonstrate that the extracts of Termitomyces robostus and Ocimum gratissimum possess immunostimulatory effects. These effects may be the reason for their use in traditional medicine.

Keywords: Cytokines, Termitomyces robostus, Ocimum gratissimum

INTRODUCTION

The immune system is a network of lymphoid organs, tissues, cells and the products of these cells whose function is to defend the organism against infective agents or foreign bodies (Anaso and Onochie, 1999; Aderem and Underhill, 2009; Lam et al., 2010; Egba et al., 2011). The immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the
pathogen has been eliminated in the form of an immune memory and allows the adaptive immune system to mount faster and stronger responses (Mayer, 2006). Inappropriate immunity has been shown as a common etiology in an ever-growing array of pathological processes including infections, allergy, aging, cancer, and a variety of disorders of various organs (Tsai et al., 2011). Many bioactive compounds are produced by plants throughout their development for a number of reasons such as defense against microorganism, insects and herbivore (Crozier et al., 2006). Ocimum gratissimum is a herbaceous plant which belongs to the Labiate family. The plant is used by the Ibo of Southern Nigeria in folklore medicine in the management of many health conditions (Prabhu et al., 2009). Pharmacological studies on the plant have shown that it has antimicrobial and antifungal (Orafidiya et al., 2006; Dubey et al., 1997), ovicidal (Pessoa et al., 2002), anti diarrheal (Ilori et al. 1996), wound healing (Orafidiya et al., 2005) and anti-inflammatory properties (Sahouo et al., 2003). Other reports have shown that it also has antimutagenic, cytotoxic and immunostimulatory effects among numerous other activities (Dubey et al., 1997; Obaseik-Ebor et al., 1993; Oladunmoye, 2006).

Our previous report showed immunostimulatory effect of the plant by the stimulation of antibody production and enhanced inflammatory response in Albino rats (Egba et al., 2014).

Termitomyces robustus belongs to the family Lyophyiceae. They are rich in minerals such as potassium, calcium, magnesium, iron and manganese, and are mostly edible (Mattila et al., 2001). The polysacharrides or glucans derived from the mushroom species have been widely recognized as immunomodulators as they are able to stimulate the host immune system and exert antitumor or antimicrobial activities along with other medicinal effects (Lindequequist et al., 2001; Wasser, 2002). It has also been suggested that higher degree of structural variability and conformational complexity of these polymeric molecules may be responsible for the potent host immune response generation (Bohn and Bemiller, 1995). Despite the structural and functional similarities of these glucans, they differ in their ability to elicit various responses, particularly cytokine expression and production (Anderson and Stasovski, 1992). Records of health promoting benefits such as antioxidant, antimicrobial, anticancer, cholesterol lowering and immunostimulatory effects have been reported for some species of mushroom (Anderson and Stasovski, 1992; Mizuno, 1995; Mau et al., 2004).

Modulation of cytokine secretion may offer novel approaches in the treatment and defense of a variety of diseases. One strategy in the modulation of cytokine expression may be through the use of herbal medicines (Kevin et al., 2006). Cytokines are now seen to be crucial to innate and adaptive inflammatory responses, cell growth and differentiation, cell death, angiogenesis and developmental as well as repair processes (Oppenheim, 2001). In addition, cytokines provide a link between organ systems, providing molecular cues for maintaining physiological stability (O’Sullivan et al., 1998). As a result of the growing recognition of cytokine activities, altering cytokine expression and targeting their receptors may offer therapeutic potential. Current pharmacological strategies include cytokine antagonist, agonist, inhibition, and stimulation models (Sommer, 1999). There is a modest body
of knowledge on the health benefit of *Ocimum gratissimum* and *Termitomyces robustus* in human health by immunomodulation. To explore the potentially health-promoting properties of *Ocimum gratissimum* and *Termitomyces robustus*, this study evaluated their immunomodulatory effects by their cytokine expression in Albino rats.

**MATERIALS AND METHODS**

**Plant Materials:** Fresh mushroom *Termitomyces robustus* and leaves of *Ocimum gratissimum* were purchased from a local farm in Umuaiga, Ikwoano LGA of Abia State, Nigeria and were identified by Dr. Omosun of Plant Science Department, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

**Preparation of Hot Aqueous and Methanol Extracts:** The dried powdered mushroom sample (10 g) was dissolved in 100 mL of hot distilled water in a beaker and left to stand overnight (12 h) with intermittent shaking and then filtered. The filtrate was concentrated in a water bath and used in the study. The hot aqueous extract of mushroom samples were continuously prepared according to demand. The methanol extract of *Ocimum gratissimum* were prepared continuously according to the demand. Also, the dried powdered leaves (150 g) of the plant were dissolved in 1500 mL of methanol in a sterile flask and swirl to ensure effective mixing and intermittent shaking. After 48 h, the mixture was decanted and solution obtained was poured into a stainless tray and allowed to evaporate to dryness at room temperature for two days using a vacuum evaporator (RC 1008 Bibby Sterilin, United Kingdom). The wet residue was freeze dried using a vacuum freezer drier and used for the study.

**Preparation of 10% Pyrogallol:** Pyrogallol reagent was prepared by dissolving 10 g of pyrogallol in 100 mL of distilled water. It was vigorously shaken for uniform mixing and the solution was used for the study.

**Antigen Preparation:** Fresh blood was collected from sheep sacrificed in a local slaughter house and preserved in EDTA bottles. It was washed three times with normal saline by centrifugation and the resulting suspension was adjusted to 1 x 10^6 SRBC/mL for immunization and challenge.

**Experimental Design:** A total of twenty male Wistar albino rats weighing between 70-110 g were randomly divided into five groups and used for the experiment. All the animals were housed in an animal house under normal conditions and acclimatized for two week. Commercial pellet diet (Vital feeds Nigeria, Ltd.) and water were fed to the animals *ad libitum*. The control groups, that is, the positive control (normal control) received normal saline via gavage while the negative control received 100 mL pyrogallol solution. Group 1 received 100 mg/kg body weight of *Ocimum gratissimum* extract and pyrogallol solution. Group 2 received 300 mg/kg body weight of *Ocimum gratissimum* and pyrogallol solution. Group 3 also received 300 mg/kg body weight aqueous hot extract of *Termitomyces robustus*. All administrations were done daily for 21 days. The protocol was approved by the experimental animal ethics committee of the College of Natural and Applied Sciences, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

**Sheep Red Blood Cell (SRBC) – Induced Humoral Antibody (Ha) Titer:** To specifically access the effects of the extracts on antibody formation, groups of four rats per treatment were immunized with 0.1 mL of Sheep Red Blood cell
suspension (1.1 × 10⁸ SRBC/mL) intraperitonially. The day of immunization was referred to as Day 0. Seven days later (Day 7), the rats were challenged by injecting 0.1 mL SRBC suspension into their left hind foot pad. Blood samples were collected from all the animals separately by ocular puncture using glass capillary tube on Day 7 (after challenge) for measurement of primary antibody titer and on day 14 for measurement of secondary antibody titer. Antibody levels were determined by the method described by Shinde et al. (1999). The collected blood was allowed to clot, and then centrifuged to get serum. 25 µL serum samples were placed into a 96-well micro titer plate. Serial two fold dilutions of the serum were made using 25 µL normal saline each time of transfer across the plate. To the 25 µL diluted serum in each well, 25 µL of 1% v/v SRBC suspension in normal saline was added. The micro titer plate was maintained at room temperature 1 h and their content examined for hemagglutination. The value of the highest serum dilution showing hemagglutination was defined as the antibody titer for the given rat.

STATISTICAL ANALYSIS

The data are expressed as mean ± standard deviation in bar charts. Comparisons were made between the groups using the one way Analysis of Variance (ANOVA) followed by post HOC LSD test. The analysis were carried out in SPSS for Windows 16. The accepted level of significance was at p < 0.05.

RESULTS

The effects of the administration of Ocimum gratissimum and Termitomyces robustus on interleukin-2 expression is shown in Figure 1. The results show that the administration of 300 mg/kg body weight of Termitomyces robustus and Ocimum gratissimum significantly increased the expression of interleukin-2 relative to the negative control group (p<0.05). Also, the administration of 100 mg/kg body weight of Ocimum gratissimum elicited a significant increase in the serum interleukin-2 expression (p<0.05). Figure 2 represents the effects of the administration of the extracts of Ocimum gratissimum and

![Figure 1: Effects of the Ocimum gratissimum and Termitomyces robustus extracts on serum interleukin-2 (IL-2)](image-url)
Termitomyces robustus on the cellular expression of interferon-γ (IFN-γ). From the results obtained, it is apparent that the administration of 300 mg/kg body weight of Ocimum gratissimum produced the highest expression of interferon (p<0.05) when compared to the negative control. Also the administration of 300 mg/kg body weight of Termitomyces robustus (group 3) significantly increased (p<0.05) the expression of serum interferon relative to the negative control group. The results in Figure 3 represent the effects of the different extracts on tumor necrosis factor (TNF-α). The results indicate a significant increase (p<0.05) upon the administration of 100 and 300 mg/kg body weight of Ocimum gratissimum compared to the
negative control group. Also from the results, we observed that administration of 300 mg/kg body weight of *Termitomyces robustus* induced a significantly (p<0.05) increased expression of serum tumor necrosis factor (TNF-α) relative to the control.

The effects of the extracts on the production of primary antibody is shown in Figure 4. The results show that the extracts induced an increased primary antibody titre relative to the negative control group (p<0.05). Also, both extracts (*Ocimum gratissimum* and *Termitomyces robustus*) were able to significantly increase (p<0.05) the secondary humoral antibody in the rats (Figure 5).

**DISCUSSION**

The limitations and paucity of currently available
pharmacologic agents that have immune boosting properties have stimulated research on novel immunostimulatory and immunomodulatory agents with different mechanisms of action. Some plant products modulate both humoral and cellular immunity, while others activate just the cellular components of the immune system. Therefore, the evaluation of plants for their capacity to promote or inhibit immunocyte proliferation is necessary to the study of immunomodulation and drug discovery (Shinde et al., 1999; Schneider et al., 2003; Smith, 1998). There are a few scientific reports on the effect of medicinal plants and mushrooms on immunity (Hardy and Chaudhri, 1997) but there is no scientific report on the effect of Ocimum gratissimum (Scent leaves) and Termitomyces robustus on cytokine expression so far. In this study, methanol extract of Ocimum gratissimum and hot water extract of Termitomyces robustus were used to examine the activity of the plant and mushroom on adaptive immunity (especially cytokine expression).

Immune response to sheep red blood cells has been widely used in the study of animal immune status (Ikeme and Adelaja, 1990). The use of Sheep Red Blood Cells (SRBC) challenge is based on the fact that responses to it is T-dependent and T-cells (T-helper cells specifically) coordinate the battle against infection by activating macrophages, B-cells and T-cells and thus, indirectly control immunoglobulin production (Sherman and Halliquest, 1990). T-cells are responsible for production of cytokines. Result of this study showed the extracts significantly (p<0.05) stimulated antibody production in the subjects compared to rats not given any extracts (Figure 4 and 5).

The results of cytokines expression (Figure 1) showed an up regulation in the serum interleukin 2 (IL-2) expression upon the administration of 300 mg/kg body weight of the extracts of Ocimum gratissimum and Termitomyces robustus (OG and TR). It is known that IL-2 has an immune stimulating activity as it supports proliferation (Anaso and Onochie, 1999; Cornish et al., 2006; Kawasaki et al., 2002) and survival (Gillis et al., 1977) of T cells (Gillis et al., 1978). The proliferation and survival could be as a result of the ability of IL-2 to induce protein synthesis in antigen-activated T-cells. Also its absence abrogates amino acids uptake and subsequent incorporation into proteins (Mitra et al., 1999).

Interferon are responsible for inducing transcription of a large group of genes which play a role in host resistance to viral infections, as well as activating key components of the innate and adaptive immune systems including antigen presentation and production of cytokines involved in activation of T cells, B cells and natural killer cells (Ngoupayo et al., 2009). From the results obtained, it is apparent that the administration of 300 mg/kg body weight of OG and TR induced the high expression of interferon (p<0.05) when compared to the negative control. These results suggest that the extracts could possess antiviral and stimulatory activities on intracellular pathogens (Egba et al., 2014; Sy et al., 2004). These results agree with our earlier finding that extract of Gongronema latifolium up regulates interferon expression in rats (Egba et al., 2014).

The increase in the expression of tumor necrosis factor on both low and high dosage groups when compared against the negative control group also supports the observed influence of OG and TR on cellular immunity.
Tumor Necrosis Factor (TNF) superfamily of cytokines represents a multifunctional group of proinflammatory cytokines which activate signaling pathways for cell survival, apoptosis, inflammatory responses and cellular differentiation (Shinde et al., 1999; Schneider et al., 2003, Krishnaraju et al., 2009). Ijeh et al. (2004) had reported that OG contains alkaloids, tannins, phytates, flavonoids and polysaccharides. A variety of phytochemicals such as polysaccharides (Shin et al., 2002) and flavonoids (Zhao et al., 2007) have been reported to modulate the immune system. Flavonoids which are polyphenols have been reported to serve as immunomodulators (Shin et al., 2002). Flavonoids stimulate human peripheral blood leukocyte proliferation. They also increase the activity of helper T cells, cytokines, interleukin-2, and macrophage and are useful in the treatment of several diseases caused by immune dysfunction (Egba et al., 2014). Thus, the probable mechanism of action of Ocimum gratissimum lies in its flavonoid component which stimulates the Helper T-cells to produce more interleukin-2 and interferon-γ. Studies have shown that mushrooms are rich sources of various bioactive molecules having anticancer and immunomodulatory potentials (Sharma et al., 2012). Among the most important constituents of mushroom Termitomyces are certain polysaccharides known as beta-glucans, which are bound to proteins (Borchers et al., 1999). Polysaccharides or beta glucans derived from mushroom species have been widely recognized as immunomodulators as they are able to stimulate the host immune system and exert antitumor or antimicrobial activities along with other medicinal effects (Lindequist et al., 2005; Wasser, 2002). The polysaccharides from the mushroom Termitomyces robustus and other mushroom extracts do not attack cancer cells and other diseases of the immune system directly; they produce their antitumor effects by activating different immune response in the host (Wasser, 2002). These reports clearly suggest that the probable immune stimulatory mechanism of TR could be the stimulation of the helper T cells.

We reported that the extracts (OG and TR) induced a significant increase on both the primary and secondary antibody titres (p<0.05) between the test groups compared to the negative control group. Humoral immune response is a type of acquired immunity. B-cell activation is a large part of the humoral immune response. Upon infusion of the SRBC in the experimental rat models, the B-cells make antibodies against the soluble sheep red blood cell. The B-cells are usually activated to produce antibodies against antigens by T-cell dependent and T-cell activation. Thus the humoral antibody titres mean values, which increased in all groups except in suppressed group (negative control), was as a result of increased immune response to the foreign sheep red blood cell.

CONCLUSION
Our observations have clearly demonstrated that Ocimum gratissimum and Termitomyces robustus extracts showed considerable immunostimulatory effects on both the innate function and adaptive immune function while exhibiting proinflammatory activity as depicted by the increase in interleukine-2 expression in albino rats. Thus, the effect of the plant and mushroom on cytokine expression may be the reason for its use in traditional medicine. Further research is needed to further characterize the active bioactive agents responsible for the immunomodulatory effects of these plants.
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