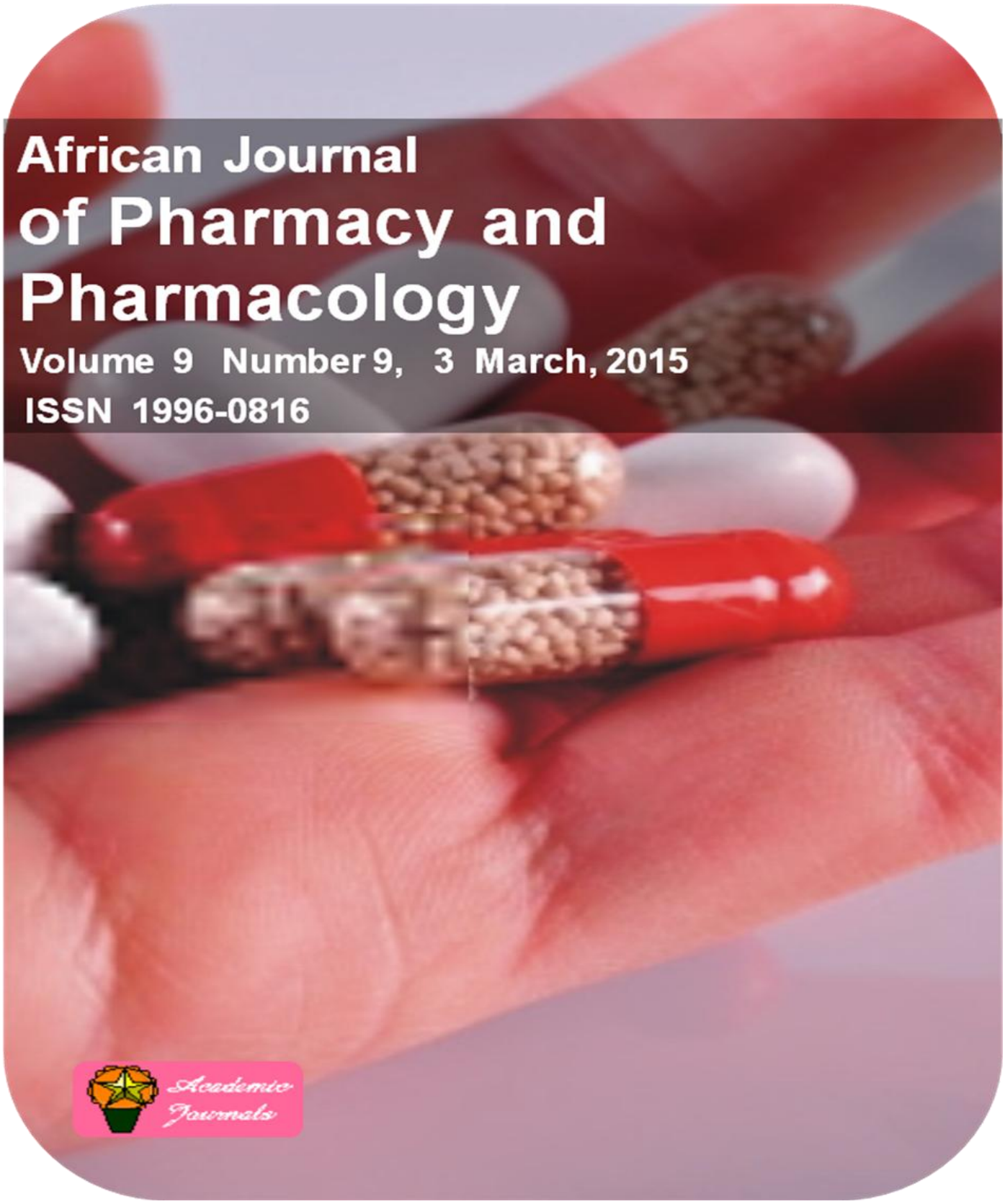

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African Journal of Pharmacy and Pharmacology

Review

A review on *Helicobacter pylori* infection and treatment

Faiza Naseer[1]*, Fatima Javid[1], Samra Sadiq[1], Muhammad Zeeshan Ashraf[1], Hira Naeem[2], Laiba Riaz[2], Aisha Shehzad[2], Huma Naz[3] and Nayab Latif[4]

[1]College of Pharmacy, GC University, Faisalabad, Pakistan.
[2]Institute of Pharmacy, Physiology and Pharmacology, Agriculture University, Faisalabad, Pakistan.
[3]Faculty of Pharmacy, University of Sargodha, Sargodha, Pakistan.
[4]University of Faisalabad, Faisalabad, Pakistan.

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***Helicobacter pylori* is a microorganism which has been the reason of various diseases, including gastritis, peptic, duodenal ulcer and cancer of the stomach. Urea breathe test (till 6 years), stool antigen test and serology testing are used for diagnosis. Triple, quadruple, sequential, salvage and levofloxacin, omeprazole, nitazoxanide, and doxycycline (LOAD) therapies are used for treatment of all *H. pylori* infections. Providing patient education regarding adherence, proper administration of drug and their adverse effects is one of the most important roles of pharmacists.**

Key words: *Helicobacter pylori*, infection, levofloxacin, omeprazole, nitazoxanide, and doxycycline (LOAD) therapy, salvage therapy.

INTRODUCTION

Helicobacter pylori, negative spiral or curved microorganism has been widely studied after his discovery, by human gastric biopsy, in 1983. *H. pylori* is commonly associated with the gastritis, peptic and duodenal ulcer as well as cancer of the stomach. The most common disease caused by *H.pylori* is peptic ulcer (Magdalena et al., 1991).

Epidemiology

H. pylorus is the most common pathogenic organism worldwide. Overall, almost 50% population of the world is infected by this organism, while the occurrence in some developing countries is as high as 80 to 90%, whereas in the U.S., 35 to 40% of the population are infected (Brian et al., 2001).

Transmission of *H. pylori*

The exact route of transmission of *H. pylori* is not fully known. *H. pylorus* is mainly transmitted by oral ingestion and it is mostly transmitted within families in developed countries. In underdeveloped countries, the prominent routes are saliva, feces and gastro-oral route (Robert et al., 2001).

Pathogenesis

The gastric mucosa shows well protection against

*Corresponding author. E-mail: faiza.naseer@ymail.com.

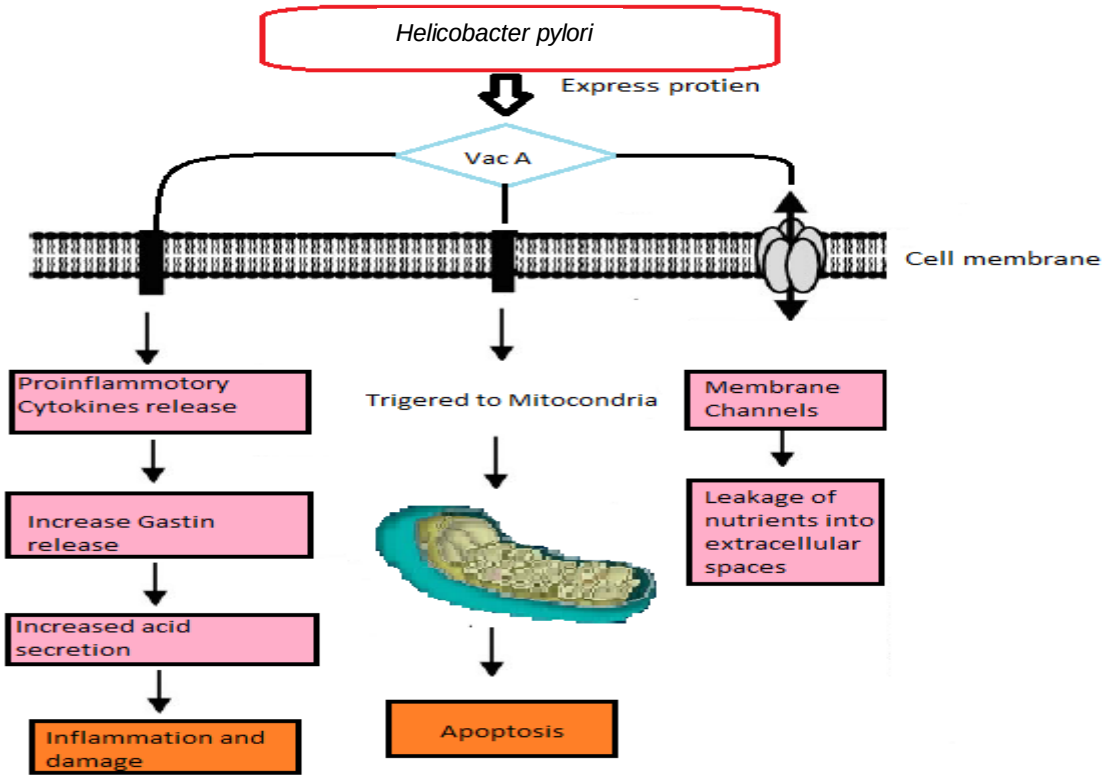

Figure 1. Pathogenesis of *Helicobacter pylori* infection triggered by VacI protein.

bacteriological infection. *H. pylorus* with distinctive properties enters into the mucus and get attached to the epithelial cells. *H. pylori* have potential to escape from the defense mechanism of the body due to which it shows persistent colonization (Sebastian and Pierre, 2002). After being entered to mucus layer, bacteria survive into the acidic pH which is due to hydrolysis of the urea by enzyme urease into ammonia and carbon dioxide. Urease activity is further regulated by urei, pH-gated urea channel (Sebastian and Pierre, 2002). Most of *H. pylori* strains discharge a 95-kDa protein named as VacA. The VacA protein plays an important role in the pathogenesis of both peptic ulceration and gastric cancer. VacA gets attach to the epithelial cell membranes and forms a voltage gated channels in the membrane, thus prompting the release of urea, bicarbonates and nutrients from the host cells (Johannes et al., 2006). Vac within the epithelial cell membrane release proinflammatory cytokines due to which there is an increase in the gastrin release and reduction in antagonist and inhibitor somatostatin. This in turns increases the acid secretion from the parietal cells and bicarbonates release is reduced which causes inflammation and damage that leads to ulcer and if untreated gastric carcinoma (Anahita and Emad, 2012). Vac also directly entered to the mitochondria where it releases cytochrome c which causes apoptosis and leads to carcinoma (Sebastian and Pierre, 2002) (Figure 1).

DIAGNOSIS

Different invasive and non-invasive diagnostic tests were performed for the detection of *H. pylori* infection. In non-invasive test for the initial diagnosis of the infection of *H. pylori*, urea breath test is performed. The specificity and sensitivity of the test is 90%. The test should be performed after 4 weeks otherwise it will give false results. Urea test can be done for children above 6 years. Another cheap and mostly used non-invasive test is serology testing. Its shows same specificity and sensitivity as urea breath test, but this test is not reliable in young children. Stool antigen test is also an alternative to urea breath test with 90% specificity and 89 to 98% sensitivity. Mostly, stool test is performed for the follow up of infection. It is the test of choice in almost all ages of children. An invasive test like endoscopy is preferred in patient with severe symptoms like gastrointestinal (GIT) bleeding as well as with age more than 50 years. An antibiotic sensitivity culture test is not routinely performed for the initial diagnosis of the infection (Sebastian and Pierre, 2002).

TREATMENT FOR *H. PYLORI* INFECTION

Triple therapy

For the treatment of *H. pylori* infection, first line

recommended therapy is a proton pump inhibitor (PPI)-based triple therapy, that is, standard PPI dose twice daily + clarithromycin 500 mg twice daily + amoxicillin 1000 mg twice daily for 10 days (Calvet, 2006). From different studies and analysis, it is proven that triple therapy shows better results with twice daily dosing of proton pump inhibitor and clarithromycin is used twice in a dose of 500 mg, rather than 250 mg. Although recommended as an alternative to patients who are penicillin allergic; the combination of clarithromycin and metronidazole should be discouraged as there is currently no effective salvage therapy if such a combination fails (Silva et al., 2008). In triple therapy regimens, clarithromycin substitution with other macrolides (e.g., erythromycin or azithromycin) is not recommended due to low efficacy (Graham and Fischbach, 2010). From recent studies, increased resistance to metronidazole and clarithromycin up to 42 and 20%, respectively is shown and the cure rate of the standard triple therapy has fallen below the acceptable rate of >80% in many regions (Vaira et al., 2009). So this therapy is not recommended as first line nowadays except local susceptibility patterns show such a treatment regimen to be highly effective (Calvet, 2006).

Quadruple therapy

Quadruple therapy is used as an alternative to the triple therapy. It includes PPI or H_2-blocker (U.S. Guidelines only) + bismuth+ tetracycline + metronidazole, that is, used for 10 to 14 days. Quadruple therapy is used as first-line therapy for eradication of *H. pylori*. Formerly, it was thought that the dosing of quadruple therapy is complex and less tolerated, so triple PPI therapy was considered the first line therapy for *H. pylori* infection treatment. Now quadruple therapy becomes first line treatment due to increased clarithromycin resistance. In standard quadruple therapy, the substitution of doxycycline for tetracycline is not recommended due to the lack of data (Calvet, 2006).

Sequential therapy

Sequential therapy is a 10 day course which starts with PPI twice daily and amoxicillin 1000 mg twice daily after which immediate 5-day course of tinidazole 500 mg twice daily, and a PPI twice daily or clarithromycin 500 mg twice daily, and metronidazole 500 mg (Calvet, 2006). European studies show a very high cure rate of this therapy, that is, 92% (Malfertheiner et al., 2012; Ruggiero, 2012). From two different analyses it is proved that this therapy is effective in macrolide resistant H. pylori (Calvet, 2006).

Salvage therapy

An eradication rate for *H. pylori* is ranging from 63 to 94% in Asian and European populations are shown with salvage therapy. Salvage therapy is a regimen of levofloxacin-based triple therapy. A meta-analysis including four randomized controlled trials showing that a 10-day levofloxacin-based triple therapy regimen had a superior eradication rate and was associated with fewer side effects as compared to a 7-day course of bismuth-based quadruple therapy. However, these results require validation in the North American population. Furthermore, the optimal levofloxacin dose 250 mg twice daily vs. 500 mg daily vs. 500 mg twice daily and duration of therapy either 7 or 10 days has yet to be determined. However, another meta-analysis did find a higher eradication rate with the 10-day over 7-day regimen. Unfortunately, resistance to fluoroquinolones is rapidly increasing. Experts now recommend using fluoroquinolone therapy only when susceptibility data are available (Calvet, 2006; Lacy et al., 2012)

Levofloxacin, omeprazole, nitazoxanide, and doxycycline (LOAD) therapy

A new four-drug regimen shows greater effectiveness with cure rate 88.9% with 10 days treatment and 89.4% with 7-day treatment in an open label study. LOAD therapy is levofloxacin 250 mg daily with breakfast + omeprazole 40 mg daily before breakfast + nitazoxanide 500 mg twice daily + doxycycline 100 mg daily for dinner. A larger randomized controlled trial is warranted to further evaluate the efficacy of this treatment regimen (Calvet, 2006; Malfertheiner et al., 2012).

Food and drug administration (FDA) approved therapy

PPIs are taken before meal. Metronidazole recommended dose is 1500 mg/day to overcome metronidazole resistance and getting better efficacy. Avoid cimetidine to reduce drug interaction. Lansoprazole + Amoxicillin combination is used only in clarithromycin allergic or resistant patients. Calvet, (2006). (Table 1).

PATIENT COUNSELLING (ROLE OF PHARMACIST)

Patient adherence is essential for successful eradication of *H. pylori*. Given the high pill burden, the increased frequency of administration and the prolonged duration of treatment, thorough understanding of the importance of completing the treatment regimen as prescribed is paramount. No adherence may be associated with awful outcomes, including treatment failure and antibiotic resistance. While adherence and proper administration of the regimen are crucial points of emphasis, patients should also be informed about potential treatment related adverse effects. Advanced notification about common adverse effects as well, providing suggestions for

Table 1. FDA approved therapy.

Drug 1	Drug 2	Drug 3	Drug 4	Duration
Esomeprazole 20 mg BID	Clarithromycin 500 mg BID	Amoxicillin 1 g BID	-	10 days
Lansoprazole 30 mg BID	Clarithromycin 500 mg BID	Amoxicillin 1 g BID	-	10-14 days
Omeprazole 20 mg BID	Clarithromycin 500 mg BID	Amoxicillin 1 g BID	-	10 days
Rabeprazole 20 mg BID	Clarithromycin 500 mg BID	Amoxicillin 1 g BID	-	7 days
Ranitidine 150 mg BID or Famotidine 40 mg/day or Nizatidine 300 mg/day (single or divided doses)	Metronidazole 250 mg QID	Tetracyclin 500 mg QID	Bismuth subsalicylate 525 mg QID	10-14 days
Omeprazole 20 mg BID	Metronidazole 375 mg QID	Tetracyclin 375 mg QID	Bismuth subcitrate potassium 420 mg QID	10 days
Omeprazole 40 mg Once daily	Clarithromycin 500 mg TID	-	-	14 days
Lansoprazole 30 mg TID	Amoxicillin 1 g TID	-	-	14 days

BID: Twice daily; TID: thrice daily; QID: four times a day.

management may help to prevent premature discontinuation of the regimen.

(1) PPIs are well tolerated, but headache, dizziness, nausea, diarrhea, constipation and abdominal pain may occur. Patients should be instructed to take PPIs 30 to 60 min prior to a meal.
(2) Hypersensitivity to any component of the regimen may occur; however, this type of reaction is most likely with amoxicillin. Amoxicillin and clarithromycin are commonly associated with GI upset (nausea, vomiting, diarrhea and abdominal pain). Amoxicillin may also be associated with headache and clarithromycin may also be associated with taste disturbances, such as a bitter or metallic taste in the mouth.
(3) Metronidazole elicits adverse effects similar to clarithromycin (that is, GI upset and metallic taste in the mouth) but also may be associated with a disulfiram-like reaction with alcohol consumption.
(4) In patients on warfarin initiating metronidazole, international internalized ratio (INR) elevations are common and require close monitoring.
(5) Tetracycline is associated with GI upset, photosensitivity and tooth discoloration. Patients should be advised to wear sunscreen and avoid prolonged exposure to sunlight.
(6) Pregnant patients and children under the age of eight should not receive tetracycline. Certain medications and foods such as calcium, antacids, iron and milk, may reduce the absorption and, thus, the effectiveness of tetracycline. .
(7) Bismuth may cause GI upset and darkening of the tongue and stool. Bismuth containing regimens should be used with caution in patients with renal impairment as accumulation may occur. Patients with aspirin (salicylate) sensitivity should avoid the subsalicylate form of bismuth (Malfertheiner et al., 2012; Ruggiero, 2012; Lacy et al., 2012).

DISCUSSION

Patient adherence is essential for successful eradication of *H. pylori*. Providing patient education regarding adherence, proper administration of drug therapy and adverse effects is one of the most important roles that pharmacists provide in caring for patients with *H. pylori* (Anahita and Emad, 2012). Additionally, pharmacists with access to prescription and/or medical records can review those records for prior clarithromycin and/or metronidazole use, which may increase the likelihood of antibiotic resistance and treatment failure. Patient allergies identified during record review should be considered in choosing a regimen. For patients with allergies, regimens containing those antibiotics should be avoided. Other factors, such as drug interactions and contraindications to drug therapy, may influence treatment. Adverse effects, ease of administration and cost may also influence the choice of an initial eradication regimen. Finally, following completion of the eradication regimen, pharmacists may assist with monitoring of persistent or recurrent symptoms. Patients should be advised to contact a health care professional if symptoms persist or recur or if they experience alarm symptoms during treatment, such as blood in the stool. Pharmacists have the opportunity to

fulfill many roles and have a valuable impact on patient care for *H. pylori* (Calvet, 2006; Silva et al., 2008; Graham and Fischbach, 2010; Vaira et al., 2009).

Conflict of interest

Authors declare that there are no conflicts of interest.

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Full Length Research Paper

Assessment of the oxidative state, related parameters and quality of muscle tissue in Nile tilapia with the application of homeopathic product Homeopatila 100® in high-density cages

Ana Paula Andretto[1], Juliana Alice Lösch[2], Geferson Almeida Gonçalves[1], Mariana Manfroi Fuzinatto[1], Denise Pastore de Lima[1], Graciela L. Braccini[2], Luiz Alexandre Filho[2], Cristiane Canan[3], Rosane Marina Peralta[1] and Lauro Vargas[1]*

[1]Food Science, Universidade Estadual de Maringá, Maringá, PR, Brazil.
[2]Department of Animal Science, Universidade Estadual de Maringá, Maringá, PR, Brazil.
[3]Department of Food Technology ,Universidade Tecnológica Federal do Paraná, Medianeira, PR, Brazil.

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The impact using Homeopatila 100® on cultured Nile tilapia in high-density cages (1240 individuals) was assessed by measuring the oxidative state and related parameters as well as the quality of muscle tissue. Males with sexual inversion from a homogenous tilapia population were randomly distributed in 10 cages with storage volume of units of 1.2 m^{-3} for a period of 91 days. Two diets were assessed: 1 (control) to 40 ml of hydroalcoholic solution (alcohol 30° GL)/kg of feed; 2 to 40 ml of the homeopathic product per kg of feed. The experiment involved the monitoring of physical and chemical parameters of water. At the final stage of the experimental period, hepatoprotective capacity of the homeopathic product Homeopatila 100® was assessed. This was done by the analyses of carbonyl protein, GSH and antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx)] in livers of Nile tilapia as well as the serum concentrations of biomarkers of hepatic damage (aspartato transaminase (AST), alanina transaminase (ALT) and FAL). In order to assess the quality of the fish' muscle tissue, the values of pH, color, texture and water retention capacity was measured. No statistical difference was observed regarding the physical and chemical parameters of water nor the analyses of the oxidative state and related parameters between treatments. Water retention capacity and texture were significantly higher ($p<0.05$) for the control treatment, while luminosity was higher for treatment with Homeopatila 100® ($p<0.05$). Nile tilapia cultivated in high density that received the homeopathic product Homeopatila 100® incorporated into their feed presented better-quality muscle tissues when compared with the group control. Homeopatila 100® did not indicate modifying effect on the oxidative state of Nile tilapia tissue cultivated in high density.

Key words: Tilapia cultivation, oxidative state, enzymes, homeopathy, stress, muscle tissue.

INTRODUCTION

Increase in fish consumption over the past few years has provided Brazil with an enormous potential for the development of fisheries and aquaculture enabling the country to become a future major fish producer worldwide. According to a Ministry of Fisheries and quaculture (MPA) Survey (2013), the country produces

approximately 2.5 million tons of fish with a population consumption of 17.3 kg per capita/year, near the world average released by the World Health Organization (WHO, 2012).

Nile tilapia is one of the most important fish species in cultivation in the country with production increasing on average 17% a year (MPA, 2013). The characteristics of tilapia are extremely favorable for cultivation (fast development, tolerance to a great variety of environmental conditions, capacity to reproduce in captivity, resistance to stress and diseases) (El-Sayed, 2006). Tilapia have meat of good acceptance in the market, good organoleptic characteristics and great industrial and culinary versatility (Furuya, 2010).

Simultaneously, with the increasing fish production, rearing animals has evolved from an artisanal to an industrial system in the world (Food and Agriculture Organization of the United Nations [FAO], 2014). The search for more profitable has led to intensive production, with the animals submitted to more stress. This has reduced the fishs' defense capacity with negative reflection on productivity and increasing occurrences of disease (Real, 2008).

To improve the production and quality of fish, homeopathy has been seen as an alternative management approach. The application of homeopathy to the herds consists in the Population Homeopathy, developed to attenuate the stressful model of animal production and assure minimum welfare to animals (Real, 2008). Several studies have demonstrated excellent results with the use of homeopathy in Nile tilapia (Andretto et al., 2014; Braccini et al., 2013; Merlini et al., 2014; Piau et al., 2012; Siena et al., 2010; Valentim-Zabott, 2008).

Fish are known for their high nutritional value (Godoy et al., 2010) and a quality muscle tissue (fillet) that can be measured through parameters such as pH, color, water retention capacity and softness, all features that can be influenced by animal stress (Koblitz, 2008).

The objective of this study was to assess the effectiveness of Homeopatila 100® on improved quality of muscle tissue of Nile tilapia in high-density cages by testing the oxidative state and related parameters.

MATERIALS AND METHODS

The experiment was approved by the Animal Experimentation Ethics Committee of the State University of Maringá with approval under Protocol 092/2013 (Annex 1).

Location and period

The experiment was conducted in Corvo River, Diamante do Norte, Paraná State (Figure 1). The 91-day experiment started in March 2014.

Physical and chemical parameters of water

The mean values of the physical and chemical parameters of water such as temperature, pH and dissolved oxygen were recorded once a week and assessed twice a day for 9 am and 4 pm at seven different points near the cages (Figure 2). Temperature and oxygen were monitored with oximeter model YSI-55/12 FT (Aquatic Eco-Systems®).

Fish, installations and feeding

Males with sexual inversion belonging to a homogenous population of Nile tilapia with mean initial weight of 50.24±8.51 g in the control treatment and 50.05±8.56 g in treatment with Homeopatila 100® were randomly distributed in 10 cages with a volume of 1.2 m^3 (1.0 × 1.0 × 1.2 m tall).

124 fish per cage were distributed. Before the beginning of the experiment, the fish were acclimated for seven days in cages, to adapt to the quality of water, density, food and management. Two treatments were assessed with five repetitions each through a fully randomized experimental design numbering 620 fish per treatment. The animals were manually fed with extruded commercial feed (5 mm) containing 32% crude protein three times a day (8:00 am, 1:00 pm and 5:00 pm) according to the recommended quantities for the species based on fish weight. The bromatological analysis of the feed was carried out at the Laboratory of Food Analysis of the State University of Western Paraná.

Treatments

Based on the results of Siena et al. (2010) who had shown the effect of Homeopatila 100® on Nile tilapia male fries with sexual inversion with better results in fish that received 40 $mlkg^{-1}$ of feed, established two treatments: control (40 ml hydroalcoholic solution per kg of feed) and *Homepatila 100* ®(40 ml per kg of feed).

Product Homeopatila 100® in the form of hydroalcoholic solution was incorporated into the feed directly using mechanically-moved sprinkler irrigation on a weekly basis, homogenized and air-dried for 24 h protected from direct sunlight. The same process was conducted for the control treatment. The complete feed was acclimated in a ventilated area without sunlight, chemical products or equipment emitting magnetic field until loose and without alcohol odor. Homeopatila 100® was developed by REAL H, a company in Campo Grande (MS); its official composition is as shown in Table 1. The product is registered in the Ministry of Agriculture, Livestock and Food Supply.

Assessment of oxidative state and related parameters

The assessment of oxidative state and related parameters was carried out at the laboratory of Hepatic Metabolism of the State University of Maringá. At the final stage of the experiment, 3 fish from each cage (15 fish per treatment/3 per cage) were anaesthetized with Benzocaine(dosage of 1 g/10 L of water) according to Stoskopf (1993). Firstly, blood sampling from Nile

*Corresponding author. E-mail: lvargas@uem.br. Tel: + 55 44 3011-8919.

Figure 1. Cages in Corvo River, Diamante do Norte, Paraná.

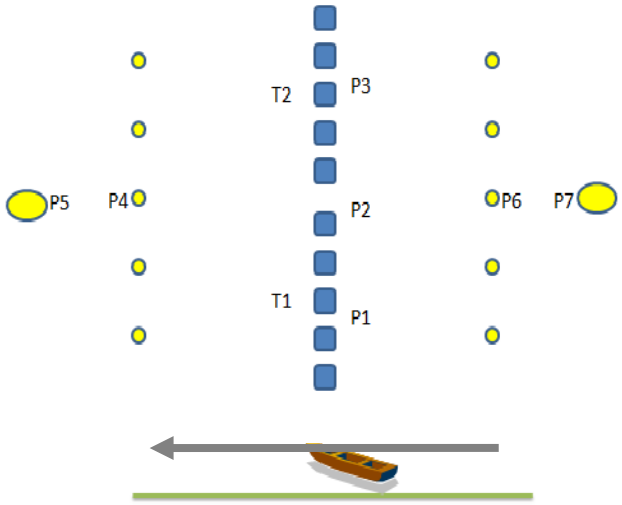

Figure 2. Monitoring points of physical, chemical parameters of water. The direction of the water flow is indicated with the arrow (right to left).

tilapia was conducted to perform enzymatic testing. On immobilization, total blood was collected through tail vase puncture, without the presence of anticoagulant to obtain serum. The serum was obtained after 3000 rpm centrifugation for 15 min. The serum was used to assess the enzymatic activities glutamate-oxaloacetate transaminase (AST), glutamate-pyruvate transaminase (ALT) and alkaline phosphatase (FAL). This was conducted with commercial kits (Gold Analisa diagnostica LTDA).

The livers were then collected after euthanasia conducted through severing the spinal cord. The abdominal cavity was surgically exposed; livers of Nile tilapia were removed, clamped in liquid nitrogen and stored at temperatures <-80°C. In order to prepare the homogenate, the clamped liver was weighted (1 g) and homogenized in Van Potter Elvehjem homogenizer with seven volumes of potassium phosphate buffer 0.1 M (pH = 7.4). The liver homogenate was used to analyze dosage of proteins and reduced glutathione (GSH). To establish the enzymatic activities of catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), dosage of protein and carbonyl protein, the homogenate was centrifuged at 10000 rpm for 15 min

Table 1. Composition of Homeopatila 100®.

Compound	Dilution
Iodum	12 CH
Sulphur	30 CH
Natrum muriaticum	200 CH
Streptococcinum	30 CH
Vehicle (Ethyl alcohol 30° GL)	Q.s.p.

Q.s.p.: Quantity sufficient for. Source: REALH – Campo Grande – MS – Brazil

using supernatant.

Protein dosage

The methodology of Lowry et al. (1951) was used on the total homogenate and centrifugation supernatant.

Establishing the level of reduced glutathione (GSH)

The level of reduced GSH was established through spectro-fluorimetry according to the description by Hissin and Hilf (1976) with alterations (Pardo-Andreu et al., 2007). The concentration of reduced glutathione was expressed as µg of GSH per mg of protein.

Carbonyl protein

The content of carbonyl proteins (CO) was established using the method of 2.4 dinitrophenylhydrazine (DNPH) as described by Levine et al. (1990). The content of carbonyl groupings was calculated based on the molar extinction coefficient of 22 mM^{-1}/cm^{-1}, and the results expressed in nmoles of carbonyl groupings per mg of protein.

Establishing catalase activity (CAT)

The activity of the catalase enzyme was assessed through enzymatic decomposition of H_2O_2 directly measured through spectrophotometry in 240 nm, according to Aebi (1974). The material used to establish the catalase was the supernatant obtained through the centrifugation of the liver homogenate. The activity of the enzyme was calculated using the molar extinction coefficient obtained from a calibration curve with H_2O_2, and the values expressed as µmols of H_2O_2 per min/mg of protein in the supernatant.

Establishing superoxide dismutase (SOD) activity

The activity of enzyme superoxide dismutase (SOD) was established through its capacity to inhibit the self-oxidation of pyrogallol reagent in alkaline medium, which can be monitored through spectophotometry in 420 nm (Marklund and Marklund, 1974). The activity in the homogenate supernatant was expressed as U of SOD per mg of protein in the supernatant.

Establishing GR activity

The activity of the GR enzyme was established through the decrease in absorbance due to the consumption of NADPH in 340 nm (Bergmeyer et al., 1974). The activity in the supernatant of the liver homogenate was expressed as µmol of NADPH per min/mg of supernatant protein.

Establishing glutathione peroxidase (GPx) activity

The activity of the GPx enzyme was established through the decrease in absorbance due to the decomposition of NADPH dependent on H_2O_2 in 340 nm at 25°C (Paglia and Valentine, 1967; Tappel, 1978). The activity in the supernatant of the homogenate was expressed in nmol of the NADPH/min/mg of supernatant protein.

Assessment of muscle tissue in Nile tilapia

To assess the quality of muscle tissue in the fish of both treatments, the analyses of pH, color, texture and water retention capacity were carried out at the Laboratory of Meats of the Federal Technological University of Paraná, Medianeira Campus, PR, Brazil.

All equipment and implements were sanitized with sodium hypochlorite solution (150 ppm) for 15 min prior to usage. The fish (15 fish per treatment/3 per cage) were gutted, had fins and skins removed and were filleted. The fillets were washed in chlorinated water (5 ppm) and immediately placed in polyethylene bags and stored (˜18°C) until the analyses.

The measurement of pH was conducted on the fillet at room temperature using potentiometer (pH 21, Hanna®, Romania). Color was measured using colorimeter (Model CR 400, Minolta®, Japan) with illuminant D65 and viewing angle of 10° in three different surface points of the fillet, corresponding to the central and lateral part of the samples (MacDougall, 1994; Perlo et al., 2006). The values of L^* (Luminosity), a^* (red-green component) and b^* (yellow-blue component) were expressed according to CIELAB color system (Minolta, 1998).

To assess texture (softness) through shearing force, the fillet was cut into 5 pieces of 1.5 cm tall × 1.0 cm wide x 2 cm long. The analyses were conducted with texturometer (TA.HD plus, Stable Micro Systems, UK) equipped with Warner-Brazler blade with a load cell of 5 kg. The blade was operated at a speed of 5.0 mms^{-1} for a distance of 20 mm (± 0.001 mm). The results of the minimum force required to perform the section were expressed in Newton (N).

The water retention capacity was measured as per Hamm (1960). The determination was based on the measure of the released water lost when applied pressure on the muscle tissue. Meat cubes (2 g) were inserted in filter papers and placed between two glass plates with a weight of 10 kg applied for 5 min. After the pressure, the fillet sample was weighted, and the difference in weight reflecting the quantity of lost water. The result was expressed in percentage of exudate water in relation to the initial weight of the sample.

Statistical analysis

To verify the existence of differences in the values of water parameters, Kruskal-Wallis H test ($p \leq 0.05$) (Ayres et al., 2000) was applied. Oxidative state and parameters, as well as the quality of muscle tissue were analyzed by Student's t-test, using GraphPad Prism® software.

RESULTS

Physical and chemical parameters of water

There was no significant difference in water temperature,

Table 2. Mean values of water parameters during the experiment.

Observed parameter	Temperature (°C)	pH	Dissolved oxygen (mg/L)
P 1	25.35±3.32[a]	8.82±0.23[a]	9.98±2.57[a]
P 2	25.80±4.10[a]	8.24±0.09[a]	9.28±2.22[a]
P 3	25.85±4.17[a]	8.15±0.12[a]	8.95±2.26[a]
P 4	25.90±4.10[a]	8.02±0.16[a]	8.87±1.89[a]
P 5	25.85±4.17[a]	8.06±0.01[a]	8.60±2.22[a]
P 6	25.90±4.24[a]	8.44±0.66[a]	9.36±3.03[a]
P 7	25.90±4.24[a]	8.59±1.29[a]	9.94±4.19[a]

Values with different letters on a single column proved significant difference through Kruskal-Wallis. Averages followed by ± standard deviation.

Table 3. Percent composition of the commercial feed 5 mm used for the experiment.

Nutrient	Commercial guarantee (%)[1]	Tested value (%)[2]
Crude protein (min)	32	32.87
Ethereal extract (min)	6	3.78
Crude fiber (max)	6.5	4.40
Ashes (max)	12	9.18

[1]Degrees of guarantee (%) according to the manufacturer. [2]Source: Laboratory of Food Analysis of the State University of Western Paraná.

Table 4. Mean values for the enzymatic activity of AST, ALT and FAL in the tilapia blood samples of the control treatment and product Homeopatila 100®.

Enzymes (U/L)	AST	ALT	FAL
Control	115.12 ± 75.03[a]	18.51 ± 7.46[a]	6.82 ± 3.44[a]
Homeopatila 100®	104.41 ± 56.03[a]	20.25 ± 8.97[a]	7.55 ± 3.21[a]

Each result is the average of 15 analyses (15 fish per treatment/3 per cage) with the respective estimates of standard deviation. Values with different letters on a single column proved significant difference through Student's t test ($p<0.05$).

pH and dissolved oxygen (Table 2) parameters between sampling locations ($p>0.05$).

Percent composition of the commercial feed used for the experiment

Protein is the major visceral and structural component in the animal organism; therefore, its maintenance and production are very important (Furuya, 2010). Costa et al. (2009) assessed differences in the crude protein (CP) during the several growth phases of tilapia (*Chitralada*) cultivated in cages, with the most efficient diet for Nile tilapia being extruded feed with 32% crude protein. The feed used for the experiment meets the percentage considered ideal for the species (Table 3).

Activity enzymatic: AST, ALT and FAL

The mean values for the enzymatic activity of AST, ALT and FAL of tilapia blood samples in the control treatment and the Homeopatila 100® presented no significant difference ($p>0.05$) according to Table 4.

Levels of reduced glutathione (GSH)

Figure 3 illustrates the levels of reduced glutathione in the liver homogenate of tilapia in both the control and the homeopathic treatment. No difference ($p>0.05$) was indicated between the studied treatments for this parameter.

Levels of carbonyl protein

No significant difference were observed ($p>0.05$) in the levels of protein oxidation in fish treated with Homeopatila 100® compared with the control group (Figure 3).

Antioxidant enzymes

The catalase, superoxide dismutase, glutathione peroxi-

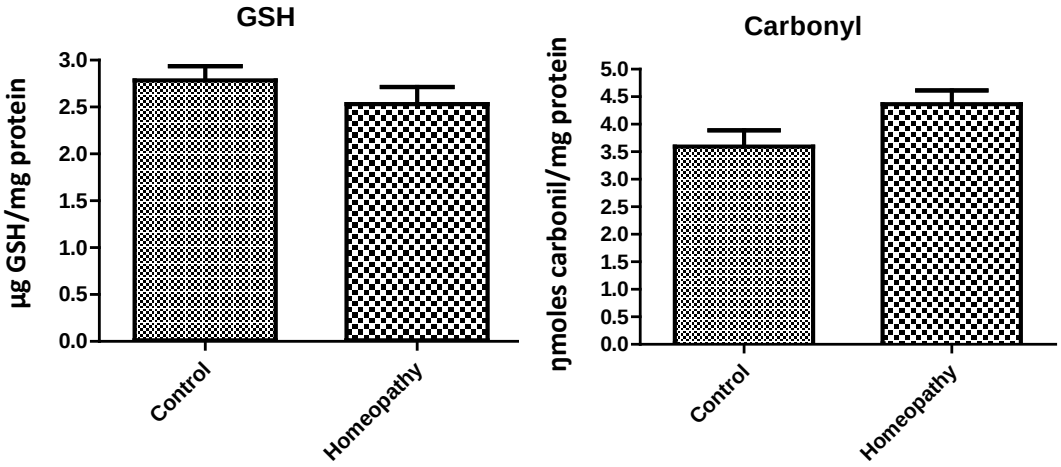

Figure 3. Levels of reduced Glutathione (GSH) and Carbonyl protein in tilapia livers.

Table 5. Enzymatic activity of catalase enzymes, superoxide dismutase, glutathione peroxidase and glutathione reductase in tilapia livers for the control group and homeopathic product.

Enzymes	Control	Homeopatila 100®
Catalase (µmoles/min.mg protein)	49.81±13.72[a]	52.02±16.55[a]
Superoxide dismutase (USOD/mg protein)	1.00±0.36[a]	0.84±0.46[a]
Glutathione peroxidase (nmoles/min.mg protein)	50.58±18.35[a]	47.19±7.51[a]
Glutathione reductase (nmoles/min.mg protein)	391.00±85.16[a]	418.07±86.38[a]

Each result is the average of 15 analyses (15 fish per treatment/ 3 per cage) with the respective estimates of standard deviation. Values with different letter on a single line proved significant difference through Student's t test ($p<0.05$).

peroxidase and glutathione reductase enzymes revealed no alterations in activity between the control and Homeopatila 100® as shown in Table 5.

Assessment of muscle tissue

The mean values of the analyses of pH, color, texture and water retention capacity of the samples of both the control and Homeopatila 100® treatments are described as shown in Table 6. For pH, parameters of color a* and b* presented no differences between the studied treatments ($p>0.05$). The water retention capacity and texture were significantly higher ($p<0.05$) for the control, while luminosity was higher for the treatment with Homeopatila 100® ($p<0.05$).

DISCUSSION

The purpose of this research was to assess whether using Homeopatila 100® for Nile tilapia in high density cages made improvements to the oxidative state and related parameters as well as the quality of the muscle tissue. This was in the light of results by Braccini et al. (2013) and Siena et al. (2010) who found a lower hepatosomatic index and histological analysis with higher amount of hepatocytes and the percentage for the indication of intracellular glycogen, presenting a more preserved liver morphologically for fish fed with using Homeopatila 100®.

Parameters related to water temperature, pH and dissolved oxygen (Table 2) are within normality according to Ribeiro (2001) for the cultivation of tropical fish such as Nile tilapia and presented similarity with the parameters found by Braccini et al. (2008) and Marengoni (2006).

As blood is a pathophysiological reflector of health of the entire body; consequently, it is an important factor regarding the diagnosis of the structural, functional conditions of fish. High levels of activity for the AST, ALT and FAL enzymes are excellent blood parameters for diagnosis of liver diseases (Motta, 2009). Firat et al. (2011) compared the effects of the exposing Nile tilapia to pesticides and metals by using enzymatic activities

Table 6. Assessment of muscle tissue quality in Nile tilapia.

Parameter	Treatments	
	Control	Homeopatila 100®
Water retention[1]	68.58±4.58[a]	66.38±4.51[b]
pH[2]	6.091±0.19[a]	6.09±0.14[a]
Color[3]		
Luminosity	45.96±1.113[a]	51.89±0.59[b]
a* (red/green)	0.98±0.33[a]	1.01±0.34[a]
b* (yellow/blue)	1.35±0.50[a]	0.955±0.33[a]
Texture/shearing force (N)[4]	5.53±2.68[a]	4.62±1.80[b]

Values with different letter on a single line proved significant difference through Student's t test ($p<0.05$). Averages followed by ± standard deviation. [1]Each result is the average of 45 analyses (15 fish per treatment/ 3 per cage/in triplicate) with the respective estimates of standard deviation. [2] Each result is the average of 45 analyses (15 fish per treatment/ 3 per cage/ in triplicate) with the respective estimates of standard deviation. [3] Each result is the average of 45 analyses (15 fish per treatment/ 3 per cage/ in three different points of the fillet) with the respective estimates of standard deviation. [4] Each result is the average of 75 analyses (15 fish per treatment/ 3 per cage/ in five different points of the fillet) with the respective estimates of standard deviation.

(ALT, AST, and FAL) and found close values for these enzymes in their control group, corroborating the absence of alterations in these parameters for both treatments in the experiment (Table 4).

Homeopatila 100® indicated no influence for the parameters tested to assess oxidative state (Figure 3; Table 5) in Nile tilapia cultivated in high density (124 fish m^3). Sevgiler et al. (2004) found similar values for catalase (antioxidant enzyme) and superoxide dismutase, and higher values for glutathione peroxidase in the liver of tilapia of the control treatment by testing the effect of a pesticide in different concentrations. Likewise Braun et al. (2013) assessed the effect of combined manipulation with high stocking density in *Salminus brasiliensis* and verified that the enzymes related to oxidative stress suffered negative alteration through manipulation. Braun et al. (2010) also found that stocking density had a slight influence, indicating that stress is an important modulator of the antioxidant response. According to Cortez and Silva (2007), the imbalance of physiological processes generated by stress can be self-sustaining; a consequence of the process of cellular oxidative stress derived from the formation of free radicals (substances with high oxidant capacity). This is due to an irregular balance between the formation of these radicals and the capacity of response of the enzymatic arsenal of antioxidant defense of living organisms.

Several pre- and post-mortem factors can influence meat final quality such as pre-slaughter impacts such as the stress associated animal handling techniques (Stien et al., 2005; Koblitz, 2008). Color of flesh is one of the factors that can be influenced by animal stress. Myoglobin is a protein present in muscle tissue that forms the metamyoglobin when submitted to oxidation causing the brown color in meats. However, the darker the fish muscle, the less desirable it is to consumers (Thiansilakul et al., 2011). Fillets of the homeopathic treatment presented better luminosity in relation to the control treatment and therefore may be more saleable to the market.

The Ministry of Agriculture, Livestock and Food Supply (2001) through the regulation for sanitary and industry inspection of products of animal origin, established that the pH of fish must be under 6.5 inside the meat. The values found in our experiments for both treatments are in accordance with the recommendations. Soares and Gonçalves (2012) found similar values for pH (5.9 to 7.11) in Nile tilapia skinless fillets (*Oreochromis niloticus*).

Despite having presented higher water retention capacity in the fish muscle tissue, the control group presented lower shearing force for treatment with Homeopatila 100®. A higher value of shearing force corresponds to greater force required to break the sample; however, the tilapia fillets treated with homeopathy presented softer texture.

Conclusion

Nile tilapia cultivated in high density receiving the homeopathic product Homeopatila 100® incorporated into their feed presented better-quality muscle tissues, especially concerning color (L^* luminosity component) and texture compared with the control group-important factors for consumers' purchasing decisions. Homeopatila 100® did not indicate modifying effect on the oxidative state of Nile tilapia cultivated in high density.

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Conflicts of interest

The authors state that there are no conflicts of interests.

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Full Length Research Paper

Pharmaceutical care in Brazilian community pharmacies: Knowledge and practice

Tiago Marques dos Reis[1*], Camilo Molino Guidoni[2], Edmarlon Girotto[2], Ricardo Radigheri Rascado[3], Patrícia de Carvalho Mastroianni[4], Joice Mara Cruciol[2] and Leonardo Régis Leira Pereira[1]

[1]School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo. Ribeirão Preto, state of São Paulo, Brazil.
[2]State University of Londrina, Londrina, state of Paraná, Brazil.
[3]Federal University of Alfenas, Alfenas, State of Minas Gerais, Brazil.
[4]São Paulo State University Júlio de Mesquita Filho. Araraquara, state of São Paulo, Brazil.

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This study aimed to assess the pharmacist's knowledge of the concept of pharmaceutical care and verify their practice in relation to this service. It involved a cross-sectional study carried out on community pharmacies in four Brazilian cities and involved pharmacists who work in community pharmacies. The pharmacists' knowledge of the concept of pharmaceutical care and their practice in respect of the service was measured. 486 pharmacies were visited and 112 pharmacists participated in the study. Of these professionals, 41% correctly identified the concept of pharmaceutical care and 70.5% said they perform it in pharmacies. The majority (n = 62) of the professionals followed standard operating procedures and four recorded data on the service offered (patient information, interventions and results). There is evidence that only 2.5% of the professionals effectively carry out the level of pharmaceutical care recommended by the Brazilian guidelines. Pharmacists are not yet ready to perform pharmaceutical care in community pharmacies. It is necessary to review the pharmacists' training and enable the professionals to properly perform this service.

Key words: Pharmaceutical care, pharmacists, knowledge, attitudes, community pharmacy.

INTRODUCTION

Pharmaceutical care (PC) was introduced in Brazil around 1995 (Lyra et al., 2000). However, this process did not consider the characteristics of the Brazilian health system, which still does not provide access to and quality care for the entire population in primary health care. Popular culture centers models of healing that focus on hospitalization instead of preventing the emergence of diseases through health prevention strategies; the models are also an obstacle to the spread of PC. Moreover, the limitation of investment from public and private sectors restricts the promotion of rational use of drugs through PC (Chaud et al., 2004; Garcia-Subirats et

*Corresponding author. E-mail: tiagomarques_farmacia@yahoo.com.br.

al., 2014).

Additionally, the deployment of PC ran into another important problem faced in the country: the lack of pharmacists in community pharmacies. This was evidenced by several authors (Farina and Romano-Lieber, 2009; França-Filho et al., 2008; Lucchetta and Mastroiani, 2010; Tomassi and Ribeiro, 2012) and represents a failure of Brazilian legislation that determines the presence of this professional during the entire period of operation of these establishments. The need for technical guidelines and training of pharmacists to perform the service was also not considered (Pereira and Freitas, 2008). Thus, the Brazilian Pharmaceutical Care Consensus (BPCC) to guide the performing of PC was developed (Ivama et al., 2002). This document defines PC as a professional practice model that seeks to achieve therapeutic outcomes defined in the health and quality of life through systematic pharmacotherapeutic monitoring of the patient. Furthermore, it establishes that this monitoring should include the registration of information relating to the patient, guidelines, pharmacotherapeutic interventions and obtained results, ensuring the measurement of the impact of comorbidities on prevention, promotion and restoration of life quality. It also proposes that macro constituents such as health education, counseling and pharmaceutical attendance are explored. Therefore it becomes essential that PC is performed in a private environment ensuring patient privacy (Ivama et al., 2002; Federal Council of Pharmacy, 2013a). In addition, a federal resolution determined that pharmacies should have protocols developed by the pharmacist to standardize the operational procedures involved in performing the service (National Health Surveillance Agency, 2009). Thus, the importance of the pharmacist to present appropriate professional conduct in relation to PC services is reinforced, implying the need for training which will promote the rational use of drugs (Pereira and Freitas, 2008).

In this context, curriculum changes were introduced in 2002 in pharmacy colleges in Brazil in an attempt to provide clinical training for future pharmacists, and enable them to perform a comprehensive and humanistic service, instead of working focused only on the management and production of drugs (Brazil, 2002). Thus, this would ensure the proper fulfillment of PC. However, little is known about the impact of these changes because there are only two studies in Brazil (Baldon et al., 2006; França-Filho, 2008) in which this type of analysis was performed and they were developed when the clinical training was being implemented.

Professional knowledge may come from the subjects learned in undergraduate and in continuing education strategies, which consist of improving professional skills through interactive workshops, courses and other activities performed after graduation (Dewulf et al., 2009; Watson et al., 2002). To measure pharmacists' knowledge and their actions in relation to PC, their performance of this service should be analyzed and this assessment should be performed in community pharmacies because there the patient has easy access to these professional (Anderson and Thornley, 2014; Pereira and Freitas, 2008).

In Brazil there are more than 70,000 community pharmacies, which extrapolate to nearly four times the ratio recommended by the World Health Organization, highlighting their importance in providing health care, and the need to assess the pharmacist who works in these health establishments (Federal Council of Pharmacy, 2013b; Zubioli, 1992). More than half of these pharmacies are private companies and they represent 85.6% of job positions occupied by pharmacists in the country (Federal Council of Pharmacy, 2013b). To work in these companies, it is necessary to be graduated in Pharmacy and this course usually lasts five years. The remuneration offered to pharmacists to work in private community pharmacies is different in each state of Brazil, ranging from two to five times the minimum wage (one minimum wage being approximately equivalent to US $285 in February, 2015).

Aim of the study

The aim was to assess the knowledge of the pharmacist on the concept of PC and verify their performance in relation to this practice in community pharmacies.

Article relevance

1. In order to perform pharmaceutical care (PC) properly in community pharmacies, the pharmacist must have knowledge of the aspects that involve the concept of this clinical practice and provide appropriate actions in relation to the therapeutic monitoring offered, following the guidelines of the service. The results of this study show that community pharmacists in socioeconomically important regions of the Brazilian territory are not prepared to perform PC.
2. Changes to the educational formation of the pharmacist and training of these professionals are required to qualify them in clinical practice which is so important to the success of treatment with medicine.
3. This study fills an important gap about the supply of PC in Brazilian community pharmacies. Besides being limited, the information available about PC held in Brazil was collected in public pharmacies and little was known about PC in private establishments.
4. Furthermore, this work is a pioneer in assessing community pharmacists from different regions of Brazil in relation to the concepts and issues involving PC.

METHODOLOGY

This is a cross-sectional study conducted with pharmacists who work in community pharmacies in the following Brazilian cities:

Ribeirão Preto and Araraquara (in São Paulo state), Londrina (Paraná state) and Alfenas (Minas Gerais state). The criteria for selection of these cities were: a population of up to one million inhabitants, be located at a maximum of 500 km away from the place of this study (Ribeirão Preto), and have at least two pharmacy colleges (one public and one private). No pharmacies sourced from public institutions or compounding pharmacies were included. All pharmacies in the four cities were visited during the data collection, which was conducted during business hours by a single researcher, to avoid bias. The addresses of the establishments were obtained from the Pharmaceutical Association of Alfenas and Regional Boards of Pharmacy of São Paulo and Paraná.

For data collection the researchers developed a semi-structured self-administred questionnaire, considering the knowledge and practice recommended for BPCC (Ivama et al., 2002). This instrument was composed of 47 questions and divided in three sections (sociodemographic information, knowledge and practices). The questionnaire was validated following models available in the literature (Armando et al., 2012; Njilele et al., 2012; Traverso et al., 2007) and validation was performed by systematic analysis of the questionnaire by two sets of judges with expertise in PC and community pharmacies. The first set consisted of ten researchers with expertise in these two areas and the second set consisted of five professors who teach the subject of PC and drug dispensation. In each of these sets, the questionnaire was modified according to guidelines issued by the judges until there was consensus among researchers and approved by the judges. Therefore the questionnaire was submitted to a pilot test with five pharmacists who work in community pharmacies, seeking to ensure reliability, clarity and effectiveness in the questionnaire. The data collected were not considered in the final analyses.

The variables analyzed were: age, gender, type of establishment where the pharmacist works (pharmacy chain or independent pharmacy), administrative role (owner, manager or employee), weekly working hours (up to 44 h or more than 44 h), remuneration (less than, equal to, or above the minimum wage, which is the minimum value established for the remuneration of the professional in each state of the federation), type of institution where they graduated (public or private), participation in continuing education activities; knowledge of the concept of PC, available resources for the realization of PC (forms, sources of drug information) and presented actions (standardization of PC operating procedures, record of interventions, privacy in attendance). Regarding education, pharmacists should respond if they graduated before or after the implementation of the curriculum changes of 2002.

The knowledge of PC concept was assessed through a multiple-choice question in which the definitions of PC (proposed by BPCC), Pharmaceutical Services and Clinical Pharmacy were available. Knowledge of the PC concept was classified as satisfactory by those who answered this question correctly. In addition, pharmacists' knowledge of the dispensing of drugs, which is considered by the BPCC as the starting point for the realization of PC, was also evaluated. Knowledge of dispensation was classified according to the number of affirmative responses in the six questions on the subject: unsatisfactory (0 to 2 affirmative responses), regular (3 to 4 affirmative responses) and satisfactory (5 to 6 affirmative responses).

Only one pharmacist in each pharmacy was invited to participate in order to avoid bias. Establishments where the pharmacist was not present received a second visit by the researcher not less than two hours following the initial visit, an acceptable time limit by the representing pharmacists' organizations, to pharmacies that function without the supervision of a professional. If the pharmacist remained absent on the second visit, the pharmacy was not included in the study. The same procedure was adopted in which pharmacies were closed down (no longer in business).

In turn, of the pharmacies included in the study, the pharmacist and the owner (or manager) of the pharmacy were informed of the objectives of the study and signed a consent form agreeing to the study. Both were required to sign the document so that the pharmacist could answer the questionnaire. The completion of the questionnaire by the participants was performed in the presence of the researcher and without permission to query any research sources. On completion, participants received an envelope with no identification to deposit the questionnaire, which was sealed before returning to the researcher. The completion of questionnaire lasted 30 min on average.

The collected data were independently double entered by different researchers using a database created by the Epi Info™ (version 3.5.4) program. Subsequently, both entries were compared and discrepancies found were corrected by the principal researchers. Data analysis was performed by the same statistical software, calculating measurements-summary as absolute frequencies, means and standard deviation. The association between "perform PC" and "knowledge of PC" was verified through logistic regression with calculations of odds ratio (OR) and confidence interval (CI) of 95%. Statistical significance was established at $p < 0.05$.

The study followed the recommendations of the STROBE statement - Strengthening the Reporting of Observational Studies in Epidemiology (Malta et al., 2010) and was approved by the Ethics Committee of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo.

RESULTS

A total of 486 pharmacies were visited for data collection, 54 of which were not included in the survey because they were either from public institutions, or compounding pharmacies, or were out of business. Seventy-three pharmacies were excluded because the pharmacist was absent during visits. In addition, 49 owners/managers did not authorize the professional to be approached, and 198 pharmacists refused to answer the questionnaire. Thus, 112 pharmacists comprised the study sample. The average age of participants was 33.4 years (SD = 8.6), 72.3% were female, 28.6% were pharmacy owners, 63.4% worked in independent pharmacies, 57.2% had a weekly workload exceeding 44 h, and 42.9% received lower pay than minimum wage determined by the associations of pharmacists in Brazil (Table 1).

More than 41% of participants were graduated according to curriculum changes from 2002. It was observed that 72.3% had graduated from private colleges. Regarding continuing education, it is shown in Table 2 that almost half of the participants attended conferences and short courses after graduation, although few pharmacists have attended activities that had as their thematic PC or related areas (such as clinical pharmacology, pharmacotherapy and drug interactions). The concept of PC was correctly identified by 41.0% of pharmacists. For every ten pharmacists who had participated in continuing education in clinical areas, seven knew about the definition of PC ($p<0.01$). However, only 40.0% of pharmacists who were trained in PC were able to identify the correct definition of the practice. The type of educational institution, year of graduation and reporting PC performing at pharmacies

Table 1. Occupational information of pharmacists who participated in the study (N=112).

Variables	Pharmacists				
	Technicians in charge N (%)	Substitutes N (%)	Assistants N (%)	Managers N (%)	Owners N (%)
Occupation in the company	62 (55.4)	14 (12.5)	4 (3.6)	13 (11.6)	32 (28.6)
Weekly workload exceeding 44 h	24 (38.7)	5 (35.7)	2 (50.0)	6 (46.2)	27 (84.4)
Lower pay to minimum wage	25 (40.3)	6 (42.9)	2 (50.0)	4 (30.8)	11 (34.4)

Table 2. Continuing education activities performed by pharmacists (N=112)[1]

Activities	Thematic areas			Total N (%)
	PC N (%)	Clinics N (%)	Others N (%)	
Improvement	1 (4.2)	3 (13.2)	19 (82.6)	23 (100.0)
Conferences and short courses	2 (3.2)	4 (6.5)	56 (90.3)	62 (100.0)
Specialization	2 (5.9)	12 (35.3)	20 (58.8)	34 (100.0)
Masters or Doctorate	-	-	2 (100.0)	2 (100.0)

[1]There were pharmacists who indicated more than one continuing education activity. PC - Pharmaceutical Care; Clinics - related to PC (clinical pharmacology, clinical pharmacy, pharmacotherapy, homeopathic therapeutics and drug interactions) areas; Other - not related to the PC (industry, teaching pharmacy and management) areas.

Table 3. Relationship between PC performing and PC knowledge (n=112)[1]

Performs PC	N (%)	Knowledge of PC		
		Satisfactory N (%)	OR (IC)	p-value
Yes	79 (73.8)	27 (34.2)	1.00	0.0069
No	28 (26.2)	18 (64.3)	3.47 (1.41-8.54)	

[1]Five pharmacists did not report if they perform PC. PC – Pharmaceutical Care; OR – odds ratio; IC – confidence interval**.**

did not show a significant relationship with knowledge of the concept of PC. Nevertheless, it is important to note that 70.5% of the evaluated professionals mentioned that they perform PC and 78.5% of them developed operating procedures to standardize the service. From Table 3 it can be observed that most pharmacists who report performing PC were those who do not have satisfactory knowledge about the concept of PC.

Considering other specific aspects, 70% of professionals with regular or satisfactory knowledge for dispensing of drugs affirmed performing PC. In turn, all pharmacists with unsatisfactory knowledge for dispensing of drugs also reported performing this service. Approximately 80% of pharmacists recognized that PC is a practice that should be documented, but only 12.5% of professionals confirmed that they record guidance, interventions, and outcomes relating to this service. According to these professionals, managers of pharmacies do not provide material resources for the recording of information, and the need to maintain patient anonymity also prevents the storage of data relating to therapeutic monitoring. One in five pharmacists responded that PC is performed at the pharmacy counter and employees (non-pharmaceutical) are responsible for running the service in 6.3% of establishments.

According to the results, there is evidence that two of the 79 pharmacists who reported performing PC run the service according to Brazilian recommendations (Figure 1). For professionals, the biggest obstacles to deployment of PC in pharmacies is the lack of space reserved for individualized patient care (53.6%) and the lack of material resources such as computers, internet access, and books for searching for information on medicines and health-related aspects (14.0%).

DISCUSSION

In this study, the number of participants was more

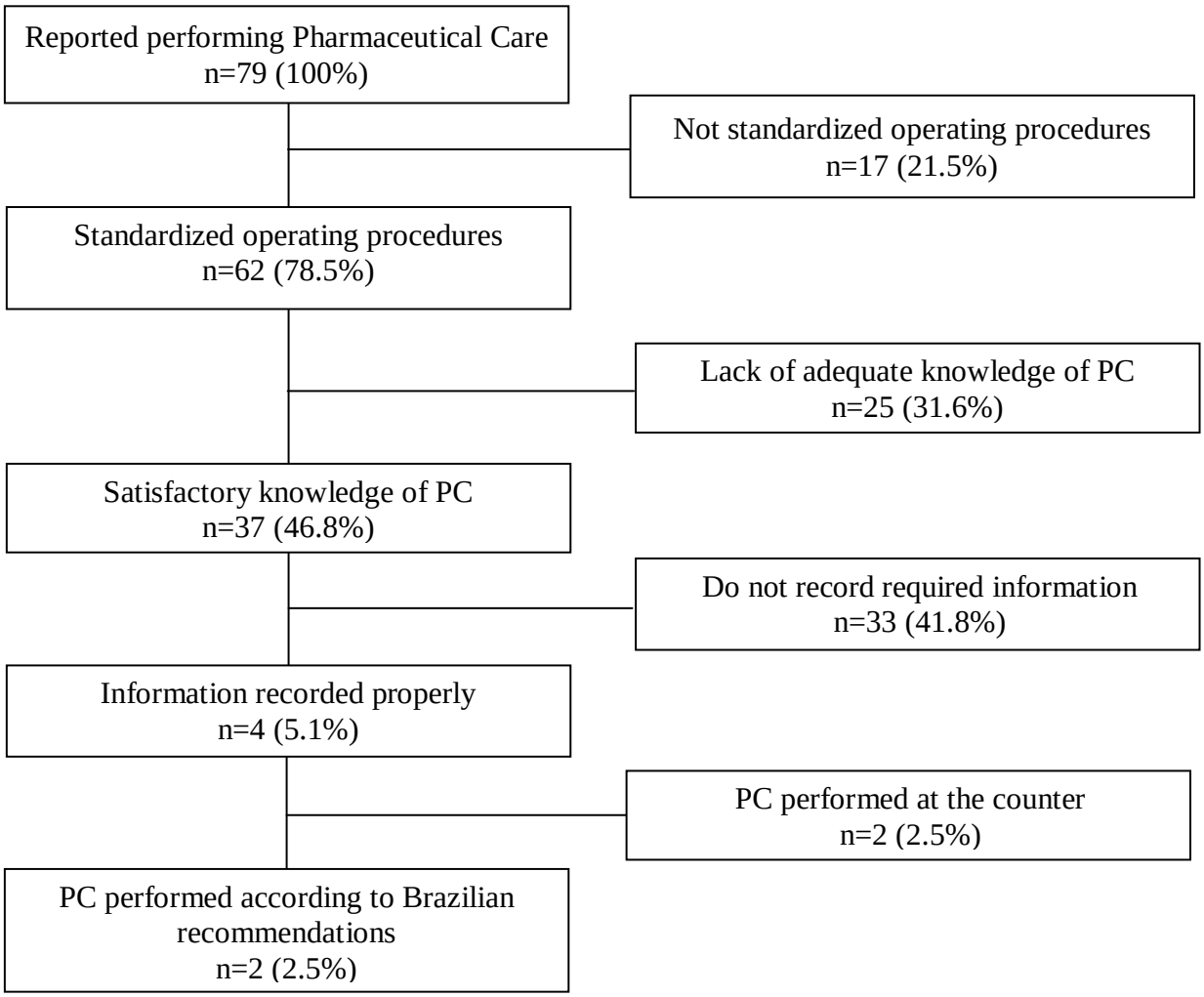

Figure 1. PC service performing flowchart by pharmacists in visited pharmacies.

influenced by the refusal of pharmacists to collaborate with research than by non-consent of the owners/managers to approach the professionals. In other research with community pharmacists, participation was also considered low by the authors (Mak et al., 2013; McIntosh et al., 2012; Tomassi and Ribeiro, 2012). This reduced participation reflects insecurity or lack of preparation of pharmacists for PC, showing deficiencies in the recent curriculum amendments from 2002. In addition, 15.0% of the pharmacies visited were functioning without the supervision of the pharmacist, revealing a failure for the mandatory presence of a responsible full time technician in these establishments (National Health Surveillance Agency, 2009). This same finding was reported by other authors (Baldon et al., 2006; Farina and Romano-Lieber, 2009; Lucchetta and Mastroianni, 2010; Tomassi and Ribeiro, 2012) and represents a barrier to the realization of PC (Al Rahbi et al., 2014).

The gender and average age of the participants were similar to the community pharmacist profiles described in previous studies (Baldon et al., 2006; França-Filho et al., 2008; Hughes and Lapane, 2011; Mak et al., 2013; McIntosh et al., 2013; Silva and Vieira, 2004; Tomassi and Ribeiro, 2012). Unlike more developed countries such as Australia, Canada and European countries where PC is in an advanced stage of implementation, it was observed in this study that less than a third of participants were owners of the pharmacies visited, reflecting how young the subjects of the study are, who might not have sufficient financial resources to possess their own pharmacy (Farina and Romano-Lieber, 2009; Farris et al., 2005; Jones et al., 2005). In this sense, the long weekly working hours (> 44 h) and the low pay, also undermine the provision of clinical services in pharmacies. Also, depletion of pharmacists and the lack of financial resources disfavor the quest for continuing education, jeopardizing the implementation and execution of PC due to lack of professional training.

In addition, it was noted that the income of pharmacists

who are the owners of the establishment is not congruent with their extensive workload. This discourages investment in services in which the benefits of such services are only obtained in the longer term, as is the case with PC (Borges et al., 2011). Again, the commercial interests in drug selling becomes the priority, forcing the pharmacists, who are employees in these establishments, to devote themselves more to selling products than the realization of services that contribute to the rational use of medication, as in PC (França-Filho et al., 2008; Silva and Vieira, 2004). It is also important to highlight that the Brazilian government does not offer incentives, fees or reimbursement for PC practice in pharmacies, contrary to what happens in countries such as Canada and the United States (Pereira and Freitas, 2008). Thus, pharmacy owners prefer to invest in commercial products than in clinical services such as PC.

Accordingly, it is noteworthy that more than half of the pharmacists did not know the definition of PC. This shows that professionals were not prepared to perform the service since they did not have a satisfactory knowledge of PC. In countries such as China and Jordan, where PC is more widespread than in Brazil, at least 60% of pharmacists correctly knew the definition of this clinical practice (Aburuz et al., 2012; Fang et al., 2011).

The lack of knowledge regarding PC could also be strengthened by the existence of pharmacies where employees (non-pharmaceutical) were responsible for the service. It is known that PC involves macro constituents that require specific knowledge and skills to identify, prevent and solve problems related to drugs (Ivama et al., 2002; Lucchetta and Mastroianni, 2010; Oliveira et al., 2013; Pereira and Freitas, 2008). In this context, trained employees are able to optimize the service performed by professionals (selecting patients who need pharmacotherapeutic follow-up by pharmacists, for example), but the lack of intrinsic pharmacist subsidies does not provide conditions for these workers to take responsibility for the service. Furthermore, the described situation reinforces the need for a rearrangement of functions between professionals and employees, to the extent that pharmacists are sometimes required to allocate part of their work time to non profession related activities (for example finalizing sales at the cashier desk or cleaning the establishment) (Blackburn et al., 2012; Davies et al., 2014; Farina and Romano-Lieber, 2009; Gregório and Velez Lapão, 2012; Van Mil and Fernandez-Llimos, 2013).

Corroborating the number of professionals who do not meet the PC concept, it was observed that pharmacists have conducted continuing education activities not related to PC (e.g. specialization courses in areas of drug industry and management of health, courses in acupuncture, hospital pharmacy workshops and handling of drugs) which obviously does not enable them for PCservice. However, it is noteworthy that less than half of the professionals who participated in activities in the area of PC adequately answered this question. This demonstrates that the graduate courses offered in the area are not yet enabling professionals to practice PC, which reinforces deficiencies in the curriculum introduced in 2002. As a consequence, the social recognition of the pharmacist is compromised, making the professional "invisible" to the health system (Dewulf et al., 2009).

In this scenario it is worrying, verifying that almost three quarters of the professionals reported performing PC (Table 3) among which, are those with–unsatisfactory knowledge for dispensing drugs. Inadequate knowledge undermines the identification of problems related to drugs and the orientation on pharmacotherapy, damaging social recognition of the profession and compromising the therapeutic relationship that should exist between pharmacists and patients for the success of PC (Agu et al., 2014; Pereira and Freitas, 2008). The opposite happens in Canada, where 71% of patients prefer pharmacist counseling to medical care when they are suffering from symptoms of lesser severity. In the example of Canada, complaints are often resolved by pharmaceutical intervention, and PC has propagated more easily (Mansell et al., 2014).

Besides not identifying the PC concept proposed by the BPCC, the conduct of one in five participants disagreed with the Brazilian recommendations because of the lack of standardization of procedures and the systematic recording of pharmacotherapeutic monitoring performed. Health surveillance of each municipality has the responsibility to oversee the standardization and compliance with procedures performed in pharmacies, and this result shows failures in the inspection of these establishments. A survey conducted in the state of São Paulo in 2009 has shown a similar situation (Farina and Romano-Lieber, 2009). According to professionals, the lack of support from pharmacy owners in providing the material resources needed to complete the service is a major obstacle to PC, showing once again that the management of some private pharmaceutical establishments focuses on profit and not the quality of life of patients (Tomassi and Ribeiro, 2012).

In addition, the realization of PC at the pharmacy counter, as reported by participants, was also identified as a barrier to the spread of PC in Argentina and Scotland (Watson et al., 2002; Uema et al., 2008). In the UK, 80% of patients on pharmacotherapy follow-up claim that PC in a private room is critical to the success of the service, providing appropriate conditions for the pharmacist to assess the health problems of the patient and plan pharmacotherapeutic interventions (Merks et al., 2014).

It was also observed that the greatest difficulties for the implementation of PC were due to lack of finance and appropriate sources to consult information related to health and medicines, as pointed out in other studies (Farina and Romano-Lieber, 2009; Farris et al., 2005). In Brazil, to subsidize the work of professionals, the

pharmacy owners usually provide free booklets distributed by pharmaceutical companies, which are designed to serve as drug advertisements. However, these tertiary sources usually have synthesized and outdated information, prevailing commercial appeal and marketing of pharmaceutical products (França-Filho et al., 2008). On the other hand, it is important to highlight that there are free access databases with recognized scientific evidence on the Internet that could easily be accessed in community pharmacies.

From the observed results, it is possible to state that the pharmacist is not yet prepared to take responsibility for the systematic pharmacotherapeutic monitoring of patients. It is noteworthy that only 2.5% of pharmacists who informed that they perform PC have the knowledge and follow the recommended actions. Based on the considerations, this study presents important data for action planning that allows training of professionals and expands the supply of PC in Brazil, either by restructuring the curriculum of the School of Pharmacy, by the intervention on the knowledge and professional conduct, or to stimulate investments required in human and material resources to perform the service.

As limitations of the study, it is recognized that the number of participants may have compromised the statistical significance of the results and the issues may have limited the scope of the stated objectives. However, it is believed that the research method avoided biases in the collection and analysis of data and the results can guide the future of PC in community pharmacies. It is important to highlight that the four municipalities involved in the study are representative of the regional and national scenario. Besides being the health reference in regions where they are located, they have on average 3.6 pharmacies per 10,000 inhabitants (similar to the Brazilian ratio), gross domestic product per capita equivalent to the national average and they are located in the south and southeast of the country (where 73.6% of the Brazilian economic activity is concentrated) (Brazilian Institute of Geography and Statistics, 2011). Moreover, all universities of Pharmacy have adopted the curriculum changes of 2002, and so it is possible to affirm that the results show the knowledge level of a representative portion of pharmacists.

Accordingly a review showed that published researches about PC in Brazil, up until 2011, were developed exclusively at public pharmacies (Ambiel and Mastroianni, 2014). Thus, this study provides data to fill an important gap about the supply of PC in the private sector; it is also a pioneer in evaluating community pharmacists from different regions of Brazil in relation to the concepts and issues involving PC.

CONCLUSIONS

Less than half of the pharmacists who work in privately owned community pharmacies in Brazil have knowledge about the concept of PC proposed by the BPCC. Moreover, the conduct shown by these professionals is not in accordance with Brazilian recommendations, because there is as lack of operational protocols standardizing the service, systematic recording of interventions and data relating to the patient, as well as physical and material resources for the proper conduct of the service. Moreover, it is possible to affirm that the curriculum changes introduced in 2002 did not achieve the aim of preparing pharmacists to perform comprehensive and humanistic services such as PC.

In light of this, it is necessary to intervene in the training of pharmacists in order to make them able to perform PC clinical services properly. In addition, it is essential to invest in resources that provide favorable conditions to implement and carry out this important practice, to promote rational use of drugs and the success of pharmacotherapy.

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Conflict of interests

There was no conflict of interest in this study.

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Full Length Research Paper

Effects of the bluish liquid (hemolymph) from the African giant snail (*Achatina marginata*) on the blood coagulation time and erythropoietic volume

S. O. Olagbende-Dada

Department of Pharmacognosy, University of Lagos, Lagos State, Nigeria.

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The bluish liquid (hemolymph) from the African giant snail designated snail bluish liquid (SBL) was investigated for its coagulatory potency *in vitro* using human blood from different patients and *in vivo* using the Wistar albino rat. Its effect on the erythrocyte volume (PCV) during the period of administration in the rat was also studied. Coagulatory potency was studied in terms of the prothrombin time (PT) by comparing the PT of the blood obtained from the patients when mixed with the SBL with that obtained when mixed with a standard coagulatory agent. The *in vivo* study administered the SBL at different doses to groups of matured Wistar rats for a period of time, determined the PT of their withdrawn blood and compared with that of a control group that did not receive SBL. At the same time the collected blood was analyzed for its red blood volume (PCV) using the haematocrit reader. All obtained data were statistically analyzed. Results showed a significant shorter PT with SBL when compared with the calcified tissue thromboplastin standard used in the *in vitro* study, and the *in vivo* result also showed dose dependent significant reduction in clotting time and increase in PCV after the SBL administration. However, the increase recorded in PCV showed a non significant increase at the low doses of 50 and 100 ml/kg; the higher doses that showed significant increases also exhibited some lethality. In conclusion, SBL is seen as a good first aid agent to arrest external bleeding. Its oral administration at low doses (<200 ml/kg) may boost blood formation but may also precipitate blood clot while the higher doses must be avoided because of toxicity.

Key words: Snail, coagulatory agent, thromboplastin, blood, hemophiliac.

INTRODUCTION

The African giant snail (*Achatina marginata*) is a land, nocturnal, invertebrate animal of the Phylum Mollusca (Ademolu et al., 2006). It is found mostly under stones or litter of decaying organic matter during the day time. In West Africa, its favorable habitat for survival is the dense high forest and the fringe of the derived Guinea Savannh (Dede et al., 2003; Ademolu et al., 2006). Apart from reported high nutritional value (Agbogidi et al., 2008; Ogogo et al., 2011) which has made it economically important in the region, it is also used in traditional

E-mail: gbendedada@yahoo.com. Tel: +234 1-8783443 or 08023635808.

medicine. It was reported that the ortho-calcium phosphate extracted from the shell of the snail could cure kidney disease, tuberculosis, anemia, diabetes and asthma (Ademolu et al., 2006). Agbogidi et al. (2008) reported that the snail meat is particularly rich in iron and good quality proteins with high levels of lysine, leucine, isoleucine and phenylalanine and that snail meat causes reduction in pain and blood loss during labour; it promotes virility and fertility in human beings and it is used in the treatment of small pox and heart-related diseases. The serotonin secreted in the snails' body has been reported to be effective in the maintenance of normal behaviour after mental depression (Agbogidi et al., 2008) and its iron content which is about 50% justifies its recommendation for the treatment of anemia. The medicinal use of the African giant snail is not limited to its meat; its bluish liquid has been reported as one of the ingredients used during traditional male circumcision surgery (Sofowora, 1984). An *in vitro* screening of the bluish liquid by Olagbende-Dada et al. (2013) showed that it lacked both antimicrobial and antifungal properties but established a coagulatory effect. This present study investigated the *in vivo* coagulatory effect of the snail bluish liquid (SBL) together with its effect on the erythropoietic volume of the blood.

Coagulation is a complex process by which blood forms clots to stop bleeding and begin repair of any damaged vessel. It is an important part of hemostasis wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot (Dahl, 2000). Coagulation involves a cellular (platelet) and a protein (coagulation factor) component. It begins almost instantly after an injury to the blood vessel has damaged the endothelium; exposure of the blood to proteins such as tissue factor initiates changes to blood platelets which immediately form a plug at the site of injury; this is called primary hemostasis. Secondary hemostasis occurs simultaneously; Proteins in the blood plasma, called co-agulation factors or clotting factors, respond in a complex cascade to form fibrin strands, which strengthen the platelet plug.

One of the tests commonly employed in the determination of blood clot is the prothrombin time (Dacie and Lewis, 2001). Prothrombin time (PT) is a blood test that measures how long it takes a blood to clot and it is an important coagulatory test because it measures the presence and activity of five different blood clotting factors (Factors I, II, V, VII and X). It is a test of extrinsic and common pathway and the normal PT for man is about 12 s (Fritsma, 2002). While the PCV gives a measure of the amount of blood cells.

These studies were designed to determine: (a) the coagulatory effects of SBL on (i) the clotting time of the blood plasma samples obtained from three different individuals (normal person, patients using warfarin and hemophilic patient) when mixed (SBL) *in vitro* and (ii) the clotting time of the obtained plasma after it (SBL) has been administered in different doses *in vivo* to rat via oral feeding for two weeks. The *in vivo* study had a control group that served as the reference with which observed results were compared; (b) the effect the bluish liquid has on the blood cell formation by measuring the packed cells volume (PVC) of the rat's blood after the two-week oral administration of the SBL.

MATERIALS AND METHODS

Sixty snails (*A. marginata*) variety ovum were purchased from snail sellers in Oyingbo market in Lagos State. They were identified by Professor R. I. Egbonmwan of the Department of Zoology, University of Lagos. Plasma from appropriate patients (three patients on warfarin and one hemophilic) were obtained from Hematology Outpatient Clinic of Lagos University Teaching Hospital. Normal (control) plasma was obtained from a healthy laboratory staff.

Lemfield centrifuge model 80-2, calcified tissue thromboplastin (Fisher Diagnostic Ltd, U.K), calcium chloride (Mayer & Baker), ethylene diamine tetraacetic acid (EDTA) bottles, haematocrit reader, haematocrit centrifuge were used for the experiment.

In vitro study

Preparation of blood plasma

Nine milliliters of blood obtained from the patient's antecubital fossa of the arm through a clean puncture at the vein wall was delivered into a 15 ml tube containing 1 ml of 0.1 M trisodium citrate. The content of the tube was properly mixed and then centrifuged at 4,000 rpm for 15 min. With the aid of a Pasteur pipette the supernatant plasma was gently removed and used immediately.

One-stage prothrombin time

A one stage prothrombin time test was carried out using calcified tissue thromboplastin (CTP) on both test and control plasma. Another series of the test were similarly carried out where the SBL as substituted for the CTP.

Exactly 0.1 ml of either fresh normal or test plasma (obtained from three patients using warfarin and one hemophilic patient) was delivered into the bottom of a 75 × 10 mm glass test-tubes placed in a water-bath at 37°C and equal volume of CTP was added. A stop watch was started immediately and the time taken for the plasma to clot was recorded. This same procedure was carried out again using 0.1 ml of the freshly obtained SBL in place of CTP. Each procedure was carried out in duplicate and the average time recorded (Table 1).

In vivo study

Twenty five adult male albino Wistar rats obtained from the animal house of College of Medicine, University of Lagos, Idi-araba Campus were allowed to acclimatize for one week and divided into five groups of 5 rats per group. The first group was orally administered 200 ml/kg of distilled water to serve as control. The remaining four groups were respectively administered with 50, 100, 200 and 300 ml/kg of the SBL orally. The SBL was collected in sterile bottle every morning and administered immediately to the rats for 14 consecutive days. After this blood was withdrawn via ocular vein puncture from the rats and plasma was prepared from

Table 1. *In vitro* study result: prothrombin time (PT) of three differently sourced blood plasma after being mixed with SBL.

Source of plasma	Normal	Patients using warfarin 5 mg/day			Haemophiliac
Patient identification	N	A	B	C	H
Mean time with CTP (Reference) (s)	11.6 ± 0.3	34.4 ± 0.2	37.5 ± 0.3	33.0 ± 0.1	No clotting after 10 min
Mean time with SBL (s)	8.0 ± 1.4	13.5 ± 2.1	15.5 ± 2.1	13.0 ± 0.2	235 ± 28.0 (6.4 min)
Mean reduction in time (s)	3.6	20.9	22.0	20.0	-
Reduction time (%)	31.0*	60.8**	58.7**	60.6**	-

Table 2. *In vivo* study result: Prothrombin time (s) of rats administered with different doses of SBL.

Group	Dose (mg/kg)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean ± SEM	Mean reduction in time
Control	200 (H_2O)	56	49	55	48	50	51.6 ± 3.26	Not applicable (Reference)
Test	50 (SBL)	51	43	50	42	44	46.0 ± 3.74	5.6 (12.2%)
Test	100 (SBL)	28	23	27	21	32	26.2 ± 3.87**	24.4 (49.2%)
Test	200 (SBL)	25	22	25	20	Died	23.0 ± 2.12**	28.2 (55.4%)
Test	300 (SBL)	Died	21	Died	23	Died	22.0 ± 1.00**	29.6 (57.4%)

Statistical evaluation (t-test) of the clotting time at 99% confidence (p = 0.01) limit for the studies indicate that the results obtained from the control (using CTP) were significantly different from the results obtained for the SBL. Values are expressed as mean ± standard error of mean. *Indicates a significant difference (p<0.05) and **indicates a highly significant difference (p<0.01).

Table 3. Percent PCV of rats dosed with different doses of SBL.

Group	Dose (ml/kg)	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean ± SEM
Control	200	32	35	38	32	36	34.6 ± 2.33
Test	50	32	36	38	33	37	35.2 ± 2.09
Test	100	34	38	40	36	38	37.2 ± 2.04
Test	200	37	41	42	38	41	39.8 ± 1.94*
Test	300	Died	45	Died	42	Died	43.5 ± 1.5*

*Indicates a significant difference (p<0.05) at 99% confident level when compared with the control.

the collected blood samples as done in the *in vitro* test; the PT test was performed in duplicate for each animal's plasma as follows: For each test, two tubes were arranged in the water bath. To each tube, 100 µl of the plasma was added and incubated at 37°C for 3 min. 200 µl of CTP was added to each sample at a time, immediately after which a stop watch was started. The prothrombin end point for each, that is, the time at which visible clot was observed was noted and the average value was recorded (Table 2).

Packed cell volume (PCV) determination

The blood collected via ocular vein puncture for each dosed animal was transferred into pre-labeled EDTA bottles and gently mixed by inversion. Three-quarter full capillary blood sample was taken from each EDTA bottle and the capillary top was sealed with plastacin. The tubes were then arranged in the haematocrit centrifuge with the sealed portion facing the outward part of the centrifuge. The samples were centrifuged at 10,000 rpm or 5 min. The micro-haematocrit reader was used to read the PCV value after the centrifugation (Table 3).

RESULTS AND DISCUSSION

Both the *in vitro* and *in vivo* results show significant reduction in the observed clotting time after the introduction of the SBL. This fact is of tremendous importance medically when the snail water is taken internally at doses higher than 50 ml/kg as it signals potential danger towards thrombosis which is majorly responsible for precipitating stroke, a condition which is becoming prevalent in Nigeria. The general consumption of snail however is limited to its meat which has been confirmed as nutritious and rich in protein and seldom is this liquid taken internally. On the other hand internal administration of this bluish water from the snail may augur well for the hemophiliacs to who blood clotting is a major problem as shown in Table 1 result. This potential effect to reduce the time for blood both *in vitro* and *in vivo* to clot may be the reason for its use to reduce blood loss during labour (delivery) in women (Agbogidi et al., 2008). The external use of the liquid which shorten the clotting time significantly (Table 1), is well desired in the cases of injuries that leave the blood vessels open like a cut, accident wounds, situations that demand quick arrest of blood flow and this justifies its use at the traditional circumcision surgery table to arrest bleeding. This

property makes snail very relevant as a first aid in arresting bleeding in any rural area (where a modern health facility is not available) when there is an injury to the blood vessel. The pro clotting property of the bluish liquid is enhanced by its high calcium content which is one of the major requirements for the process of blood clotting.

The packed cell volume (PCV) recorded an insignificant dose dependent increase in value and showed lethality at 300 ml/kg (as three out of the five rats died in the course of the study) and this is the dose at which a significant increase was recorded. This positive effect on PCV value is expected for it has been shown by Olagbende-Dada et al. (2013) that the SBL contains both iron (Fe) and copper (Cu) elements that are required for blood cell formation. The observed increase in PCV suggests that the SBL could be of use (within its safety margin) in preventing or treating mild anemia; however, its use is not advisable because of its tendency to form blood clot.

Conclusion

SBL can be used as a good first aid agent to arrest external bleeding of damaged blood vessels. Its oral administration at low doses (<200 ml/kg) may boost blood formation but may also precipitate blood clot while the higher doses must be avoided because of toxicity.

Conflict of interest

Authors declare that there are no conflicts of interest.

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Full Length Research Paper

In vitro evaluation of the antifungal activity of extracts of *Baillonella toxisperma* (Pierre), a Sapotaceae, on the growth of some human pathogenic yeasts

S. H. Riwom Essama[1]*, M. A. Nyegue[2], C. Ndoye Foe[1,2], S. P. Bouopda Tamo[1] and F. X. Etoa[1]

[1]Laboratory of Microbiology, Department of Microbiology, University of Yaoundé I, PO Box 812 Yaoundé, Cameroon.
[2]Laboratory of Phytobiochemistry and Plants Study, Department of Biochemistry, PO Box 812 Yaoundé, Cameroon.

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An ethnopharmacological survey concerning medicinal properties of *Baillonella toxisperma* (Pierre) was carried out by interview with the indigenous population of Dimako village situated in the East region of Cameroon. The result showed that the plant is implicated in the treatment of many infections among which is fungal infections. To confirm the antifungal property of *B. toxisperma* (Pierre), the barks and leaves of the plant were collected and serial extractions in water, hydro-ethanol mixture (3:7), ethanol, methanol and ethyl acetate were performed *in vitro*. One part of the hydro-ethanol (3:7) extract was degreased by mixing in water-hexane mixture (1:1). The extracts were then tested *in vitro* against *Candida albicans, Candida parasilopsis, Candida* sp. responsible for superficial, deep or systemic mycosis and against *Cryptococcus neoformans* responsible for sub-acute meningitis in immunodeficient individuals. The susceptibility of yeasts to plant extracts was evaluated using the wells diffusion method and yeasts growth inhibition parameters were evaluated according to the proposed National Committee for Clinical Laboratory Standards (NCCLS) M27-A2 standard guidelines (2002). The minimal inhibitory concentrations (MIC) and minimal fungicidal concentrations (MFC) determined were between 0.93 and 30.0 mg/ml. The extracts were fungicidal on clinical yeasts tested with MFC/MIC ratio of 1 or 2. The hexane phase HT2 from the hydro-ethanol crude extract of the barks gave the best antifungal activity on *C. neoformans,* with a MIC of 0.93 mg/ml and a MFC of 1.87 mg/ml. This activity was similar to the one obtained with fluconazole. Phytochemical screening revealed the presence of polyphenols, phenols, tannins, flavonoids, steroids, alkaloids, saponins, phlobatannins, triterpenes, anthocyanins, cardiac glycosides, leucoanthocyanins and fats, which are bioactive substances. The results could explain scientific validation to the traditional medical uses of *B. toxisperma* (Pierre) to treat fungal infections.

Key words: Cameroon, *Baillonella toxisperma* (Pierre), ethnopharmacology survey, fungal infections, bioactive compounds.

INTRODUCTION

Fungal infections are global public health problems in Africa and particularly in Cameroon. The prevalence of

*Corresponding author. E-mail: sarariwom@yahoo.fr

resistance to antifungal agents has significantly increased in the past decade (Tasleem et al., 2011). Furthermore, the problem of fairly higher toxicity and limited numbers of effective drugs now available underline the necessity to discover new antifungal compounds. Medicinal plants have been used for centuries as remedy for the treatment of various diseases and are the major sources of drugs by virtue of their high secondary metabolites content (Nostro et al., 2000). The World Health Organization had recognized traditional herbal medicine as a building block of primary health care. Many investigators have evaluated the bioactivity of plant extracts and the isolated constituents against the serious infectious organisms (Parekh and Sumitra, 2006). History shows that plants have been an important source of medicines against microbial infections. The compounds isolated from plants such as 2-decanone, hydroxydihydrocornin-aglycones, various indole derivatives and isoflavanones are reported to have interesting antifungal activities (Tasleem et al., 2011).

Baillonella toxisperma (Pierre) is a plant used in traditional medicine by many communities in Cameroon. This plant is used in the treatment of over 50 diseases among which are microbial infections. *B. toxisperma* (Pierre) grows in primary tropical rain forest in hot and humid climates (Louppe, 2005; Angerand, 2006). In Cameroon, it is found abundantly in the East and South regions. Several studies have already been conducted on *B. toxisperma* (Pierre) but to the best of our knowledge the antimicrobial property of this plant has never been studied before. Nothing has been published on its antifungal activity. Debroux (1998) and Ngueguim et al. (2009) reported, respectively that this plant is traditionally used to cure microbial infections like rheumatism, toothache, hemorrhoids, injury, sexually transmitted infections, diarrhea and malaria. Decoction of barks or leaves is traditionally drunk to treat systemic candidiasis or is rubbed on the skin to prevent and to treat vaginal and oral mycoses (Bouopda, 2013). Almond oil of fruits of *B. toxisperma* (Pierre) is used to cure superficial infections like ringworms (Laird, 2000).

To validate this ethnomedicinal information, we have performed an ethnopharmacological survey on the antimicrobial properties of *B. toxisperma* (Pierre) in Dimako village situated in the east region of Cameroon. This village was chosen according to the socio-cultural importance that the indigenous population of this locality confers to this plant and according to the frequency of its utilization to treat microbial infections by the indigenous population.

Candida species and *Cryptococcus neoformans* remain the most common causes of invasive opportunistic fungal infections (Kao et al., 1999). Among *Candida* species, *Candida albicans* and *Candida parasilopsis* are responsible for extremely varied clinical manifestations, ranging from superficial to deep or systemic mycosis and *C. neoformans* (Pierre) is the causative agent of sub-acute meningitis in immune depressed persons. These infections can lead in certain cases to the death of the patient. In this framework, the antifungal activity of *B. toxisperma* (Pierre) extracts was evaluated *in vitro* against some clinical yeast among which *C. albicans, C. parasilopsis, Candida* sp. and against *C. neoformans*.

MATERIALS AND METHODS

Ethnopharmacology survey

The ethnopharmacological survey was carried out from the 11th to the 29th August, 2012. One hundred dwellers in Dimako village among whom 70 men, 21 women and 9 children were submitted to an interview concerning local names of *B. toxisperma* (Pierre), description of the part of the plant used as a remedy (for example, barks, leaves, roots or fruits), mode of preparation (for example, decoctions or infusions) and mode of administration of the recipes. They were equally questioned because of their equal access to natural medicines. The provided information was collected using a questionnaire approved by Department of Biochemistry of University of Yaoundé I. The data collected permits us to determine number of citations for the medicinal uses and to calculate percentage of citations of each part of *B. toxisperma* (Pierre) in the treatment of ailments.

$$\text{Percentage of citations of each part} = \frac{\text{Number of citations of each part}}{\text{Total number of citations}} \times 100$$

Plant

Barks and leaves of *B. toxisperma* (Pierre) were harvested in Dimako on September 15th, 2012. The botanical identification was confirmed at the National Herbarium of Yaoundé-Cameroon, by comparison with specimen No. 54060/HNC.

Fungal strains

Fungi strains were gratefully given by “Centre Pasteur du Cameroun” (Yaoundé) and were made up of four yeasts which are *C. albicans, C. parasilopsis, Candida* sp and *C. neoformans*. These yeasts were clinically isolated in patient, identified and stored at ± 4°C in a refrigerator. *Candida* sp was identified like *Candida* genre but her specie has not been determined.

Methodology for extraction and antifungal screening

Plant extracts

About 2000 g of leaves and barks each was dried in the shade and away from moisture and then crushed using an electric grinder. The extraction was performed in ethanol, methanol, water-ethanol mixture (3:7), ethyl acetate and water. Samples of 200 g of bark powder and 120 g of leaf powder were weighed and macerated in 1000 ml of solvent for every system for the bark and 600 ml in each solvent system for the leaves, for a period of 3 days at room temperature. The macerate obtained was filtered through Watman No. 1 filter paper and the filtrate was concentrated using a rotary evaporator. The extractions were repeated three times. A portion of 10 g of the ethanol extract of leaves or barks was subjected to liquid-liquid partition in 400 ml of water-hexane mixture (1:1) by emulsion. The aqueous and hexane phases obtained from this

partition were separated and concentrated. Four hundred milligrams of each extract were weighed out exactly and dissolved in 4 ml of dimethyl sulphoxide (DMSO) 10% to give a stock solution at 100 mg/ml used for the biological tests.

Phytochemical screening

Phytochemical tests were carried out using standard procedures described by Harborne (1998) and Edeoga et al. (2005).

Antifungal screening

Preliminary susceptibility test to plant extracts: From a 48 h culture of each clinical yeast, the inoculum was prepared by suspending in 1 ml of 0.85% NaCl saline solution, a pure colony of clinical yeast (National Committee for Clinical Laboratory Standards (NCCLS), 2002). Seeding of 100 µl of each inoculum was realized on the surface of Sabouraud dextrose agar (SDA) medium previously cast to a thickness of 4 mm, in Petri dishes of 90 mm in diameter. The inoculated dishes were dried at room temperature under a fumes cupboard. After 15 min, vertical cylindrical wells of 6 mm of diameters were made on these media using pasteur pipettes. To limit the spread of extracts in the agar, the bottom of the wells were blocked with a few drops of SDA, then the fixed volumes of 50 ml stock solutions of the extracts (100 mg/ml) or of fluconazole (40 mg/ml) were distributed in each well separately. Influence of DMSO 10% on the fungal growth was evaluated (negative control). After a pre-diffusion of the test substances for 20 min at room temperature, the plates were incubated at 35°C for 48 h. The diameters of halos of inhibition around the wells were measured using a caliper. Each test was performed three times (Singh et al., 2009).

Evaluation of the inhibition parameters: The inhibition parameters of yeast growth were evaluated according to the modified M27-A2 broth microdilution method described by NCCLS (2002). This involved preparing double dilutions of tested substances in 100 µl of SDB medium into the wells of a microtiter. The range of final concentrations tested were 0.46 to 30.0 mg/ml for each plant extract and 0.03 to 3.75 mg/ml for fluconazole. Each serial dilution was performed in triplicate. The fungal inoculum was prepared in 0.85% NaCl saline solution from a 48 h of clinical yeast culture on SDA and adjusted to 2.5×10^5 CFU/ml. Volumes of 30 µl of this inoculum were distributed to all the wells of the microtiter. A line of the plate without plant extract served as a control for the growth of the organism (negative control) and another (without plant extract and without inoculum) served as sterility testing medium (positive control). The plates were sealed with parafilm paper and the preparations were incubated at 35°C for 48 h. Phenol red was used as a color indicator and after incubation; the minimum inhibitory concentration (MIC) was retained as the lowest concentration for which no color change of the medium was observed. MFC were determined by subcultures. 50 µl of the contents of wells with concentration greater than or equal to MIC were introduced in 150 µl of new SDB medium. After 72 h of incubation at 35°C, subcultures for which there was no resumption of growth corresponded to MFC.

Statistical analysis of data

Statistical package for social sciences (SPSS) 16.0 software for Windows was used for statistical analysis. The inhibition diameters of the growth of yeasts by extracts during susceptibility tests were analyzed using one-way analysis of variance (ANOVA). P values < 0.05 were considered as significant. The results were expressed as means ± standard deviations.

RESULTS

Ethnopharmacological information

The inquiry about local names and the indigenous medicinal properties of *B. toxisperma* (Pierre) was performed in Dimako village. Commonly called Moabi, Ayap and Adjap by indigenous population of this village, *B. toxisperma* (Pierre) is traditional medicinal plant implicated in the treatment of many diseases among which microbial infections like Fungal infections, malaria, rheumatism, gastro-intestinal infections, ringworm, toothache, pulmonary infections and hemorrhoids. Parts of the plant commonly used are barks, leaves, latex and almond oil. Percentage of citations of medicinal uses of different parts has been calculated and the result show that barks and almond oil are the most used parts with proportions of 42.91 and 22.38%, respectively. Table 1 reports ailments, parts of the plant used to prepare medicinal potions, mode of administration and number of citations of parts of the plant in the treatment of theses ailments. Figure 1 reports percentage of citations of each part of *B. toxisperma* (Pierre) in the treatment of ailments.

Extraction and antifungal screening

The barks and leaves of *B. toxisperma* (Pierre) were extracted and their antifungal activities were tested. Table 2 reports the results obtained from phytochemical screening of the different extracts. The results obtained from preliminary susceptibility tests of yeast to extracts and from antifungal screening tests showed that the yeasts were sensitive to all plant extracts and fluconazole. The inhibition diameters obtained from barks crude extracts varied from 7.11 ± 0.54 to 17.65 ± 0.54 mm and those obtained from leaves crude extracts varied from 7.33 ± 0.12 to 15.76 ± 0.17 mm. The solvent of dissolution of the extracts DMSO 10% has not influenced the yeasts growth (absence of inhibition zone around of well). The results obtained from inhibition parameters showed that MIC_s determined ranged from 0.93 and 40 mg/ml and MFC from 1.87 to 40 mg/ml. The antifungal activities of different extracts and fluconazole are reported in Tables 3 and 4, respectively. The MFC/MIC ratio was calculated. According the classification of Fauchère and Avril (2002) adapted to fungi by Nyegue (2006), an antifungal is fungicidal when MFC/MIC = 1 or 2, fungistatic when 4 < MFC/MIC < 16 and tolerant when MFC/MIC > 32. The extracts of *B. toxisperma* (Pierre) are fungicidal on tested strains with MFC/MIC = 1 or 2.

DISCUSSION

Ethnopharmacological survey showed that *B. toxisperma*

Table 1. Traditional medicinal property of *B. toxisperma* (Pierre).

Ailment	Part used	Preparation/mode of administration	Number of citations
Fungal infections	Barks or leaves	Decoction/oral or rub	17
Pulmonary infections	Latex	Inhalation	05
Malaria and fever	Leaves	Decoction/oral	20
	Barks	Steam bath and bath	20
Backache	Latex	Rub	14
Wounds			09
Rheumatism	Almond oil	Rub	07
	Barks	Decoction or infusion/oral	16
	Latex	Inhalation	10
Gastro-intestinal infections	Latex		02
	Barks	Decoction/oral	21
	Leaves		21
Ringworm	Almond oil	Rub	13
Itches			23
Scabies			17
Toothache	Barks	Decoction or infusion/oral	15
Leucorrhea			11
Hemorrhoids			15
Sinusitis	Latex	Inhalation	12
Total number of citations			268

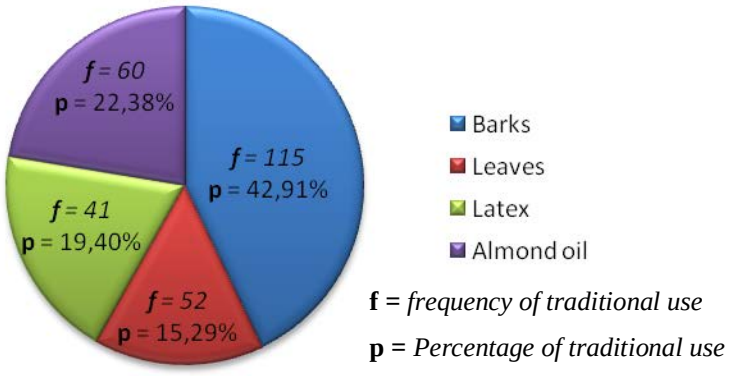

Figure 1. Frequency and percentage of traditional use of each part of *Baillonella toxisperma* (Pierre).

(Pierre) is of great importance in the treatment of various ailments. Ailments recorded in the interviews with dwellers in Dimako village were in majority infectious diseases like fungal infections, malaria, pulmonary infections, rheumatism, itches, scabies, toothache, hemorrhoids, leucorrhea, wounds, gastro-intestinal infections and ringworm. According to the number of citations for medicinal uses (Table 1), fungal infections appear to be one of the most important group of ailment to be treated using *B. toxisperma* (Pierre) extracts. Decoction or infusion of barks and leaves has been cited in the treatment of fungal diseases. This potion is

Table 2. Phytochemical screening of *Baillonella toxisperma* (Pierre) extracts.

Phytomolecules tested	Vegetal extracts													
	Barks							Leafs						
	T1	T2	ET2	HT2	T3	T4	T5	S1	S2	ES2	HS2	S3	S4	S5
Saponosides	+	+	+	+	+	+	-	+	-	-	-	-	-	-
Polyphenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tannins	+	+	+	-	+	+	+	+	+	+	-	+	-	+
Phlobatannins	+	+	-	-	+	+	+	-	-	-	-	-	-	-
Flavonoids	+	+	+	-	+	+	-	-	+	+	-	-	+	-
Anthocyanins	+	+	-	+	+	+	+	-	-	-	-	-	+	+
Leucoanthocyans	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Triterpenes	+	+	+	-	+	+	+	-	-	-	-	-	+	-
Steroids	+	+	+	+	+	-	-	-	+	-	+	-	+	+
Cardiac glycosides	+	+	+	-	+	+	-	-	-	-	-	-	-	-
Lipids	+	+	-	+	+	+	+	-	+	-	+	-	+	+

T1 and S1 = aqueous extracts, T2 and S2 = hydro-ethanol extracts, T3 and S3 = ethanol extracts, T4 and S4 = methanol extracts, T5 and S5 = ethyl acetate extracts, ET2 and ES2 = aqueous phase of water-hexane partition, HT2 and HS2 = hexane phase of water-hexane partition, T = barks, S = leaves, (+) = presence and (-) = absence.

traditionally drink to treat systemic candidiasis or rub on the skin to prevent or to treat superficial mycosis such as vaginal, oral or cutaneous candidiasis. Almond oil of fruits has been too cited in treatment of skin diseases like ringworms due to filamentous fungi. This suggests that, *Baillonella toxisperma* (Pierre) contains antifungal substances which could be used for therapeutic purposes. According the data collected, barks was the part of plant most cited in the preparation of traditional remedies with a percentage of citations of 42.91%, follow by almond oil (22.38%), latex (19.40%) and leaves (15.29%) (Figure 1).

The results obtained from chemical composition (Table 2) of extracts of *B. toxisperma* (Pierre) revealed that *B. toxisperma* (Pierre) contains metabolic groups like polyphenols, phenols, saponins, tannins, phlobatannins, flavonoids, anthocyanins, leucoanthocyanins, triterpenes, steroids, cardiac glycosides and lipids. These bioactive compound groups are cited in many studies for theirs antifungal properties. Several phenolic compounds such as 3-geranyloxy-6-methyl-1,8-dihydroxyanthraquinone, vismiaquinone, bivismiaquinone and chlorogenic acid, isolated from plants, demonstrated significant antifungal activities (Kuete et al., 2007a; Jassim and Naji, 2003). Terpenoids like cymbopogonol and 3-oxo-(20S, 24S)-epoxydammarane-19,25-diacetate isolated from the barks of *Caesalpinia pulcherrina* exhibited a prominent antifungal activity (Nasimul et al., 2003). They have the property of precipitating fungal proteins (Sher, 2009; Kansole, 2009). Flavonoids like angusticornin B and bartericin A were reported to be very active against yeasts such as *C. albicans*, *C. glabrata* and *C. krusei* (Kuete et al., 2007b). They have the ability to complex with the polypeptides of microbial cell wall, leading to the loss of function and consequently to the death of the pathogens (Boris, 1996). Steroids, tannins, phlobatannins, anthocyanins, leucoanthocyanins, Saponins and cardiac glycosides have been also reported by several authors for their antifungal activity.

Preliminary susceptibility tests (Table 3) showed effective inhibition of micro-organisms growth. At 100 mg/ml, the inhibition diameters obtained from barks crude extracts varied from 7.11 ± 0.54 mm (aqueous crude extract of barks on *C. albicans*) to 17.65 ± 0.54 mm (hydro-ethanol extract of barks on *C. parasilopsis*) and those obtained from leaves crude extracts varied from 7.33 ± 0.12 mm (aqueous crude extract of leaves on *Candida* sp) to 15.76 ± 0.17 mm (hydro-ethanol crude extract of leaves on *Candida* sp). The analysis of these results shows that hydro-ethanol crude extracts of the barks and leaves exhibited on the whole the better inhibition activities on the yeasts tested, comparing to those obtained from aqueous, ethanol, methanol and ethyl acetate extracts. However, theses inhibition diameters obtained from crude extracts remain lower compared to those obtained with fluconazole (17.84 ± 0.42 to 21.44 ± 0.54 mm). Difference of antifungal efficiency of crude extracts could be explained by the fact that the solvent system used for the extraction plays a significant role in the solubility of the active principles of plant materials which in turn influences the antimicrobial activities of the extracts and can also be explained by the composition of each extract which could influences her diffusion in the gel (Bouopda, 2013).

Table 3. Preliminary susceptibility of yeasts against *Baillonella toxisperma* (Pierre) extracts, fluconazole and DMSO 10%

Yeasts	Vegetal extracts										Ref	Negative control
	Barks					Leafs						
	T1	T2	T3	T4	T5	S1	S2	S3	S4	S5	Fluc	DMSO 10%
C. albicans	7.11^{a}±0.54	16.48^{b}±0.71	11.17^{ab}±0.31	10.84^{a}±0.25	9.54^{a}±0.54	9.14^{a}±0.11	15.38^{b}±0.31	11.22^{a}±0.41	11.24^{a}±0.24	9.84^{a}±0.21	17.84^{a}±0.42	6.00^{a}±0.00
C. parasilopsis	10.85^{b}±042	17.65^{b}±0.54	12.11^{b}±0.62	11.45^{ab}±0.38	10.57^{a}±0.47	9.52^{a}±0.58	12.54^{a}±0.32	10.41^{a}±0.78	11.29^{a}±0.23	11.25^{a}±0.17	18.63^{a}±0.11	6.00^{a}±0.00
C. neoformans	7.41^{a}±0.45	12.54^{a}±0.38	9.55^{a}±0.47	13.20^{b}±0.34	9.66^{a}±0.84	9.65^{a}±0.75	12.68^{a}±0.74	9.83^{a}±0.62	10.58^{a}±0.24	10.84^{a}±0.42	21.44^{b}±0.54	6.00^{a}±0.00
Candida sp.	12.34^{b}±0.34	11.47^{a}±0.29	10.33^{ab}±0.27	13.29^{b}±0.19	11.33^{a}±0.18	7.33^{a}±0.12	15.76^{b}±0.17	9.16^{a}±0.62	11.42^{a}±0.18	10.88^{a}±0.14	18.82^{a}±0.32	6.00^{a}±0.00

T1 and S1 = aqueous extracts, T2 and S2 = hydro-ethanol extracts, T3 and S3 = ethanol extracts, T4 and S4 = methanol extracts, T5 and S5 = ethyl acetate extracts, T = barks, S= leaves, Ref = reference, Fluc = fluconazole and DMSO = dimethylsulfoxide. Means of inhibition diameter ± standard deviations.

Table 4. MIC, MFC and MFC/MIC values of *Baillonella toxisperma* (Pierre) extracts and fluconazole.

Yeasts	Inhibition parameters (mg/ml)	Vegetal extracts														Ref
		Barks							Leafs							
		T1	T2	ET2	HT2	T3	T4	T5	S1	S2	ES2	HS2	S3	S4	S5	Fluc
C. albicans	MIC	30	7.50	30	3.75	30	15	15	30	15	15	7.50	30	30	30	3.75
	MFC	N/D	7.50	30	7.50	30	30	30	30	30	15	7.50	30	30	N/D	3.75
	MFC/MIC	N/D	1	1	2	1	2	2	1	2	1	1	1	1	N/D	1
C. parasilopsis	MIC	30	7.50	30	3.75	30	15	30	30	15	30	3.75	15	15	30	1.87
	MFC	30	15	N/D	7.50	30	30	30	30	15	30	3.75	30	30	30	1.87
	MFC/MIC	1	2	N/D	2	1	2	1	1	1	1	1	2	2	1	1
C. neoformans	MIC	N/D	15	30	0.93	30	15	30	15	7.50	30	7.50	15	15	30	0.93
	MFC	N/D	15	N/D	1.87	30	30	30	30	15	30	7.50	15	30	N/D	1.83
	MFC/MIC	N/D	1	N/D	2	1	2	1	2	2	1	1	1	2	N/D	2
Candida sp	MIC	15	7.50	15	3.75	15	15	30	30	30	30	15	30	30	15	3.75
	MFC	30	7.50	30	7.50	30	30	30	N/D	30	N/D	15	N/D	30	30	3.75
	MFC/MIC	2	1	2	2	2	2	1	N/D	1	N/D	1	N/D	1	2	1

T1 and S1 = aqueous extracts, T2 and S2 = hydro-ethanol, T3 and S3 = ethanol extracts, T4 and S4 = methanol extracts, T5 and S5 = ethyl acetate extracts, ET2 and ES2 = aqueous phase of water-hexane partition, HT2 and HS2 = hexane phase of water-hexane partition, T = barks, S = leaves, Ref = reference and Fluc = fluconazole, N/D = undetermined.

The results obtained from inhibition parameters (Table 4) showed that after 48 h of incubation and at 35°C, MIC_s determined varied from 0.93 to 30 mg/ml and MFC_s determined varied from 1.87 to 30 mg/ml. Hydro-ethanol crude extracts of barks and of leaves were the most active crude extracts on the yeasts tested with 7.50 mg/ml < MFC_s < 15 mg/ml for the barks and 15 mg/ml < MFC_s < 30 mg/ml for the leaves. These results are in accordance with those obtained in preliminary susceptibility tests. The hexane phase HT2 of the hydro-ethanol barks crude extract gave the most significant antifungal activity obtained in this work on *C. neoformans* with inhibition parameters similar to those of fluconazole (MIC = 0.93 mg/ml and CMF = 1.87 mg/ml). The effectiveness based on the MFC (MFC of the ethanol crude extract/MFC of hexane phase from water-hexane

partition of ethanol crude extract) illustrates that the hexane phase HT2 of the hydro-ethanol barks crude extract was 4 times more active than this latter one on *C. albicans*, 2 times more active against *C. parasilopsis*, 8 times more active against *Candida* sp and *C. neoformans*. The hexane phase of the HS2 hexane-water partition of the basic ethanol leaves extract was 4 times more active on *Candida* sp, and 2 times more active against *C. albicans* and *C. parasilopsis*. This amelioration of antifungal activity would be due to concentration of non-polar substances into hexane phase. Hexane is a non-polar solvent which extracts non-polar substances (Boulenouar et al., 2009). The hydro-ethanol extraction followed by the hexane-water partition extracts proved therefore to be a promising way for concentrating the active principle of *B. toxisperma* (Pierre).

According the classification of Fauchère and Avril (2002) adapted to fungi by Nyegue (2006), extracts of *B. toxisperma* (Pierre) were fungicidal on yeasts tested with MFC/MIC ratio of 1 or 2; this justifies the use of this plant in traditional medicine. The activity of a plant substance depends on several factors including the concentration of active ingredients (Achraf, 2012). According to the results obtained in this study, *B. toxisperma* (Pierre) contain many useful substances some of which are biologically active and can be used for therapeutic purposes or to serve as precursors for the synthesis of new drugs. Further isolation and characterization of metabolic compounds of *B. toxisperma* (Pierre) could lead to discovery of new antimicrobial substance.

Conclusion

The numerous adverse side effects reported with the use of many current antifungal substances calls for an urgent need to search for new therapeutic agents. Present preliminary *in vitro* antifungal properties of *B. toxisperma* (Pierre), a plant of the Cameroon traditional pharmacopoeia, revealed that the extracts of this plant were found to be effective for the growth control of test fungi. The phytochemical screening showed that the plant extracts contain bioactive metabolic groups cited for their antimicrobial activities by many authors. These extracts could be then used for further isolation and purification of active compounds. This study is an important step towards clinical evaluation in order to produce improved phytomedicine in the treatment of microbial infections or to produce new therapeutic drugs.

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Conflict of interest

There is no conflict of interest as regard this study.

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