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Full Length Research Paper

Exploration of the neurotoxicity of ciprofloxcin or gatifloxacin single dose in rat cortex and hippocampus

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The study aimed to evaluate the neurotoxicity of ciprofloxacin (Cip) or gatifloxacin (Gati) single oral dose in male albino rats weighing (100 ± 20 g) grouped as control-administered water, ciprofloxacin (80 mg/kg) and gatifloxacin (32 mg/kg) each of 12 rats. The frontal cortex of both groups revealed decrease in glutamate, GABA, taurine, histidine and serotonin levels and elevation of aspartate, glycin and serine and AChE activities. While noradrenaline and dopamine levels reduced significantly in Gati group, noradrenaline increased significantly in Cip group. Hippocampus of either Cip or Gati group's results revealed elevation of all detected amino acids and monoamines except the reduction of glutamate, aspartate and dopamine in Cip group. In the meantime, AChE activities significantly reduced in both treatments. Serum results showed elevation of glucose in both treated groups. The histological examination of Gati brain tissue showed neuronal degeneration in the cerebral cortex and congestion in the blood vessels and capillaries in hippocampus tissue without histopathological alteration observed in Cip group tissue. Overall, the data showed the effect of the quinolones single dose towards hyperglycemia and shift in balance of neurotransmitters and acetylcholinesterase as well as the histopathological alterations in the tested brain areas.

Key words: Ciprofloxacin, gatifloxacin, cortex, hippocampus, neurotransmitters, glucose.

INTRODUCTION

Gatifloxacin is one of the broad-spectrum fluoroquinolones available and approved by the US food and drug administration (FDA) in December 1999. Ever since its release in the market, there have been numerous reports implicating gatifloxacin as a cause of hypoglycemia and hyperglycemia. This prompted Bristol-Meyer Squibb Co. to list diabetes mellitus as a contraindication to gatifloxacin use in the US product labeling and Health Canada to issue an advisory against the use of gatifloxacin in patients with diabetes (Jose et

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al., 2007). Gatifloxacin showed to be equivalent to ciprofloxacin for the treatment of acute uncomplicated lower urinary tract infections (Naber et al., 2004). In extensive *in vivo* and *in vitro* experiments performed in an attempt to explain the central nervous system (CNS) side effects of quinolones sometimes observed under therapeutic conditions, they are like dizziness, restlessness, tremor, insomnia, hallucinations, convulsions, anxiety and depression. However, the molecular target or receptor for such effects is still not exactly known. Extensive toxicological and biochemical experiments were performed to explain the CNS effects observed under therapeutic conditions (Akahane et al., 1993; De Sarro et al., 1999, De Sarro and De sarro 2001).

Seizure activity is associated with a wide range of local biochemical changes, affecting various neurotransmitters (monoamines, amino acids) (Freitas et al., 2004; Cavalheiro et al., 2006). Cortex and hippocampus areas appeared to be important in the expression of early convulsive seizures (Kelly et al., 1999; Ang et al., 2006) in addition to the important functional association between cortical regions and the hippocampus in seizure propagation (Kelly et al., 2002) and suggested playing a role in inducing convulsions by quinolones (Motomura et al., 1991). The US Food and Drug Administration (FDA) Safety Announcement (8-15-2013) has recently issued a warning about fluoroquinolone antibacterial drugs; serious side effect of peripheral neuropathy may occur soon after these drugs are taken and may be permanent.

The study designed using single oral dose of the tested quinolones to explore its neurotoxicicity as the single dose in accordance with previous studies where it was used for treatment (Loo et al., 1985), randomized controlled trials (Boy et al., 2004; Kaushik et al., 2010; Heidari Bateni et al., 2014) and its prophylactic activity (Terzi et al., 2005; Alborzi et al., 2008). The study aims to ascertain the effect of oral single dose of either Cip or Gati in male albino rat on the concentrations of amino acid and monoamine neurotransmitters and acetylecholinesterase activities in the frontal cortex and hippocampus brain areas and the histopathological examination of both areas, in addition to the determination of serum glucose level.

MATERIALS AND METHODS

Experimental animals

This study carried was out on thirty-six adult male albino rats (*Rattus norvegicus*) with average body weight of range 100 ± 20 g obtained from the Egyptian Institution of Serum and Vaccine (Helwan). The experiment was conducted in the Department of Physiology in National Organization for Drug Control and Research (NODCAR). The male albino rats were housed in iron mesh cages with seven rats each. Clean sawdust was used to keep the animals dry and clean throughout the experimental period. The experimental animals were allowed acclimating under the laboratory conditions two weeks before the beginning of the experiments. The animals were kept under controlled temperature of 21°C and 12 h light/12 h dark cycle throughout the course of experiment. A commercial pelleted diet was used during the experiment and allowed water *ad libitum*.

Drugs

Ciprofloxacin (Cipro) ($C_{17}H_{18}FN_3O_3$•HCl•H_2O), manufactured by Bayer healthcare pharmaceuticals, Ciprofloxacin hydrochloride tablets and Gatifloxacin (TEQUIN) ($C_19H_{22}FN_3O_4$•1.5 H_2O) manufactured by Bristol-Myers Squibb Company. The antibiotics were administered by gastric intubation technique and doses calculated equivalent to the human therapeutic dose according to the Guidance for Industry and Reviewers (2002).

Experimental design

Animals were divided into three groups using random selection; the first group (n = 12 rats) was administered 2 ml of distilled water, the second group (Cip) was administered 80 mg/100 g body weight ciprofloxacin dissolved in 2 ml water. The Gati-treated rat groups (n = 48 rats) was administered 32 mg/100 g body weight gatifloxacin dissolved in 2 ml water. Animals were sacrificed after 12 h from dose administration by rapid decapitation. Blood samples were collected and sera separated for assessment of glucose using the BioAssay Systems' glucose assay kit (QuantiChromTM Glucose Assay Kit). The brains were dissected out quickly, weighed and cleaned. Four brains from each treated group were served for the histopathological examination according to Banchroft et al. (1996) and the rest eight brains for the biochemical analysis. The frontal cortex and hippocampus areas were separated and divided into two halves; the first half was served for acetylcholinesterase activity assay according to the modification of Ellman et al. (1961) method as described by Gorun et al. (1978). The second half was homogenized in 75% high performance liquid chromatography (HPLC) methanol (1/10 weight/volume) using a homogenizer surrounded with an ice jacket. The homogenates were used for the determination of the brain contents of amino acids using the precolumn PTC derivatization technique according to method of Heinrikson and Meredith (1984) and monoamines neurotransmitters according to method described by Pagel et al. (2000).

Statistical analysis

Reported values were represented as means ± SE. Statistical analysis was evaluated by one-way ANOVA. Once a significant F-test was obtained, least significance difference (LSD) comparisons was performed to assess the significance of differences among various groups using statistical processor system support "SPSS" for Windows software, Release 20.0 (SPSS, Chicago, IL).

RESULTS

The data as presented in Figure 1 as percentage change from control about frontal cortex showed decrease in

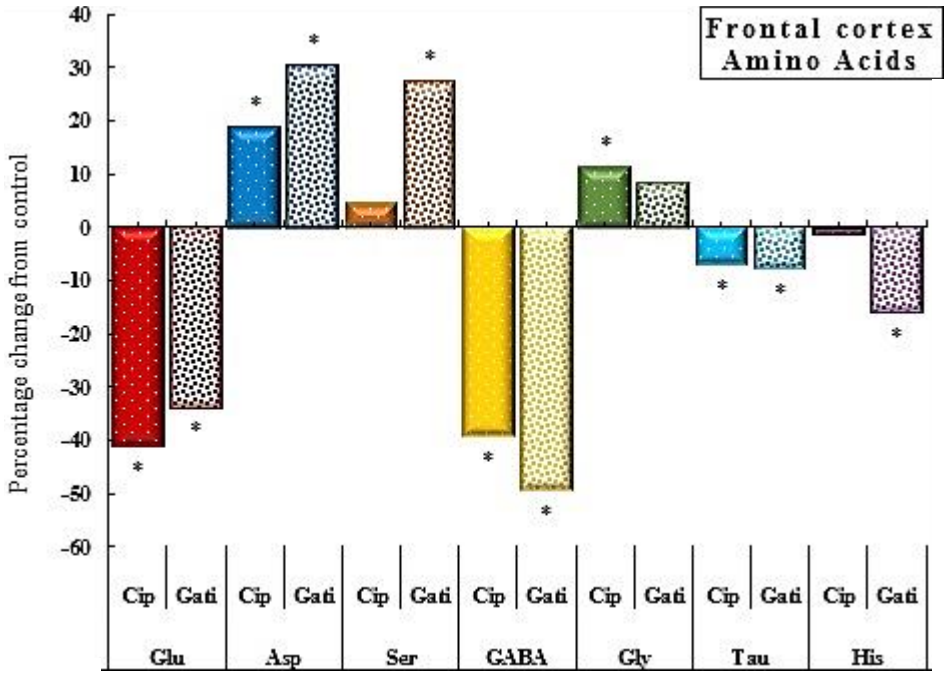

Figure 1. Percentage change from control of amino acids in cortices of rats treated with either ciprofloxacin (Cip) (80 mg/kg) or gatifloxacin (Gati) (32 mg/kg) single dose.

levels of glutamic acid, GABA, taurine and histidine and increase in aspartic, glycine and serine levels post administration of either ciprofloxacin or gatifloxacin. In ciprofloxacin group, glutamic acid, GABA, taurine and histidine data as mean ± SE is given as 5.32 ± 0.17 (-40.97%), 1.51 ± 0.05 (-39.02%), 1.86 ± 0.04 (-6.85%) and 1.20 ± 0.01 (-1.15%), respectively, while in gatifloxacin group, results are given as 5.99 ± 0.15 (-33.60%), 1.26 ± 0.003 (-49.21%), 1.85 ± 0.06 (-7.45%) and 1.03 ± 0.02 (-15.76%) different from control values 9.02 ± 0.25, 2.47 ± 0.04, 2.00 ± 0.05 and 1.22 ± 0.03, respectively. Aspartic acid increased significantly after ciprofloxacin and gatifloxacin: 3.37 ± 0.06 (18.49%) and 2.84 ± 0.06 (30.10%), respectively, from control value 2.18 ± 0.04. Serine level increased significantly after gatifloxacin: 0.60 ± 0.02 (27.23%) and not statistically different after ciprofloxacin administration: 0.49 ± 0.01 (4.47%), respectively, from control value 2.18 ± 0.04. Glycine level increased significantly after ciprofloxacin: 1.90 ± 0.04 (10.97%) and not statistically different after gatifloxacin administration 1.85 ± 0.06 (8.05%) from control value 1.71 ± 0.06.

The data represented in Figure 2 as percentage change from control about hippocampus showed no statistically different decrease in glutamic acid level and significant decrease in level of aspartic acid in ciprofloxacin group recording 9.17 ± 0.20 (-6.72%) and 1.53 ± 0.06 (-20.67%), respectively from control values 9.83 ± 0.30, 1.93 ± 0.05, respectively. Meanwhile, serine, GABA, glycine and histidine levels increased significantly in ciprofloxacin group recording values, 0.25 ± 0.005 (11.89%), 2.46 ± 0.08 (27.67%), 1.14 ± 0.01 (13.91%) and 2.70 ± 0.09 (9.65%), respectively from control values 0.23 ± 0.01, 1.92 ± 0.07, 1.00 ± 0.02 and 2.47 ± 0.07, respectively. Amino acids level in hippocampus of gatifloxacin group showed no statistically different increase in glutamic acid recording: 10.39 ± 0.26 (5.60%). Meanwhile, it showed significant increase in all detected amino acids recording values as: 1.98 ± 0.03 (2.37%), 0.28 ± 0.01 (22.03%), 3.17 ± 0.10 (64.59%), 1.44 ± 0.05 (44.04%), 3.62 ± 0.07 (7.75%) and 2.71 ± 0.06 (10.02%) for aspartic, serine, GABA, glycine, taurine and histidine, respectively.

Monoamines level and acetylcholinesterase activities recorded percentage change from control in frontal cortex and hippocampus of treated groups as well as serum glucose presented in Figure 3. In ciprofloxacin group, noradrenaline increased significantly in frontal cortex and hippocampus as mean ± SE by 1.19 ± 0.03 (12.44%) and 0.65 ± 0.03 (15.60), respectively. While in gatifloxacin group, noradrenaline decreased significantly in frontal cortex recording 0.78 ± 0.03 (-26.48%) and significantly

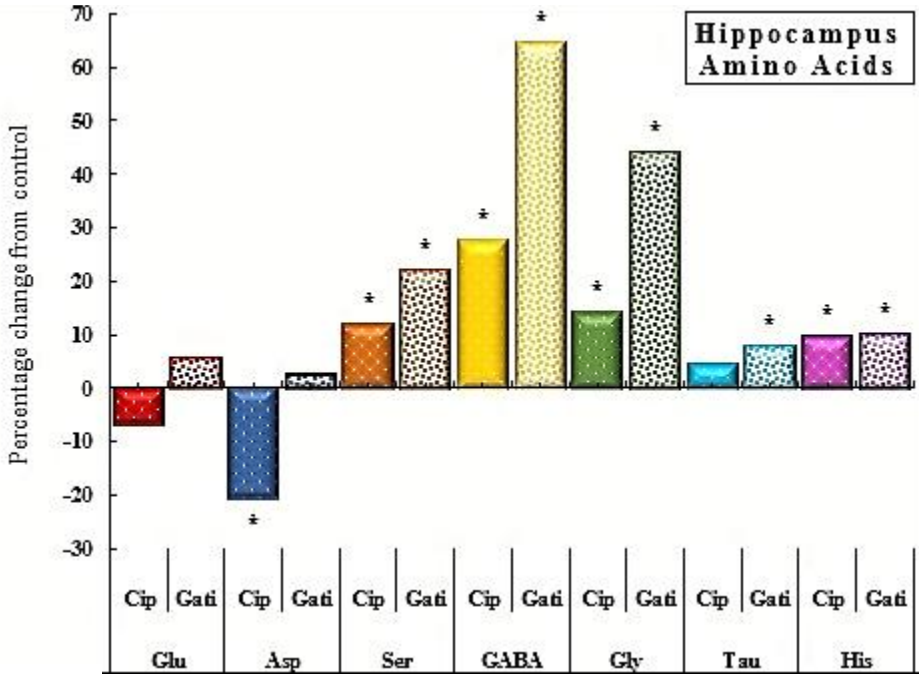

Figure 2. Percentage change from control of amino acids in hippocampi of rats treated with either ciprofloxacin (Cip) (80 mg/kg) or gatifloxacin (Gati) (32 mg/kg) single dose.

increased in hippocampus 0.87 ± 0.01 (54.79) from control values 1.06 ± 0.004 and 0.56 ± 0.05, respectively. Dopamine decreased significantly in frontal cortex of gatifloxacin group 2.95 ± 0.05 (12.21%) from control value 3.36 ± 0.11. Serotonin level decreased significantly in cortical area of ciprofloxacin and gatifloxacin groups 0.07 ± 0.001 (-15.30%) and 0.48 ± 0.002 (-43.53%), respectively from control value 0.08 ± 0.003. Meanwhile it increased significantly in hippocampus of gatifloxacin group 0.29 ± 0.004 (19.95%) from control value 0.25 ± 0.006.

Acetylcholinesterase activities increased significantly in frontal cortex while it decreased significantly in hippocampus of both treatments recording in frontal cortex activities of 16.7 ± 0.61 (21.82%) and 15.64 ± 0.91 (17.84%) in ciprofloxacin and gatifloxacin groups, respectively from control value 13.27 ± 0.33. While in hippocampus the data recorded was 15.47 ± 0.55 (-11.70%) and 16.23 ± 0.61 (-7.36%) in ciprofloxacin and gatifloxacin groups, respectively from control value 17.52 ± 0.25. In addition, serum glucose level increased, recording 16.7 ± 0.61 (21.82%) and 121.88 ± 3.50 (40.69%) in ciprofloxacin and gatifloxacin groups, respectively from control value 86.63 ± 0.93. With regards to the hispopathological examination, the response of cortex and hippocampus cells to Cip and Gati administration is represented in Figure 4A to D. Figure 4A and B showed normal histology of cerebral cortex and hippocampus in control group. There was no histopathological alteration observed in hippocampus of Cip group in Figure 4C, while in Gati group there was neuronal degeneration in the cerebral cortex (Figure 4D) associated with congestion in the blood vessels and capillaries of the hippocampus (Figure 4E).

DISCUSSION

Fluoroquinolones had structural similarities to kynurenic acid and other similar compounds which are endogenous ligands of the glutamate receptor, which might suggest an interaction of quinolones with ligand-gated glutamate receptors as well (Schmuck et al., 1998), and may explain the effect on quinolones subjected groups. The excitatory potency of fluoroquinolones is based on activation of the N-Methyl-d-aspartate (NMDA) receptor by abolishing the Mg^{2+} block in the ion channel which would prolong the opening time of the channel, thus increasing intracellular Ca^{2+} concentration in the neurons (Sen et al., 2007). The characteristics of gatifloxacin transport across blood brain barrier were investigated using primary cultured rat brain microvessel endothelial cells (rBMECs) as an *in vitro* model and study suggested that gatifloxacin transport across rBMECs involves a

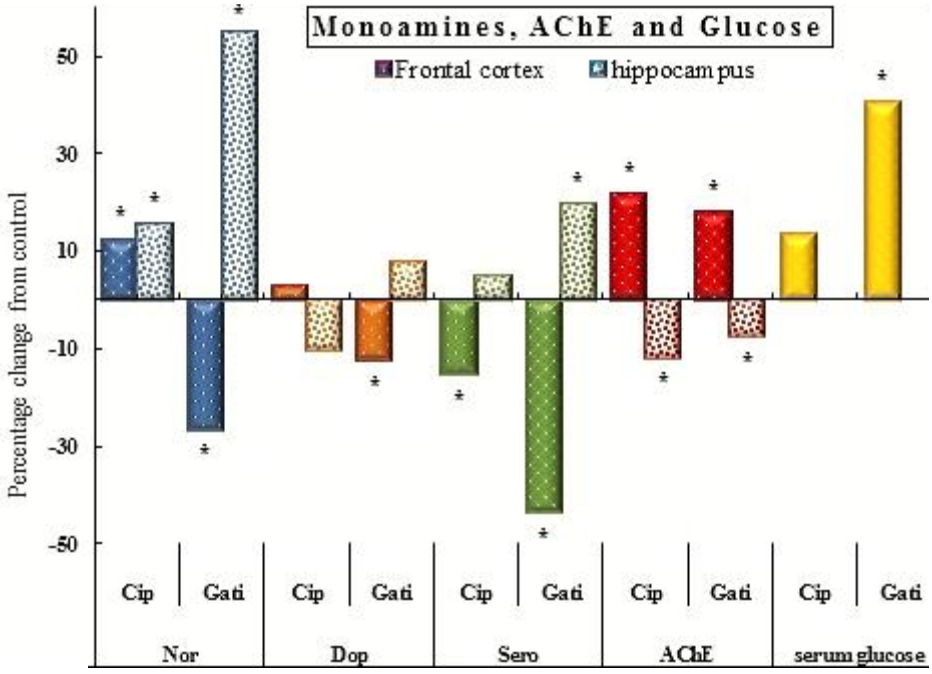

Figure 3. Percentage change from control of monoamines and acetylcholinesterase (AChE) in cortices and hippocampi and serum glucose of rats treated with either ciprofloxacin (Cip) (80 mg/kg) or gatifloxacin (Gati) (32 mg/kg) single dose.

Na+/Ca^{2+} exchange mechanism and extracellular Ca^{2+} (Li et al., 2009). The effect on Ca^{2+} may declare the effect of both antibiotics on taurine levels detected favoring recovery after neuronal hyperactivity (Rawi et al., 2011). Elevated aspartate, serine and glycine might suggest to the excitatory potencies of fluoroquinolones through their activation role on N-Methyl-D-aspartate-type glutamate receptor (NMDAR) (Curras and Dingledine, 1992; Wolosker, 2006; Wolosker et al., 2008).

The regional differences in GABA levels and acetyl-cholinesterase activities recorded decrease of GABA level and increase of AChE activity in the cortical area. Meanwhile, increase of GABA level and decrease of AChE activity in the hippocampal area in both treatments mimics that predicted in rat epileptic models (Appleyard et al., 1986) and support the proconvulsant effect of the quinolones previously discussed (Smolders et al., 2002; Abdel-Rahman et al., 2013; Arafa et al., 2013). Biochemical studies proposed role for AChE in brain mechanisms in development of status epilepticus through decrease in the AChE activity in the hippo-campus (Freitas et al., 2006). The effect of ciprofloxacin and gatifloxacin on GABA levels and acetyl-echolinesterase activities in cortex and hippocampus and their relation to anixiety and seizure generation was discussed in our previous study (Rawi et al., 2011; Abdel-Rahman et al., 2013). Seizure induction or decrease seizure threshold related effect to either ciprofloxacin or gatifloxacin single dose was previously declared (Darwish, 2008; Quigley and Lederman, 2004). In addition, serine elevation might be related to hippocampal serotonin increment detected in our study (Santini et al., 2014). Histidine content decreased in the frontal cortex and increased in hippocampus of ciprofloxacin and gatifloxacin treated groups. Histamine synthesis rate is a function of histidine content and histidine raises the possibility of a profound direct effect on CNS function (Yoshimatsu et al., 2002) and the herein results support the anaphylactoid reactions and hypotensive action of quinolones under therapeutic conditions as reported by Furuhata et al. (1998), Johannes et al. (2007) and Jones et al. (2013).

Fluoroquinolone-associated anaphylaxis may occur after first-ever intake of the agent (Sachs et al., 2006). In addition, drugs that release histamine may provoke headache, asthma, hypotension, arrhythmia, urticaria, pruritus, flushing and other conditions in patients with histamine intolerance (Maintz and Novak, 2007). Monoamines levels recorded in the tested antibiotics shows elevation in noradrenaline and reduction in dopamine and serotonin in the frontal cortex in the Cip and Gati groups except reduced level of noradrenalin in Gati group. However, in hippocampus there are elevations in monoamines levels in both groups except reduction of dopamine level in Gati group. These data

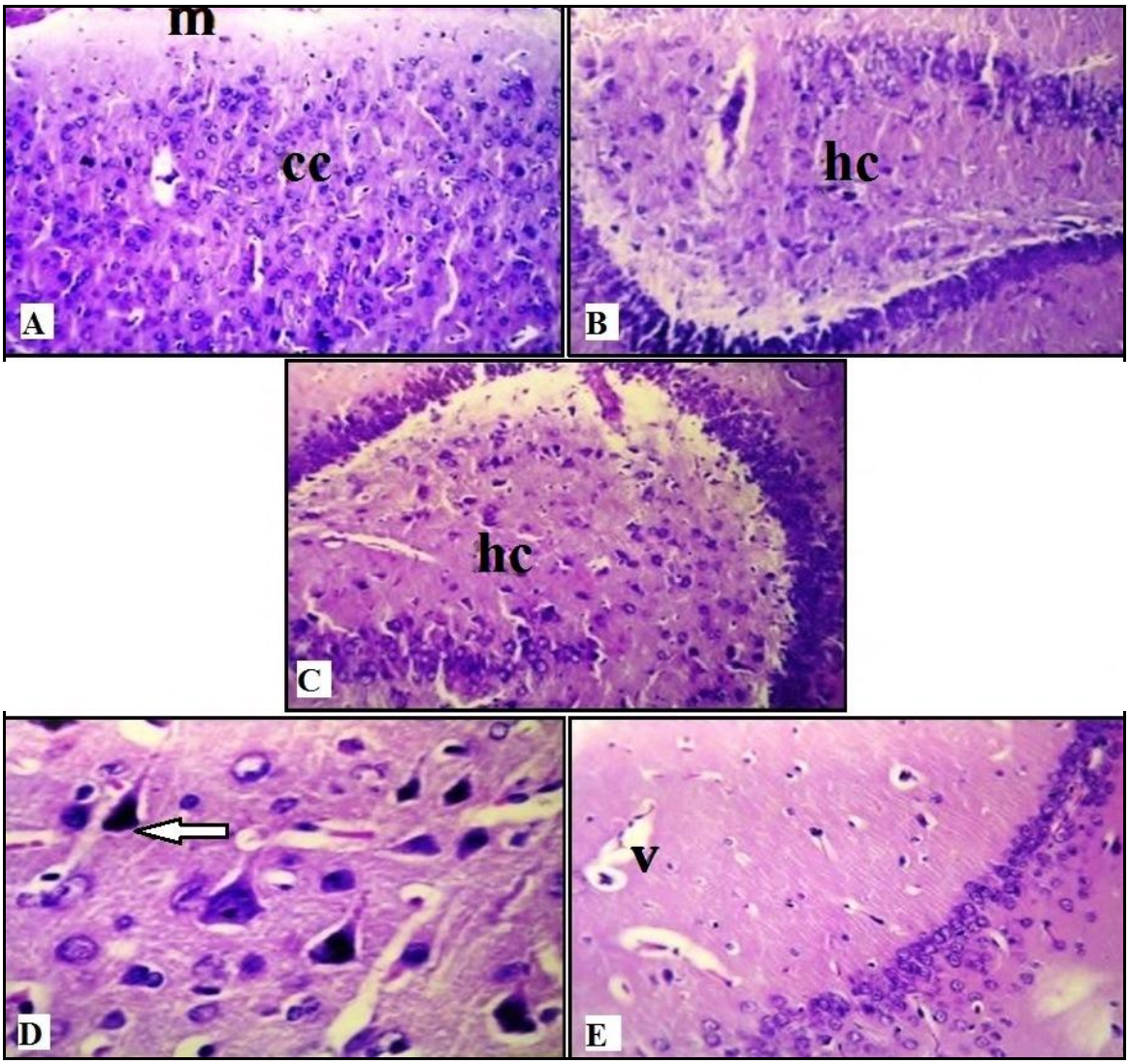

Figure 4. Light micrographs of brain sections in treated groups showing in control group normal histology of cerebral cortex (CC) and covering meninges (m) (H&E × 40) (A) and normal rat histology of hippocampus (hc). (H&E × 40) (B). Normal histological structure observed in the hippocampus tissue (hc) in ciprofloxacin group. (H&E × 40) (C). Neuronal degeneration in the cerebral cortex (arrow). (H&E × 160) (D), and congestion in the blood vessels and capillaries of the hippocampus (H&E × 64) (E) in gatifloxacin group.

may be validated by the seizure induction through the assumption about the pharmacological treatments that lowering monoamine levels in the brain generally increase the susceptibility to seizures, while treatments that increase monoamines decrease the susceptibility (Kiyofumi and Akitane, 1977). The data recorded about monoamines in the tested antibiotics may be a supplement data to the previously mentioned seizure inducing activity of quinolones (Ooie et al., 1997; Moorthy et al., 2008; Agbaht et al., 2009). The involvement of prefrontal cortex in depression and the link between reduced serotonin level in prefrontal cortex and depression symptoms as previously stated (Juckel et al., 1999; Koenigs and Grafman, 2009) is in accordance with the levels detected in our study. So the increment in the intracellular Ca^{2+} ions led to the rupture of the vesicles in the presynaptic terminals and increased the release of the neurotransmitters (Bullock et al., 1995) as a result, the content of catecholamine is decreased. The neuro-transmitters alterations support the hyperexcitability which reflected on the histopathology of cortex and hippocampus mainly in the most affected Gati group in

line with several previous studies discussed in Rawi et al. (2011) and Arafa et al. (2013).

As regard to the effect on glucose level in tested groups, Yamada et al. (2006) reported the effect of gatifloxacin on insulin secretion and islet insulin content by using isolated mouse pancreatic islets. Islet insulin content significantly decreased by gatifloxacin already at day one; however, there are some case reports that show that only one or two doses of gatifloxacin can induce hyperglycemia (Biggs, 2003; Arce et al., 2004). Gatifloxacin was withdrawn from clinical use after reports of drug-induced hyperglycemia and other fluoroquinolones reported to interfere with glucose homeostasis (Telfer, 2014). Onyenwenyi et al. (2008) indicated that non-diabetic gatifloxacin treated patients appeared to have an increased risk of hyperglycemia and the risk reduced in diabetics. Ghaly et al. (2009) previously documented that fluoroquinolones did not stimulate insulin secretion in the presence of a basal glucose concentration; rather, they only enhanced the secretion elicited by a stimulatory glucose concentration.

Recent study by Ghaly et al. (2014) explained why fluoroquinolones produce hypo- and hyperglycaemias, because fluoroquinolones affect the function of the mitochondria in pancreatic beta cells, which may diminish the insulinotropic effect of KATP channel closure and contribute to the hyperglycaemic episodes. In addition, ciprofloxacin and gatifloxacin cause oxidative stress and decrease the mitochondrial membrane potential (Lowes et al., 2009; Talla and Veerareddy, 2011; Rawi et al., 2011; Arafa et al., 2013). Gatifloxacin acutely diminish gluconeogenesis by inhibition of mitochondrial pyruvate transport (Drozak et al., 2008) since pancreatic beta cells have an exceedingly low antioxidant capacity (Lenzen et al., 1996) and inhibition of pyruvate transport may interfere with nutrient stimulation of insulin secretion. The study of Telfer (2014) extended to suggest a connection between the ingestion of fluoroquinolones antibiotics and the development of type 2 diabetes and advice that follow-up longitudinal studies to be undertaken to examine the history of individual diabetic patients for previous fluoroquinolone exposure. Glucose resuscitation resulting in hyperglycaemia activates the NADPH pathway in neurons, causing cytotoxic oxidative stress. The same phenomenon could also adversely affect oligodendrocytes (Suh et al., 2007). The study concluded from the recorded results that the excitatory potency of ciprofloxacin and gatifloxacin could be achieved from the first dose.

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Conflict of interests

The authors have declared that no competing interest exists.

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Full Length Research Paper

Drug use pattern in out-patient children: A comparison between primary and secondary health care facilities in Northern Nigeria

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Children are more vulnerable to adverse events related to use of drugs. It is therefore important to study drug use in children in order to optimize pharmacotherapy. The aim of this study was to compare drug utilization in paediatric outpatient departments of primary and secondary health care facilities. The patient and drug information of 600 patients was analyzed for World Health Organization (WHO) recommended prescribing indicators. The average number of drugs per prescription was significantly ($p < 0.0005$) lower in secondary (2.97) compared to primary (3.62) facilities, while average consultation time was shorter ($p < 0.0005$) in primary than secondary facilities. Percentages of drugs prescribed from Nigerian Essential Drug List (EDL, primary {89.78%}; secondary {91.79%}) and by generic name (primary {55.04%}; secondary {57.88%}) were insignificantly different between the facilities. The use of injectables was low (8.32% in primary versus 3.74% in secondary facilities) while antibiotic use was high (54.14% in primary to 60.28% in secondary facilities). Analysis of the dispensing indicators showed that the secondary facilities were significantly ($p < 0.05$) better than the primary facilities, even though not a single drug was adequately labeled in both the primary and secondary facilities. Prescription from EDL was found to be fair in the study area while use of injections was low. There is a need for improvement in case of medicines prescribed by generic name.

Key words: Drug utilization, out-patients, children, health care facility.

INTRODUCTION

Rational drug use has been defined as using the right drug in the right patient, for the right indication, in the right dose and dosage form, for the right duration of time. The rational use of drugs seeks to avoid the frequent problems of over- and under-prescription, inappropriate prescription and the use of new, expensive drugs when equally effective, well tried, safe, high quality and cheaper alternatives are available (NEDP, 1993). Unfortunately, in most cases, prescribing and dispensing patterns do not always conform to these criteria. The consequences of such inappropriate use of drugs cannot be overlooked especially in children. This is because children differ greatly from adults, not merely in size but also in the proportions and constituents of their bodies as

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Table 1. Characteristic of the general outpatient department prescribers.

Facility type	Total	Doctors [n (MYT)]	Nurses [n (MYT)]	CHO/CHEW [n (MYT)]
Secondary (n=8)	38	18 (5.2)	14 (20.4)	6 (13.3)
Primary (n=12)	39	0 (0)	0 (0)	39 (13.4)
Total [n (%)]	77	18 (23.4)	14 (18.2)	45 (58.4)

CHO: Community health officer; CHEW: community health extension worker; MYT: mean years of training.

well as functioning of their physiological systems. These differences are reflected in the way the body handles and responds to drugs and are relevant to medication (Laurence et al., 1999). No single rule or formula suffices for all paediatric cases; the dose is therefore established partly by scaling for body weight and/or surface area and by making pharmacokinetic and pharmacodynamic measurements when opportunities arise.

Studies conducted in most developing countries have shown that a high percentage of consultations end with prescriptions regardless of the necessity to prescribe (Kroenke, 1985). Numerous studies have also described irrational patterns of drug use that include polypharmacy, use of drugs that were not related to the diagnosis, patient non-compliance, overuse and misuse of antibiotics, and unnecessary use of injectable drugs (Quick et al., 2002). Such practices may result in a waste of resources, inappropriate patient demand, antimicrobial resistance, and increased drug-related morbidity and mortality. This study is therefore aimed at investigating the prescribing and dispensing practices at a representative sample of health care facilities in Kano State, Nigeria using WHO drug use indicators and comparing the results obtained between the primary and secondary health care facilities in the state.

METHODOLOGY

Study design

This was a comparative, cross sectional study involving paediatric outpatient departments of twenty (8 secondary and 12 primary) public health care facilities selected by multistage sampling technique in Kano State Northwestern Nigeria. The study was conducted between June 2009 and December 2009. The Ethical Committee of the Aminu Kano Teaching Hospital and Kano State Ministry of Health approved the study protocol.

Inclusion and exclusion criteria

The patients included in this study were those presented with general illness, aged 11 years and below. Patients presenting to the health care facilities for follow-up of chronic diseases, and patients presenting to receive services such as vaccination, and other specialized care services, were excluded.

Study sample

Based on the WHO recommended methodology (WHO, 1993), stratification of health care facilities according to senatorial districts and systematic random sampling were used to select a total of 20 health care facilities across the state. In each health care facility, 30 paediatric outpatient prescriptions were collected using systematic random sampling.

Data collection

The principal investigator (PI) collected data on both prescribing and dispensing indicators prospectively from each facility due to difficulties encountered in the availability of retrospective records. Demographic data of each patient, diagnosis, antimicrobial sensitivity test and detailed prescription were recorded on a modified WHO core prescription indicators form. Data collected were coded and de-identified. These recorded forms were used to analyze the average number of drugs per prescription, number of encounters with antibiotics, percentage of drugs prescribed by generic name/listed in the Nigerian Essential Drug List (EDL), percentage of drugs actually dispensed and adequately labeled. The consultation and dispensing times (WHO, 1993) were collected using a disguise technique (the investigator stayed outside the consultation room and the pharmacy). At each facility, the consultation and dispensing times were counted for 30 patients, and 30 parents were interviewed upon exit after the drug(s) had been dispensed to investigate dispensing practices and patient knowledge.

Analysis

Data were entered into Microsoft excel 2007 and Statistical Package for Social Sciences (SPSS version 15) and a descriptive analysis was performed. Drug utilization indicators were computed and compared between the primary and secondary facilities by unpaired Student's t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

In total, 77 health care providers prescribed at paediatric outpatients of the 20 selected facilities during the study period out of which only 23.4% (18/77) were qualified medical doctors (Tables 1 and 2). Among the 600 patients that participated in the study, 44.7% (286) were male and the average age of the patients was 3.7 ± 0.3 years. A total of 2016 drugs were prescribed in 600 prescriptions, giving an average of 3.36; and the range of drugs per encounter varied from 2 to 5. Three drugs were prescribed in most of the patients (57.3%) and there was not a single prescription wherein no drug was prescribed. Values of the prescription indicators are as shown in Figures 1 to 6. Dispensing and other patient care indi-

Table 2. Characteristic of the general outpatient department dispensers.

Facility type	Total	Pharmacists [n (MYT)]	Pharm Tech [n (MYT)]	CHO/CHEW [n (MYT)]
Secondary (n=8)	51	6 (6.8)	42 (13.2)	3 (7.3)
Primary (n=12)	5	0 (0)	0 (0)	5 (12.8)
Total [n (%)]	56	6 (10.7)	42 (75.0)	8 (14.3)

Pharm Tech: Pharmacy technician; CHO: community health officer; CHEW: community health extension worker; MYT: mean years of training.

Table 3. Dispensing indicators for paediatric outpatients' departments of 20 health care facilities in Kano State, Nigeria.

Indicator	Health care facilities	
	Primary (n=12)	Secondary (n=8)
Average dispensing time (s)	25.73 ± 1.18	38.68 ± 2.17[b]
Percentage of drugs actually dispensed	56.17 ± 2.36	95.91 ± 2.08[c]
Percentage of drugs adequately labeled	0.00 ± 0.00	0.00 ± 0.00[ns]
Percentage of parents who claim to have had adequate knowledge	79.03 ± 1.45	89.26 ± 2.08[a]

ns: Not significant; a ≤ 0.05; b ≤ 0.005; c ≤ 0.0005.

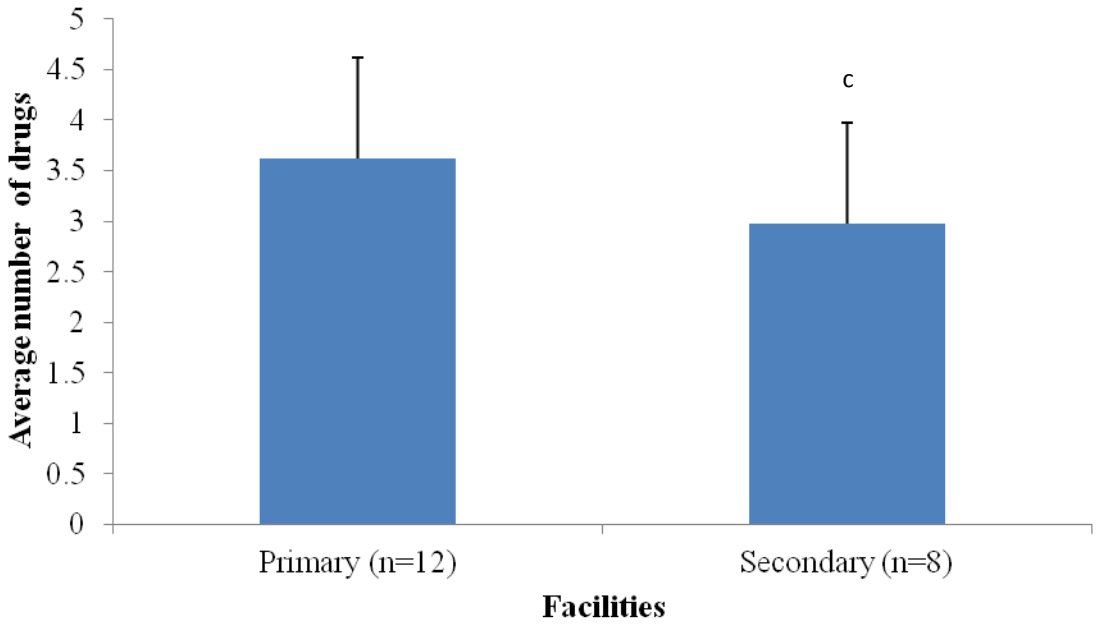

Figure 1. Average number of drugs per prescription for paediatric outpatients' departments of 20 health care facilities in Kano State, Nigeria. ns: Not significant; c = < 0.0005.

cators for the primary and secondary facilities are listed in Table 3.

DISCUSSION

This study revealed that the mean number of drugs prescribed per patient was significantly lower ($p < 0.0005$) in secondary (2.97) compared to primary facilities (3.62). Values of 2.3 to 3.7 drugs per encounter in secondary facilities have been reported from India (Dimri et al., 2008), Nigeria (Odusanya, 2004) and Ghana (Owusu-Dakuu and Sablah, 2004). In primary facilities, rates of 2.5 to 3.13 drugs per encounter have been reported in India (Anuja et al., 2010), Nigeria (Nwolisa et al., 2006) and Yemen (Bashrahil, 2010). In the present study, three or more drugs were prescribed in 72.4% of prescriptions, which reflects a trend towards polypharmacy, as it has

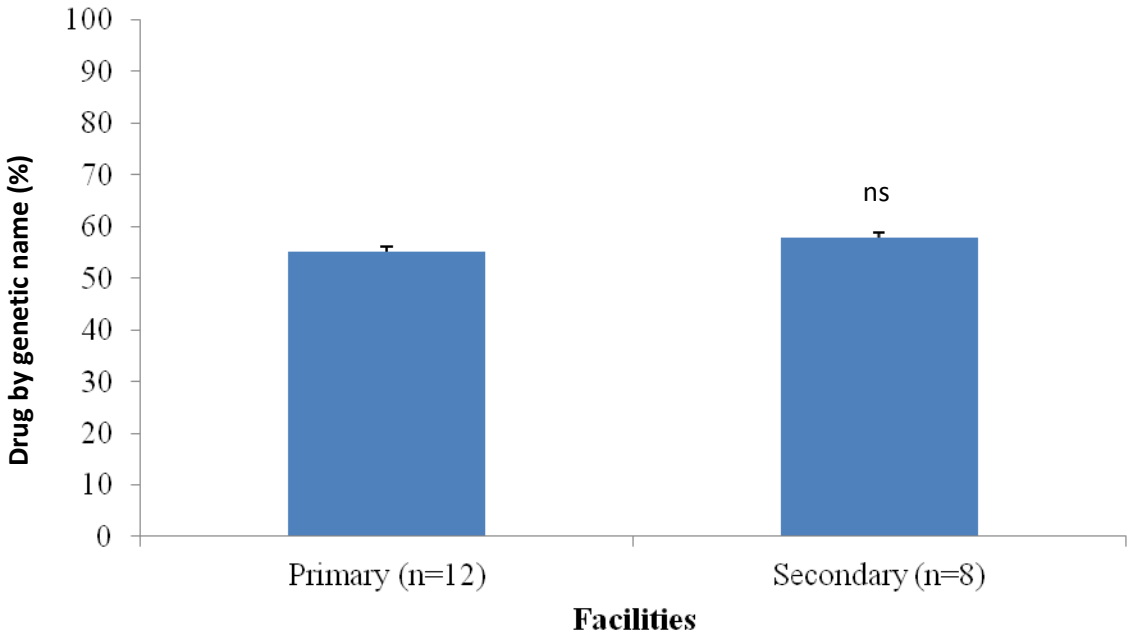

Figure 2. Percentage of drugs prescribed by generic name for paediatric outpatients' departments of 20 health care facilities in Kano State, Nigeria. ns: Not significant.

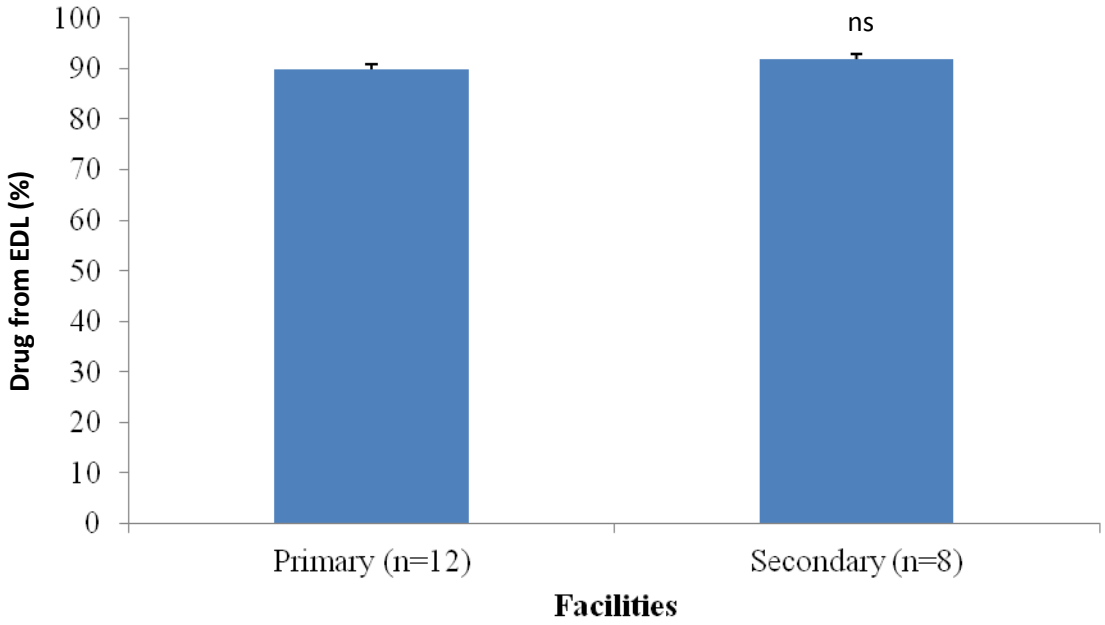

Figure 3. Percentage of drugs prescribed from EDL for paediatric outpatients' departments of 20 health care facilities in Kano State, Nigeria. ns: Not significant.

been proposed that the average number of drugs per prescription should be 1.6 to 1.8 (Isah et al., 2006). Prescriptions of high numbers of drugs may be attributed to patient demand; patients believe that the prescribing of more drugs will ensure improvement and facilitate the cure of their conditions more quickly. Other possible factors could be that the treatment is based on pure symptoms instead of a proper diagnosis due to lack of adequately trained personnel, laboratory facilities and overcrowdings in the health care centres. Although

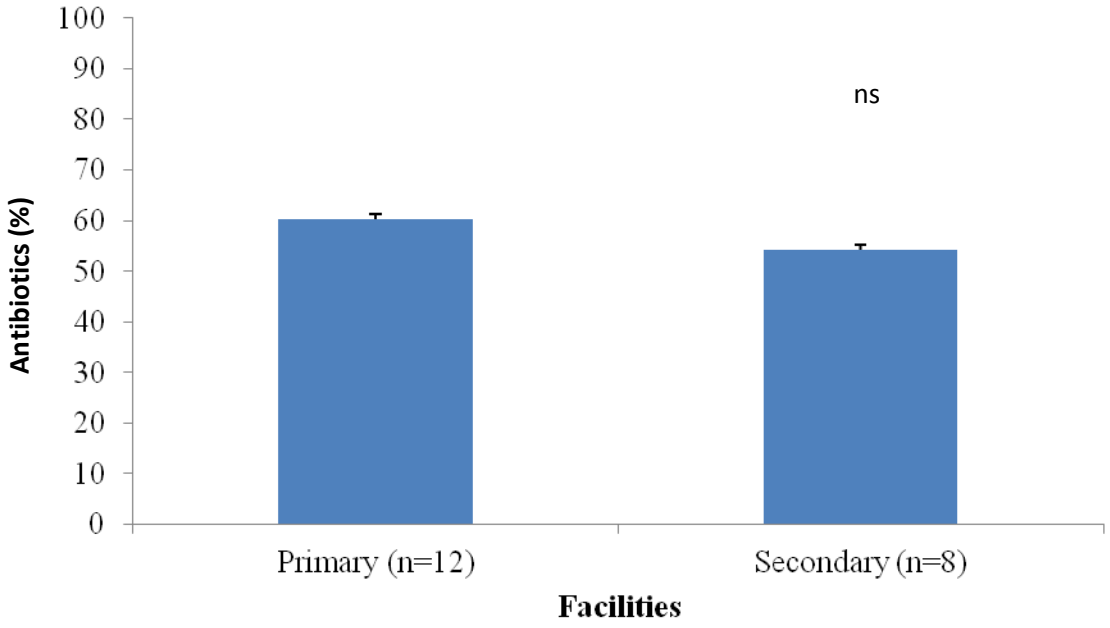

Figure 4. Percentage of antibiotics prescribed per encounter for paediatric outpatients' departments of 20 health care facilities in Kano State, Nigeria. ns: Not significant.

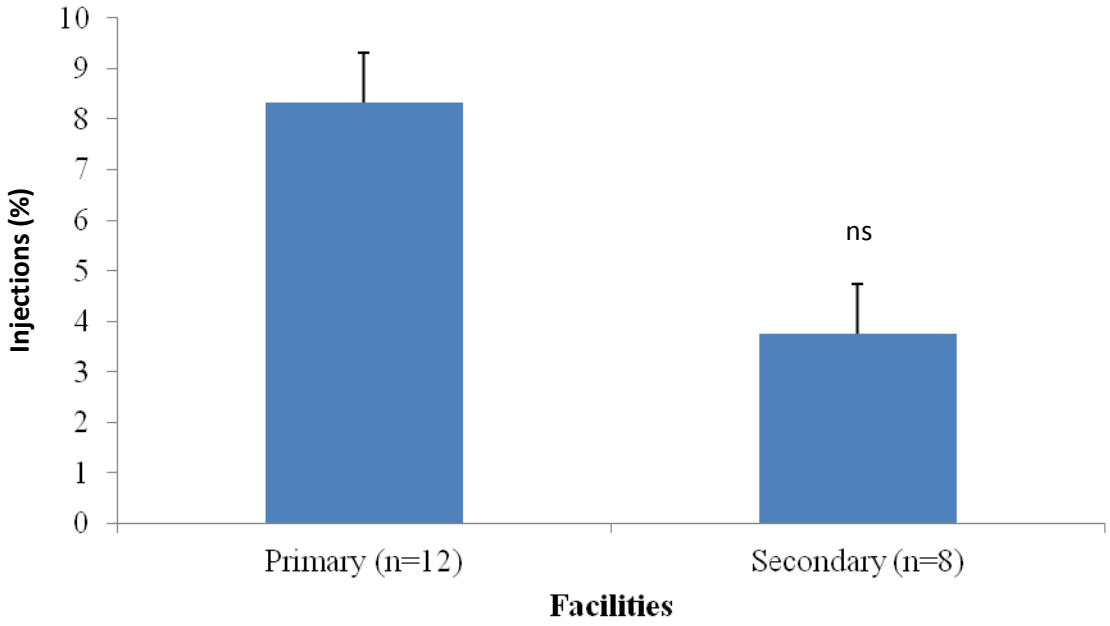

Figure 5. Percentage of injections prescribed per encounter for paediatric outpatients' departments of 20 health care facilities in Kano State, Nigeria. ns: Not significant.

increasing efforts are being made to improve drug-use practices in developing countries, it should be noted that some of these patients visit the hospitals with other diseases such as anaemia, malnutrition and at times some other infections. These make poly pharmacy inevitable.

Prescribing by generic name is known to reduce the cost of drug treatment and rationalizing drug therapy. This varies from 13.3 to 93% across the globe (Nsimba, 2006). Prescribing by generic name in our study was similar (55 to 61%) in most facilities studied. Other studies have reported even lower percentages, ranging from 25 to 60%, (Bashrahil, 2010; Chedi et al., 2010), while the optimal percentage should be close to 100%

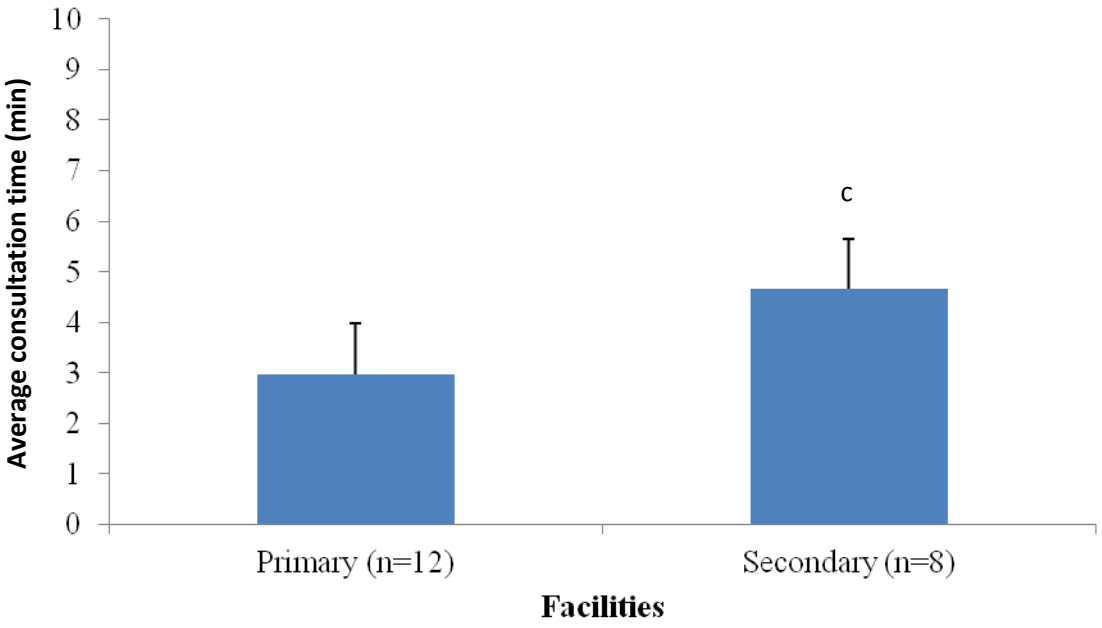

Figure 6. Average consultation time (min) for paediatric outpatients' departments of 20 health care facilities in Kano State, Nigeria. ns: Not significant; c ≤ 0.0005.

(Isah et al., 2006). The low rate of generic prescribing may be due to the fact that the information on medicines that prescribers usually receive is mostly provided by the drug companies. Another contributing factor for low generic prescribing by prescribers is their beliefs that the brand names are easy to remember as well as the frequent use of brand names during their training (Awad et al., 1999). Generic prescribing is strongly recommended, as it facilitates education and knowledge, and it also allows the pharmacist to maintain a more economic stock control system based on a smaller range of reasonably priced drugs (Awad and Himad, 2006).

The percentages of drugs prescribed from EDL in this study were lower among the primary facilities (89.78%) compared with secondary facilities (91.79%) but this was not significant ($p > 0.05$). These figures were higher than the values previously reported in the same area (Chedi et al., 2010) but lower than the optimal value of 100% (Isah et al., 2006). When the prescribers were asked concerning their low number of prescriptions from the EDL, some of the arguments forwarded by them were that, not all the drugs for various diseases are available in the EDL and resistance had developed to some of the drugs on the list. Appropriate use of antibiotics is necessary to prevent emergence of drug resistant bacteria. It has been recommended that fewer than 30% of prescriptions should contain an antibiotic (Isah et al., 2006). Percentages of antibiotics prescribed per encounter obtained in this study compared favourably with those reported from Ghana (Bosu and Ofori-Adjei, 2000), Cambodia (Chareonkul et al., 2002) and other parts of Nigeria (Chukwuani et al., 2002). The major factors that influence high antibiotic prescribing at health centers have been reported to be a lack of knowledge about appropriate antibiotic use, including overestimation of the severity of illness to justify antibiotic prescribing by prescribers, and pressure from patients who believe that antibiotics provide rapid symptomatic relief of the disease (Awad et al., 2006). The same factors may possibly play a role in health care centres of Kano. Antibiotics are essential drugs, but the overuse may increase antibiotic resistance, which will endanger their therapeutic effectiveness, increase treatment failure and, as a result, lead to longer and more severe illness episodes with higher costs and mortality rates.

Although inappropriate high levels of injection prescribing (17.1% to 80%) have been reported in Tanzania (Massele et al., 2001) and Zimbabwe (Trap et al., 2002), in the present study, overall injection use was low (5.0 to 8.0%). The proportion was higher in primary as compared to secondary facilities, but not significant. During the period of this study, prescribers spent a mean of 4.3 to 5.0 min in the secondary and 2.9 to 3.1 min in the primary facilities with patients. These figures correspond well with values measured in other developing countries (Hogerzeil et al., 1993). Health care providers have attributed the short time of contact with patients to their work overload, namely, a large number of patients. Though it is difficult to present optimal standards for consultation time, prescribers should take enough time to provide the patient with the necessary information regarding his/her condition and instructions/warnings related to the prescribed drug.

The mean dispensing time was found to be shorter ($p <$

0.005) in primary (24.8 to 36.1 s) than that observed in secondary (35.8 to 44.8 s) facilities. These figures are slightly higher than the average value obtained (12.5 s) from other studies in twelve developing countries (Hogerzeil et al., 1993) but far shorter than 86.1 s recorded in Nepalese pharmacies (Kafle et al., 1992). Although, from a management point of view, a short dispensing time could imply a very efficient dispensing system, from a clinical point of view, such a short time would not be expected to provide adequate counseling on medication, and may infer less attention to detail and a greater potential for errors. Factors that may account for a short dispensing time include pre-packed and pre-labeled drugs as well as a heavy flow of patients or even shortage of staff.

The percentage of drugs that were actually dispensed in secondary facilities was close to 100%, as recommended (Isah et al., 2006). This indicates a good stock control system in the secondary health care centers in Kano. In contrast, only 56.17% was actually dispensed in the primary facilities studied. The significant difference ($p < 0.0005$) between the secondary and primary facilities could be explained by the fact that the Drug Revolving Fund (DRF) scheme of all the secondary health care facilities in the state was supported by the Partnership for Transformation in the Health Sector in Nigeria (PATH), while only one primary facility out of the twelve selected was under the programme during the study period. Although the WHO (1993) and National Drug Policy of Nigeria (NDP) (2005) recommend that each drug label should contain the dose regimen, generic name of the drug and patient's name. In the study area, not a single dispensed drug was adequately labeled in all the facilities. Similar result was obtained in Cambodia (Chareonkul et al., 2002) and India (Rishi et al., 2003). At the end of the study, when the dispensers were asked about the inadequate labeling, they stated that given their typical workload they hardly had time to interact with the parents; hence, they prefer to draw pictograms and explain how the individual drugs should be taken.

About 90 and 80% of parents in the secondary and primary health care facilities, respectively claimed to know the correct dosage schedule. These figures, though higher than 55 to 68.3% reported in Bangladesh (Guyon et al., 1994), Burkina Faso (Krause et al., 1999), Cambodia (Chareonkul et al., 2002) and India (Rishi et al., 2003), did not necessarily reflect reality since the response "Yes, I know the dose" was accepted as positive answer.

Conclusion

Conclusively, this study provides few insights into the drug use patterns in children outpatient departments of primary and secondary public health care facilities in Kano State, Nigeria. The prescription from EDL was fair, the use of injections was low and there is a scope for improvement in case of medicines prescribed by generic name.

Conflict of interest

Authors declare that there are no conflicts of interest

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Performance, parasitic infections, hematology and hepatic histology of *Colossoma macropomum* (tambaqui) fed on homeopathic product

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Homeopathic products may act in an organism by stimulating the immune system, allowing the restoration of balance and encouraging organic response under stress. This study investigated the performance, blood, morphological and parasitological parameters in *Colossoma macropomum* (tambaqui) fed diets containing different concentrations of Homeopatila 100®. Juvenile tambaqui underwent four treatments with three replicates: 0, 20, 40 and 60 ml Homeopatila 100®/kg of extruded feed with 32% crude protein for 60 days. At the end of 60 days the growth performance, blood parameters, gill parasites and hepatic histology in fish fed homeopathic product were evaluated. Treatment with Homeopatila 100® did not improve the growth performance of fish. There was no difference in the prevalence and abundance of monogeneans and protozoans in the gills of fish, except in those fed with 60 ml homeopathic product. The plasma glucose levels were higher in fish fed diet containing 40 and 60 ml homeopathic product. The mean corpuscular volume and hematocrit levels in fish fed 20 and 60 ml were higher than in controls. In fish with fed 40 ml increased number of neutrophils and reduced number of lymphocytes was found. However, 60 ml of the product caused increase in the number of monocytes and reduced the number of lymphocytes, eosinophils and PAS-positive granular leukocytes. Under conditions in this study, the Homeopatila 100® did not improve fish performance or reduce parasitic infections, but showed a relative improvement in blood response of fish fed on 40 ml of this homeopathic complex.

Key words: Fish farming, homeopathy, monogenea, parasites, protozoa, blood.

INTRODUCTION

Currently, almost half of the fish production comes from aquaculture in Brazil, but due to demand for products

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based on fish, the production will increase in the coming decades, mainly by socioeconomic and health reasons. The country has a huge potential for expansion of this activity, such as favorable climate, possibility of use of Union's water for fish farming, both reservoirs and estuary, plus a large number of biodiversity species with livestock potential (Rocha et al., 2013). Moreover, it produces native species of great economic interest as *Colossoma macropomum* (tambaqui), a Serrasalmidae from the Amazon River basin.

Tambaqui is the most cultivated native fish in Brazil; its production was 111,084.1 tons in 2011, accounting for 20.4% of total domestic production. This production represents an increase of over 100% compared to 2010 (MPA, 2013). This increase in domestic production is mainly due to its zootechnical characteristics that favor the production of this Amazonian fish, such as rapid growth, relative resistance to diseases and good tolerance to high temperature and to low levels of dissolved oxygen in the water (Araujo-Lima and Gomes, 2005; Santos et al., 2013). It has regular supply of fingerlings and good yield of fillet without skin, its flesh is of good nutritional quality and its production cycle in cages is short, six to eight months (Oliveira et al., 2013).

Growth in fish farming is one of the most important and commonly used criteria to measure the fish response to the diet or ingredients used in the feed. Because growth is the measure of greatest applicability in the production systems to assess the growth performance of cultured fish, once it is closely related to productivity and profitability (Fracalossi et al., 2013), being assessed in different ways. With the intensification of the tambaqui fish farming, the disease problems have increased mainly by the infections of protozoan *Ichthyophthirius multifiliis* and monogenean helminthes, which may impair the production and productivity (Tavares-Dias et al., 2013). Such problems of parasitic diseases require constant need of treatment to reduce and control the parasites during intensive production of this fish. Prophylactic care should be permanent in fish farming of tambaqui, due to the difficulty of treating infectious and parasitic diseases when installed. Homeopathic products may act in the body of animals by stimulating the immune system, allowing the restoration of balance and encouraging organic responses in reducing stress. The use of such products, besides contributing to the prophylaxis by reducing the management stress, can reduce the use of chemotherapy and antibiotics, avoiding risks to the environment, animals and consumers (Siena et al., 2010). However, the use of homeopathic products and their potential benefits are virtually unknown in fish farming.

In *Oreochromis niloticus* (Nile tilapia), 40 ml Homeopatila 100®/kg diet improved the survival of fingerlings, feed conversion, hepatosomatic index, increased the number of muscle fibers, number of hepatocytes and hepatic glycogen (Vargas and Ribeiro, 2009; Braccini et al., 2013). However, there are few studies on the effects of homeopathic products in fish. This study evaluated the growth performance, blood and morphological parameters and gill infections in *C. macropomum* fed diets containing different concentrations of Homeopatila 100®.

MATERIALS AND METHODS

Experimental design

In this experiment, 300 fingerlings of *C. macropomum* (12.0 ± 0.5 cm and 42.7 ± 3.1 g) obtained from commercial fish farming (Macapá, AP, Brazil) were acclimated for seven days in water tanks. Fish were randomly distributed in water tanks (500 L), containing 400 L useful volume, maintained at a density of 25 fish per tank. The design was completely randomized with four treatments (20 ml hydroalcoholic solution - controls, 20, 40 and 60 ml Homeopatila 100®/kg diet) and three replications. The homeopathic product was added to the extruded commercial diet containing 32% crude protein and fish fed for 60 days.

Preparation of diets with homeopathic product

We used commercial diet containing 32% crude protein, 65 g ether extract, 70 g crude fiber, 100 g mineral matter, 12 g calcium, 6000 mg phosphorus,16000 IU vitamin A, 250 IU vitamin E, 4500 IU vitamin D3, 30 mg vitamin K3, 325 mg vitamin C and 32 mg thymine (B1) for each kg of diet. Weekly, Homeopatila 100® in the form of hydroalcoholic solution was incorporated to the commercial feed using a hand sprayer. Composition of the complex Homeopatila 100®, for 1000 ml: 250 ml of iodum 12 cH, 250 ml of sulphur 30 cH, 250 ml of natrummuriaticum 200 cH, 250 ml of streptococcinum 30 cH and q.s medium (ethylalcohol 30%). Subsequently, the feed was homogenized and dried at room temperature, removing it periodically for 24 h. The feed was stored in a cool dry place without any incidence of sunlight, chemicals and equipment that emitted magnetic field until being loose and without alcohol odor (Siena et al., 2010). The same inclusion process was conducted to the control treatment using 20 ml of 30% alcohol per kg feed. The amount of feed provided to fish was *ad libitum*, and three times a day (9:00, 13:00 and 17:00 pm).

Parameters of growth performance

In the initial and final experiment, all fish were weighed (g) in a digital scale and measured in total length (cm) using caliper to determine the following parameters of the body growth performance:

1. Weight gain (g) = WG = ($W_1 – W_2$)
2. Daily weight gain (DWG) = WG/t
3. Specific growth rate (%) = (SGR) = 100 × ($LnW_1 – LnW_2$) / t
4. Feed conversion rate (FCR) = Amount / weight gain

Where W_1 = mean weight (g) in the final experiment; W_2 = mean weight (g) in the initial experiment; t = time (days) of experiment

5. Relative condition factor (Kn) (Le-Cren, 1951).

Procedures for collection and analysis of blood parameters

After 60 days of feeding with 20 ml of hydroalcoholic solution

Table 1. Physical and chemical parameters of the farming water of *Colossoma macropomum* fed on different concentrations of Homeopatila 100®.

Treatments (mg/kg)	Oxygen (mg/L)	pH	Temperature (ºC)	Conductivity (µs/cm)	Total ammonia (mg/L)
Control	5.57±0.58[a]	6.05±0.41[a]	29.82±0.41[a]	0.036±0.003[a]	0.85±0.57[a]
20	5.43±0.64[a]	6.12±0.38[a]	29.90±0.42[a]	0.037±0.003[a]	0.66±0.37[a]
40	5.49±0.61[a]	6.09±0.39[a]	29.93±0.42[a]	0.038±0.005[a]	0.71±0.54[a]
60	5.46±0.63[a]	6.09±0.38[a]	29.98±0.43[a]	0.036±0.003[a]	0.69±0.37[a]

Means followed by different letters in the same column indicate differences between treatments by the Tukey test ($p < 0.05$). Values expressed as mean ± standard deviation.

(control), 20, 40 and 60 ml Homeopatila100®/per kg feed, five fish per replicate of the different treatments were anesthetized with eugenol (15 mg/L water) (Inoue et al., 2011) for blood collection. An aliquot of blood collected by puncture of the caudal vessel was collected from the 60 fish with the aid of syringes containing Ethylenediaminetetraacetic acid (10%). The blood was used to determine the total number of erythrocytes in a Neubauer chamber, concentration of hemoglobin using Drabkin's reagent and reading on a spectrophotometer at 540 nm absorbance and hematocrit by the microhematocrit method. With this data, the Wintrobe erythrocytic indices were calculated: mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). Blood smears were confectioned and stained with a combination for differential leukocyte counts in up to 100 cells of interest, in each extension and also for determining the total number of leukocytes and total thrombocytes (Ranzan-Paiva et al., 2013). Leukocytes were identified and classified in lymphocytes, monocytes, neutrophils, eosinophils and PAS-positive granular leukocytes (LG-PAS), following the recommendations of Tavares-Dias et al. (1999). A portion of blood was centrifuged to obtain the plasma and determination of the concentrations of glucose and total protein using kits from Doles (GO, Brazil) and spectrophotometric reading.

Parasitological analysis

After 60 days of feeding on 20 ml hydroalcoholic solution (control), 20, 40 and 60 ml Homeopatila100® per kg diet, 10 fish of each repetition of the different treatments were anesthetized with eugenol (15 mg/water) (Inoue et al., 2011), then the gills were collected for parasitological analysis. The gills of 120 fish were collected, fixed in 5% formalin and used for collection, fixation, quantification and preparation for the identification of parasites (Eiras et al., 2006). The prevalence and mean abundance were calculated (Bush et al., 1997) for each treatment.

Biometric and histological analyses of the liver

After 60 days of feeding on 20 ml of hydroalcoholic solution (control), 20, 40 and 60 ml Homeopatila100® per kg diet, 10 fish of each replication of the different treatments were anesthetized with 15 mg/L of eugenol (Inoue et al., 2011) for liver collection. The liver of 120 fish was removed, weighed on a precision scale used to determine the Hepatosomatic index (HSI) (Tavares-Dias et al., 2000). For each treatment, liver of two other fish of each repetition was collected, totaling 24 fish, washed in sodium chloride (0.85%) and fixed in Bouin solution for 12 h. Then, the fragments were washed in 70% ethanol for removal of picric acid, dehydrated and embedded in paraffin, following routine procedures. After making the slides (in duplicate) for morphological analysis and determination of the number of hepatocytes per area, the staining was performed with hematoxylin and eosin (Behmer et al., 1976) and for histochemical staining of hepatic glycogen (Beçak and Paulete, 1976); in order to quantify the percentage occupied by this inclusion/area, the periodic acid-Schiff (PAS) + hematoxylin was used. The morphometric analyses of liver tissue were performed from images captured on Olympus BX41 optical microscope coupled with camera Olympus Q-Color 3, using a 40× magnification objective. Fifty images of each fish were analyzed, totaling 300 images/treatment; the standardized floor area was 20914.7 μm^2. Such measurements were performed with the aid of image analysis of software Image Pro Plus® (Version 4.5, Media Cybernetics, USA).

Physical and chemical parameters of water in the tanks

In the experimental tanks, water renovation was constant and the accumulated debris was siphoned every two days. The water temperature, pH, dissolved oxygen levels, ammonia and electrical conductivity were measured daily, using digital devices (YSI, USA) for each purpose.

Statistical analyses

All data have been previously evaluated under assumptions of normality and homoscedasticity using Shapiro-Wilk and Bartlett tests, respectively. For data with normal distribution the analysis of variance (ANOVA - one way) was used, followed by Tukey test for means comparison. For data that did not follow this pattern of distribution the Kruskal-Wallis test was used, followed by Dunn's test for means comparison.

RESULTS

The physical and chemical parameters of the water in the farming tanks of tambaqui fed on different concentrations of Homeopatila 100® showed no differences between them (Table 1). Initial and final values of length and weight of tambaqui are in Figure 1. There was no significant difference ($p > 0.05$) among treatments after 60 days of feeding on homeopathic product. After 60 days of fish feeding, the weight gain, feed conversion ratio and specific growth rate were different only in fish kept at 60 ml Homeopatila 100®/kg diet compared to control and other treatments. However, the Kn of fish was not influenced by treatments with homeopathic product (Table 2). The gills of the fish were parasitized by

Table 2. Parameters of production performance of *Colossoma macropomum* fed on different concentrations of Homeopatila 100® for 60 days.

Treatments (ml/kg)	WG (g)	DWG(g/day)	FCR	SGR (%)	Kn	S (%)
Control	154.4 ± 34.3^{ab}	2.6 ± 0.57^{a}	1.52	2.51 ± 0.33^{ab}	0.99 ± 0.01^{a}	100
20	167.1 ± 41.3^{ab}	2.8 ± 0.68^{a}	1.53	2.63 ± 0.34^{a}	1.00 ± 0.01^{a}	100
40	169.8 ± 47.69^{b}	2.8 ± 0.79^{a}	1.57	2.65 ± 0.40^{a}	1.00 ± 0.03^{a}	100
60	150.57 ± 42.03^{a}	2.5 ± 0.70^{b}	1.72	2.47 ± 0.35^{b}	0.99 ± 0.01^{a}	100

WG: Weight gain; DWG: Daily weight gain; FCR: Feed conversion rate; SGR: specific growth rate; Kn: relative condition factor; S: Survival. Means followed by different letters in the different rows indicate differences between treatments by the Tukey test ($p < 0.05$). Values expressed as mean ± standard deviation.

Table 3. Prevalence (P%) and mean abundance (MA) of parasites in the gills of *Colossoma macropomum* fed different concentrations of Homeopatila 100®/kg feed for 60 days.

Parasite species	Control		20 ml/kg		40 ml/kg		60 ml/kg	
	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA
Ichthyophthirius multifiliis	100	7.144.3 ± 4033.6^{a}	100	13.310.9 ± 4997.0^{b}	100	10.401.8 ± 6853.3^{ab}	100	54.108.3 ± 70851.9^{c}
Piscinoodinium pillulare	0	0^{a}	0	0^{a}	0	0^{a}	30	362.5 ± 615.43^{b}
Anacanthorus spatulathus	100	24.1 ± 18.0^{a}	100	40.6 ± 59.5^{ab}	100	24.9 ± 14.2^{a}	100	53.2 ± 33.1^{b}
Notozothecium janauachensis	100	27.1 ±15.3^{a}	100	30.5 ± 30.60^{a}	100	36.0 ± 26.2^{ab}	100	51.9 ± 31.0^{b}
Mymarothecium boegeri	100	15.3 ± 4.8^{a}	100	14.7 ± 10.0^{a}	100	22.8 ± 21.0^{a}	100	43.4 ± 27.7^{b}
Linguadactyloides brinkimanni	23.3	0.96 ± 2.8^{a}	13.3	0.63 ± 2.3^{a}	3.3	0.1 ± 0.5^{a}	10	54.108.3 ± 70851.9^{c}

Values expressed as mean ± standard deviation. Means followed by different letters in the same row indicate difference between treatments by the Dunn test ($p < 0.05$), for mean abundance.

Ichthyophthirius multifiliis Fouquet, 1876 (Ciliophora), *Piscinoodinium pillulare* (Schäperclaus, 1954) Lom 1981 (Dinoflagellida), *Anacanthorus spathulatus* Kritsky, Thatcher and Kayton, 1979, *Notozothecium janauachensis* Belmont-Jegu, 2004, *Mymarothecium boegeri* Cohen and Kohn, 2005 and *Linguadactyloides brinkimanni* Thatcher and Kritsky, 1983 (Dactylogyridae). There was no difference in the prevalence of these parasites among groups of fish fed on different concentrations of homeopathic product. However, *P. pillulare* occurred only in fish maintained on diets containing 60 ml of Homeopatila100® (Table 3).

In hosts, the highest parasite abundance was of *I. multifiliis* and the lowest abundance was of *L. brinkimanni*. The abundance of *I. multifiliis* was higher in fish fed on diets containing 20 and 60 ml Homeopatila 100® compared to control fish. The abundance of *A. spatulathus*, *N. janauachensis* and *M. boegeri* was higher in fish fed diets containing 60 ml of homeopathic product when compared with controls (Table 3). Plasma glucose levels were higher in fish fed on diets containing 40 and 60 ml Homeopatila 100® when compared to controls. Hematocrit and Mean corpuscular volume (MCV) of fish fed on diets containing 20 and 60 ml of homeopathic product were higher than in controls. In fish fed diet contaning 20 ml of homeopathic product, the total number of thrombocytes and monocytes were higher than in controls. However, the number of lymphocytes was reduced in all groups fed on different concentrations of homeopathic products. The number of neutrophils was increased only in fish with 40 ml Homeopatila 100® when compared to controls, while the number of PAS-LG and eosinophils were smaller in fish fed on this homeopathic product 100® (Table 4).

The percentage of glycogen did not differ among treatments with homeopathic product. However, the number of hepatocytes was lower in

Table 4. Blood parameters of *Colossoma macropomum* fed on different concentrations of Homeopatila 100®/kg feed for 60 days.

Parameters	Control	20 mL/kg	40 ml/kg	60 ml/kg
Glucose (mg dl^{-1})	93.4 ± 5.4[a]	99.9 ± 8.6[a]	110.9 ± 11.5[b]	113.6 ± 12.4[b]
Protein (mg dl^{-1})	3.6 ± 0.5[a]	3.6 ± 0.3[a]	3.6 ± 0.4[a]	3.6 ± 0.4[a]
Hematocrit (%)	19.7 ± 1.5[a]	22.5 ± 3.7[b]	20.7 ± 2.3[ab]	22.9 ± 1.8[b]
Hemoglobin (g/dl^{-1})	7.2 ± 1.3[a]	7.25 ± 0.97[a]	7.0 ± 0.7[a]	7.5 ± 0.9[a]
RBC (number $10^6/\mu l^{-1}$)	0.99 ± 0.17[a]	0.87 ± 0.20[a]	0.95 ± 0.2[a]	0.96 ± 0.26[a]
MCV (fL^{-1})	204.5 ± 33.9[a]	263.9 ± 55.6[b]	224.5 ± 39.3[ab]	251.3 ± 55.8[b]
MCHC (g/dl^{-1})	36.8 ± 6.1[a]	32.8 ± 4.7[a]	33.9 ± 3.6[a]	32.8 ± 2.3[a]
Thrombocytes (number μl^{-1})	19.000 ± 6577[a]	12.682 ± 3856[b]	30.8747 ± 8073[c]	27.230 ± 14599[ac]
Leukocytes (number μl-^{1})	48.877 ± 9706[a]	30.683 ± 9667[b]	43.235 ± 12115[a]	42.062 ± 12675[a]
Lymphocytes (number μl^{-1})	20.938 ± 5151[a]	11.762 ± 5059[b]	13.209 ± 6887[b]	11.193 ± 4164[b]
Monocytes (number μl^{-1})	18.397 ± 6083[ac]	11.040 ± 3980[b]	15.359 ± 4826[a]	22.330 ± 8524[c]
Neutrophils (number μl^{-1})	4589 ± 2927[a]	4611 ± 3330[a]	9646 ± 5586[b]	6.565 ± 3654[ab]
Eosinophils (number μl^{-1})	3269 ± 2494[a]	2.029 ± 943[ab]	3349 ± 2225[a]	1.380 ± 721[b]
PAS-LG (number μl^{-1})	1802 ± 9618[a]	1431 ± 1042[ab]	1671 ± 1163[a]	743 ± 334[b]

Means followed by different letters in the different rows indicate differences between treatments by the Dunn test ($p < 0.05$). Values expressed as mean ± standard deviation. PAS-LG: PAS-positive granular leukocytes.

Table 5. Number of hepatocytes, hepatic glycogen and hepatosomatic index (HSI) of *Colossoma macropomum* fed on different concentrations of Homeopatila 100® for 60 days.

Treatments (ml/kg)	No. hepatocytes	Glycogen (%)	HSI (%)
Control	247.8 ± 38.1[a]	19.5 ± 2.7[a]	2.17 ± 0.15[ab]
20	251.8 ± 38.1[a]	19.8 ± 2.6[a]	2.17 ± 0.20[a]
40	223.3 ± 34.1[b]	19.4 ± 1.6[a]	1.99 ± 0.34[b]
60	246.9 ± 25.7[a]	19.0 ± 2.2[a]	2.05 ± 0.29[ab]

Means followed by different letters in the same column indicate differences by the Dunn test ($p < 0.05$). Values expressed as mean ± standard deviation.

fish treated with 40 ml of Homeopatila 100®/kg of feed when compared to the other treatments, but the hepatosomatic index was lower than that in fish treated with 20 ml of homeopathic product (Table 5).

There was no morphological change in the liver of fish fed on diets containing homeopathic product and controls. Fish treated with 20 ml Homeopatila 100®/kg diet showed diffuse melanomacrophage centers in the hepatocytes and blood vessels, while in fish kept in 40 ml homeopathic product, such structures were only observed in blood vessels. However, in control fish and with 60 ml homeopathic product melanomacrophage centers were observed (Figure 2).

DISCUSSION

In Tambaqui fed control diet 20 ml of hydroalcoholic solution, 20, 40 and 60 ml of Homeopatila100® there was no mortality for 60 days. Such concentrations of Homeopatila 100® when added to the diet of Nile tilapia increased the survival of fingerlings for 60 days (Siena et al., 2010; Braccini et al., 2013). 20 and 40 ml Homeopatila 100®/kg did not affect the performance parameters of tambaqui similarly to that described for Nile tilapia fed the same concentrations of that homeopathic product in commercial feed (Siena et al., 2010). This homeopathic product had no benefic effect on feed conversion of tambaqui, since the best results were observed in the control group, unlike the expected, as it improved feed conversion in Nile tilapia fed on diets containing 40 ml of Homeopatila 100®/kg feed (Siena et al., 2010; Braccini et al., 2013). In addition, feeding on 60 ml Homeopatila 100® reduced weight gain, mean daily gain, specific growth rate and increased feed conversion of tambaqui. Moreover, this concentration did not affect apparent feed conversion, weight and length of Nile tilapia (Siena et al., 2010; Braccini et al., 2013) kept under similar conditions to the present study.

The condition factor, a quantitative indicator of fish's

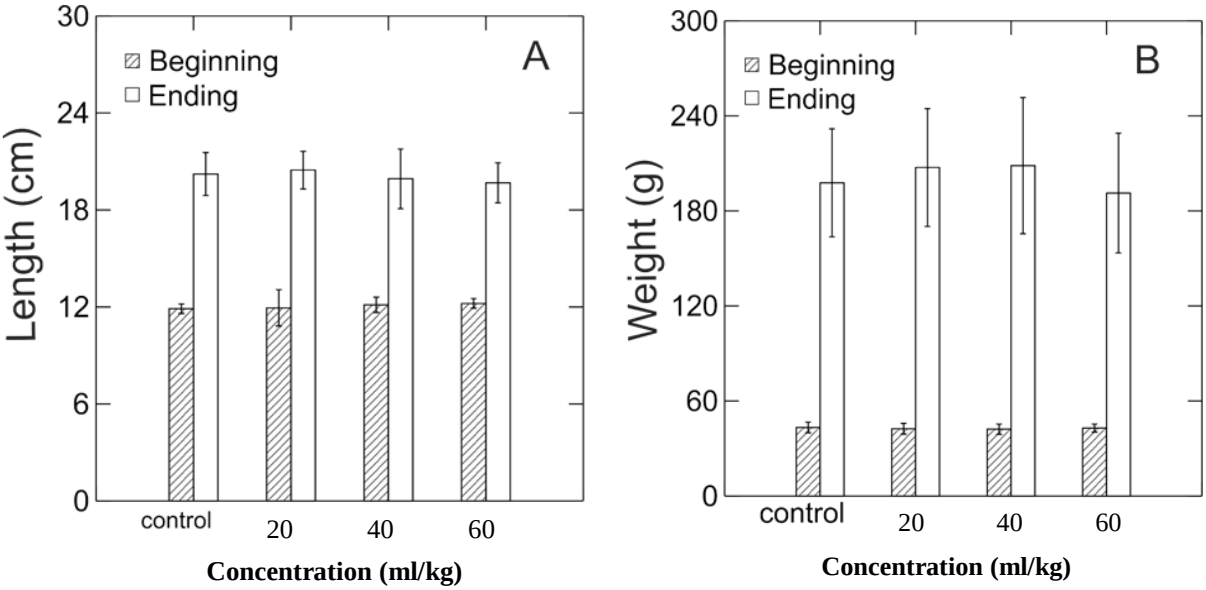

Figure 1. Initial and final length (**A**) and initial and final body weight (**B**) of *Colossoma macropomum* fed during 60 days with different concentrations of Homeopatila 100®. Values expressed as mean ± standard deviation.

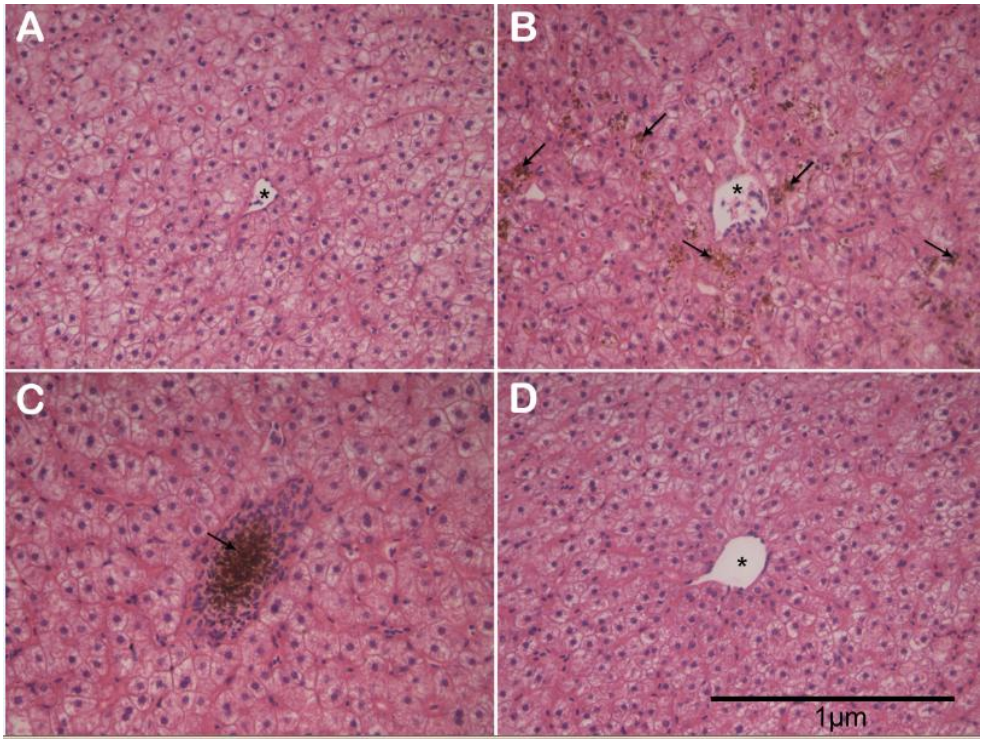

Figure 2. Liver morphology of *Colossoma macropomum* fed 0 mL of Homeopatila 100®/kg. (A) 20 ml, (B) 40 ml, (C) and 60 ml (D), highlighting the presence of melanomacrophage centers (arrows). *Indicate blood vessel.

body condition used to evaluate the different feeding conditions, interference of population density and other environmental conditions (Le-Cren, 1951; Guidelli et al., 2011) showed no difference among treatments of Homeopatila 100® in tambaqui which was similar to that found in Nile tilapia fed this same homeopathic product

and at the same concentrations (Valentim-Zabott et al., 2008).

No difference in mean daily gain and specific growth rate was observed in tambaqui (42.7 ± 3.1 g) fed diet containing 32% crude protein and 20 ml of hydroalcoholic solution (control), 20 and 40 ml of Homeopatila 100®. Such growth performance parameters were higher than those described by Lemos et al. (2012) for the same fish (7.7 ± 0.2 g) when fed diets containing 26% crude protein; but the feed conversion was better. Moreover, the weight gain was similar to that reported by Pereira-Junior et al. (2013), for tambaqui (6.6 ± 0.1 g) fed on diets containing 38.3% crude protein, but apparent feed conversion was also lower. However, such differences are attributed to the initial fish size, which were larger in this study, and also to the different levels of protein used in the diet. Therefore, these results indicate that, during growth, tambaqui seems to have better feed conversion than at the beginning of fattening.

The gills of tambaqui fed diets containing 20 ml of hydroalcoholic solution (controls), 20, 40 and 60 ml Homeopatila 100®/kg diet were found infected by *I. multifiliis, P. pillulare* and four monogenean species (*A. spathulatus*, *N. janauachensis, M. boegeri* and *L. brinkimanni),* but *I. multifiliis* was the most abundant parasite and *P. pillulare* was only found in fish treated with 60 ml of this product. However, with the use of homeopathic product only *L. brinkimanni* showed reduction in prevalence, because regardless of treatment, all fish were parasitized by *I. multifiliis* and monogenean species, but the abundance of these parasites was higher in fish treated with 60 ml of product homeopathic. In addition, there was no difference in the prevalence of *Trichodina* sp. and Gyrodactylidae species as well as the mean intensity of parasites for Nile tilapia maintained for 60 days with diets containing these same concentrations of Homeopatila 100® (Braccini et al., 2013).

Tambaqui fed 60 ml Homeopatila 100® had lower growth performance and hence were the most parasitized by monogeneans and *I. multifiliis*, since the latter is an opportunistic protozoan. Moreover, infection with *P. pillulare*, another opportunistic protozoan, occurred only in those fish. However, for goats using homeopathic products, the number of eggs of gastrointestinal helminthes was reduced (Neves et al., 2012), but for sheep these drugs had no antiparasitic efficacy (Cavalcante et al., 2007; Signoretti et al., 2008), as has occurred in fish in the present study. Although the use of homeopathic products do not always have efficacy against helminthes, homeopathy can help to reduce the effects of parasitic infections, balancing the host-parasite relationship (Cavalcante et al., 2007; Signoretti et al., 2008).

In this study, only fish fed diets containing 20 and 60 ml Homeopatila 100® had higher hematocrit and higher MCV, while the levels of plasma protein, hemoglobin concentration, hematocrit, red blood cell count and MCHC were not affected by treatment with the homeopathic product. However, Nile tilapia also fed with Homeopatila 100® showed reduction in plasma levels of cortisol, glucose, hematocrit, hemoglobin, red blood cell count and MCHC (Vargas and Ribeiro, 2009), then featuring a macrocytic-hypochromic anemia. On the other hand, no sign of anemia occurred in all fish of this study, but there was a higher concentration of glucose in fish of the highest concentrations of Homeopatila 100®, showing signs of stress. However, fish treated with 60 ml of Homeopatila 100® had higher parasitism, caused possibly by stress (Tavares-Dias et al., 2009b).

The thrombocytes are multifunctional cells of fish, since they primarily participate in the coagulation process and secondly, assist the defense mechanism (Ranzani-Paiva et al., 2013; Santos and Tavares-Dias, 2011), thus being in constant movement between the hematopoietic organs and circulation. Reduction in the number of these blood cells may indicate a hemostatic disorder. In this study, fish fed 20 ml Homeopatila 100® showed reduced number of thrombocytes, while those fed 40 ml showed an increase. Vargas and Ribeiro (2009) reported increased percentage of thrombocytes for Nile tilapia fed with this same homeopathic product.

In fish, the leukocyte count is an important tool to infer the state of health and immune system because of the many functions of these cells. Lymphocytes are white blood cells involved in a variety of immune functions such as immunoglobulin production and modulation of defense. Neutrophils are the first phagocytic leukocytes in response to infection. Monocytes and PAS-LG are phagocytes that perform migration to the inflammatory site during infectious processes. Eosinophils are white blood cells that may participate in the defense process against parasites (Ranzani-Paiva et al., 2013; Santos and Tavares-Dias, 2011). In *C. macropomum*, 20 ml of Homeopatila100® caused leukopenia due to lymphocytopenia and monocytopenia, while 40 ml of this product led to a Neutrophilia accompanied by lymphocytopenia. However, treatment with 60 ml of Homeopatila 100® resulted in monocytophilia accompanied by lymphocytopenia, eosinopenia and reduced number of LG-PAS. Similarly, the use of Homeopatila 100® also caused a reduction in the percentage of lymphocytes and eosinophils in Nile tilapia (Vargas and Ribeiro, 2009). Neutrophilia has been reported to fish with parasitic infections and sometimes can be accompanied by lymphocytopenia, depending on the stage of infection and effects of stress caused by parasitism (Santos et al., 2011).

In fish, the liver is a hematopoietic organ, but also stores large amount of glycogen and fat, then such reserves can influence its weight, also interfering with the hepatosomatic index (Tavares-Dias et al., 2000; Barbosa et al., 2011). In *C. macropomum*, 60 days of feeding on diets containing different concentrations of Homeopatila 100® did not affect the Hepatosomatic index and per-

centage of hepatic glycogen, but 40 mL reduced the amount of hepatocytes. However, in *O. niloticus* treated with Homeopatila 100®, there was decreased amount of lipid inclusion in the liver and consequently in the HSI, but it has been reported to increase on hepatocytes number (Siena et al., 2010; Vargas and Ribeiro, 2009).

The liver also controls many vital functions as it has important role in physiology and immunity. It has perisinusoidal cells of the reticulum-endothelial system that, when present, have a phagocytic function, because they are a type of macrophage known as melano-macrophages (Bombonato et al., 2007). The melano-macrophages have several functions in fish, such as phagocytosis or pathogens, antigen processing in the immune response, destruction, detoxication or recycling of endogenous and exogenous materials, deposits of metabolites of dead cells, including red blood cells, as well as response to different antigens (Campos et al., 2008; Manrique et al., 2014). *Colossoma macropomum* maintained with 20 ml Homeopatila 100®/kg diet showed diffuse melanomacrophages centers in hepatocytes, but in fish kept with 40 ml of this homeopathic product, such structures were restricted to vascular channels (Campos et al., 2008).

Conclusion

For *C. macropomum* the Homeopatila 100® did not reduce the infections of parasites in the gills, but showed a relative improvement in blood parameters of fish fed on 40 ml. As the Homeopatila 100® has not improved the performance of the fish; therefore, the use of this homeo-pathic product is an additional cost in fish production for the fish farm. However, this homeopathic product was prepared in principle aiming at requirements of Nile tilapia, African fish with different biology from *C. macropomum*, an Amazonian fish.

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Conflict of interest

Authors declare that there are no conflicts of interest

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In vitro evaluation of the antibacterial activities of the methanol, aqueous and n-hexane extracts of *Ocimum lamiifolium* from Ethiopia

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Ocimum lamiifolium **(local name Dama Kesse, Amharic) is a medicinal plant in Ethiopia. Its leaves are squeezed and sniffed to treat coughs and colds. They are also used to treat eye infections and to stop nose bleedings. In the present study, leaves of *O. lamiifolium* were collected from their growing habitats. Dried leaf powders were extracted using methanol, distilled water and n-hexane. 25, 50, and 100 mg/ml doses of the extracts made in Tween 80 (2%) were screened for their antimicrobial activities against *Staphylococcus aureus*, *Escherchia coli, Pseudomonas aeruginosa* and *Shigella boydii* using disk diffusion assay. The inhibition zones due to the methanolic extract ranged from 0 (in *S. aureus* due to 25 mg/ml*)* to 12 mm (in *E. coli* due to 100 mg/ml). Inhibition zones due to the aqueous extract ranged from 8 mm in *S. aureus* and *S. boydii* to 12 mm in *S. boydii* at concentrations of 25 and 100 mg/ml, respectively. The n-hexane extract at 25 mg/ml resulted in inhibition zone that ranges from 7 mm (against *S. aureus*) to 11 mm (against *E. coli*) at 50 and 100 mg/ml doses. The minimum inhibitory concentration of *S. boydii* and *E. coli* was 10 mg/ml due to all the extracts. The minimum inhibitory concentrations on *S. aureus* were 10, 20 and 50 mg/ml due to the aqueous, n-hexane and methanolic extracts, respectively. *P. aeruginosa* was minimally inhibited at 10 mg/ml due to the methanol and aqueous extracts and 15 mg/ml due to the n-hexane extract. The methanol, aqueous, and n-hexane extracts of *O. lamiifolium* leaf extracts inhibited the test bacteria with significantly higher levels of inhibition zones than the negative control (T80). The positive controls (Tetracycline and Chloramphenicol) also showed significantly higher inhibition zones than the 100 mg/ml concentration of the extracts and T80 except that Chloramphenicol failed to inhibit *S. aureus* and *P. aeruginosa.* However, combination of Chloramphenicol with plant extracts raised their inhibition zones from zero to 23 and 25 mm in *S. aureus* and *P. aeruginosa*, respectively.**

Key words: *Ocimum lamiifolium*, antibacterial activity, methanolic extract, aqueous extract, n-hexane extract.

INTRODUCTION

The genus *Ocimum* (Lamiaceae) consists of about 30 species distributed in the tropics and subtropics of the

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Old and New worlds, with some species cultivated in temperate areas. *Ocimum lamiifolium* Hochst.ex Benth(local name Tossign, Amharic) is mostly found in clearings and edges of primary and secondary mountain forests and bushlands, tall grasslands, abandoned fields, at altitudes between 1200 and 2900 m. Traditionally, the fresh leaves are squeezed and the juice is sniffed to treat cough and cold. The juice is also used as eye rinse to treat eye infections. At the same time, the crushed leaves are put in the nostrils to stop nose bleeding (Asfaw and Demissew, 2009).

Biologically, different extracts of the genus *Ocimum* are known for their antibacterial (Nakamura et al., 1999; Nascimento et al., 2000; Adebolu and Oladimeji, 2005; Adiguzel et al., 2005; Ahmad and Aqil, 2007; Goyal and Kaushik, 2011; Patil et al., 2011; Sneha et al., 2011; Prasannabalaji et al., 2012), antifungal (Amadioha, 2001), and antioxidant (Hakkim et al., 2008) activities. *O. lamiifolium* extracts are also known to have antibacterial, antifungal, insecticidal and insect repellent (Dagne, 2009), antiinflammatory (Kashyap et al., 2011) activities. The chemical composition of essential oils of six *Ocimum* species from East Africa including *O. lamiifolium* were majorly phenyl propane derivatives or terpenoids, including methyl eugenol, 1, 8-cineole, camphor, bornyl acetate, germacrene-D, E-myroxide, germacrene-B, caryophylene oxide and p-cymene (Kashyap et al., 2011). In another study by Tchoumbougnang et al. (2014), 85.7% of *O. lamiifolium* essential oils were monoterpenes [sabinene (33.8%), (Z)-β-ocimene (17.2%), terpinen-4-ol (8.4%) and others] and sesquiterpenes (8.7%) [β-caryophyllene (5.6%), germacrene D (1.1%), (E)-β-farnesene (1%) and others)]. Sabinine is known to have inhibition effects against Gram negative and Gram positive bacteria (Wiart, 2006; Uinoiseau et al., 2010). In addition, eugenol, a component of *Ocimum* has antibacterial and antihelmintic activities (Adebolu and Oladimeji, 2005). Components of *Ocimum basilicum* like apigenin, linalool and ursolic acid, exhibit a broad spectrum of antiviral activity and are used as remedies for treating disorders such as viral ocular, respiratory and hepatic infections (Chiang et al., 2005). The aim of the present study, however, was to test the antibacterial activities of the methanol, aqueous, and n-hexane extracts of the leaves of *O. lamiifolium*.

MATERIALS AND METHODS

Dried and powdered leaves of *O. lamiifolium,* methanol (Reagent chemical Services Ltd., United Kingdom), n-hexane (Uni-Chem Chemical Reagents), nutrient agar (Oxoid LTD., Bsingstoke, Hampshire, England), Muller-Hinton agar (Oxoid LTD., Bsingstoke, Hampshire, England), sulfuric acid (SDFCL Fine Chemical Ltd., Mumbai, India), Tween 80 (Uni-Chem Chemical Reagents), sodium chloride (Nike Chemical, India), cotton swab (Nataso, India), tetracycline (Oxoid Ltd., United Kingdom), chloramphenicol (Oxoid Ltd., United Kingdom), barium chloride (BDH Chemicals Ltd. Poole, England), an autoclave (Express autoclave, Dixons surgical Ltd.), petri dishes, and distilled water (Biomedical Laboratory, Addis Ababa University, Ethiopia).

Plant collection and identification

O. lamiifolium leaves were collected from their natural habitats Central and North East Ethiopia. The plants were not flowering during the period of collection. The collected specimens were authenticated by botanists from the National Herbarium of Addis Ababa University and voucher specimens were deposited at the same herbarium of Addis Ababa University.

Extraction of plant

Collected leaves of *O. lamiifolium* were washed by distilled water and subjected to shade drying at 25°C. Then the dried leaves were pulverized to get course powder. 100 g of the powder was added to 1 L (1:10, w:v) of three solvent types, namely, methanol (absolute), n-hexane (absolute), and distilled water and each mixture was shaked for 48 h at 120 rotations/min. The solutions were filtered by Whatman No. 1 filter paper. Finally, the methanol and hexane extracts were concentrated under vacuum in a rotary evaporator (Büchi Laboratoriums-Tchnik AG CH-9230 Flawil/Schweiz) to give gummy residues and the aqueous extracts using a lyophylizer (Bioblock Scientific, Illkirch Cedex, France). The crude extracts were then weighed and the yield of the each extract was calculated as 17.8, 12 and 6.7% (methanol, aqueous and n-hexane extract, respectively).

Bacterial strains

Clinical isolates of *Staphilococcus aureus*, *Shigella boydii*, *Escherichia coli*, and *Pseudomonas aeruginosa* were obtained from the Ethiopian Public Health Institute (EPHI). These isolates were screened for their susceptibility towards different doses of the different extracts of *O. lamiifolium* as well as two standard antibiotics [Tetracycline (30 µg/disk) and Chloramphenicol (30 µg/disk)]. In order to perform the antimicrobial screening, the bacterial isolates were cultured overnight at 37°C on Nutrient Agar medium. Colonies collected from each 24 h old bacterial culture were diluted in sterile saline and the optical density was adjusted in comparison with 0.5 McFarland' scale to prepare a standardized inoculum (1.5×10^8 cfu/ml). The bacteria from saline solutions were spread on Müller Hinton Agar plates using sterile cotton swabs.

The paper disc diffusion technique was applied to determine the antimicrobial activities of the tested plant extracts. Sterile paper discs (5 mm in diameter) immersed in stock solutions containing 25, 50 and 100 mg/ml prepared in 2% Tween 80 of plant extracts were placed on the surface of inoculated Nutrient Agar plates. Plates were then incubated for 24 h at 37°C, and diameters of the inhibition zones were recorded. All assays were applied in triplicates and the results are given as means ± standard error of the mean.

Determination of minimum inhibitory concentration (MIC)

MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of microorganisms after overnight incubation (Yilmaz, 2012). MICs were defined as the lowest concentration of the aqueous, methanol and n-hexane extracts of *O. lamiifolium* inhibiting visible growth of the bacteria. On the other hand, the MBC was defined as the lowest concentration of the extracts of *O. lamiifolium* required to kill all the test bacteria (Yilmaz, 2012). The MIC was determined using agar dilution method which is described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and

Table 1. Minimum inhibitory concentrations (MIC) of *Ocimum lamiifolium* leaf extracts.

Bacterial species	Gram type	MIC (mg/ml)		
		Methanol extract	Aqueous extract	n-Hexane extract
S. boydii	-	10	10	10
S. aureus	+	50	10	20
E. coli	-	10	10	10
P. aeruginosa	-	10	10	15

Infectious Diseases (ESCMID, 2000). The following procedure was followed to determine the MIC; 20 ml agar was used in 9-cm Petri dishes for agar dilution. Nineteen-milliliters molten agar was added to 1 ml of each plant extract to make the total volume 20 ml. Müller Hinton agar was prepared as recommended by the manufacturer. The sterilized agar was set to cool to 50°C in a water-bath. Extracts of *O. lamiifolium* were prepared into doses of 5, 10, 15, 20, 25, 50 and 100 mg/ml in 25 to 30 ml containers. Nineteen-milliliters of molten agar was added to each container and mixed thoroughly, and finally poured into pre-labeled sterile Petri dishes on a level surface. The plates were allowed to dry at room temperature so that no drops of moisture remain on the surface of the agar.

Bacterial suspensions were prepared in 0.85% normal saline and were standardized by 0.5 McFarland standards to 1.5×10^8 colony forming units (CFU)/ml. The inocula were inoculated on the dry plates. The inoculum spots were then allowed to dry at room temperature before inverting the plates for incubation. Finally, the plates were incubated at 37°C in air for 18 h. The MIC (the lowest concentration of the extracts that completely inhibited visible growth) was judged by the naked eye.

Determination of susceptibility test of bacteria towards standard antibiotics and their combinations with *O. lamiifolium* extracts

Susceptibility of the test bacteria towards Chloramphenicol, Tetracycline and their combinations with the leaf extracts of *O. lamiifolium* was determined according to the classification indicated by Bauer et al. (1966). Based on this literature, inhibition zones due to Chloramphenicol (30 µg) can be classified as resistant (≤12 mm), intermediate (13 to 17 mm), and sensitive (≥18 mm) and zones of inhibition for Tetracycline (30 µg) are interpreted as resistant (≤14 mm), intermediate (15 to 18 mm), and sensitive (≥19 mm).

RESULTS

Antibacterial activities of *O. lamiifolium* leaf extracts

The methanol extract inhibited the test bacteria in a dose dependent manner (Figure 1). At 25 mg/ml, it did not inhibit *S. aureus* while the rest bacteria were inhibited by this dose with inhibition zones just below 10 mm. The 50 mg/ml concentration of the methanol extract on the other hand inhibited all the bacteria with mean inhibition zones ranging from 6 mm (*S. aureus*) to over 10 mm (*E. coli*). At 100 mg/ml concentration, the methanol extract inhibited three of the test bacteria with mean inhibition zones above 10 mm and *S. aureus* with mean inhibition zone close to 10 mm. The antibacterial activity of the methanol extract was generally lower than that of Tetracycline and Chloramphenicol. However, it was better than Chloramphenicol in inhibiting *S. aureus* and *S. boydii*. The aqueous extract inhibited all the test bacteria at 25, 50 and 100 mg/ml doses minimally inhibiting *S. aureus* and *E. coli* each with mean inhibition zones of 8 mm at 25 mg/ml dose and maximally *S. boydii* (12 mm) at 100 mg/ml. The aqueous extracts too were generally less effective than Tetracycline and Chloramphenicol although Chloramphenicol resistant strains (*S. aureus* and *P. aeruginosa*) were sensitive to these extracts. Like that of the aqueous extract, the n-hexane extract inhibited all the test bacteria at the three dose levels, *S. aureus* being inhibited minimally (7 mm) at 25 mg/ml and *E. coli* being inhibited maximally (11 mm) at 100 mg/ml. In general, the trend of inhibition of the test bacteria by the three extracts of *O. lamiifolium* showed that the aqueous extract is the best followed by its methanol and n-hexane extracts, respectively.

Determination of the MIC

The MIC concentrations of O. *lamiifolium* leaf extracts ranged from 10 to 50 mg/ml (Table 1). The 50 mg/ml concentration of its methanol extract inhibited all the bacteria and its 10 mg/ml inhibited 75% of them. The aqueous extract, on the other hand, inhibited all the bacteria at a concentration of 10 mg/ml and the n-hexane extract inhibited the microorganisms with a range of MICs from 10 to 20 mg/ml of which the 20 mg/ml inhibited the entire, 15 mg/ml inhibited 75%, and 10 mg/ml inhibited 50 percent of them.

Antibacterial effects of the combinations of Tetracycline (30 µg/ml) and Chloramphenicol (30 µg/ml) with *O. lamiifolium* leaf extracts at 100 mg/ml

Chloramphenicol (30 µg) resulted in inhibition zones of 31 and 33 mms in S. *boydii* and *E. coli*, respectively (Figure 2). On the contrary, it did not inhibit *S. aureus* and *P. aeruginosa*. Tetracycline (30 µg) inhibited *S. aureus* with inhibition zone of 11 mm and the rest bacteria with inhibition zones above 19 mm. Combination of these antibiotics to *O. lamiifolium* extracts at a dose of 100

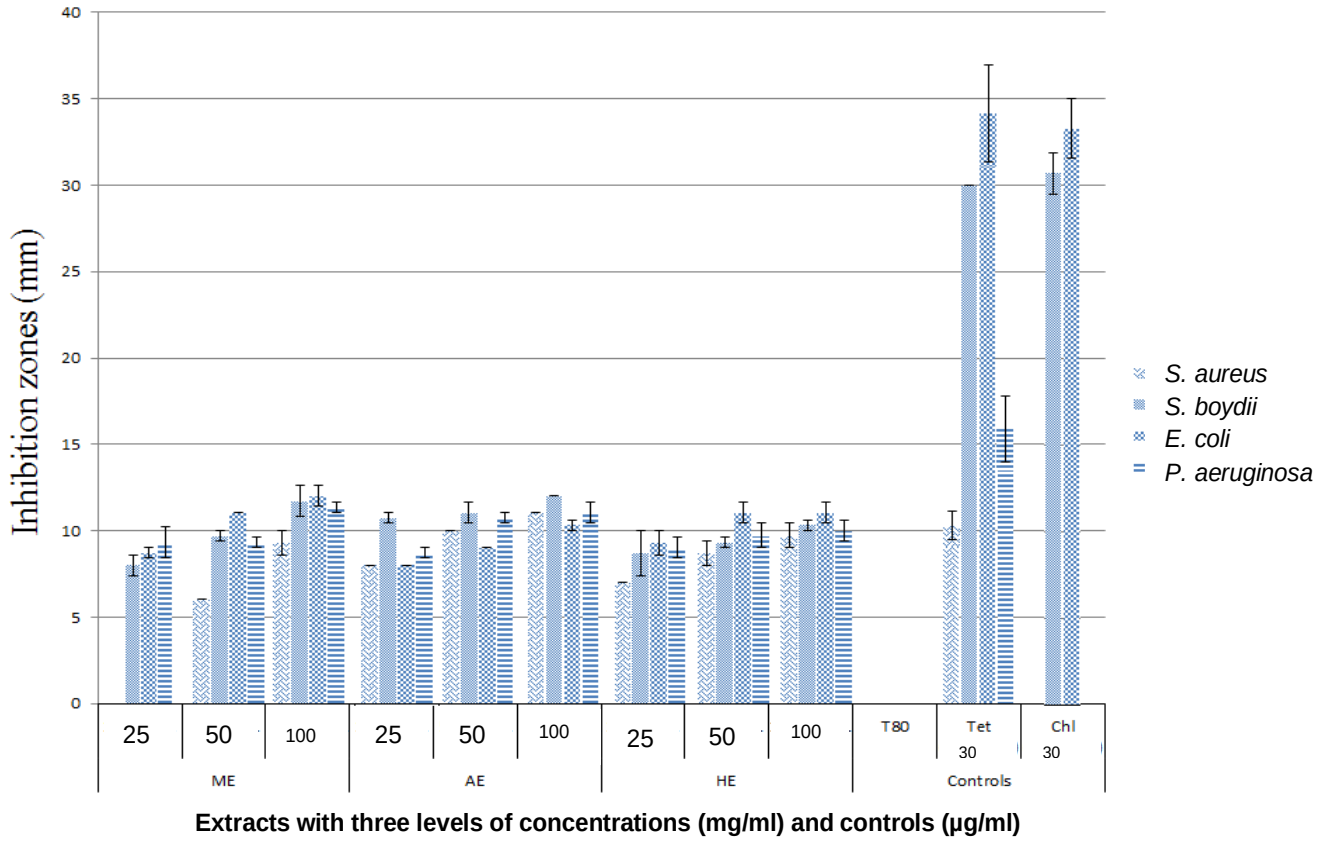

Figure 1. Inhibition zone (mm) of *O. lamiifolium* leaf extracts against Gram positive and negative bacteria. Tet: Tetracycline; Chl: Chloramphenicol; T80: Tween 80; ME: Methanol extract; AE: Aqueous extract; HE: n-hexane extract.

mg/ml, however, increased the inhibition zones of the test bacteria. Inhibition of *S. boydii* and *E. coli* due to Chloramphenicol surpassed inhibition due to the combination of Chloramphenicol and plant extracts (100 mg/ml) (Figure 2). On the contrary, inhibition of *S. aureus* and *P. aeruginosa* was found to be higher than either caused by Chloramphenicol or extracts. In the same manner, inhibition of *S. boydii* and *E. coli* by Tetracycline were higher than inhibition by combination of plant extracts (100 mg/ml) and Tetracycline while *S. aureus* and *P. aeruginosa* were more sensitive to the combinations than individual parts. Generally, combining standard drugs with plant extracts boosted the inhibition of *S. aureus* and *P. aeruginosa* than that of either the drugs or the extracts.

Differences in zones of inhibition among the methanol, aqueous, and n-hexane extracts of *Ocimum lamiifolium* at concentrations of 100 mg/ml

Differences in inhibition zones due to the different extracts of *O. lamiifolium* against the test bacteria are presented in Table 2.

DISCUSSION

Inhibition was concentration dependent in all of the bacteria with *S. boydii* being the most sensitive bacterium followed by *E. coli*, *P. aeruginosa* and *S. aureus* in decreasing order of sensitivity. Similar findings were demonstrated in a study by Gebrehiwot and Unakal (2013) where *E. coli* was the most sensitive followed by *S. aureus* and *P. aeruginosa*, respectively to the aqueous and ethanol extracts of *O. lamiifolium*. This antibacterial activity may be due to the occurrence of antibacterial active components like eugenol and sabinene in the extracts (Adebolu and Oladimeji, 2005; Wiart, 2006; Uinoiseau et al., 2010). In the present study, *S. aureus* and *P. aeruginosa* were found to be resistant to Chloramphenicol may be due to the ability of these bacteria to inactivate Chloramphenicol by enzymes coded by the *cat* genes or the ability of *P. aeruginosa* to inactivate Chloramphenicol by Chloramphenicol acetyltransferase enzyme and decreased outer membrane permeability or active efflux of this drug (Byarugaba, 2009). *S. aureus* was resistant to tetracycline may be due to its abilities like active efflux of the antibiotic and ribosome protection or modification of

Table 2. Inhibition zones of *Ocimum lamiifolium* extracts at different concentrations.

Extract	Concentration	Mean inhibition zone (mm) ± SEM			
		S. aureus	***S. boydii***	***E. coli***	***P. aeruginosa***
ME	25	0.00 ± 0.00	8.00 ± 0.58*^{d}	8.67 ± 0.33*^{d}	9.33 ± 0.88*^{d,e}
	50	6.00 ± 0.00*^{d,e}	9.67 ± 0.33*^{d}	11.00 ± 0.00*^{d}	9.33 ± 0.33*^{d,e}
	100	9.33 ± 0.67*^{d,e}	11.67 ± 0.88*^{d}	12.00 ± 0.58*^{d}	11.33 ± 0.33*^{d,e}
AE	25	8.00 ± 0.00*^{a,d,e}	10.67 ± 0.33*^{d}	8.00 ± 0.00*^{d}	8.67 ± 0.33*^{d,e}
	50	10.00 ± 0.00*^{a,d,e}	11.00 ± 0.58*^{d}	9.00 ± 0.00*^{d}	10.67 ± 0.33*^{d,e}
	100	11.00 ± 0.00*^{d,e}	12.00 ± 0.00*^{d}	10.33 ± 0.33*^{d}	11.00 ± 0.58*^{d,e}
HE	25	7.00 ± 0.00*^{a,d,e}	8.67 ± 1.33*^{d}	9.33 ± 0.67*^{d}	9.00 ± 0.58*^{d,e}
	50	8.67 ± 0.67*^{d,e}	9.33 ± 0.33*^{d}	11.00 ± 0.58*^{d}	9.67 ± 0.67*^{d,e}
	100	9.67 ± 0.67*^{d,e}	10.33 ± 0.33*^{d}	11.00 ± 0.58*^{d}	10.00 ± 0.58*^{d,e}
Controls	Tet	#11.33 ± 0.88*^{a,b,c,d,e}	30.00 ± 0.00*^{a,b,c,d}	34.00 ± 3.06*^{a,b,c,d}	16.00 ± 2.08*^{a,b,c,d,e}
	Chl	0.00 ± 0.00	30.67 ± 1.20*^{a,b,c,d}	33.33 ± 1.67*^{a,b,c,d}	0.00 ± 0.00
	T80	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

ME: Methanolic extract; AE: aqueous extract; HE: n-hexane extract; [a]ME; [b]AE; [c]HE; [d]T80; [e]Chl; T80: Tween 80; Tet: Tetracycline (30 µg/disk); Chl: Chloramphenicol (30 µg/disk); *Significantly higher inhibition than; [#]Not significantly different from b and c at 50 mg/ml and a, b and c at 100 mg/ml extracts.

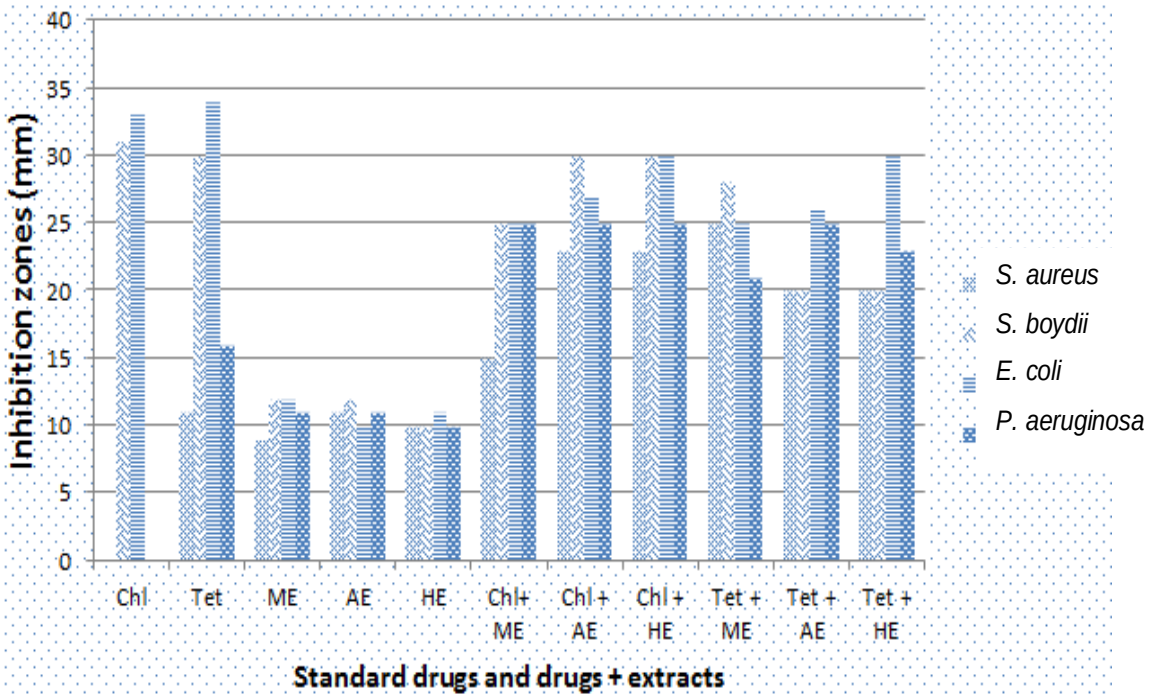

Figure 2. Antibacterial activities of the combinations of Tetracycline (30 µg) and Chloramphenicol (30 µg) with *O. lamiifolium* extracts with dose levels of 100 mg/ml. Tet: Tetracycline; Chl: Chloramphenicol; ME: Methanol extract; AE: Aqueous extract; HE: n-hexane extract.

the antibiotic (Byarugaba, 2009). All the extracts of *O. lamiifolium* showed better inhibition than the negative control (Tween 80) and were less effective than Tetracycline and Chloramphenicol. However, the leaf extracts were more effective than Chloramphenicol against *S. aureus* and *P. aeruginosa*. On the other hand,

these extracts were less effective than Chloramphenicol against *S. boydii* and *E. coli*. Application of the aqueous extract inhibited even the most resistant bacterium (*S. aureus*) at the lowest concentration (25 mg/ml).

E. coli and *S. boydii* were the most sensitive to the extracts followed by *P. aeruginosa* and the least sensitive of all was *S. aureus* showing that the Gram negative bacteria were more sensitive to the plant extracts than the Gram positive one (*S. aureus*). The results confirmed that *O. lamiifolium* extracts are important to inhibit *S. boydii* and *E. coli*, followed by *P. aeruginosa*, and least effective against *S. aureus*. The aqueous extract of *O. lamiifolium* seem to be effective than its methanol and n-hexane extracts against these bacteria. This result clearly distinguishes the importance of the aqueous extract which contains the most effective components to inhibit bacterial growth contradicting to the finding by Goyal and Kaushik (2011) where the methanolic extract of *Ocimum sanctum* L. showed comparatively higher activity than other organic and aqueous extracts. On the other hand, this result agrees with the work of Gebrehiwot and Unakal (2013) where the aqueous extract was found to be more effective than its ethanol extract against *S. aureus*, *E. coli* and *P. aeruginosa*. Generally, differences in activities of *O. lamiifolium* extracts may be due to the differences in their chemical compositions which are determined by different factors such as climate, plant nutrition, stress (Carson and Hammer, 2011), fertilizer application (Duke, 2009), plant organs used, plant developmental stage, plant origin, chemotypes, and methods used (Zuzarte et al., 2011).

Sometimes, the effectiveness of antibiotics can be increased by coupling them with plant extracts (Kekuda, 2012). In the present study, combination of Tetracycline (30 µg/disc) to the methanol extract of *O. lamiifolium* (100 mg/ml) increased the sensitivity of *S. aureus.* On the other hand, combination of Chloramphenicol (30 µg/disc) with all the extracts increased the sensitivity of *S. aureus* and *P. aeruginosa*. The implication of this finding is that the use of plant extracts in combination with less effective antibiotics can increase the susceptibility of bacteria to these antibiotics and can be solutions to bacterial resistance to antibiotics.

The majority of the dosages of all the extracts of *O. lamiifolium* inhibited the test bacteria with inhibition zones significantly higher than that of tween 80. The positive control (Tetracycline) on the other hand inhibited all the bacteria with inhibition zones significantly higher than all the extracts except the 50 mg/ml and the 100 mg/ml concentrations of the methanol, aqueous, and n-hexane extracts of *O. lamiifolium* which resulted in inhibition zones on *S. aureus* which were not significantly different from that exerted by Tetracycline. This leads to the conclusion that the aqueous, methanol and n-hexane extracts of *O. lamiifolium* have comparable activities with Tetracycline against *S. aureus.*

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Full Length Research Paper

Chemical composition of essential oils from the stem barks of *Croton conduplicatus* (Euphorbiaceae) native to the Caatinga biome

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***Croton conduplicatus* is a species popularly known in the Brazilian Caatinga (semi-arid vegetation) as "quebra faca". Essential oils from the stem barks of *C. conduplicatus* were obtained by hydrodistillation after 2, 3 and 4 h of extraction with a very high percentage (95.93, 96.69 and 98.45%, respectively) of identified total components present in crude essential oils. Analyses were made by gas chromatography/mass spectrometry (GC/MS). The most abundant constituents were α-pinene, β-pinene, camphor and (*E*)-caryophyllene. The occurrence of α-pinene and β-pinene has been reported in essential oils of several other species of *Croton*, indicating that this species is typically of the Euphorbiaceae family.**

Key words: *Croton conduplicatus*, essential oil, volatile constituents, medicinal plants, Caatinga.

INTRODUCTION

Croton (Euphorbiaceae) is one of the largest genera of flowering plants, with nearly 1300 species of herbs, shrubs and trees that are ecologically prominent and often important elements of secondary vegetation in the tropics and subtropics worldwide (Simionatto et al., 2007).

Some species of the genus *Croton*, such as *Croton cajucara*, *Croton zambesicus*, *Croton nepetaefolius* and *Croton celtidifolius*, have been described as medicinal plants with their biological activities assessed. Amongst such plants studied to date, many have been revealed to display multiple biological activities, such as anti-inflammatory, antioxidant, antinociceptive, anticonvulsant and anxiolytic activities (Zhao et al., 2012).

Plants belonging to the genus *Croton* are well known for producing a variety of diterpenoids including pimarane, kaurane, labdane, cembrane, cleisthantane, and clerodane diterpenoids, with a wide range of

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Table 1. Chemical composition of essential oils of stem bark of *Croton conduplicatus* subjected to hydrodistillation for 2, 3 and 4 h of extraction.

Order	Compounds	RI*	GC/MS (%)		
			After 2 h	After 3 h	After 4 h
1	Tricyclene	922	0.39	0.78	0.50
2	α-Thujene	924	0.75	0.55	0.13
3	α-Pinene	932	32.87	35.35	25.84
4	Camphene	948	4.01	8.01	5.66
5	Sabinene	971	0.06	---	---
6	β-Pinene	977	13.56	16.77	7.79
7	Myrcene	988	0.72	0.50	0.24
8	*p*-Cymene	1024	0.72	0.56	0.44
9	Limonene	1028	1.24	1.40	0.72
10	1,8-Cineole	1031	1.46	0.29	0.54
11	γ-Terpinene	1056	0.15	0.21	0.14
12	*cis*-Linalool oxide	1069	0.13	---	---
13	*trans*-Linalool oxide	1086	0.56	0.81	0.36
14	Linalool	1100	2.18	1.24	0.86
15	NI	1104	0.24	---	---
16	*trans*-Pinocarveol	1140	---	0.31	---
17	Camphor	1146	7.30	9.32	6.37
18	Camphene hydrate	1154	---	0.19	0.15
19	Borneol	1171	0.33	0.33	0.64
20	Terpinen-4-ol	1179	0.87	0.52	0.29
21	α-Terpineol	1194	1.40	0.87	0.70
22	Thymol methyl ether	1228	0.70	---	0.49
23	Cyclosativene	1365	0.66	0.63	0.46
24	α-Copaene	1373	0.62	1.14	0.68
25	β-Elemene	1387	0.30	0.16	0.25
26	α-Gurjunene	1399	---	---	0.28
27	(*E*)-Caryophyllene	1416	7.80	4.66	5.07
28	β-Cubebene	1426	0.64	---	0.34
29	NI	1445	---	---	0.33
30	α-Humulene	1452	1.47	0.84	1.18
31	NI	1464	---	---	0.51
32	γ-Muurolene	1472	2.08	0.19	2.76
33	Germacrene D	1477	0.36	---	0.80
34	α-Selinene	1492	---	---	0.37
35	α-Muurolene	1496	3.66	3.21	1.79
36	β-Bisabolene	1506	---	0.30	0.27
37	δ-Amorphene	1509	---	---	0.38
38	γ-Cadinene	1509	0.23	---	---
39	NI	1511	---	---	0.44
40	δ-Cadinene	1515	0.31	0.26	1.78
41	NI	1538	0.18	---	---
42	Elemol	1545	0.30	---	---
43	Hedycariol	1545	---	---	0.57
44	Germacrene B	1554	---	0.20	0.32
45	Caryophyllene oxide	1578	6.63	3.88	6.91
46	Viridiflorol	1585	---	---	0.22
47	Guaiol	1593	0.66	0.41	0.83
48	Globulol	1600	---	---	1.12
49	Humulene epoxide II	1605	0.84	0.50	1.16

Table 1. Cont'd.

50	NI	1622	2.16	1.70	---
51	Aristolene	1629	---	1.00	1.32
52	NI	1633	0.39	0.48	---
53	Hinesol	1636	---	0.76	0.76
54	NI	1652	0.65	0.63	---
55	NI	1667	0.45	0.50	---
Total		-	95.93	96.69	98.45

*RI: Retention indices on DB-5MS column (relative to *n*-alkanes); NI: not identified compound; (---): Not detected.

biological activities (Pudhom et al., 2007). On the other hand, essential oils are other important class of secondary metabolites in this genus. Recently, Salatino et al. (2007) reported the study of the essential oils of about thirty species of *Croton*. The results indicated that some of these oils are rich in terpenoids and phenylpropanoids, and others are rich only in terpenoids (Salatino et al., 2007).

Despite of the large array of data on other *Croton* species, the knowledge about *Croton conduplicatus* is scarce. This species is popularly known in the Brazilian Caatinga as "quebra faca". Decoction of its leaves and stem barks are used in folk medicine to treat influenza, headache, indigestion, stomach problems and stomachache (Cartaxo et al., 2010). To the best of our knowledge, no phytochemical and pharmacological studies have previously been reported on this species.

This paper presents for the first time the chemical composition of *C. conduplicatus* stem barks essential oils by gas chromatography/mass spectrometry (GC/MS).

MATERIALS AND METHODS

Plant

Stem barks of *C. conduplicatus* Kunth. were collected from a single individual in September 2012 in Petrolina (Coordinates: S 09°03'54"; W 40°19'12"), State of Pernambuco, Brazil. A voucher specimen (HTSA2421) was deposited at the Herbário do Trópico Semiárido (HTSA) of the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). Considering that the plant is a shrub, the stem barks were cut close to the ground.

Extraction of essential oils

The fresh stem barks (100 g) were cut into pieces, and subjected to hydrodistillation for 2, 3 and 4 h in a modified Clevenger-type apparatus. The oils were dried over anhydrous sodium sulfate. The essential oils obtained have yellow color and characteristic odor. The oils were stored in a refrigerator until the analysis by GC/MS.

Analysis of essential oils

The substances present in the essential oil of *C. conduplicatus* were investigated on a Shimadzu QP-2010 gas chromatograph interfaced to a mass spectrometer (GC/MS). The following conditions were used: DB-5MS column Agilent Technologies (30 m × 0.25 mm × 0.25 µm); helium (99.999%) carrier gas at a constant flow of 1.1 ml/min; 1.0 µl injection volume; injector split ratio of 1:10; injector temperature 250°C; electron impact mode at 70 eV; ion-source temperature 280°C and transfer line temperature 260°C. The oven temperature was programmed from 60°C, with an increase of 3°C min^{-1} to 240°C.

A mixture of linear hydrocarbons (C_9H_{20}–$C_{21}H_{40}$) was injected under the same experimental conditions as samples, and identification of the constituents was performed by comparing the spectra obtained with those of the equipment database (Wiley 7 lib and Nist 08 lib) and by using the Kovats Index, each constituent was calculated as previously described (Adams, 1995; Van den Dool and Kratz, 1963). The data were acquired and processed with a PC with Shimadzu GC/MS Solution software.

RESULTS AND DISCUSSION

In every extraction, 100 g of *C. conduplicatus* stem barks were used and the crude oils yield was found to be 0.90, 0.97 and 0.97 ml, for 2, 3 and 4 h of extraction, respectively. The CG/MS analysis led to the identification of 95.93, 96.69 and 98.45% of the total components present in crude essential oils.

The chemical constituents of the essential oil of *C. conduplicatus* were identified by comparing their mass spectral data with reference spectra in the computer library. The identified compounds are as shown in Table 1 according to their retention indexes.

The main compounds found in the oil of the stem barks after 2, 3 and 4 h of extraction were α-pinene (32.87, 35.35 and 25.84%, respectively), β-pinene (13.56, 16.77 and 7.79%, respectively), camphor (7.30, 9.32 and 6.37%, respectively) and (*E*)-caryophyllene (7.80, 4.66 and 5.07%, respectively). Variation in extraction time was performed to verify their influence on the yield and the chemical composition of the essential oil. Depending on the compound of interest, the time of extraction can be adjusted.

In light of these chemical evidences, some authors purpose to consider that the co-occurrence of α and β-pinene might be a characteristic of the genus *Croton*, however, in the light of a larger number thus far studied, taxa, β-caryophyllene and linalool seem to be equally a

frequent major constituents of many *Croton* spp. (Radulovic et al., 2006).

Particular relevance should also be given to the presence of minor, but not negligible compounds detected in our samples as caryophyllene oxide, camphene and α-muurolene. The relatively high content of camphene in the stem oil differentiates *Croton decaryi* from the other members of the genus *Croton* since there are no published data on a *Croton* spp. containing this monoterpene hydrocarbon as one of the major constituents (Radulovic et al., 2006). Thus, camphene could be considered as chemotaxonomic marker for *C. decaryi*.

Conclusion

C. conduplicatus has been examined for the first time for the essential oil obtained by hydrodistillation of fresh stem barks. GC/MS has been provided in order to chemically characterize the essential oil, evidencing a monoterpene prevalence. In comparison to the others essential oils from *Croton* spp., the oil of this species shows constituents that are present in other species of this genus.

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Conflict of interest

Authors declare that there are no conflicts of interest.

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