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Research Article

Phytochemical Analysis and Evaluation of the Antibiotic and Anti-inflammatory Activities of the Ethanolic Extract of the Dried Fruit of *Tetrepleura tetraptera* in Wistar Rats Infected with a Clinical Strain of *Staphylococcus aureus*

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Abstract

The use of medicinal plants with antibiotic and immuno-modulating properties could constitute an alternative in the fight against antibiotic resistance. *Tetrapleura tetraptera* is a plant of the Fabaceae family whose fruits are used in food, cosmetics and therapy. The objective of this work was to evaluate the antibiotic properties of the ethanolic extract of the dried fruits of *Tetrapleura tetraptera* after an infection with a clinical strain of *Staphylococcus aureus.* Phytochemical screening of *T. tetraptera* fruits was carried out by precipitation and staining reactions. The determination of total phenols was done by the colorimetric method of Folin-Ciocalteu; the determination of flavonoids by the aluminum trichloride method. Antibiotic activity was assessed by numeration of blood cells involved in the natural and specific immune response (leukocytes, neutrophils and lymphocytes). Anti-inflammatory activity was demonstrated by the presence or absence of C-reactive protein in the blood of infected rats. The results obtained were compared with those obtained in rats infected with *Staphylococcus aureus* and treated with Bactrim (Sulfamethoxazole+Trimethoprim) 40 mg/kg. Our results showed that the fruits of *T. tetraptera* contained several secondary metabolites and were rich in flavonoids and total phenols. The ethanolic extract of the fruits of *T tetraptera* had an antibiotic activity which allowed to eliminate the infection by *Staphylococcus aureus* germs after 21 days and the inflammation induced by the infection after 7 days.

Keywords: Medicinal Plant; Bacteria; Phytochemical Analysis; Antimicrobial Activity

Introduction

Antimicrobial resistance is a growing threat to health and development. The misuse aand overuse of antimicrobials is the main factor that has led to the emergence of drug-resistant pathogens. Resistant bacterial infections are indeed associated with nearly 4.95 million deaths each year and 1.27 million deaths are directly attributed to antimicrobial resistance [1]. *Staphylococci* are gram-positive bacteria that are very present in the environment, they can be found in water, in the air, on the ground, in food and on animals. Man is the main tank of *Staphylococcus aureus*. Thus,

it is normally and frequently found in the nasal cavities (nostrils) or ears, on the surface of the skin (hands), in the folds or moist areas (armpits, perineum, external genitalia) [2]. *Staphylococcus* occupies a very important place in human and animal pathology. *Staphylococcus aureus* commands an impressive arsenal of virulence factors, many of which challenge the immune system [3]. The defense against this pathogen relies above all on the innate immune system in which phagocytes, mainly neutrophils, act in concert with the complement system [4,5]. The adaptive immune system, composed of T cells, B cells, and antibodies, can facilitate, focus, and enhance innate immunity [6]. *Staphylococcus aureus* is a dreadful pathogen that has been able to develop resistance to each new antibiotic introduced for half a century. The plasticity of its genome gives it the ability to adapt to all environmental conditions, and in particular to acquire antibiotic resistance genes and to develop regulatory mechanisms to adapt to increasing concentrations of antibiotics [7]. Resistance to penicillin, initially restricted to the hospital environment, very quickly spread to the community environment and currently affects more than 90% of S. aureus strains. The introduction in 1959 of methicillin, a semi-synthetic derivative of penicillin for the treatment of staphylococcal infections raised great hopes. But barely a year later, the first hospital strains of methicillin-resistant *S. aureus* (MRSA) appeared [8,9]. In May 1996, the first clinical documentation of infection by an S. aureus with decreased susceptibility to glycopeptides (to vancomycin) was reported. Since then, several similar cases have been documented all over the world [10]. Faced with the situation of antibiotic resistance, the alternative of traditional medicine is to be taken seriously. African forests represent the main source of medicinal plants in rural areas and contribute to local and national economies. The use of plants for therapeutic purposes is a centuries-old practice that is currently experiencing renewed interest among the public. It is possible to use whole plants or the extraction products they provide. *Tetrapleura tetraptera* of the Fabaceae family is a species from tropical Africa that grows in semi-deciduous forest. It is a tree with deciduous leaves that grows about 20 to 25 meters in height. Its fruits are pods surmounted by 2 fleshy, shiny brown wings. Still called Aridan, the plant is highly sought due to its medicinal and aromatic values. Its different parts are used for culinary and therapeutic purposes. Culinarily, the fruits of *T. tetraptera* are used as flavorings and spices. The leaves, stems, bark, fruits and roots of the plant are used as a contraceptive, anti-inflammatory, anti-ulcer, hypotensive, anti-anaemic and especially anti-microbial. Infusions of the pods of *T. tetraptera* are consumed by mothers of newborns for the reconstruction of blood lost during childbirth and to prevent inflammation. It is reported that the fruits of *T. tetraptera* strengthen the immune system and fight against bacterial infections [11-13]. In this work we determined the secondary metabolites contained in the fruits of *T. tetraptera*, assayed the phenolic compounds and followed the evolution of blood cells involved in the immune reaction in rats infected with S. aureus and untreated, infected rats treated with Bactrim and the infected rats treated with the ethanolic extract of the fruits of *T. tetraptera* in order to evaluate the antibiotic activity of this extract.

Materials and Methods

Materials

Plant Material

The plant material consisted of the dried fruits of *Tetrapleura tetraptera* which were harvested on a farm in Porto-Novo in the department of Oueme. The fruits were identified at the National Herbarium of the National University of Abomey-Calavi (Benin). After collection, the samples were dried at laboratory temperature (20-25°C) for about two months until their mass stabilized. The fruits were cut into small sized pieces before grinding in a coarse grinder resulting in a coarse powder.

Animal material

The experimental animals wmale and female Wistar rats weighing between 150 and 250 g. All animals were of SOP health status (Exempt from specific pathogenic organisms). Upon receipt, the rats were randomly placed in groups of five (5) in standard cages for an acclimatization period (2 weeks) before being used in the various experiments. During this period the animals had free access to food and water and remained kept in the animal facility of the Histology Laboratory of the Institute of Applied Biomedical Sciences (ISBA) at a constant temperature (22 ± 2) °C. They were subjected to a 12/12h light/dark cycle. The dark phase of this cycle begins at 12 p.m. and the different experiments always took place from 11 a.m. to 6 p.m. due to the nocturnal activity of the animal (active phase).

Extraction of bioactives

The extraction was carried out by the technique of maceration. 50 mg, coarse fruit powder was weighed and poured into 500ml

of 95% ethanol. The mixture is kept stirred for 24 hours at room temperature with manual stirring every 30 minutes to obtain the ethanolic extract. The extract was then filtered on a Buchner apparatus under vacuum. The filtrate was then recovered. The total volume of the filtrate is concentrated under vacuum at 60° C. using a Heidolph-type rotary evaporator. The dry extract was then collected, weighed and labeled. The dry solid was resuspended in dimethyl sulfoxide (DMSO) to prepare various concentrations of the hydroethanolic phase, which were used in the bioassay. The dried extract was weighed and the yield was calculated as a percentage using the following formula:

R = Mass of recovered sample x 100/Mass of initial sample

Phytochemical screening:

It consisted in exploring the chemical potential of plants through the search for the main families of chemical compounds by coloring and precipitation reactions in tubes. The method used was that of Houghton supplemented and improved [14].

Treatment of rats

The rats were weighed in order to find the average weight and the calculated dose of extract to be administered. The rats were force-fed daily and at the same time for 21 days.

- Batch 1 (negative control batch) received plain water orally for 21 days;
- Batch 2 (the positive control batch) consisted of 5 infected rats which received 0.9% physiological saline for 21 days;
- Batch 3 (reference control) consisted of 5 rats infected and treated with Sulfamethoxazole + Trimethoprim (Bactrim) 40 mg/kg body weight for 21 days
- Batch 4 consisted of 5 rats infected and treated with the ethanolic extract of *Tetrapleura tetraptera* at 300 mg/kg of body weight for 21 days
- Batch 5 consisted of 5 rats infected and treated with the ethanolic extract of *Tetrapleura tetraptera* at 500 mg/kg of body weight for 21 days.

Rat infection

The rats of batches 2, 3, 4, 5 received the injection of the pathogen *Staphylococcus aureus*. Three germ colonies are diluted in 3 ml of physiological water for injection.

White blood cell, neutrophil and lymphocyte numeration

It was made on the blood collected using a hematology automaton (System KX). The number of blood cells was given by the automaton for each batch of rats. The number of blood cells of the rats infected and treated with the extracts of *T. tetraptera* was compared with the number of blood cells in control rats, untreated infected rats and infected rats treated with Bactrim.

C-reactive protein (CRP) test

The CRP-Latex particles were coated with antibodies, anti-rat CRP. The CRP-LATEX reagent was standardized to detect serum CRP levels around 6mg/L, which was considered to be the lowest concentration of clinical significance. Mixing the latex reagent with the serum containing CRP leads to an antigen-antibody reaction which resulted in easily visible agglutination within two minutes. The presence or absence of visible agglutination indicated the presence or absence of CRP in the specimen. It was performed using a manual technique.

Results

Phytochemical analysis of fruits of *Tetrapleura tetraptera*

The results of the phytochemical screening are summarized in table 1. These results show that the ethanolic extract of the fruits of Tetrapleura tetraptera contains phytochemicals such as catechic tannins, flavonoids (anthocyanins, leucoanthocyanins and free flavonoids), reducing sugars, mucilages, coumarins, saponosides and anthraquinones combined o-heterosides.

Secondary metabolites		**Fruit**
Tannins	Total Tanins	+
	catechic Tannins	+
	Gallic Tannins	-
Flavonoids	Anthocyanins	+
	Free Flavonoids	+
	Leucoanthocyanins	+
Mucilage		+

Reducing sugar		+
Alkaloids		-
Combined Anthraquinones O-heterosides with reduced genins	O-heterosides	+
	O-heterosides with reduced genins	-
	C-heterosides	-
Coumarins		+
Free Anthraquinones		-
Saponosides		+

Table 1: Secondary metabolites present in the fruits of *Tetrapleura tetraptera.*

+ present – Absent.

Content of phenolic compounds in the dried fruits of *Tetrapleura tetraptera*

The dosage of total polyphenols, condensed tannins and total flavonoids was carried out by establishing calibration curves with gallic acid solutions for total polyphenols, quercetin solutions for total flavonoids and cachetin solutions for catechic tannins. The quantitative analysis in phenolic compounds is made by UV-visible spectrophotometry. The fruits of T tetraptera contain 3.27 mg/g of dry matter of total polyphenols; 4.30 mg/dry matter of condensed tannins and 33mg/g of dry matter of total flavonoids.

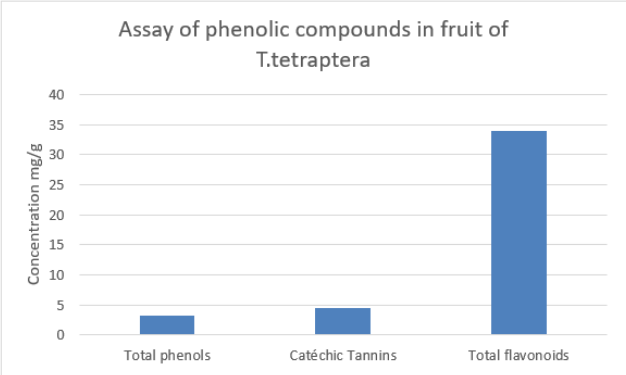

Figure 1: Concentration of Phenolic compounds in dried fruit of *Tetrapleura tetraptera*.

Antibiotic activities of Tetrapleura tetraptera fruits

Numeration of leukocyte on days 0, 7, 14 and 21 after infection with *S. aureus*

The leukocyte numeration was made on the batches of negative control rats which had not been infected, the untreated infected positive control rats, the infected rats treated with Bactrim, the infected rats treated with doses of 300 mg and 500 mg per kilogram of body weight of the ethanolic extract of *T tetraptera.*

Day 0, the average leukocytes in the different batches of rats were approximately equivalent and were around 7.5 Giga/Liter of blood.

Day 7 after infection, the number of leukocytes was: control rats 8.075 ± 1.9 G/L; untreated infected rats 25.48 ± 3.5 G/L; infected rats treated with bactrim 12.5 ± 1.5 G/L; infected rats treated with *T tetraptera* 300 mg/kg 17.68 ± 3.6 G/L; infected rats treated with *T tetraptera* 500 mg/kg 13.68 ± 2.3 G/L. It was observed increased rate in all infected rats with highest rate in untreated infected rats.

Day 14 after infection, the number of leukocytes was: control rats 8.6 ± 2.1; untreated infected rats 17.8 ± 2.3; infected rats treated with bactrim 8.5 ± 1.2; infected rats treated with *T tetraptera* 300 mg/kg 13.68 ± 1.7 G/L; infected rats treated with *T tetraptera* 500 mg/kg 12.22 ± 2.6 G/L. It was observed equivalent leukocyte levels in uninfected rats and rats treated with Bactrim.

Day 21 after infection, the number of leukocytes was: control rats 7.5 ± 0.9; untreated infected rats 13.22 ± 1.4; infected rats treated

with bactrim 7.76 ± 0.7; infected rats treated with T tetraptera 300 mg/kg 7.6 ± 1.2 G/L; infected rats treated with T tetraptera 500 mg/kg 7.6 ± 1.1G/L. It was observed similar leukocyte levels in uninfected rats, infected rats treated with Bactrim and infected rats treated with both doses of the ethanolic extract of the dried fruit of *T. tetraptera.*

Figure 2: Numeration of leukocytes in the different batches of rats on days 0,7,14 and 21.

Neutrophil numeration at days 0, 7, 14 and 21 after infection

The neutrophil numeration was made on the same batches of rats.

On day 0, the average neutrophil number in the different batches of rats were between 0.28 and 0.38 Giga/Liter of blood.

Day 7 after infection, the number of neutrophils was: control rats 0.39 ± 0.17G/L; untreated infected rats 1.37 ± 0.29G/L; infected rats treated with bactrim 0.73 ± 21G/L; infected rats treated with *T. tetraptera* 300 mg/kg 1.22 ± 0.22 G/L; infected rats treated with *T tetraptera* 500 mg/kg 1.21 ± 0.29G/L. The level of neutrophils increased in all infected rats with the highest level in untreated infected rats.

Day 14 after infection, the number of neutrophils was: control rats 0.43 ± 0.08; untreated infected rats 1.09 ± 0.26; infected rats treated with bactrim 0.41 ± 0.13; infected rats treated with *T tetraptera* 300 mg/kg 0.84 ± 0.21 G/L; infected rats treated with *T tetraptera* 500 mg/kg 1.09 ± 0.26G/L. It was observed similar neutrophil numbers in uninfected rats and rats treated with Bactrim.

Day 21 after infection, the number of neutrophils was: control rats 0.27 ± 0.07; untreated infected rats 0.55 ± 0.1; infected rats treated with bactrim 0.29 ± 0.1; infected rats treated with *T tetraptera* 300 mg/kg 0.30 ± 0.08G/L; infected rats treated with T tetraptera 500 mg/kg 0.42 ± 0.16G/L. It was observed similar neutrophil levels in uninfected rats, infected rats treated with Bactrim and infected rats treated with a dose of 300 mg/kg of the ethanolic extract of the dry fruit of *T. tetraptera*.

Figure 3: Numeration of neutrophils in the different batches of rats on days 0, 7, 14 and 21.

T lymphocytes numeration at days 0, 7, 14 and 21 post infection

The numeration of T lymphocytes was made on the same batches of rats.

On day 0, the average T lymphocytes in the different batches of rats were between 6.2 and 7 Giga/Liter of blood.

Day 7 after infection, the number of T lymphocytes was: control rats 6.97 ± 1.71G/L; untreated infected rats 21.70 ± 2.99G/L; infected rats treated with bactrim 10.18 ± 1.33G/L; infected rats treated with *T tetraptera* 300 mg/kg 14.23 ± 2.62 G/L; infected rats treated with *T tetraptera* 500 mg/kg 10.92 ± 2.61G/L. The number of T cells Increased in all infected rats with highest number in untreated infected rats.

Day 14 after infection, the number of T lymphocytes was: control rats 7.46 ± 1.93; untreated infected rats 15.17 ± 2.02; infected rats treated with bactrim 6.95 ± 1.00; infected rats treated with *T. tetraptera* 300 mg/kg 11.27 ± 1.55 G/L; infected rats treated with *T. tetraptera* 500 mg/kg 9.83 ± 2.18G/L. the number of T cells lymphocyte decreased in treated infected rats. T cells levels were similar in control rats and rats treated with Bactrim. A dose-response effect was observed in rats treated with the extract.

Day 21 after infection, the number of T lymphocytes was: control rats 6.53 ± 0.78; untreated infected rats 11.40 ± 1.18; infected rats treated with bactrim 6.68 ± 0.71; infected rats treated with *T. tetraptera* 300 mg/kg 6.60 ± 1.17G/L; infected rats treated with *T. tetraptera* 500 mg/kg 6.33 ± 0.88G/L. The rate of T lymphocytes was similar in the control rats, the infected rats treated with Bactrim and the infected rats treated with the 2 doses of the ethanolic extract of the dry fruit of *T. tetraptera*.

Figure 4: Numeration of T lymphocytes in the different batches of rats on days 0,7,14 and 21.

Evaluation of the anti-inflammatory activity of *T. tetraptera* dried fruit extract

Table 2 showed the presence or absence of C-reactive protein on days 0 and 7 post-infection. On day 0, none of the infected batches had an inflammatory reaction, but on the 7th day inflammation was observed in all the untreated infected rats and only a very insignificant number in the infected batches treated with various substances (Bactrim and extract at doses of 300 mg/kg and 500 mg/kg).

Batches of rats	**Batch 1 Control rats (n = 4)**	**Batch 2 Infected non treated rats (n = 5)**	**Batch 3 Infected and treated rats with Bactrim (n = 5)**	**Batch 4 Infected and treated rats with *Tt* 300 mg/ Kg (n = 5)**	**Batch 5 Infected and treated rats with *Tt* 500 mg/ Kg (n = 5)**
Number of CRP-positive rats at D0	0	0	0	0	0
Number of CRP-positive rats at D7	0	5	1	2	1

Table 2: Number of C-Reactive Protein (CRP) positive rats according to batches of rats.

Discussion

Our results showed that the ethanolic extract of the fruits of *Tetrapleura tetraptera* contained phytochemical compounds such as catechic tannins, flavonoids (anthocyanins, leucoanthocyanins and free flavonoids), reducing sugars, mucilages, coumarins, saponins and the combined o-heteroside anthraquinones. The quantitative

study showed that the fruits of T. tetraptera were rich in flavonoids (33 mg EQ/g of dry matter) and less rich in total phenols (3.27 mg EC/g of dry matter) and in catechic tannins (4.30 mg EGA/g dry matter). Flavonoids have immunostimulant and anti-inflammatory properties, tannins have antibacterial properties [15,16].

The leukocyte numeration showed that at the 7th day after infection, the number of leukocytes increased in all the infected rats but this increase was very significant in the untreated infected rats. This suggests that the infection stimulated the proliferation of leukocytes and the different treatments attenuated the infection in the treated rats. At the 14th day the number of leukocytes decreased in all the infected rats compared to the 7th day and the number of leukocytes in the rats infected and treated with Bactrim became comparable to the number of leukocytes in the uninfected rats, which suggests that on the 14th day the reference tablet (Bactrim) was able to eliminate the infection. At day 21 after infection, the number of leukocytes became comparable in all batches of rats, uninfected rats and infected rats treated with Bactrim and the doses of the extract of *T tetraptera*. These results suggest that after 21 days, the two doses of the ethanolic extract of *T tetraptera* are able to eliminate the infection of the germs of staphylococcus aureus like the Bactrim tablet. Leukocytes protect the body against infections. They locate the site of an infection, fight off the invader by producing antibody proteins to attach to fix and destroy the foreign organism. The group of leukocytes is composed of several cells including neutrophils, lymphocytes, macrophages etc.

The neutrophil numeration showed that on day 0 after infection with the clinical strain of *S. aureus*, the number of neutrophils was low in the 5 groups of rats. At day 7 after infection, the number of neutrophils increased in all infected rats, with the greatest number in untreated infected rats. This result suggests the activation of neutrophil proliferation after infection. On day 14, the number of neutrophils decreased in infected rats. The number of neutrophils in rats infected and treated with Bactrim is equivalent to that of uninfected control rats. This suggests that after 14 days of treatment Bactrim cleared the infection. On day 21, the number of neutrophils was low and equivalent in all batches of rats, suggesting that the ethanolic extract of *T tetraptera* eliminated the bacterial infection in 21 days of treatment. This confirms the result obtained with the leukocyte numeration. The increase in the number of neutrophils after an infection corresponds to the natural immune response which is the first line of natural defence, its implementation is immediate, spontaneous and rapid as soon as a foreign element enters the organism and whatever the aggressor (virus, bacteria, parasite). Bacteria secrete bacterial products such as lipoproteins on their surface. These conserved bacterial products are commonly known as pathogen-associated molecular models (PAMPs) and are recognized directly by recognition receptors expressed on the surface of neutrophils. Activation of these receptors activates signal transduction pathways that prolong neutrophil survival, facilitate neutrophil adhesion and phagocytosis of pathogens. Additionally neutrophils produce cytokines and chemokines that cause degranulation and promote the production and release of reactive oxygen species (ROS), ultimately contributing to microbicidal activity [17-19].

Regarding the T lymphocyte numeration, their level varied in the same way as the blood cells mentioned above. The high concentration of T lymphocytes in infected rats on day 7 and its fall only in the infected treated rats on day 21. This result suggests that the infection with *S. aureus* induced a specific immune reaction and the ethanolic extract of the dried fruit of *T. tetraptera* had an antibiotic effect which induced the suppression of infection like Batrim tablets. Lymphocyte secretion is a factor indicating that the induced infection has elicited an adaptive response. They produce specific antibodies that bind to the surface of microbes and facilitate their phagocytosis after opsonization. T cells recruit neutrophils and macrophages to the site of infection. They also produce cytokines to eliminate bacteria or viruses inside cells or directly eliminate cells by acting as cytotoxic cells [20,21].

On day 7 after infection, all the untreated infected rats were positive for C-Reactive Protein, which was not the case for the infected rats treated with Bactrim and the two doses of the fruit extract of *T. tetraptera*. The anti-inflammatory activity of the ethanolic extract of the fruits of the plant is similar to that of Bactrim the reference tablet. Inflammation is induced by the invasion of macrophages and neutrophils at the site of infection and the production of pro-inflammatory cytokines [22,23].

Our results showed that the ethanolic extract of the dried fruit of *T. tetraptera* reduced the proliferation of cells involved in the immune response in infected rats to values observed in uninfected rats 21 days after infection. These observations suggest a total

elimination of *Staphylococcus aureus* germs as observed with the antibiotic Batrim. In addition, the anti-inflammatory activity of the extract is comparable to that of Bactrim. The extract therefore has antibiotic and anti-inflammatory activity. These properties could be linked to the presence of flavonoids and tannins in the fruit of *T. tetraptera* [24-27].

Conclusion

The dry fruit of *T. tetraptera* contains several secondary metabolites and is very rich in flavonoids. Its ethanolic extract has antibiotic and anti-inflammatory effects comparable to those of the reference antibiotic Bactrim. These activities could be linked to the presence of flavonoids. The dry fruit of T. tetraptera could therefore be used as an antibiotic in the event of infection by S. aureus germs.

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