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Quercetin improves antioxidant response in diabetes through maintenance of reduced glutathione levels in blood

Mariza Olvera Nájera, Imelda Silvar Tinajero, Lorena I. Rodriguez Páez, Sergio E. Meza Toledo and José L. Muñoz Sánchez

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Accepted 23 September, 2013

Oxidative stress is involved in development of diabetes and its complications. Quercetin has shown to have antioxidant activity against oxygen free radicals and the ability to induce a better antioxidant response in healthy animals. The aim of the present study was to investigate the effect of daily administration of quercetin, in two different doses, on the levels of reduced glutathione (GSH) and lipid peroxidation in rats, two weeks after induction of diabetes. Both doses of quercetin significantly increased blood GSH levels and reduced lipid peroxidation in liver. Low quercetin doses increased superoxide dismutase activity (SOD) levels in liver and reduced lipid peroxidation in the heart. High quercetin doses induced recovery of GSH levels in pancreas. In the present study, we provide evidence that daily administration of quercetin improves the antioxidant response in diabetic rats.

Key words: Diabetes, glutathione (GSH), quercetin, oxidative stress.

INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder that is the result of total or relative deficiencies in insulin secretion and/or insulin action. DM is characterized by high blood glucose levels and disturbances of carbohydrate, lipid and protein metabolism (Maritim et al., 2003). Long term uncontrolled hyperglycemia also increases oxidative stress and reactive oxygen species (ROS) formation, causing tissue damage and an elevated risk of development of complications (Mohora et al., 2007). The cell capacity to resist oxidative damage is determined by its antioxidant defense systems, where reduced glutathione (GSH) is the most ubiquitous and readily available within cells. GSH is a tripeptide that regulates an important number of biological processes, such as apoptosis initiation (Circu and Aw, 2012), gene expression (Sen, 2000), immune response (Droge and Breitkreutz, 2000), cell differentiation (Huh et al., 2006) and antioxidant defense (Kidd, 1997).

GSH is depleted in erythrocytes from human diabetics (Sekhar et al., 2011), pre-diabetics (Nwose, 2006), individuals with metabolic syndrome (Cardona et al., 2008) and gestational diabetic patients (Rajdl et al., 2005). GSH influences the redox status of the cell, which is critical for biochemical functions and in turn, a redox imbalance, may predispose cells to oxidative damage and whole-organism diabetic complications (Matough et al., 2012). Several reports indicate that flavonoids have the capacity to regulate genes to be involved in the GSH biosynthesis and related enzymes in healthy and some pathological conditions (Kang et al., 2009). In particular...
pre or post treatment with flavonoids up to 72 h after induction of diabetes in rats prevents the decrease of GSH levels and antioxidant enzymatic activity in some internal organs (Quine and Raghu, 2005).

Quercetin (3,3′,4′,5,7-pentahydroxyflavone, Figure 1) is a polyphenolic compound found in a great variety of vegetables and fruits. It is also a potent antioxidant agent that inhibits pro-oxidant enzymes and up-regulates the expression of genes encoding enzymes involved in drug metabolism and antioxidant defense such as γ-glutamylcysteine synthetase, the rate limiting enzyme in glutathione synthesis (Nijveldt et al., 2001; Myhrstad et al., 2002). It is possible that the treatment with quercetin decreases the oxidative damage in diabetes by improving the antioxidant response through maintenance of suitable GSH levels. While quercetin is rapidly absorbed in the small intestine, its clearance from plasma is slow (Walle et al., 2005). Hyperglycemia decreases glucose uptake and leads to an insulin resistance. It also produces changes in the expression, distribution and function of glucose transporters in the different tissues (Kahn, 1992). The uptake of quercetin into the erythrocytes is mediated through glucose transporter 1 (GLUT1) and it is dependent on intracellular glucose (Cunningham et al., 2005). GLUT1 is widely expressed both in insulin and in noninsulin-responsive tissues. Other GLUT’s are expressed specifically in other tissues, that is, GLUT2 is present in gut, liver and pancreatic islets; GLUT3 in the central nervous system and brain; and GLUT4 in insulin-responsive tissues, skeletal muscle, adipose tissue and heart (Wood and Trayhurn, 2003).

The present study was designed to investigate the effect of quercetin on GSH levels of diabetic rats that received the treatment two weeks after that the induced diabetic state was confirmed, as well as to evaluate the effect of continuous administration of low and high doses of quercetin on lipid -peroxidation in organs and antioxidant response.

**MATERIALS AND METHODS**

**Animals**

Male albino Wistar rats 110 to 130 g were acclimatized for about 7 days prior to dosing with streptozotocin (STZ). The animals were fed with a standard diet and water ad libitum under standard laboratory conditions; temperature 25±2°C and relative humidity 55±10%, dark and light cycles of 12 h each were maintained. The experiments were carried out according to the guidelines for animal care and use of the National Institutes of Health (Bethesda, Maryland, USA) and were approved by our institutional scientific research review board.

**Drugs and Chemicals**

Streptozotocin (STZ), quercetin, thiobarbituric acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), glutathione peroxidase (EC 1.11.1.9), hydrogen peroxide, xanthine oxidase (EC 1.1.3.22) and xanthine, were purchased from Sigma-Aldrich.

**Diabetes induction**

Diabetes was induced in rats by a single intramuscular injection of STZ at 50 mg/kg, diluted in 0.1 M citrate-buffer (pH 4.5) as described by Nakhoda and Wong (1979). The diabetic state was checked 72 h after induction with STZ. Blood samples were drawn from the tail vein to determine glucose levels in an automatic analyzer (YSI 2700 Select). Animals were considered diabetic when blood glucose levels exceeded 250 mg/dl and were used in the experimental group.

**Experimental protocol**

Three groups of diabetic animals and a healthy control group (designated SC) were formed. Group size varied from five to ten rats. The administration of quercetin was performed in doses 15 (DQ15) and 30 (DQ30) mg/kg starting two weeks after the induction of experimental diabetes. Two quercetin doses were used in order to study the dose-dependent effect. Quercetin was administered orally as a suspension in vegetable oil by intragastric (i.g.) route for four weeks. Diabetic control (DC) and SC groups were administered only with vegetable oil at the same conditions.

At the end of the experimental period, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/ kg). In order to avoid a decrease in GSH levels rats were not subjected to fasting prior to sacrifice. Liver, kidney, heart and pancreas were removed and blotted; tissue samples were used for GSH assay immediately after dissection, thus preventing GSH oxidation. Remaining tissues were stored at -70°C until further use. GSH blood samples were drawn from the abdominal aorta in a heparin tube and processed immediately.

**Blood glucose and body weight determination**

Glucose was measured using an automated (YSI 2700 SELECT Biochemistry Analyzer) and the body weight was assessed daily during the four weeks of treatment.

**Determination of reduced glutathione in blood and rat organs**

Reduced glutathione (GSH) was using the method described by Ellman (1959), in which the reduction of Ellman’s reagent (DTNB) by a thiol group produces 2-nitro-5-mercaptobenzoic acid, an intensely yellow compound whose absorbance was measured at
Determination of lipid peroxides

Malondialdehyde (MDA), a reactive aldehyde that is generated by lipid peroxidation, was determined according to the method of Mihara and Uchiyama (1978). The adducts formed following the reaction of tissue homogenate with thiobarbituric acid were extracted with n-butanol. The difference in the absorbances at 535 nm and 520 nm was used to measure tissue MDA content, which was expressed as µM/mg protein.

Measurement of antioxidant enzyme activities

Tissues were briefly washed in ice-cold 0.9% (w/v) saline and stored at -70°C, until used. Protein concentration was measured in tissue homogenate by the method of Lowry and Rosenbrough (1951), using bovine serum albumin as standard.

For the superoxide dismutase (SOD) assay, tissue samples were homogenized in 0.05 M phosphate buffer (pH 7.4), in a 1:10 tissue/buffer, the supernatant was carefully separated and a mixture of chloroform-ethanol (3:5 v/v) was added. This suspension was centrifuged at 5000 × g at 4°C for 2 h. The supernatant was used for SOD and GPx assays. SOD activity was measured using the xanthine/oxidase system as superoxide generator (Sun et al., 1988). The results were expressed as units per mg protein tissue. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%.

Glutathione peroxidase activity was measured indirectly using the procedures of Wendel (1980) in which the amount of unconsumed GSH remaining at specific time intervals is determined in the presence of small amounts of peroxide. In the appearance of product assay, the rate of glutathione oxidation by peroxide is measured by monitoring the conversion of NADPH + H+ to NADP+ as oxidized glutathione (GSSG) is converted to GSH. The results were expressed as units per mg protein. One GPx unit catalyzes the oxidation by H2O2 of 1.0 μmole of reduced glutathione to oxidized glutathione per minute at pH 7.0 at 25°C.

Statistical analysis

Data are present as the mean ± SE for the number of animals in each group (n= 5-10). Graphs were plotted and statistical analysis was performed by one-way variance analysis (ANOVA) followed by Dunnett’s test for multiple comparisons. p-values smaller than 0.05 were considered to be statistically significant.

RESULTS

Effect of quercetin on blood glucose concentration and body weight

Blood glucose concentrations were significantly higher in groups having induced diabetes respect to the non-diabetic control (392.5±25.2 and 141.62±18.42 mg/dl, respectively; p<0.001). Quercetin (15 and 30 mg/kg) treatment of diabetic rats did not decrease their blood glucose values (363.5 ± 36.18 and 362 ± 38.01 mg/dl, for groups DQ15 and DQ30, respectively; Figure 2a).

There was significant reduction in the body weight of diabetic groups treated with quercetin (DQ15 and DQ30) and the untreated diabetic control (223.93±4.62, 233.83±9.64 and 228.55±3.69 g, respectively; p<0.011) as compared to the non-diabetic control group (342.24±17.75 g, Figure 2b). No deaths occurred in any group of rats tested during the experiment.

Glutathione levels in organs from rat administrated with quercetin

Two oral doses of quercetin were used (15 and 30 mg/kg b.w) for evaluation of their effect on GSH levels in organs of diabetic animals. Our results indicate that diabetic animals have a decreased levels of pancreatic glutathione compared with the healthy control group (3.51±0.11 and 4.03±0.11 μg/g tissue, respectively; p<0.007). In diabetic rats, quercetin at 30 mg/kg b.w significantly increased the concentration of pancreatic glutathione (group DQ30) as compared to animals in the diabetic control group (4.14±0.278 and 3.51±0.11 μg/g tissue, respectively; p<0.045). Results are presented in Table 1.

Effect of the administration of quercetin on blood glutathione levels

The concentrations of glutathione in the blood of diabetic control rats were markedly diminished (4.75±1.16 mg/dl) as compared to those animals in which diabetes had not been induced (21.75±1.87 mg/dl; p<0.017). In rats treated with quercetin at either 15 or 30 mg/kg the glutathione concentration significantly increased (14.51±2.52 and 14.73±0.69 mg/dl, respectively; p<0.001) when compared with results from the diabetic control group (Figure 3).

Lipid peroxidation

The basal concentration of malondialdehyde in the liver of diabetic control rats was found to be greater than that in the healthy control group (1.66±0.741 and 0.102±0.015 µM/mg protein, respectively; p<0.001, Figure 4a). Rats treated with quercetin at either 15 or 30 mg/kg b.w. were found to have a lower liver concentration of malondehyde...
Table 1. Effect of quercetin on GSH levels in different organs of diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Liver µM/g tissue</th>
<th>Kidney µM/g tissue</th>
<th>Heart µM/g tissue</th>
<th>Pancreas µM/g tissue</th>
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<tr>
<td>SC</td>
<td>9</td>
<td>5.32±0.35</td>
<td>4.81±0.10</td>
<td>3.60±0.100</td>
<td>4.03±0.112</td>
</tr>
<tr>
<td>DC</td>
<td>8</td>
<td>5.13±0.15</td>
<td>4.27±0.29</td>
<td>3.14±0.112</td>
<td>3.51±0.112</td>
</tr>
<tr>
<td>DQ15</td>
<td>5</td>
<td>4.70±0.31</td>
<td>3.65±0.07</td>
<td>3.62±0.191</td>
<td>3.27±0.191</td>
</tr>
<tr>
<td>DQ30</td>
<td>5</td>
<td>5.64±0.62</td>
<td>4.72±0.45</td>
<td>4.00±0.135</td>
<td>4.14±0.278</td>
</tr>
</tbody>
</table>

SC, healthy control group; DC, diabetic control group; DQ15 and DQ30, diabetic rats treated with quercetin at 15 or 30 mg/kg bw, respectively. Values were expressed as mean ± SD. a, Significant vs. healthy control, p < 0.05; b: significant vs. diabetic control; c: significant vs. DQ15.

Figure 2. Glucose levels (A) and body weight (B) in rats after treatment with quercetin. Data are means ±SEM (error bars), n = 5 to 10 animals for each treatment group. SC, healthy control group; DC, diabetic control group; DQ15 and DQ30, diabetic rats treated with quercetin at 15 or 30 mg/kg bw, respectively; *Significant vs. SC.

Figure 3. GSH contents in blood of rats administered with quercetin. Data are means ±SEM (error bars), n = 5 to 10 animals for each treatment group. SC, healthy control group; DC, diabetic control group; DQ15 and DQ30, diabetic rats treated with quercetin at 15 or 30 mg/kg bw, respectively; *Significant vs. SC, ** Significant vs. DC.

(0.508±0.160 and 0.491±0.094 µM/ mg protein, respectively) as compared to values in the livers from animals in the diabetic control (p<0.004).

In the heart of rats treated with quercetin at 15 mg/kg b.w., a decrease in malondialdehyde concentration was observed with respect to animals in the diabetic control; p<0.003 (Figure 4b). This effect was not observed in kidney and pancreas (Figure 4c and d). Conversely, in rats subjected to treatment with quercetin at 30 mg/kg b.w., we observed an increase in heart malondialdehyde concentration (0.199±0.018 and 0.447±0.06 µM/mg protein, respectively; p<0.003 Figure 4b) when compared with the group treated with quercetin 15 mg/kg.

Superoxide dismutase and glutathione peroxidase activities

The basal enzymatic activity of SOD was higher in the livers of rats in the diabetic control group, as well as in the livers from rats treated with quercetin (DQ15 and DQ...
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Figure 4. Effect of quercetin doses on malondialdehyde levels in rat homogenates from liver (A), heart (B), kidney (C) and pancreas (D). Data are means ±SEM (error bars), n = 5 to 10 animals for each treatment group. SC, control group; DC, diabetic control group; DQ15 and DQ30, diabetic rats treated with quercetin at 15 mg/kg or 30 mg/kg bw, respectively; *Significant vs. SC, ** Significant vs. DC, *** Significant vs. DQ15.

30), compared with that of animals in the healthy control group (8.41±0.55, 10.9±1.058, 7.23±0.77 and 1.55±0.149 U/mg protein; p<0.001 respectively). In particular, the DQ group (administer with quercetin at 15 mg/kg) showed a significant increase in the activity of SOD (10.90±1.06 U/mg protein) with respect to the DC group (8.38±0.52 U/mg protein; p<0.042, Figure 5a).

The basal activity of glutathione peroxidase (GPx) in diabetic rats (not treated with quercetin) decreased with respect to that in the healthy control (3.38±0.32 and 7.26±0.63 U/mg protein, respectively, p<0.001). Animals treated with quercetin (groups DQ15 and DQ30) did not present an increase in GPx relative to that of the diabetic control group (3.19±0.46, 2.78±0.56 and 3.38±0.32 U/mg protein respectively, Figure 5b).

**DISCUSSION**

It has been found that reduced glutathione (GSH) is de-
pleted in erythrocytes from human diabetics (Sekhar et al., 2011) and pre-diabetics (Nwose, 2006); from metabolic syndrome sufferers (Cardona et al., 2008) or from females undergoing gestational diabetes (Rajdl et al., 2005). GSH influences the redox status of the cell, therefore affecting biochemical functions. The lack of GSH or its decrease could lead to physiological imbalance and may predispose cells to oxidative damage and eventually diabetic complications (Matough et al., 2012). In our study, STZ-diabetic rats showed significant blood GSH depletion. Previous reports indicate that as part of its diabetogenic action STZ produces hemolysis and a fast decrease of the GSH in red blood cells (RBCs) through oxidative stress (Slonim et al., 1976). Chronic hyperglycaemia leads to the generation of reactive oxygen species and glycooxidation products lowering glucose uptake into erythrocytes by changes in GLUT 1, mostly in its outer domain (Moussa, 2008; Hu et al., 2000; Ciuchi et al., 1996). Quercetin treatment significantly increased the concentration of GSH in blood of experimental animals. These results are an indication that the quercetin might have a protective effect on RBCs, guarding off oxidative stress generated by diabetes conditions. There are two possible contributing factors to the protective action of quercetin: First the ability of quercetin to bind the plasma membrane of erythrocytes could inhibit the oxidation of lipids exposed at high glucose levels, therefore preventing direct membrane damage (Koren et al., 2010).

An alternative explanation is that hyperglycemia is accompanied by a decrease in glucose entry into erythrocytes without affecting its extrusion. In this mechanism quercetin could be imported via GLUT1 therefore inhibiting the exit of glucose via the same transporter, thus leading to the partial restoration of glucose homeostasis. An increase in intracellular glucose may lead to an elevation in NADPH synthesis through the pentose phosphate pathway, in turn, preventing GSH depletion inside the erythrocyte with the ultimate consequence of an overall decrease in oxidative stress (Kang, 2009; Martin et al., 2003).

The depletion of GSH in liver and pancreas of rats with STZ-induced diabetes has been associated with STZ toxicity (Bastar et al., 1998). In our study no changes in liver GSH levels were observed in STZ-treated animals. This result could be due to the fact that animals were not sacrificed under fasting conditions, which is in agreement with the findings of McLennan et al. (1991) who showed that GSH depletion in liver of STZ-diabetic rats that only consumed 10 g/day during 24 days in the initial stage is a consequence of food restriction rather than diabetes alone. Diabetic control rats showed a substantial reduction in their pancreatic GSH levels. In fact, STZ has been demonstrated to deplete the antioxidant pool in target cells, making them more susceptible to oxidative damage. Additionally, in STZ-induced diabetic rats, hyperglycemia has been shown to induce typical apoptotic changes in the pancreas and oxidative stress (Kim et al., 2002). Treatment with quercetin 30 mg/kg increased the amounts of GSH relative to STZ-diabetic rats not treated with quercetin, suggesting that quercetin ameliorates the oxidative stress produced by STZ and hyperglycemia via a third possible mechanism where quercetin-mediated induction of GSH synthesis through
interaction with Nrf2 (nuclear factor erythroid 2-related factor), a transcription activator that regulates the inducible expression of numerous detoxifying and antioxidant genes. Nrf2 binds to a specific DNA sequence known as ARE (antioxidant response element), which can be activated by several electrophilics and oxidant compounds of diverse chemical nature. ARE sequences can be found on the promoters of γ GCSH and other antioxidant genes. Nrf2 is inactive in the cytosol where it is bound to Keap 1 cytoskeleton-associated protein. The Nrf2-Keap 1 binding is thought to be dependent on specific Keap 1 sulfhydryl groups, which in turn have been postulated to be sensors of cellular redox state. Therefore, release and translocation of Nrf2 to the nucleus is linked to cellular oxidative stress, as well as the presence of thiol-reactive compounds and antioxidants. Quercetin-o-quinone, a product of oxidation of quercetin by free radicals, may react with critical Keap 1 sulfhydryl groups. Subsequently Nrf2 could be translocated to the nucleus, inducing the transcription of antioxidant-responsive genes (Arredondo, 2010; Moskaug et al., 2005).

We did not observe any changes in heart and kidney GSH levels, confirming prior observations by Wohlaeb and Godin (1987) who proposed that the absence of GSH depletion in the heart may be partly related to increased glutathione reductase (GSSG-RD) activity.

We did not find any differences in the extent of pancreatic lipid peroxidation across the three groups of STZ-diabetic rats. These results concur with those reported by Tatsuki et al. (1997), who observed that increased lipid peroxidation in pancreas takes place at early stages (2 to 7 h) of the onset of STZ-induced diabetes but normalizes after a long period (7 weeks), probably due to a cellular antioxidant response to the effects of STZ attack. Increases in lipid peroxidation in kidney and heart were not found in rats administered with STZ. A decrease in lipid peroxidation was observed in the hearts of animals treated with quercetin (15 mg/kg), when compared with diabetic control group, as evidenced by a reduction in malondialdehyde concentrations. These results corroborate the findings of Edremitiloglu et al. (2012).

Increments in hydroxyl radical production associated with hyperglycaemia, as well as alterations of trace element concentrations (that is, increases in iron and copper mean levels and decreases of zinc and magnesium), have been correlated with higher lipid peroxidation and lower GSH concentrations in the liver of STZ-diabetic rats (Ozcelik et al., 2011; Francés et al., 2010). We found that quercetin treatments at 15 or 30 mg/kg were able to decrease lipid peroxidation in the liver, demonstrating the flavonoid’s antioxidant properties. These results confirm previous reports that have shown reduction of liver lipid peroxidation in STZ-diabetic rats treated with curcumin or (−)-epicatechin (Sotikno et al., 2012; Sabarimuthu and Pamakanthan 2005). We propose that the antioxidant effect observed in the liver of diabetic rats treated with quercetin is the result of this compound’s ability to induce the expression of metallothionein (MT) (Kuo et al., 2001). Moreover, the additional activities of quercetin such as scavenging reactive free radicals, chelating trace metal ions and regenerating membrane-bound antioxidants, such as α-tocopherol, can contribute to the overall antioxidant response (Hussein, 2011). It would be also interesting to measure MT gene expression in the liver of rats treated with quercetin.

Various causes have been proposed for diabetes-induced alterations of antioxidant enzymatic activities. These include response to oxidative stress, allosteric effects (glycation), and changes in the concentration of cofactors (Fujisawa and Kadoma, 2006). In our study, the hepatic SOD activity was significantly increased whereas GPx activity was significantly diminished in STZ-diabetic rats compared to control, in agreement with the work of Cho et al. (2002). Superoxide radicals have been implicated in cell dysfunction (Kakkar et al., 1998) and they may be capable of inducing the production of hepatic SOD. Generation of free radicals and the presence of high concentrations of H2O2 decrease activity of GPx during diabetes, GPx could eliminate H2O2 when present at low concentrations. Streptozotocin/-quercetin (15 mg/kg) treatment increased SOD activity in the liver. This finding is consistent with previous reports which indicated that quercetin increased the antioxidant activity of SOD and catalase without increasing the GPx activity in several tissues (Jeong et al., 2012).

In this study, continuous administration of quercetin did not reduce blood glucose levels in the animals, perhaps because the animals were not given quercetin at day of sacrifice. Jadhav and Puchchakayala (2012) reported that quercetin administration 30 min before an oral glucose test in both healthy and STZ nicotinamide-diabetic rats produces a hypoglycemic effect and suggested that the possible mechanisms of action of quercetin involve inhibition of glucose transport in the rat intestinal sacs, enhanced insulin secretion or increased peripheral glucose uptake.

Conclusion

Our findings suggest that orally administered quercetin leads to the recovery of blood and pancreas levels of reduced glutathione. It would be interesting to investigate the mechanism by which quercetin favors GSH increase and evaluate whether the combination of quercetin and other flavonoids could lead to an even more beneficial synergistic effect against hyperglycemia-related damages.
damages via radical oxygen species.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ABTS, 2,2’-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; NBT, nitro blue tetrazolium; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin.

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Full Length Research Paper

Characterization and controlled release of gentamicin from novel hydrogels based on Poloxamer 407 and polyacrylic acids

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Parenteral administration of gentamicin, a hydrophilic aminoglycoside antibiotic commonly used in the control of Gram positive and Gram negative infections, is limited by adverse effects such as nephrotoxicity, ototoxicity and neurotoxicity. In this study, topical hydrogels of gentamicin were produced using three polymeric hydrogels of Poloxamer 407 and polyacrylic acids (Carbopols® 971P and 974P), and evaluated in terms of drug content, pH, swellability in different media, viscosity, spreadability, skin irritation on rats and time-resolved stability. The in vitro permeation of gentamicin from the hydrogel formulations was carried out in phosphate buffered saline using a modified Franz diffusion apparatus. Results obtained indicate that gentamicin-loaded hydrogels showed good encapsulation, stability, pH-dependent swelling, tolerability on rats, greater percentage drug release than the commercially available gentamicin ointment and pure sample of gentamicin. Overall, Poloxamer 407 hydrogels of gentamicin gave the most desirable properties in terms of drug permeation, spreadability, pH, swellability and viscosity, superior to polyacrylic acids hydrogels of gentamicin. This study has shown that Poloxamer 407 hydrogels of gentamicin could offer a promising approach for topical delivery of gentamicin for the treatment of skin infections caused by gentamicin-resistant bacteria.

Key words: Poloxamer 407 (P407), topical hydrogels, gentamicin, polyacrylic acids (Carbopols® 971P and 974P), antimicrobial activities.

INTRODUCTION

The solubility characteristics of a substance greatly influence its ability to penetrate biological membranes (Eljarrat-Binstock et al., 2004). Both the aqueous solubility of a drug at the absorption site and the partition coefficient strongly influence the rate of transport across the biological membrane. The enhancement of drug dosage form formulation is connected with the application of auxiliary substances or with new technological possibilities, which may be intended for controlled drug delivery (Shazly et al., 2012). Controlled release of antibiotics at the site of infection is a new strategy being employed to treat chronic infections (El-Gendy et al., 2009). Localized delivery systems, based on biodegradable polymers are capable of slow and...
controlled release of drug for a required period of time, with initial burst effect to circumvent the infection (Kopeczek, 1984; Graham and McNeill, 1984). Important factors, which influence drug release are polymer composition, drug diffusion, osmotic pressure, and bioerosion (Coppi et al., 1996; Khare and Peppas, 1995). The major advantage of these systems is steady and extended release of the antibiotics directly to infected tissue without systemic toxicity. Furthermore, no surgical intervention or procedures are required for their removal (Stephens et al., 2000).

Hydrogels, swollen three-dimensional networks of hydrophilic polymers held together by association bonds or cohesive forces, are suitable carriers for drug delivery (Eljarrat-Binstock et al., 2004). They are of special interest in controlled release applications, because of their soft tissue biocompatibility, the ease with which drugs are dispersed in matrix and the high degree of control achieved by selecting the physical and chemical properties of polymer network. Hydrogels have a high water content and rubbery nature similar to natural tissue, which make them desirable for biomedical applications. Hydrogels have been investigated extensively for application as carriers in diffusion-controlled release devices (Mayol et al., 2008; Raja et al., 2011; Changez et al., 2003; Ayhan and Özkan, 2007; Sokmen et al., 2008). In this type of device, a drug or protein is incorporated into the system and then released in response to a change in the environment. The hydrogels are rendered insoluble due to the cross-linking which may be physical or chemical. These cross-links are responsible for providing the physical integrity and network structure of the hydrogels. The polymeric biomaterials are used to delay drug dissolution at a slower rate depending on exposure of drug molecules to aqueous environment surrounding the drug delivery system. Their usage is advantageous in safety, ease of manufacture, cost effectiveness, biocompatibility and biodegradability (Mayol et al., 2008). In general, the gelation of a polymeric solution can be triggered by a number of factors such as variations in temperature, as for Poloxamers (Desai and Blanchard, 1998), pH, as for Carbopol (Srividya et al., 2001; Cho et al., 2012), or the presence of cations, as for alginates (Cohen et al., 1997). Topical drug administration generally refers to topical application of agents to healthy intact skin either for localized treatment of tissues underlying the skin or for systemic therapy (Brown et al., 2006). It delivers drugs to the circulatory system and have been shown to possess controlled release rate, enhanced efficacy, stable plasma concentration, non-invasive administration, increased safety, reduced dosing frequency and simplicity of use, to mention few (Allen Jr., 2011). Yet, a common problem with it is permeation across stratum corneum (sc), which limits the size and property of drug molecules that pass through (Prausnitz and Langer, 2008). Flux across the skin is therefore dependent upon skin hydration, partitioning, transport as well as concentration gradient across the skin (Azarmi et al., 2007). Hydrogels (including topicals) achieve sustained release by diffusion from a reservoir through microporous membrane into the skin (Mayol et al., 2008; Raja et al., 2011; Changez et al., 2003; Ayhan and Özkan, 2007; Sokmen et al., 2008).

Gentamicin sulphate is an aminoglycoside antibiotic commonly used topically in the control of severe Gram positive and Gram negative microbial infections especially in burns and wounds as well as for treating bone and soft tissue infections (Chang et al., 2006). Topical gentamicin is often used in the treatment of impetigo, infected bed sores, burns, nasal staphylococcal carrier state, pyoderma, infections of the external eye and adenexa (Nishijima and Kurokawa, 2002). Despite its benefits, bacterial barriers and adverse effects such as nephrotoxicity, ototoxicity and neurotoxicity upon prolonged use limit gentamicin daily dosage (Robert and Walters, 1998). In fact, many clinicians are reluctant to use it, even for a short term (Drusano et al., 2007). Efforts have been made to determine its optimal therapeutic regimens in order to increase its overall efficacy while minimizing drug toxicity. These include liposomes (Jia et al., 2008), solidified reverse micellar drug delivery systems (Umeyor et al., 2011, 2012a, b), hydrogels (Eljarrat-Binstock et al., 2004; Changez et al., 2003; Ayhan and Özkan, 2007; Sokmen et al., 2008) and more recently, gentamicin transdermal microgels (Nnamani et al., 2013), and gentamicin-gold nanospheres for antimicrobial drug delivery to Staphylococcal infected foci (Ahangari et al., 2013). Topical hydrogels could be employed as an alternative low dose regimen aimed not only at reducing toxicity associated with prolonged use of gentamicin but also assuring proper utilization of the benefits of gentamicin, especially its rapid bactericidal activity, particularly in blood stream infections.These hydrogels can retain large quantities of gentamicin solution and can be directly applied to the skin without need for sophisticated equipment. Thus, this work seeks to design a gentamicin-loaded topical hydrogel and evaluate its physicochemical characteristics in an attempt to achieve predictable and reproducible gentamicin delivery. Gentamicin-loaded hydrogels of poloxamer 407 and polyacrylic acids (Carbopol® 971 and 974) were prepared and evaluated with respect to physicochemical properties, swelling and in vitro drug permeation. The hydrogel takes the form of a topical dosage system, containing a defined drug loading available for release within a defined surface area. Such a system is expected to be self-adhesive, backed with a protective material and capable of delivering a drug dose comparable to that delivered by Geby the proprietary gentamicin creams and ointments.

MATERIALS AND METHODS

The following materials were used: gentamicin pure powder (a kind
University of Nigeria, Nsukka, following the 18th Assembly Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

Three gelling agents were employed: Poloxamer 407 (P407), and two polyacrylic acids (Carbopol ® 971P and 974P, that is, C971 and C974). Poloxamer 407 was dissolved completely in purified water pre-cooled to approximately 5°C. Graded concentrations of gentamicin (0.03, 0.06 and 0.09 w/w) were dissolved in aliquots of purified water and propylene glycol added and mixed until a homogenous mass was formed. For the polyacrylic acid hydrogels, the same procedure was followed except that room temperature purified water was used instead of pre-cooled water and that the hydrogels were further neutralized by triethanolamine. All products were adjusted to pH 5.5 and kept at room temperature for 24 h to ensure absence of air-bubble before they were dispensed into an all-glass still. All experiments involving the use of animals were conducted in accordance with Ethical Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and Use Committee (Research Ethics Committee) of the University of Nigeria, Nsukka, following the 18th WMA General Assembly Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

Preparation of gentamicin hydrogels

<table>
<thead>
<tr>
<th>Composition (g)</th>
<th>Plain hydrogels (w/w)</th>
<th>Drug-loaded hydrogels (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poloxamer 407</td>
<td>Carbopol® 971P 974P</td>
</tr>
<tr>
<td></td>
<td>13.2</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Propylene glycol (a)</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>Triethanolamine (b)</td>
<td>40.0</td>
</tr>
</tbody>
</table>

Propylene glycol (a) means quantity in Poloxamer 407 hydrogel; Amount of purified water in drug-loaded Poloxamer 407 hydrogels was 40 ml. Propylene glycol (b) and Triethanolamine (b) mean quantity in Carbopol hydrogels only; Amount of purified water in drug-loaded Carbopol hydrogels was 44 ml.

Rheological evaluation

Viscosity assessment of the hydrogels was done using a Brookfield viscometer (GallenKamp, England). Due to the viscous nature of the formulations, 1, 3 and 5 g quantities of the semi-solid formulations were dissolved in 25 ml of purified water for 24 h and their viscosities determined. An average of three readings was carried out for validity of statistical analysis.

Spreadability determination

About 1 g of each formulation was sandwiched between two glass slides; a lower calibrated slide marked into 5 cm spaces and an unmarked upper slide. Different weights (50, 100, 200 and 300 g) were placed over the upper slide at 1 min intervals. The diameter occupied by the spreading gel was finally measured to determine the area of spread (length × width). The sample weight was fixed in order to have same assay for all the samples and to limit the glass from sliding.

Drug content analysis

About 0.5 g of hydrogel was dissolved in 10 ml of water, centrifuged (TDL-4 B. Bran Scientific and Instru. Co., England) at 1000 rpm for 30 min, filtered through a Whatman No. 1 filter paper, adequately diluted and the concentration of gentamicin determined spectrophotometrically (Shimadzu UV-1601 UV/Vis double beam spectrophotometer, Japan) after derivatization with o-phthaldialdehyde reagent by Zhang’s method (Zhang et al., 1994). Briefly, the o-phthaldialdehyde reagent was formulated by adding 2.5 g o-phthaldialdehyde, 62.5 ml methanol and 3 ml of 2-mercaptoethanol to 560 ml sodium borate in distilled water solution. The reagent was stored in a brown bottle in a dark chamber for at least 24 h before use. This reagent could be used only up to three days. Gentamicin sulphate solution, o-phthaldialdehyde reagent, and isopropanol (to avoid precipitation of the products formed) were mixed in similar proportions and stored for 30 min at room temperature. The homologous aromatic dialdehyde, o-phthaldialdehyde is essentially non-fluorescent until it reacts with a primary amine of gentamicin in the presence of excess sulfhydryl such as 2-mercaptoethanol to yield a fluorescent isoidnone whose absorbance was then measured at 332 nm (Chang et al., 2006).

Evaluation of the formulations

Physical examination

The semi-solid formulations were physically examined for colour, homogeneity, and consistency. The pH was also re-evaluated (before each use) to make sure that it was stable within the skin pH of 5.5 (El-Gendy et al., 2009).
Study of swelling behaviour of the hydrogels

The effect of pH on swelling of hydrogels was investigated as follows: 0.5 g quantity of hydrogel from each batch of hydrogels was weighed and placed in 10 ml of buffer solutions of different pH [0.1 N HCl (pH 1.2), double distilled water (pH 7.0) and phosphate buffered saline (PBS, pH 7.4)]. The hydrogels were removed from their respective swelling media at predetermined time intervals; blotted dry with tissue paper and their weights were observed on analytical balance. This process was continued until the sample appeared to be dissolved. The equilibrium weight swelling (ESW) of each hydrogel was calculated using the following equation (Raja et al., 2011):

\[
ESW = \frac{W_2 - W_1}{W_1} \times 100
\]

where \( ESW \) is the equilibrium swelling, \( W_2 \) represents the weight of the swollen hydrogel at time \( t \) and \( W_1 \) is the weight of the hydrogel before swelling.

Skin irritation test

After obtaining the study protocol approval from the University of Nigeria Committee on safe handling of experimental animals, experiments were performed on male albino Wistar rats weighing 150 to 180 g (Department of Pharmacology and Toxicology, UNN). The rats were maintained under controlled environmental conditions and 12-h light-dark cycle. All animals received standard laboratory diet and water ad libitum. The dorsal hair of the rats was carefully removed using electric clippers, while they acculturated. Formulations (0.5 g) were applied to the shaved dorsal surface of the rats and spread until complete absorption. The rats (n = 18) were divided into four groups of test (5 rats per batch of 3 test formulations) and control experiment [commercial gentamicin ointment, drug solution (gentamicin ampoule), and the control group that did not receive any treatment], altogether involving two rats. All formulations had equal drug concentration of 1% with the commercial gentamicin ointment. After 1 h application of all treatments, the treated skin was each time examined visually for erythema and/or oedema while the treatment lasted for 5 days.

Preparation of the rat abdominal skin

Male Wistar rats were sacrificed with prolonged anesthesia and the abdominal skin of each rat was excised. Abdominal hairs on the skin of animal were removed with clipper and full thickness skin was surgically removed and dermis side was wiped with isopropyl alcohol to remove residual adhering fat. Heat separation technique (Nnamani et al., 2013) was used to separate the epidermis. The technique involved soaking the entire abdominal skin in water at 60°C for 1 min followed by careful removal of the epidermis from dermis with a blunt forceps. The epidermis was washed with water and wrapped with aluminium foil and stored at -20°C until used. The stored epidermis was allowed to thaw, cut into 4.5 × 4.5 cm² pieces and hydrated by placing in phosphate buffer saline (PBS, pH 7.4) overnight before use.

Permeation studies

The skin permeation studies were performed by using a modified Franz diffusion cell following an established method (Nnamani et al., 2013). The effective diffusion area was 2.27 cm² and the receptor chamber has a capacity of 32 ml. The excised rat abdominal skin (Wistar albino) was mounted between the donor and receptor compartment of the diffusion cell. After equilibration for 30 min, 500 mg of the gentamicin-loaded hydrogel was placed in the donor compartment containing 5 ml of PBS. The receptor compartment of the diffusion cell was filled with PBS. The whole assembly was fixed on a magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 50 rpm; the temperature was maintained at 32 ± 0.5°C. The samples were withdrawn at different time intervals, filtered through a 0.45 µm pore size cellulose membrane filter and analyzed for drug content spectrophotometrically at 341 nm, against the blank (PBS). The receptor phase was replenished with equal volume of PBS at each sample withdrawal. The experiment was carried out in replicates. The cumulative percentages of drug permeated per square centimeter of the rat skin were plotted against time. Control experiments were also performed in each case using pure sample of gentamicin and commercially available gentamicin ointment.

Permeation data analysis

The flux (µg cm⁻² h⁻¹) of gentamicin was calculated from the slope of the plot of the cumulative amount of gentamicin permeated per cm² of skin at steady state against time using linear regression analysis (El-Gendy et al., 2009; Nnamani et al., 2013). The permeation coefficients were obtained from the steady-state flux values making use of the following equations.

\[
P = J/C_0 (cm/h)
\]

where \( P \) is the permeation coefficient; \( C_0 \) is the initial drug concentration in the drug compartment: \( J \) represents the steady-state flux obtained from equation 5.

\[
J = dQ/Adt (µg/cm².h)
\]

where \( Q \) indicates the quantity of substances crossing the rat skin, \( A \) is the area of the rat skin exposed and \( t \) is the time of exposure.

Kinetic modeling of drug release

To analyze the mechanism of drug release from the hydrogels, the release data were fitted to various release kinetic equations and models to determine the in vitro release kinetic models and mechanisms. Three kinetic models including the zero-order, first-order, and Higuchi square root models were applied to process the release data to find out the equation with the best fit (Stephens et al., 2000; Mayol et al., 2008; Raja et al., 2011; Nnamani et al., 2013).

\[
Q = K_1 t
\]

\[
Q = 100(1 - e^{-K_2 t})
\]

\[
Q = K_3 (t)^{1/2}
\]

where \( Q \) is the release percentage at time, \( t \), \( K_1 \), \( K_2 \), and \( K_3 \) are the rate constants of zero-order, first-order and Higuchi models, respectively.

Stability study of hydrogel formulations

Stability study was carried out for the hydrogel formulations as per ICH guidelines (Nnamani et al., 2013) at 40°C in a humidity chamber having 75% relative humidity (RH) for three months. After
hydrogel formulations. Hydrogels of Carbopol possessed the best physical assessment among all the Poloxamer 407 hydrogels were the most flexible and All the hydrogels were transparent, clear and colourless.

Physiological evaluation and pH measurements

Three months, samples were withdrawn and the drug content of the hydrogels re-evaluated.

RESULTS AND DISCUSSION

Characterization of gentamicin hydrogels

Physical evaluation and pH measurements

All the hydrogels were transparent, clear and colourless. Poloxamer 407 hydrogels were the most flexible and possessed the best physical assessment among all the hydrogel formulations. Hydrogels of Carbopol ®974P were more consistent than those of Carbopol ®971P. The pH values of the gentamicin hydrogels are presented in Table 2. The pH of the hydrogels of Carbopol ®971P and 974P before adjustment were more acidic than Poloxamer 407 (Table 2) that produced entirely basic hydrogels, hence the need to buffer the former with triethanolamine to pH of 5.5. This would enhance the efficiency of the formulations as topical drug delivery systems for gentamicin as well as make the formulations safe for use on the skin, consistent with previous report (El-Gendy et al., 2009).

Table 2. pH of the gentamicin hydrogels.

<table>
<thead>
<tr>
<th>Batch</th>
<th>0 mg</th>
<th>30 mg</th>
<th>60 mg</th>
<th>90 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poloxamer 407</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial pH</td>
<td>5.41</td>
<td>4.67</td>
<td>4.72</td>
<td>6.50</td>
</tr>
<tr>
<td>Adjusted pH</td>
<td>5.50</td>
<td>5.50</td>
<td>5.40</td>
<td>5.50</td>
</tr>
<tr>
<td>Carbopol ®971P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial pH</td>
<td>4.0</td>
<td>1.39</td>
<td>0.95</td>
<td>1.08</td>
</tr>
<tr>
<td>Adjusted pH</td>
<td>5.51</td>
<td>5.53</td>
<td>5.50</td>
<td>5.42</td>
</tr>
<tr>
<td>Carbopol ®974P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial pH</td>
<td>1.16</td>
<td>1.13</td>
<td>1.04</td>
<td>1.06</td>
</tr>
<tr>
<td>Adjusted pH</td>
<td>5.48</td>
<td>5.49</td>
<td>5.56</td>
<td>5.44</td>
</tr>
</tbody>
</table>

Buffers used: Acid – pH H₄, Base – pH H₃, Triethanolamine – drops.

Viscosity measurement

Figure 1a to c shows the results of viscosity determination of hydrogels based on P407, C971P and C974P, respectively. Results of the study indicate that hydrogels of C974P (batches C₀–C₃ of Figure 1c) were the most viscous whereas hydrogels of P407 (batches A₀–A₃ of Figure 1a) were the least viscous among the polymeric hydrogels. This is justified since C974P which is a highly cross-linked polymer produced highly viscous gels with rheology similar to mayonnaise (Srividya et al., 2001; Cho et al., 2012; Wagner et al., 2000). On the other hand, P407, being the least cross-linked polymer among the polymeric hydrogels (Shazly et al., 2012; Graham and McNeill, 1984; Desai and Blanchard, 1998) employed in this study produced the least viscous hydrogels.

The results equally revealed concentration-dependent increase in the viscosities of all hydrogel formulations (that is, 5 g >3 g >1 g/25 ml of distilled water). It was also observed that the drug-loaded hydrogels (batches A₁–A₃, B₁–B₃ and C₁–C₃) were more viscous than the plain hydrogels (batches A₀, B₀ and C₀), which may be related to the additional drug content in the formulation compositions of the former. Among the drug-loaded hydrogels, viscosities decreased with increase in drug loading irrespective of the polymeric hydrogel employed in the formulation, and as such hydrogel formulations loaded with 30 mg of gentamicin (batches A₁–C₁) were the most viscous whereas hydrogels loaded with 90 mg of gentamicin (batches A₃–C₃) were the least viscous. The reason for this is unknown but may be related to enhanced drug entrapment by the hydrophilic polymers. Viscosity of the gel matrix is an important factor to consider in evaluation of drug penetration from gels across the skin or artificial membrane (Azarmi et al., 2007) and is used to measure the extrudability of a gel (Nnamani et al., 2013). Decreased viscosity of the hydrogels at higher drug loadings (batches A₂–C₂ and A₃–C₃) not only implied that these hydrogel formulations would be easily extruded from their containers or packages, but also indicated potential improvement in the diffusivity of gentamicin within the hydrogel network which in turn would facilitate flux, consistent with previous reports (Azarmi et al., 2007; Nnamani et al., 2013).

Spreadability determination

The results of spreadability determination of the hydrogel formulations (Figure 2a to c) generally credited hydrogel formulations based on P407 as the best followed by hydrogel formulations of C971P and lastly C974P hydrogel formulations. This is in agreement with the results of viscosity determination, as the most viscous formulations were the least spreadable and vise versa. It was not surprising that hydrogel formulations based on C971P NF polymer were more spreadable than those of C974P NF polymer, because C971P polymer hydrogel is a lightly cross-linked polymer with long rheology which would result in flow-like honey (high spreadability) in a semi-solid formulation (Desai and Blanchard, 1998), as seen in batches B₀–B₃. The C974P polymeric hydrogel, which is a highly cross-linked polymer, produces highly viscous hydrogels (batches C₀–C₃) with rheology similar to mayonnaise (low spreadability) (Nnamani et al., 2013; Wagner et al., 2000). P407 is the least cross-linked polymer among the polymeric hydrogels (Shazly et al., 2012; Graham and McNeill, 1984; Desai and Blanchard,
hence the most spreadable and the least viscous hydrogel formulations (batches A1-C3) were obtained from it. The results also revealed that drug-loaded hydrogel formulations (batches A1-A3, B1-B3 and C1-C3) were comparatively less spreadable than the plain hydrogels (A0-C0), since the latter were less viscous than the former, as earlier explained. Furthermore, it was observed from Figure 2a to c that there was load (weight)-dependent (300 g > 200 g > 100 g > 50 g) as well as drug concentration-dependent (90 mg > 60 mg > 30 mg) increases in areas spread across all batches. Among the drug-loaded hydrogel formulations, batches A2-C3 containing 90 mg of gentamicin were the most spreadable, thus justifying the viscosity results (the least viscous drug-loaded batches); whereas those containing 30 mg of gentamicin (batches A1-C1) were the least spreadable, because they were the most viscous gentamicin-loaded hydrogel formulations. Overall, batch A3 hydrogel formulation containing P407 and the highest quantity of gentamicin (90 mg) (Figure 2a) was the least...
Figure 2. Spreadability of P407 hydrogels (a), C971P hydrogels (b) and C974P hydrogels (c) based on 50, 100, 200 and 300 g weights (n=3). A₁-C₁, A₂-C₂ and A₃-C₃ contain 30, 60 and 90 mg of gentamicin sulphate respectively while A₀-C₀ are drug-free hydrogels.

Table 3. Drug content and kinetic models of drug release from gentamicin-loaded hydrogels.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
<th>Drug content (%) (^{a,b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>0.9269</td>
<td>0.8899</td>
<td>0.9935</td>
<td>68.52 ± 1.88</td>
</tr>
<tr>
<td>A₂</td>
<td>0.8551</td>
<td>0.8793</td>
<td>0.9802</td>
<td>85.76 ± 4.00</td>
</tr>
<tr>
<td>A₃</td>
<td>0.9427</td>
<td>0.9338</td>
<td>0.9955</td>
<td>77.05 ± 3.98</td>
</tr>
<tr>
<td>B₁</td>
<td>0.8843</td>
<td>0.7275</td>
<td>0.9809</td>
<td>82.89 ± 2.83</td>
</tr>
<tr>
<td>B₂</td>
<td>0.9660</td>
<td>0.8791</td>
<td>0.9904</td>
<td>93.00 ± 4.04</td>
</tr>
<tr>
<td>B₃</td>
<td>0.9365</td>
<td>0.9516</td>
<td>0.9835</td>
<td>86.27 ± 3.75</td>
</tr>
<tr>
<td>C₁</td>
<td>0.9891</td>
<td>0.7217</td>
<td>0.9981</td>
<td>79.10 ± 2.08</td>
</tr>
<tr>
<td>C₂</td>
<td>0.8925</td>
<td>0.8942</td>
<td>0.8999</td>
<td>90.66 ± 3.90</td>
</tr>
<tr>
<td>C₃</td>
<td>0.9955</td>
<td>0.9781</td>
<td>0.9966</td>
<td>84.91 ± 2.72</td>
</tr>
</tbody>
</table>

\(^a\)Means\(±\)SD, \(^b\)n=3 after 3 months, A₁-A₃, B₁-B₃, and C₁-C₃ are Poloxamer 407, Carbopol\(^®\) 971P and Carbopol\(^®\) 974P hydrogels containing 30, 60 and 90 mg of gentamicin sulphate respectively.
viscous and most spreadable, thus necessitating further development of the latter as topical dosage form.

**Drug content analysis**

The content of gentamicin in the hydrogel formulations is as shown in Table 3. The results indicate that drug encapsulation efficiency (EE) increased with increase in the concentration of gentamicin up to 0.06% w/w for all batches, yielding maximum EE (85.76, 93.00 and 90.66%) for hydrogels formulated with P407, C971 and C974, respectively. Thereafter, the drug encapsulation efficiency decreased with increased drug loading. So, the hydrogels loaded with 0.06% w/w gentamicin resulted in higher EE percent while those loaded with 0.09% w/w gentamicin gave the least. The implication is that gentamicin attained saturation solubility in the hydrogel matrices at 0.06% w/w loading for all batches, and so, more of the drug could not be solubilized and encapsulated, consistent with earlier reports (Umeyor et al., 2011; Umeyor et al., 2012a). However, all the batches had good EE percent. Overall, batch B₂ formulated with C971P polymeric hydrogel and 0.06% w/w gentamicin gave the best drug content of 93.00%. Since gentamicin is freely soluble in water (hydrophilic), the result agrees with Küchler et al. (2009) that much of the drug was present in the aqueous phase of the formulations, loosely attached at or near the particle surface, because the more hydrophilic the substance, the weaker the interaction with particle surface, and eventually the compound could be localized in the surfactant layer. When more drug particles at the periphery of the particle surface eventually encounter the polymeric cross-linked gel-matrices of polyacrylic acid resins and P407, stabilization would occur (Souto et al., 2011). Thus, to improve the skin uptake and delivery of such a drug, a semi-solid vehicle such as topical hydrogels would be a better approach as it would exhibit high stability in addition to low toxicity.

**Swelling behaviour of the hydrogel formulations**

Swelling parameter is a vital factor for characterization of hydrogels, because there is fundamental relationship between swelling of the polymer and nature of the swelling medium (Raja et al., 2011). Swelling studies for the concentration of gentamicin up to 0.06% w/w for all batches, yielding maximum EE (85.76, 93.00 and 90.66%) for hydrogels formulated with P407, C971 and C974, respectively. Thereafter, the drug encapsulation efficiency decreased with increased drug loading. So, the hydrogels loaded with 0.06% w/w gentamicin resulted in higher EE percent while those loaded with 0.09% w/w gentamicin gave the least. The implication is that gentamicin attained saturation solubility in the hydrogel matrices at 0.06% w/w loading for all batches, and so, more of the drug could not be solubilized and encapsulated, consistent with earlier reports (Umeyor et al., 2011; Umeyor et al., 2012a). However, all the batches had good EE percent. Overall, batch B₂ formulated with C971P polymeric hydrogel and 0.06% w/w gentamicin gave the best drug content of 93.00%. Since gentamicin is freely soluble in water (hydrophilic), the result agrees with Küchler et al. (2009) that much of the drug was present in the aqueous phase of the formulations, loosely attached at or near the particle surface, because the more hydrophilic the substance, the weaker the interaction with particle surface, and eventually the compound could be localized in the surfactant layer. When more drug particles at the periphery of the particle surface eventually encounter the polymeric cross-linked gel-matrices of polyacrylic acid resins and P407, stabilization would occur (Souto et al., 2011). Thus, to improve the skin uptake and delivery of such a drug, a semi-solid vehicle such as topical hydrogels would be a better approach as it would exhibit high stability in addition to low toxicity.

**Skin irritation and in vitro drug permeation**

The skin irritability test was carried out to evaluate the tolerability of the gentamicin hydrogels after application. The results obtained agreed with literature knowledge of the useful polymers not adversely affecting the skin (Kopecek, 1984; Desai and Blanchard, 1998; Srividya et al., 2001; Cho et al., 2012; Wagner et al., 2000; Küchler et al., 2009; Souto et al., 2011). There was no erythema or edema observed generally across all batches. Based on this, the gentamicin hydrogel formulations were well-tolerated by the rat.

The results of the permeation study, which is an important tool that predicts in advance how a drug would behave in vivo (El-Gendy et al., 2009; Nnamani et al., 2013; Ahangari et al., 2013). The faster swelling of drug-loaded hydrogels prepared from P407 (batches A₁-A₃) at 8 h especially in PBS (Figure 3Aa) suggests faster release of the incorporated drug and perhaps, faster antibacterial activity whereas lower degrees of swelling associated with gentamicin-loaded hydrogels of the polyacrylic acids batches (B₁-B₃ and C₁-C₃) suggest possibility of prolongation of release of the incorporated drug; since drug release is directly related to polymer swelling (Raja et al., 2011). Meanwhile, polymer swelling is controlled by many other factors (Geever et al., 2008).
Figure 3. Swelling profiles of P407 hydrogels (A), C971P hydrogels (B) and C974P hydrogels (C) in (a) alkaline medium (PBS, pH 7.4), (b) neutral medium (double distilled water, pH 7.0), and (c) acidic medium (0.1N HCl, pH 1.2) (n=3). A<sub>1</sub>-C<sub>1</sub>, A<sub>2</sub>-C<sub>2</sub> and A<sub>3</sub>-C<sub>3</sub> contain 30, 60 and 90 mg of gentamicin sulphate respectively while A<sub>0</sub>-C<sub>0</sub> are drug-free hydrogels.
Figure 4. Permeation profile of gentamicin sulphate from (a) P407 hydrogels, (b) C971P hydrogels, (c) C974P hydrogels in PBS, pH 7.4 (n=3). A1-C1, A2-C2 and A3-C3 contain 30, 60 and 90 mg of gentamicin sulphate respectively while S1 and S2 are commercial gentamicin sulphate ointment and pure sample of gentamicin, respectively.

(2013) are shown in Figure 4a to c, whereas the permeation data (permeation coefficients and steady-state permeation fluxes) were presented in Table 4. It is evident from the figures that there was controlled permeation of gentamicin from the topical hydrogels without a burst effect in all the formulations.

In vitro permeation was performed in PBS which was the medium that recorded the highest swelling of the hydrogel formulations. The permeability assessment of gentamicin from the hydrogel formulations across the rat skin (Table 4) showed permeation fluxes of 4.917, 5.161 and 5.239 µg/cm².h for P407 hydrogel formulations containing 30, 60 and 90 mg of gentamicin, respectively (batches A1-A3); 4.126, 4.572, and 4.717 µg/cm².h for C971P-based hydrogel formulations containing 20, 60 and 90 mg of gentamicin sulphate, respectively (batches B1-B3); then 4.761, 4.839 and 4.961 µg/cm².h for C974P-based hydrogel formulations containing 30, 60 and 90 mg of gentamicin sulphate, respectively (batches C1-C3). The permeation fluxes for commercial gentamicin
Table 4. Permeation parameters of gentamicin-loaded hydrogel formulations.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Flux ($J$) ($\mu$g/cm$^2$.h)$^{a,b}$</th>
<th>Permeation coefficient ($P$) (cm/h)$^{a,b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A$_1$</td>
<td>4.917</td>
<td>$9.834 \times 10^{-7}$</td>
</tr>
<tr>
<td>A$_2$</td>
<td>5.161</td>
<td>$1.032 \times 10^{-6}$</td>
</tr>
<tr>
<td>A$_3$</td>
<td>5.239</td>
<td>$1.781 \times 10^{-6}$</td>
</tr>
<tr>
<td>B$_1$</td>
<td>4.126</td>
<td>$9.048 \times 10^{-7}$</td>
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<tr>
<td>B$_2$</td>
<td>4.572</td>
<td>$9.395 \times 10^{-7}$</td>
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<td>B$_3$</td>
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<td>$9.496 \times 10^{-7}$</td>
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<tr>
<td>C$_1$</td>
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</tr>
<tr>
<td>C$_2$</td>
<td>4.839</td>
<td>$9.678 \times 10^{-7}$</td>
</tr>
<tr>
<td>C$_3$</td>
<td>4.961</td>
<td>$9.857 \times 10^{-7}$</td>
</tr>
<tr>
<td>S$_1$</td>
<td>3.837</td>
<td>$8.329 \times 10^{-7}$</td>
</tr>
<tr>
<td>S$_2$</td>
<td>2.354</td>
<td>$7.106 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

$^a$Mean±SD, $^b$n=3, A$_1$-A$_3$, B$_1$-B$_3$, and C$_1$-C$_3$ are Poloxamer 407, Carbopol® 971P and Carbopol® 974P hydrogels containing 30, 60 and 90 mg of gentamicin sulphate respectively; S$_1$ and S$_2$ are commercial gentamicin ointment and pure sample of gentamicin, respectively.

ointment BP and pure sample of gentamicin were 3.839 and 2.354 $\mu$g/cm$^2$.h, respectively (batches S$_1$ and S$_2$). The permeation coefficients of P407 based hydrogel formulations in PBS were $9.834 \times 10^{-7}$, $1.032 \times 10^{-6}$ and $1.781 \times 10^{-6}$ cm/h for hydrogels containing 30, 60 and 90 mg of gentamicin, respectively (batches A$_1$-A$_3$); the permeation coefficients of C971P-based hydrogel formulations were $9.048 \times 10^{-7}$, $9.395 \times 10^{-7}$ and $9.496 \times 10^{-7}$ cm/h for hydrogels containing 30, 60 and 90 mg of gentamicin sulphate, respectively (batches B$_1$-B$_3$); the permeation coefficients of C974P-based hydrogel formulations were $9.522 \times 10^{-7}$, $9.678 \times 10^{-7}$ and $9.857 \times 10^{-7}$ cm/h for hydrogels containing 30, 60 and 90 mg of gentamicin sulphate, respectively (batches C$_1$-C$_3$); while the permeation coefficients of gentamicin ointment BP and pure sample of gentamicin were: $8.329 \times 10^{-7}$ and $7.106 \times 10^{-7}$ cm/h, respectively. These values were within the range obtained for some lipophilic and lipophobic drugs (Nnamani et al., 2013; Küchler et al., 2009; Souto et al., 2011). Since drug-loaded hydrogel formulations prepared with P407 especially batch A3 gave the highest permeation flux and coefficients, it implies that sustained release gentamicin dosage form might be developed with this polymer, consistent with similar hydrogel formulations based on this polymeric hydrogel (Mayol et al., 2008; Desai and Blanchard, 1998). Also, the high swelling suggests fast and high drug release of the incorporated drug (Raja et al., 2011; Geever et al., 2008). Generally, topical hydrogels based on P407 gave greater permeation fluxes and coefficients than corresponding hydrogel formulations based on Carbopols® 971P and 974P. Comparing these with the fluxes and coefficients of unformulated gentamicin sulphate (pure sample of gentamicin) and gentamicin ointment BP, the gentamicin-loaded hydrogel formulations demonstrated enhanced permeation attesting further to improvement in the in vitro performance of the formulations. The drug permeation result presented here strongly indicates improved permeation of the drug through the rat skin membrane which is known to mimic permeation in vivo (El-Gendy et al., 2009; Nnamani et al., 2013) as stated earlier. Flux across the skin is dependent upon skin hydration, drug partitioning and transport as well as concentration gradient across the skin (Azarmi et al., 2007). By implication, polymeric hydrogels (P407, C974P and C971P) facilitated release of drug from the hydrogels and its subsequent transport across the rat skin, more than the commercially available gentamicin ointment and the unformulated drug (pure sample of gentamicin). This enhanced skin delivery of gentamicin would be of immense benefit in treatment of topical microbial infections caused by gentamicin-susceptible organisms since the topical formulations could be employed either for localized treatment of tissues underlying the skin (Brown et al., 2006).

Kinetic modeling of drug release

Different mathematical models were used to describe the kinetics of gentamicin release from hydrogels. The criterion for selecting the most appropriate model was chosen on the basis of goodness-of-fit test (Stephens et al., 2000; Mayol et al., 2008; Raja et al., 2011; Nnamani et al., 2013; Melero et al., 2012). The result is presented in Table 3. A comparative evaluation of the coefficient of determination ($r^2$) for hydrogel formulations shows that drug release from the formulations obeyed the Higuchi square root model better than other models. By implication, the release of gentamicin sulphate from hydrogels was governed predominantly by a diffusion mechanism.

Stability study

Assessment of the stability of novel formulations is
always very important in drug product design and development. Stability could be viewed from the degradation of the active ingredients or physical property of the formulation (Nnamani et al., 2013). In order to determine the change in drug content on storage, stability study was carried out. Table 3 shows insignificant difference in drug content of gentamicin before and after storage for three months. The result indicates that the formulations were stable at the required storage condition.

Conclusions

The design and preparation of hydrogels is an exciting field of research that seeks to exploit the attractive properties of different polymeric carriers to improve the delivery of therapeutic molecules. In this research work, gentamicin-loaded topical hydrogels based on Poloxamer 407 and Carbopol® (971P and 974P) were developed and evaluated for improved skin delivery of gentamicin. All the formulations showed pH-dependent swelling, good drug encapsulation, tolerability on rats and stability, as well as good in vitro property performance, which were generally better than commercial gentamicin ointment and pure sample of gentamicin. The Poloxamer 407 based-hydrogels exhibited the best in vitro performance. It follows, therefore, that this delivery system could offer a promising approach for the treatment of topical infections caused by gentamicin-susceptible bacteria.

ACKNOWLEDGEMENT

The authors thank BASF, Ludwigshafen, Germany for the provision of Poloxamer 407 used in this study. They also acknowledge the support of the Lubrizol Corporation, USA for samples of Carbopol® 971P and 974P.

Abbreviations: P407, Poloxamer 407; C971P, Carbopol® 971P; C974P, Carbopol® 974P.

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Full Length Research Paper

Galenic formulation and antimicrobial activities of tablets made from essential oil of *Lippia multiflora* Moldenke (Verbenaceae)

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Essential oil-containing tablets made from essential oil of *Lippia multiflora* Moldenke have been developed with an intention to treat buccal and pharyngeal infections after perlingual administration. Tablets were accomplished with Arabic gum, mannitol and *L. multiflora* essential oils (2.5% w/w) and tested for antimicrobial activity using agar dilution techniques. The tablets were tested against strains from buccal and pharyngeal flora like *Streptococcus β hemolyticus A*, *Streptococcus α hemolyticus*, *Staphylococcus aureus*, *Staphylococcus* non *aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and fungi *Candida albicans*. Galenical and biogalenical controls proved to be satisfactory. Essential oil-containing tablets possessed significant antimicrobial effect. Gram-positive bacteria and fungi were more sensitive to test tablets than Gram-negative bacteria. The essential oil of *L. multiflora* formulated in tablets retained its antimicrobial activity against oral and pharyngeal pathogen.

Key words: *Lippia multiflora*, essential oil, essential oil-containing tablets, antimicrobial.

INTRODUCTION

Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century. In the developing countries, bacterial infections are still the main cause of deaths (Iwu et al., 1999). Down the ages, essential oils and other extracts of plants have evoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases (Tepe et al., 2004). According to the World Health Organization (WHO), about 80% of the people in developing countries rely primarily on medicinal plants for their primary health care (Wood-Sheldon et al., 1997). Medicinal and aromatic plants are widely used as medicine and constitute a major source of natural organic compounds. It has long been acknowledged that some plant essential oils exhibit antimicrobial properties and it is necessary to investigate these plants scientifically. Essential oils are potential sources of novel antimicrobial compounds, especially against bacterial pathogens (Azizi et al., 2009). *Lippia multiflora*, a member of the Verbenaceae family, is an aromatic and medicinal plant of West Africa (Adjanohoun and Aké, 1979). African traditional medicine healers use their leaves as tea and in the treatment of malaria, hyper-
tension (Koffi, 1995), boils, diarrhea (Nacoulma-Ouédraogo, 1996), antipyretic and diuretic (Kanko, 1995), antifungal (Baba-Moussa et al., 1997) and as a mouth disinfector (Kerhaho and Adam, 1974). This plant’s essential oil compositions and its antimicrobial properties have previously been investigated. The main constituents of the investigated *L. multiflora* oils were identified as 1,8-Cinéol, geranial, terpinène, and thymol (Oussou et al., 2004; Kunle et al., 2004; Bassolé et al., 2003).

A number of studies have demonstrated the antimicrobial properties of *L. multiflora* oils and its major components against a wide range of microorganisms, the same microorganisms of the buccal flora (Bassole et al., 2003; Oladimeji et al., 2004; Oussou et al., 2004; Kunle et al., 2003; Pelissier et al., 1994).

Our research focused on developing an oral pharyngeal formula processed into tablets containing this essential oil of *L. multiflora* and to evaluate its antimicrobial activity against oral clinical isolates of Gram positive (*Staphylococcus aureus*, *Staphylococcus negative*-coagulase, Streptococcus A, Streptococcus a, *Enterococcus faecalis*) and Gram negative (*Escherichia coli*, Klebsiella pneumoniae, Pseudomonas aeruginosae) bacteria, in addition to a pathogenic fungus, *Candida albicans*. The main aim of this study was therefore to demonstrate whether the essential oil of *L. multiflora* when associated with prior judiciously chosen excipients within a galenical formulation could retain its antimicrobial activity.

**MATERIALS AND METHODS**

**Collection of plant material and extraction process of essential oils**

The leaves of *L. multiflora* were collected in Boundiali, Northeren Côte d’Ivoire, in March, 2006. Taxonomic identification of the plant was performed by a Botanist, Prof. Ake Assi of the Centre National de Floristique d’Abidjan (CNF). *L. multiflora* was dried for 10 days at 28 and 30°C in the dry drain board of the laboratory. Air-dried plant material (100 g) of *L. multiflora* was extracted by hydro-distillation for 2 h in a modified Cleveinger-type apparatus (yield 0.5% v/w).

Three kinds of distillations were brought to the boiler for 2 h, using air-dried vegetal material (100 g) in 1 L of water; the whole mixture was put in a 2 L balloon, topped by a 60 cm long airstream and connected to a refrigetar. The essential oil thus obtained was protected from light and stored at 4°C till further use.

**Excipients**

All excipients used for the galenical formula were obtained from Cooper Pharmaceuticals, (France).

**Microbial strains**

A panel of twenty six common pathogenic organisms was used for antibacterial tests (Table 1), which includes gram-positive bacteria, gram-negative bacteria and fungi. These clinical isolates were obtained from oral pharyngeal samples of the patients suffering from pharingitis, from the external Ear Nose and Throat (ENT) Department, CHU-Cocody, Côte d’Ivoire. All strains were purified by three successive streaking and re-isolation on Mueller Hinton agar or Saboraud chloramphenicol agar. The purity and identity were confirmed by standard bacteriological methods (Feron, 1994). Media: Nutrient broth No 2, pH 7.4; nutrient agar, pH 7.4; malt extract broth, pH 5.6; Mueller Hinton agar (MHA) are all products from Bio-Rad Laboratories, (France) used for the experiment.

**Formulation of essential oil-containing tablets**

Some pre formulation tests carried out and taken into account organoleptic features, colour and consistency helped constitute a basic formula. A unique permanent formula was adopted so that the final concentration in essential oil of *L. multiflora* could stand to 150 µg/ml. Pounded in a Chinese made mortar, a pasty mixture was obtained and prepared in water with all excipients. The essential oil was then added to this pasty mixture, homogenized and allowed to solidify. After 3 to 5 days, solidified homogeneous paste was chopped off into pieces of 1 g unit.

**Mascroscopical and biogalenical tests**

Ten volunteers were given the tablets and questioned for their opinion on its colour, taste and odour. The pH value was set by using pH meter. Ten gram (10 g) of tablets were weighed and dissolved in 90 ml of germ free distilled-water. After filtration, the homogeneous solution thus obtained allowed the identification of

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**Table 1. Profile of resistance of the Cocci positive Gram to antibiotics.**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Antibiotics</th>
<th>OXA R</th>
<th>AM R</th>
<th>CTX R</th>
<th>CAZ R</th>
<th>GM R</th>
<th>E R</th>
<th>L R</th>
<th>VAN R</th>
<th>LVX R</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> (N=3)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><em>S. non aureus</em> (N=1)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Streptococq A (N=1)</td>
<td></td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>Rn</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Streptococq a (N=8)</td>
<td></td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Rn</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><em>E. feacalis</em> (N= 5)</td>
<td></td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>Rn</td>
<td>4</td>
<td>Rn</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

OXA: Oxacilline 5 µg; AM: amoxicilline 20 µg; CTX: cefotaxime 30 µg; CAZ: ceftazidine 30 µg; GM : gentamycine 15 and 500 µg; E: erytromycine 15 UI; L: lincosamine 15 µg; VAN: vancomycine 30 µg; LVX: levofl oxacine 5 µg; R: resistant; Rn: low natural resistance; Rn: natural resistance.
the pH rate; five measurements were performed to achieve this stage. The biogalenical test consisted in determining the disintegrative duration. This was realized with an Erweka set of apparatus equipped with an oscillating basket. A unit quantity of 10 g of tablets and a disc were put in the different 6 tubes of the oscillating basket apparatus, then it was immersed in a 1 liter Becher flask by using a method of to and fro vertical movements (28 per minute with an amplitude of 5 cm). The final stage of disintegration was reached at a point where no sediment (remaining paste) was observed on the grating. The disintegration tests were immediately carried out upon tablets just after its preparation (Day 0) and also on each consecutive day after its preparation (that is, Day 1 to 7).

Antibacterial tests

The agar dilution method was used to assess the antimicrobial activities of the essential oil-containing tablet. One series of dilutions of tablets was prepared in peptoned thinner previously buffered with tween (monopotassium phosphate: 3.5 g; disodium phosphate: 7.25 g; sodium Chloride: 4.3 g; peptone 1; tween 80: 3 g; water qsp: 1000 ml). Each concentrations of tablets incorporated in Mueller Hinton agar (bacteria) or Sabouraud-chloramphenicol (fungus) were 18.75, 30, 37.5 and 60 µg/ml. The diluent was also used as the negative control. A suspension of the organism is prepared to be tested equivalent to $10^6$ cfu/ml (Oussou et al., 2008), and 10 µl of this suspension is placed on each of the series of plates using a micro-pipette (final inoculum were $10^5$ cfu/spot). Four different bacterial isolates (plus quality control organisms) can be tested simultaneously on each agar plate. For fungi, two spot were placed on each of the series of plates containing Sabouraud-chloramphenicol. Two fungi have been tested simultaneously on each agar plate. The plates were incubated for 18 to 24 h at 37°C (bacteria) and 72 h at 30°C (fungi). Amoxicillin (20 µg) was used as positive controls for bacteria and Amphotericin B (100 µg) for fungus.

Each test was performed in triplicate and repeated thrice. Growths in plates were estimated in term of inhibition percentage reckoned from a proportion of 100% survival in the reference plates of growth control. The calculation method of the inhibition percentage of each bacterium at various tested concentrations could be summed up by the following formula:

$$I = 100 - \frac{n}{N} \times 100$$

Where I: Inhibition of microbacterial isolates (in percentage %), N: number of cultured microbacterial isolates, n: number of positively grown microbacterial.

RESULTS AND DISCUSSION

Macroscopical and biogalenical tests

The tablets formula consisted of 40% pulverized Arabic gum, 40% of mannitol, 250 µl of L. multiflora essential oil and distilled water qsp 10 ml. The tablets displayed a homogeneous particularity, a slightly sweet taste and citronella like fragrance, with an average pH of 7.4. The biogalenical results (desintegration duration) carried out upon the tablets are presented in Figure 1, and the tablets disintegration curve testified the evolution from day 0 to day 7. The curve stayed stationary with dissolution duration of the tablets less than 1 min from day 0 to day 7; from day 2 to day 5, this duration altered.

Figure 1. The tablets disintegration curve
Table 2. Profile of resistance of negative the Gram bacilli to antibiotics.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>AM&lt;sup&gt;n&lt;/sup&gt;</th>
<th>AMC&lt;sup&gt;n&lt;/sup&gt;</th>
<th>AN&lt;sup&gt;n&lt;/sup&gt;</th>
<th>TIC&lt;sup&gt;n&lt;/sup&gt;</th>
<th>CIP&lt;sup&gt;n&lt;/sup&gt;</th>
<th>CTX&lt;sup&gt;n&lt;/sup&gt;</th>
<th>CXM&lt;sup&gt;n&lt;/sup&gt;</th>
<th>GM&lt;sup&gt;n&lt;/sup&gt;</th>
<th>IMP&lt;sup&gt;n&lt;/sup&gt;</th>
<th>PIP&lt;sup&gt;n&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae (N=2)</td>
<td>Rn</td>
<td>1</td>
<td>1</td>
<td>Rn</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli (N=2)</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (N=2)</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

AMC: amoxicilline + acide clavulanique 20 + 10 µg; AN: amikacine 30 µg; TIC: ticarcilline 75 µg; CIP: ciprofloxacine 5 µg; CXM: cefadroxyl 30 µg; IMP: imipineme 10 µg; PIP: piperacilline 75 µg.

Table 3. Antibacterial activities upon bacterial clones by the tablets (% of inhibition).

<table>
<thead>
<tr>
<th>Bacteria clones</th>
<th>Dilution of the tablets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2/5</td>
</tr>
<tr>
<td>Streptocoque β hemolytique A (N=1)</td>
<td>100</td>
</tr>
<tr>
<td>Streptocoque α hemolytique (N=8)</td>
<td>100</td>
</tr>
<tr>
<td>S. aureus (N=3)</td>
<td>100</td>
</tr>
<tr>
<td>S. non aureus (N=1)</td>
<td>100</td>
</tr>
<tr>
<td>E. faecalis (N=5)</td>
<td>100</td>
</tr>
<tr>
<td>E. coli (N=2)</td>
<td>100</td>
</tr>
<tr>
<td>K. pneumoniae (N=2)</td>
<td>100</td>
</tr>
<tr>
<td>P. aeruginosa (N=2)</td>
<td>0</td>
</tr>
</tbody>
</table>

from 1 min to 1 min 24 s. While from day 5 to day 7, the disintegration time remained basically constant at 1 min 30 s.

The biogalenical study of the preparation indicated an increase of disintegration duration of tablets during their conservation. This duration rose by 30 s after seven days of their preparation. As disintegration plays an important role in maintaining porosity and hardness of the galenical shape, it was deduced that during the conservation, either the hardness had increased or the porosity had decreased. It might be related to the presence of the Arabic gum which becomes hard in presence of humidity and reduces porosity of the galenical shape by increasing the connections between particles. Thus, it is advisable to keep these tablets in water-tight packaging (bearing a weak humidity rate).

Antibacterial tests

Plant essential oil extracts have been used for many thousands of years, especially in food preservation, pharmaceuticals, medicine and natural therapies (Hazzit and Baaliouamer, 2009). It is necessary to investigate those plants scientifically which have been used in traditional medicine to improve the quality of healthcare. Essential oils are potential sources of novel antimicrobial compounds (Mitscher et al., 1987), especially against bacterial pathogens.

The antimicrobial profile of essential oil-containing tablets is shown in Table 1. The figures in the table are calculated mean percentage of inhibition. In general, essential oil-containing tablets displayed varying degrees of antimicrobial activity against the tested microorganisms. At the concentration of 37.5 µg/ml, tablets inhibited the growth of S. β hemolyticus A, S. α hemolytique, S. aureus, S. non aureus and E. faecalis (Table 2). They also inhibited the growth of all the strains of E. coli and K. pneumoniae at 60 µg/ml. The tablet had strongest activity against C. albicans at 30 µg/ml (Table 3). Pseudomonas aeruginosa was the most resistant bacteria since the essential oil-containing tablets showed no inhibition towards it. For tablets, the gram-positive bacteria and fungi C. albicans were more sensitive compared to the gram-negative bacteria (Table 4).

The in vitro activities tests carried out with the tablets shows an inner considerable antibacterial activity. The L. multiflora essential oil-containing tablets have therefore retained their antibacterial properties when probed upon pathogenic bacteria of buccal flora. The results obtained were in perfect comformity with those reported by Pelissier et al. (1994) and Adon (1993), who used L. multithora essential oil-mouth rinse and essential oil-toothpaste, respectively. The galenical formulation was reported to be highly active against isolated microorganisms (bacteria and fungi) of the buccal flora and thus supporting its traditional use in mouthwash in some communities.
In general, the antimicrobial activity of the essential oils tested was more pronounced against Gram-positive than against Gram-negative bacteria, a general observation derived from studies with essential oils from many other plant spices (Nostro et al., 2000). This generally higher resistance among Gram-negative bacteria could be ascribed to the presence of their phospholipidic membrane, almost impermeable to lipophilic compounds (Nikaido and Vaara, 1985). The absence of this barrier in Gram-positive bacteria allows the direct contact of essential oil’s hydrophobic constituents with which they bring about their effect, causing either an increase of ion permeability and leakage of vital intracellular constituents, or impairment of the bacterial enzyme system (Cowan, 1999).

P. aeruginosa was definitively too robust. This resistance is not astounding, for the bacterium is endowed with an intrinsic resistance to biocides because of the nature of its external membrane. The latter consists of lipopolysaccharides which build up a barrier impervious to hydrophobic composites. Once it is tested by permeabilising agents, this external membrane’s inactive substances against P. aeruginosa become active (Ratledge and Wilkinson, 1988). Noted to be implicated in various infections whose evolutions are more or less acute, depending on the status of the environment, and very often dramatic for acquired immune deficiency sick people (Siqueira et al., 1985), C. albicans fungi turned out to be very sensitive compared to other microorganisms.

The essential oil of L. multiflora originated from Côte d’Ivoire is composed of a majority of oxygenated monoterpenes. Kanko et al. (2004) and Oussou et al. (2004) identified 1,8-cineol, geraniol and α terpinene as its major composites, whereas Pelissier et al. (1998) reported tagetone, myrcène and ipsénone to be its major composites. Antimicrobial properties of the essential oil of L. multiflora are well-known, as well as its composites which are ascribed with strong antibacterial activity. Amvam et al. (1998) asserted that these composites probably would operate in occasional synergistical manner.

The interest of the tablet formulation resides in the fact that the tablet form will allow sustained release time of the essential oil and therefore have the following advantages: Prolonged efficacy, and better adherence because the number of doses administered is reduced, while the administration of the essential oil directly has the following disadvantages of large number of firms that makes compliance difficult and risk of significant toxicity.

**Conclusion**

With the intention of verifying if a galenical form responding to a perlingual administration with active constituent of L. multiflora essential oil could still keep its antibacterial properties, we ventured on formulating a formula in which the active constituent was interacting with prior judiciously chosen excipients. The essential oil of L. multiflora Mold is a part of these natural substances whose natural antibacterial activity had largely been proved. Its occurrence in the formulation of tablets for therapeutical proposes has shown its efficiency upon all isolated organisms of the oral pharyngeal cavity except P. aeruginosa. Essential oil-containing tablets with essential oil of L. multiflora was found to have significant antibacterial activity and must further be subjected to stability testing and clinical studies before they can be prescribed as phytomedicines in the treatment of oral mucous infections and the oropharynx.

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**Table 4. Inhibitive activity of the tablets upon the C. albicans (% of inhibition).**

<table>
<thead>
<tr>
<th>Mycotic clone</th>
<th>Amphotericine B</th>
<th>Dilution of the tablets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2/5</td>
</tr>
<tr>
<td>C. albicans (N=2)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
composition of *Thymus palloscens* de Noe from Algeria. J. Essent. Oil Res. 21(2):162-165.


Factor I mutation in Tunisian patient with atypical hemolytic uremic syndrome

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Atypical hemolytic uremic syndrome or the Shiga toxin-producing *Escherichia coli* (STEC) negative hemolytic uremic syndrome (HUS) is a rare disorder typically classified as familial or sporadic. Recent literature has suggested that approximately 50% of patients have mutations in factor H (CFH), factor I (CFI), or membrane cofactor protein (encoded by CD46). Importantly, results of renal transplantation in patients with mutations in either CFH or CFI are dismal, with recurrent disease leading to graft loss in the majority of cases. In this study, a case was described of a patient who developed atypical hemolytic uremic syndrome waiting for a kidney graft. A patient suffering from aHUS and his family was screened for CFI, CFH and membrane cofactor protein (MCP) mutations. The sequencing results of CFH, CFI, and CD46 genes revealed that the patient was heterozygous for a missense mutation, a substitution of a proline residue for a leucine residue at amino acid 64 in CFI. However, the molecular investigation for the family showed the presence of the same mutation in the CFI gene in two members. In silico study demonstrate a functional consequence of this abnormal protein. This study reemphasizes the importance of screening patients with atypical hemolytic uremic syndrome for mutations in the CFI, CFH and MCP genes before renal transplantation and shows the challenges in the management of these patients.

Key words: Hemolytic uremic syndrome (Ahus), complement proteins, factor I mutation, transplantation, in silico.

INTRODUCTION

Hemolytic uremic syndrome (HUS) is characterized by the triad of thrombocytopenia, Coomb’s test negative microangiopathic hemolytic anemia, and acute renal failure. In recent years, mutations in genes coding for regulators of the complement system, factor H (CFH), membrane cofactor protein (MCP, CD46), factor I (CFI), C3 complement and Factor B (FB), have been associated with development of aHUS (Caprioli et al., 2006; Sanchez-Corrall, 2004; Bresin et al., 2013). Mutations in the gene encoding thrombomodulin, a membrane-bound glycoprotein with anticoagulant properties that modulates complement activation on cell surfaces, have also been

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described in aHUS (Noris et al., 2010). In addition to inherited defects in CFH, acquired abnormalities affecting factor H function are also seen in the form of inhibitory autoantibodies. Factor H auto-antibodies are reported in 5 to 10% of aHUS patients.

CFI is an 88 kDa glycoprotein that circulates in plasma in the active form at a concentration of 35 mg/ml (Catterall et al., 1987). During post-translational modification, CFI is proteolytically cleaved into the heavy chain (50 kDa) and the light chain (38 kDa), which are covalently linked via a disulfide bond (Fearon, 1977). The heavy chain is composed of five domains: the FI membrane attack complex domain (FIMAC), the CD5-like domain, the low-density lipoprotein receptor 1 and 2 domains (LDLR1 and 2) and finally a region of no known homology (amino acids 277 to 295). The serine protease (SP) FI is special since it degrades C4b and C3b in the presence of specific cofactors like C4BP, FH, MCP or CR1. CFI is a unique protease since it has no natural inhibitors and works only together with its cofactors (Nilsson et al., 2010). The crystal structure of the complement regulatory enzyme human factor I was determined (Roversi et al., 2011).

Heterozygous mutations within the CFI gene have been identified in individuals with atypical hemolytic uremic syndrome (aHUS). Mutations consequence on the proteins and symptoms of aHUS is not clear but poor regulation of the alternative pathway of complement activation in the kidney has been suggested to be a causative factor (Noris et al., 2005). The majority of CFI mutations induce a lack of protein synthesis (Vyse et al., 2010), and only few mutations have been associated with a functional deficiency (Kavanagh et al., 2008).

In this study, we described one patient with severe aHUS with a heterozygous mutation in the CFI gene. The effect on CFI protein function was showed using in silico studies. In fact, it is important to understand how the complement system is regulated in this patient, especially with a view to developing therapeutic options.

**MATERIALS AND METHODS**

Patient HUS161 was a 35-year-old man presenting typical signs of aHUS. He had two relatives with glomerulosclerosis, one of them being dead. Currently, the patient is undergoing chronic hemodialysis and waiting for a kidney graft (Leban et al., 2011). C3, C4, CFH and CFI proteins levels were measured by nephelometry and sensitive enzyme linked immunosorbent assay (ELISA) methods as described (Perez-Caballero et al., 2001; Gonzalez-Rubio et al., 2001). All protein levels were normal in the patient and all family members (Table 1). All exons of the CFI, CFH, MCP and C3 genes were amplified by the polymerase chain reaction (PCR). PCR products were purified with alkaline phosphatase and exonuclease (Amersham, The Netherlands). Subsequently, a sequence reaction was performed using the ready reaction sequence mix (Applied BioSystems). After precipitation, the fragments were sequenced by the ABI Prism 3130 Genetic Analyzer. Analysis of sequence of all exons encoding for CFH, MCP, CFI, C3 and FB genes (Esparza-Gordillo et al., 2006; Rodriguez de Cordoba et al., 2008) were performed using Chromas 2. Fifty (50) healthy controls and his family were analyzed for the mutations. The PolyPhen server (http://www.bork.emb-heidelberg.de/PolyPhen) was used to predict the possible impact of amino acid substitution observed in patient HUS161.

**RESULTS**

Serum levels of C3, C4, CFH and CFI are shown in Table 1. All protein levels were normal in the patient and all family members. In this study we describe one patient with severe aHUS and a new heterozygous mutation in exon 2 (c.191C>T) (Figure 1) resulting in an amino acid substitution from proline to leucine acid at position 64 in the FIMAC domain of the CFI protein (Figure 2). The unaffected father and one brother of patient have the same heterozygous CFI mutation. The CFI mutation was not found in 50 healthy controls. In patient, we found a heterozygous risk polymorphism in CFH, -257C > T (promoter region) which has been described to predispose to aHUS (Caprioli et al., 2006). No MCP, CFH, C3 and FB mutations were found in the patient.

**DISCUSSION**

Atypical HUS is a disease that during the last years has been associated with impaired regulation of the alternative pathway of complement. In more than 50% of aHUS patients, one or several genetic abnormalities have been identified in complement inhibitors (Nilsson et al., 2010). A heterozygous mutation in CFI, a regulatory protein of the complement system, was detected in the studied patient, his father and his brother (Figures 1 and 2). This mutation was described by Maga et al. (2010) and resulted in an amino acid substitution from proline to leucine acid at position 64 in the FIMAC domain.

Proline usually imposes greater conformational constraints on the polypeptide backbone than other amino acids. It makes a positive contribution to protein stability through entropic effects, especially in regions where it can be tolerated, such as in loops and turns (Nilsson et al., 2010). In the current situation, while this mutation could slightly destabilize the domain, it could not be structurally tolerated at this position. In fact, the CFI concentration in the serum of our patient was found to be normal. This indicates that the heterozygous mutation P64L in CFI does not significantly influence the CFI serum concentration. Previous researches have showed that mutations in CFI, especially in the FIMAC domain induce reduced cleavage of fluid-phase C4b and C3b (Nilsson et al., 2010; Maga et al., 2010). These studies...
Table 1. Complement data in patient with aHUS.

<table>
<thead>
<tr>
<th>Patient</th>
<th>C3 mg/dl</th>
<th>C4 mg/dl</th>
<th>CFH mg/dl</th>
<th>CFI%</th>
<th>AP</th>
<th>CH50</th>
<th>RVA</th>
<th>MCP%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>97</td>
<td>29.9</td>
<td>32.5</td>
<td>100</td>
<td>normale</td>
<td>normale</td>
<td>normale</td>
<td>121</td>
</tr>
</tbody>
</table>

Normal values: C3 (77 to 135 mg/dl), C4 (14 to 47 mg/dl); CFH (12 to 56 mg/dl); CFI (71 to 115%); MCP (91 to 109%).

Figure 1. Pedigree of the patient family.

Figure 2. (a) Wild-type DNA sequence. (b) Sequence of patient HUS161. A single base pair change c.191C>T in exon 2 of CFI gene.

described that eight patients have presented a mutation in CFI gene and have progressed to end stage renal disease (ESRD); one of them showed a P64L mutation in CFI gene.

We can suggest that the mutation altered the function of the CFI protein. This deduction is supported by the results previously described by Nilsson et al. (2010) who reported impaired function of several mutations in the FIMAC domain towards degradation of both C4b and C3b especially the mutation R62A. The R62A localization is very near from our mutation P64L (two amino acids). This study analyzed binding of the FI mutant to C3b. R62A
was the only mutant in the FIMAC domain, which still showed detectable but decreased activity compared to wild type. However, the mutant protein showed substantially impaired ability to degrade C3b deposited on cell surfaces. To study the mutation structure and/or function effect on protein, the PolyPhen server was used to predict the possible impact of amino acid substitution observed in patient HUS161.

PolyPhen predicts the p.P64L mutation to be probably damaging with a percentage of 99.9% (sensitivity: 0.11; specificity: 99%). This result can be explained by the presence of the proline at position 64 that can perturb interdomain contacts or form new interactions with a FI ligand when C3b is part of a deposited C3-convertase. The mutation P64L reduced function in degrading C4b and C3b in solution and only when C4BP and CFH were used as cofactors. This result confirm that the mutation P64L perturb the functional regulation of the CFI protein and consequently the alternative complement pathway. Further in vitro studies remain necessary to confirm our finding.

Many studies found significant associations between several known variants in CFH in aHUS patients (Dragon-Durey, 2004; Fremeaux-Bacchi et al., 2005; Pickering et al., 2007). Four of the SNPs genotyped in that study were included in our analysis (c.257C > T, c.184G > A, c.1204C > T, c.2016A > G and c.2808G > T). In patient, we found a heterozygous risk polymorphism in CFH, -257C > T (pro-moter region) which has been described to predispose to aHUS (Caprioli et al., 2003). The polymorphisms identification in the patient family is under study to explain the non appearance of the disease especially the father showing the P64L mutation in CFI gene.

In general, renal transplantation is prone to failure in patients with a heterozygous mutation in CFI. Considering mutations in CFI, one of the patients reported by Fremeaux-Bacchi et al. (2005) had a recurrence of HUS in two transplants. The first patient of Kavanagh et al. (2008) had initially an excellent renal function after transplantation, but a recurrence and deterioration of the function occurred after 2 months. The second patient had a recurrence of HUS, which was noted 20 months after the transplantation. The patient with CFI or CFH mutations have much worse prognoses since the CFI and CFH proteins are mainly produced in the liver. There have been some successful combined renal and liver transplantation where the patients with a CFH mutation received extensive plasma therapy before, during and after the operation and as consequences do not show any evidence of disease in the renal graft (Jalanko et al., 2008).

The patient HUS161 progressed to end-stage renal disease and required renal transplantation. The renal graft is not recommended by the family because of the presence of the P64L mutation in two members. After the identification of the molecular abnormality in the CFI gene, the therapeutic treatment of the patient was directed to a plasmapheresis. Indeed, the renal transplantation was contraindicated and the combined renal and liver transplantations are not available in Tunisia. Genetic analyses of complement proteins are not yet possible on the research and clinical levels in Tunisia. These studies merit to be developed in Tunisian research laboratories in order to decrease the percentage of the recurrence of aHUS in the Tunisian patients. Also, it is important to assess the functional impact of mutations/polymorphisms identified in aHUS patients because this knowledge can affect the mode of treatment and that which we have found in the current study.

This study has important clinical implications. It is important in patients who are being considered for transplantation that it be known whether they have a CFH, CFI, or MCP mutation so that they can be informed appropriately of the risks for recurrence. We would not recommend live related transplantation in our patient who is known to have either a CFI mutation unless the donor has been screened for the same mutation. The description of mutations in CFI, CFH, and MCP genes has established that atypical HUS is a disease of complement dysregulation. Understanding the molecular mechanisms that are responsible for this disease allows us now to examine the potential of complement inhibition as a means of therapy.

ACKNOWLEDGMENTS

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Full Length Research Paper

Bioassay guided isolation and characterization of antimicrobial and anti-trypanosomal agents from *Berberis glaucocarpa* Stapf.

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Different fractions of *Berberis glaucocarpa* Stapf were tested for their antibacterial and anti-trypanosomal properties using the well established Alamar blue™ 96 well microplate assays. Then eight known compounds (1 to 8), including five alkaloids (1 to 5), were isolated and characterized from this plant for the first time. All the compounds were tested for their antimicrobial and antitrypanosomal properties. Most of the pure compounds were also found to possess good antibacterial and anti-trypanosomal properties. Berberine (minimum inhibitory concentration (MIC) = 12.5 µg/ml), berberine chloroform (MIC = 25 µg/ml) and syringaresinol (MIC = 12.5 µg/ml) were found to have anti-bacterial and anti-trypanosomal properties. The MIC values were determined for only active compounds.

**Key words:** *Berberis glaucocarpa*, alkaloids, anti-trypanosomal, antibacterial.

INTRODUCTION

Plants are the major source of biologically active compounds that can be used as medicines. In recent years, the interest in the use of medicinal plants have increased rapidly due to the resistance developed by the pathogens against the existing antibiotics (Arias et al., 2004). The genus *Berberis* L. has 500 living species around the world. They are mostly shrubs having 3-5 branched spines and have simple leaves (Ahrendt, 1961). Onion extracts have been found to have antioxidant properties. Onion prevents cancer, coronary heart diseases, cataract and diabetes. Fresh onion juice have been found to have analgesic and anti-inflammatory properties (Sima et al., 2012).

*Peganum harmala* L. extracts have been found to have antiviral, antidiabetic, antifungal, antioxidant, anti-septic, antitumor, insecticidal, cytotoxic, heptaprotective and antileishmanial properties (Jinous and Fereshteh, 2012). Different *Berberis* species are important folk medicines for the treatment of allergies, fever, metabolic disorders, eye diseases, osteoporosis, joint pain and menopause problems (Dipti et al., 2012). Berberine is one of the major and most important isoquinoline alkaloid found in all berberis species and has a wide range of pharmacological uses. (Dipti et al., 2012) The alcoholic extracts of *Berberis* have isoquinoline alkaloids and bisbenzyl isoquinoline alkaloids. The alkaloids are mainly responsible for the bioactivities of *berberis* species (Slavik and Slavikova, 1995). Keeping in mind the phytochemical importance of the genus *Berberis*, we decided to explore *Berberis glaucocarpa* Stapf for its...
phytoconstituents and bioactivity.

MATERIALS AND METHODS

General experimental procedures

Ultraviolet (UV) data was collected with Thermo Spectronic Unicam UV-300 spectrophotometer. IR (Infrared) analysis was recorded with JASCO FTIR 4200, 1D and 2D NMR was collected with Bruker Avance DRX - 400 operating at a frequency of 400.13 MHz ($^1$H) and 100.62 MHz($^{13}$C) at a temperature of 296.0 K.

Plant material

Berberis glaucocarpa Stapf was collected from Azad Kashmir Pakistan during July 2009 (in blooming period) and was identified by Prof Dr. Tanveer Akhtar (chairperson Botany Department University of Azad Jammu and Kashmir). A voucher specimen number 9615-A was allotted and a specimen was deposited in the herbarium of Botany Department University of Peshawar.

Extraction and isolation

B. glaucocarpa Stapf root wood (4 kg) was dried at room temperature and then grinded with a grinder. The powdered plant material was soaked in commercial grade ethanol (95 %) at room temperature for ten days. The dilute crude extract was concentrated under reduced pressure using rotary evaporator to yield a gummy residue (308 g). The residue was treated with 5 % aqueous HCl solution and filtered. The filtrate was allowed to stand overnight to yield a brownish yellow precipitate (384 g). The precipitate was washed with water and then dried to yield a residue (308 g). The residue was treated with 5 % aqueous NaOH solution and filtered. The filtrate was basified with NH$_3$ to PH 10 and then repeatedly extracted with dichloromethane several times to afford Fraction B (Fr.B) (dichloromethane fraction) (23 g). The acidic filtrate was subjected to silica gel column chromatography to afford Fraction A (Fr.A) (acidic fraction) (87 g). The acidic filtrate was combined with the basic filtrate to yield a combined filtrate (330 g). The combined filtrate was extracted with dichloromethane several times to yield Fraction C (Fr.C) (basic fraction) (67 g). The remaining aqueous solution was Fraction D (Fr. D) (water fraction).

Fraction A (Fr.A)

Fr. A was subjected to silica gel column chromatography to afford berberine using chloroform as solvent system and gradually increasing the polarity with methanol.

Fraction B (Fr.B)

This fraction was subjected to silica gel column chromatography eluted with n-hexane and ethyl acetate mixture at first and then followed by elution with chloroform and methanol mixture. After repeated column and preparative chromatography, syringaracinol, syringic acid and gallic acid were purified.

Fraction C (Fr.C)

It was subjected to silica gel column chromatography. The column was eluted with different solvents in increasing order of polarity. Palmatine, oxoberberine, berberine chloroform and columbamine were purified using repeated column, prep chromatography and sephadex LH-20.

RESULTS AND DISCUSSION

Ethanolic extract of B. glaucocarpa Stapf root wood (4 kg) resulted in the isolation of seven known compounds and a known alkaloidal artifact for the first time from this plant. The isolated compounds were found to be berberine [1] (Lenka et al., 2007), palmatine [2] (Radek et al., 2003), columbamine [4] (Tian-Jye et al., 2004), oxoberberine [5] (Jiri et al., 2004), syringaresinol [6] (Wisit et al., 2011), syringic acid [7] (Sheng-Ming et al., 2006) and gallic acid [8] (Eladahshan, 2011) while the isolation artifact was found to be berberine chloroform [3] (Radek et al., 2003).

One of the prominent clinical uses of Berberis vulgaris is in bacterial infection such as bacterial diarrhea and parasitic intestinal and ocular infections. (Birdsall and Kelly, 1997) Berberine is a major constituent alkaloid of all berberis species. Berberine has shown very significant antimicrobial properties against different strains of bacteria such as S. aureus, Candida spp (Freile et al., 2003). Subfractions containing berberine and syringaresinol were found to have good activity and hence it can be said that berberine and syringaresinol are mainly responsible for the antibacterial and anttrypanosomal activities of the sub fractions also. Furthermore, as all the compounds were already known compounds therefore their structures were confirmed by comparing their 1D and 2D NMR data with the literature (Figure 1).

Berberine

Yellow needles; m.p = 208-209°C; UV $\lambda_{max}$ (MeOH) = 266, 349, 427 nm; IR (v cm$^{-1}$) = 3020, 1634, 1601, 1458. EI-MS m/z (rel. int. %) = 336 (6, M$^+$), 335 (7), 321 (100), 306 (38), 292 (32), 278 (75), 263 (10), 248 (6), 219 (7), 191(7), 138(7), 95(3), 44(11). $^1$H NMR (MeOD) (Table 1), $^{13}$C NMR (MeOD) (Table 3).

Palmatine

Brownish yellow needles, 202-203°C; UV $\lambda_{max}$ (MeOH) = 266, 347, 430 nm; IR (v cm$^{-1}$) = 2992, 1624, 1522, 1501, 1390, 1240; EI-MS m/z (rel. int. %) = 352 (11, M$^+$), 351 (35), 337 (100), 294 (46), 278 (34), 138(5). $^1$H NMR (DMSO-d$_6$) (Table 1), $^{13}$C NMR (DMSO-d$_6$) (Table 3).

Berberine chloroform

Light yellow crystals, m.p = 186-188°C; UV $\lambda_{max}$ (MeOH) = 231, 285, 381 nm; IR (v cm$^{-1}$) = 2980, 2400, 1610, 1501, 1290, 1240; EI-MS m/z (rel. int. % ) = 420 (78), 383 (82), 368 (75), 336 (100), 321 (26), 292 (24), 278 (27)
Figure 1. Structures of compound 1 to 8.
Table 1. $^1$H Chemical shifts (mult, $J$ in Hz) of compounds 1 to 5.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Beberine (MeOD)</th>
<th>Palmatine (DMSO-d$_6$)</th>
<th>B/Chloroform (CDCl$_3$)</th>
<th>Columbamine (MeOD)</th>
<th>Oxoberberine (DMSO-d$_6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1</td>
<td>7.62, (s)</td>
<td>7.72, (s)</td>
<td>7.14 (s)</td>
<td>7.35 (s)</td>
<td>7.40 (s)</td>
</tr>
<tr>
<td>H-4</td>
<td>6.94, (s)</td>
<td>7.08, (s)</td>
<td>6.59 (s)</td>
<td>6.54 (s)</td>
<td>6.63 (s)</td>
</tr>
<tr>
<td>H-5</td>
<td>3.24 (t, 10.0 Hz)</td>
<td>3.20 (t, 6.6 Hz)</td>
<td>2.73 (m); 3.3 (m)</td>
<td>3.20 (m)</td>
<td>2.91 (t, 8.2 Hz)</td>
</tr>
<tr>
<td>H-6</td>
<td>4.92 (t, 10.0 Hz)</td>
<td>4.95 (t, 6.4 Hz)</td>
<td>3.71 (m); 3.8 (m)</td>
<td>4.87 (m)</td>
<td>4.13 (t, 8.8 Hz)</td>
</tr>
<tr>
<td>H-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-8</td>
<td>9.75, (s)</td>
<td>9.89, (s)</td>
<td>5.62, (s)</td>
<td>9.52, (s)</td>
<td>-</td>
</tr>
<tr>
<td>H-9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
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<td>H-10</td>
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<tr>
<td>H-11</td>
<td>8.10 (d, 9.0 Hz)</td>
<td>8.20 (d, 9.2 Hz)</td>
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<td>H-12</td>
<td>7.98 (d, 9.0 Hz)</td>
<td>8.04 (d, 9.2 Hz)</td>
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<td>7.94 (d, 8.0 Hz)</td>
<td>7.23 (d, 8.4 Hz)</td>
</tr>
<tr>
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<td>6.08 (s)</td>
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<td>7.01 (s)</td>
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<td>3.92 (s)</td>
<td>4.05 (s)</td>
<td>3.74 (s)</td>
</tr>
<tr>
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<td>4.15 (s)</td>
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<td>2-OCH$_3$</td>
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<td>3.93 (s)</td>
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<td>3.90 (s)</td>
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<td>2'-O-CH$_2$-O-</td>
<td>6.09 (s)</td>
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<td>6.03 (s)</td>
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</table>

Table 2. $^1$H Chemical shifts (mult, $J$ in Hz) of compounds 6 to 8.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Syringaresinol (CDCl$_3$)</th>
<th>Syringic ACID (CDCl$_3$)</th>
<th>Gallic acid (DMSO-d$_6$)</th>
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<tbody>
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<td>H-1</td>
<td>3.12 ( m)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-2</td>
<td>4.76 (d, 4.0 Hz)</td>
<td>7.34 (s)</td>
<td>6.94 (s)</td>
</tr>
<tr>
<td>H-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-4a</td>
<td>4.31 (dd, 9.2 and 6.8Hz)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-4b</td>
<td>3.94 (m)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-5</td>
<td>3.12 (m)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-6</td>
<td>4.76 ( d, 4.0 Hz)</td>
<td>7.34 (s)</td>
<td>6.94 (s)</td>
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<tr>
<td>H-7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-8a</td>
<td>4.31 (dd, 9.2 and 6.8Hz)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-8b</td>
<td>3.94 (m)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3-OCH$_3$</td>
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<td>3.90 (s)</td>
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<td>5-OCH$_3$</td>
<td>-</td>
<td>3.90 (s)</td>
<td>-</td>
</tr>
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<td>H-1', 1''</td>
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<td>-</td>
</tr>
<tr>
<td>H-2', 2''</td>
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<td>-</td>
</tr>
<tr>
<td>H-4', 4''</td>
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<td>-</td>
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</tr>
<tr>
<td>H-5', 5''</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-6', 6''</td>
<td>6.60 (s)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3'-OCH$_3$</td>
<td>3.92 (s)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4'-OH</td>
<td>5.52 (s)</td>
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</tr>
<tr>
<td>5'-OCH$_3$</td>
<td>3.92 (s)</td>
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<tr>
<td>3'-OCH$_3$</td>
<td>3.92 (s)</td>
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<td>-</td>
</tr>
<tr>
<td>4'-OH</td>
<td>5.52 (s)</td>
<td>-</td>
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<tr>
<td>5'-OCH$_3$</td>
<td>3.92 (s)</td>
<td>-</td>
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</tbody>
</table>
Table 3. $^{13}$C data of compounds 1 to 5.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Beberine (MeOD)</th>
<th>Palmatine (DMSO-d$_6$)</th>
<th>B/Chloroform (CDCl$_3$)</th>
<th>Columbamine (MeOD)</th>
<th>Oxoberberine (DMSO-d$_6$)</th>
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</thead>
<tbody>
<tr>
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<td>150.86</td>
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<td>110.20</td>
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<td>159.11</td>
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<td>146.41</td>
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<td>149.69</td>
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(27); $^1$H NMR (CDCl$_3$) (Table 1), $^{13}$C NMR (CDCl$_3$) (Table 3).

**Columbamine**

Yellow needle like crystals, m.p = 281-283°C; UV $\lambda_{max}$ (MeOH) = 224, 276, 349 nm; IR (v cm$^{-1}$) = 3310, 1605, 1510, 1456, 1316; EI-MS m/z (rel. int. %) = 338 (7, $M^+$), 321 (100), 306 (35), 291 (30), 278 (73), 264 (10); $^1$H NMR (MeOD) (Table 1), $^{13}$C NMR (MeOD) (Table 3).

**Oxoberberine**

Yellow needles, m.p = 191-192°C; UV $\lambda_{max}$ (MeOH) = 284, 315, 370 nm; IR (v cm$^{-1}$) = 3310, 1605, 1510, 1456, 1316; EI-MS m/z (rel. int. %) = 338 (7, $M^+$), 321 (100), 306 (35), 291 (30), 278 (73), 264 (10); $^1$H NMR (MeOD) (Table 1), $^{13}$C NMR (MeOD) (Table 3).

**Syringaresinol**

Colorless crystals, m.p = 183-185°C; UV $\lambda_{max}$ (MeOH) = 240, 276 nm; IR (v cm$^{-1}$) = 3325, 1612, 1520, 1456, 1378, 1320; EI-MS m/z (rel. int. %) = 418 (100, $M^+$), 210 (20), 193 (26), 181 (99), 167 (70); $^1$H NMR (CDCl$_3$) (Table 2), $^{13}$C NMR (CDCl$_3$) (Table 4).

**Syringic acid**

Grey amorphous solid, m.p = 204-205°C; UV $\lambda_{max}$ (MeOH) = 229, 286 nm; IR (v cm$^{-1}$) = 3380, 1675, 1564, 1480; EI-MS m/z (rel. int. %) = 198 (100, $M^+$), 183 (34), 167 (17), 153 (11), 127 (19), 97 (27), 83 (36), 71 (34), 69 (41); $^1$H NMR (MeOD) (Table 2), $^{13}$C NMR (MeOD) (Table 4).

**Gallic acid**

Off white amorphous powder; m.p = 269-270°C; UV $\lambda_{max}$ (MeOH) = 273 nm; IR (v cm$^{-1}$) = 3370, 2996, 1715, EI-MS m/z (rel. int. %) = 170 (98, $M^+$), 154 (94), 127 (15), 106 (7), 79 (18); $^1$H NMR (DMSO-d$_6$) (Table 2), $^{13}$C NMR (DMSO-d$_6$) (Table 4).
Table 4. $^{13}$C data of compounds 6 to 8.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Syringaresinol (CDCl$_3$)</th>
<th>Syringic acid (MeOD)</th>
<th>Gallic acid (DMSO-d$_6$)</th>
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<tr>
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<td>C-1', 1''</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-6', 6''</td>
<td>102.76</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3'-OCH$_3$</td>
<td>56.40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4'-OH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5'-OCH$_3$</td>
<td>56.40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3''-OCH$_3$</td>
<td>56.40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4''-OH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5''-OCH$_3$</td>
<td>56.40</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5. Anti-trypanosomal results.

<table>
<thead>
<tr>
<th>Compound/crude</th>
<th>T. brucei</th>
<th>20 µg/ml</th>
<th>10 µg/ml</th>
<th>5 µg/ml</th>
<th>%D control</th>
<th>%D control</th>
<th>%D control</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGA (Fr.A)</td>
<td>21.0</td>
<td>82.0</td>
<td>109.2</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BGSD (Fr.B)</td>
<td>21.6</td>
<td>78.5</td>
<td>93.4</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BJSET (Fr.C)</td>
<td>18.7</td>
<td>69.2</td>
<td>89.7</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beberine</td>
<td>1.8</td>
<td>46.5</td>
<td>76.1</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berberine chloroform</td>
<td>1.1</td>
<td>1.9</td>
<td>86.8</td>
<td>12.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syringaresinol</td>
<td>23.7</td>
<td>83.4</td>
<td>98.4</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmatine</td>
<td>85.0</td>
<td>97.0</td>
<td>93.0</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Columbamine</td>
<td>85.0</td>
<td>97.0</td>
<td>93.0</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxoberberine</td>
<td>8.5</td>
<td>83.2</td>
<td>86.8</td>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

%D control, Percentage of DMSO control; NC, not calculated.

**Anti-bacterial and anti-trypanosomal activities**

All the isolated compounds and their fractions were tested for their anti-bacterial and anti-trypanosomal properties using Alamar blue™ 96 well microplate assays (Tables 5 and 6). Four strains of bacteria namely SMRSA (200 µg/ml), EMRSA (200 µg/ml), *Mycobacterium marinum* (100 µg/ml) and *Escherichia coli* (500 µg/ml) and one strain of *Trypanosoma* namely *Trypanosoma brucei*. *brucei* (20, 10 and 5 µg/ml) were used for assays. Berberine (MIC = 12.5 and 25 µg/ml), berberine chloroform (MIC = 25 and 12.5 µg/ml) and syringaresinol (12.5 µg/ml) were found to be very active against SMRSA, *Mycobacterium marinum* and *T. brucei. brucei*. 
Table 6. Anti-bacterial results.

<table>
<thead>
<tr>
<th>Compound/crude</th>
<th>100 ug/ml</th>
<th>200 ug/ml</th>
<th>500 ug/ml</th>
<th>100 ug/ml</th>
<th>200 ug/ml</th>
<th>500 ug/ml</th>
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<tr>
<td></td>
<td>% D control</td>
<td>% D control</td>
<td>MIC</td>
<td>% D control</td>
<td>MIC</td>
<td>% D control</td>
<td>MIC</td>
<td>% D control</td>
<td>MIC</td>
</tr>
<tr>
<td>BGA (Fr.A)</td>
<td>4.0</td>
<td>2.0</td>
<td>50</td>
<td>78.8</td>
<td>NC</td>
<td>49.7</td>
<td></td>
<td></td>
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<tr>
<td>BGSD (Fr.B)</td>
<td>12.2</td>
<td>-2.2</td>
<td>25</td>
<td>14.9</td>
<td>NC</td>
<td>54.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BJSET (Fr.C)</td>
<td>15.5</td>
<td>1.4</td>
<td>50</td>
<td>86.5</td>
<td>NC</td>
<td>56.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beberine</td>
<td>-2.9</td>
<td>-0.1</td>
<td>12.5</td>
<td>72.0</td>
<td>NC</td>
<td>51.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berberine chloroform</td>
<td>0.6</td>
<td>50.2</td>
<td>25</td>
<td>70.5</td>
<td>NC</td>
<td>19.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syringaresinol</td>
<td>12.2</td>
<td>-0.6</td>
<td>12.5</td>
<td>84.9</td>
<td>NC</td>
<td>52.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmatine</td>
<td>78.1</td>
<td>71.8</td>
<td>NC</td>
<td>75.3</td>
<td>NC</td>
<td>52.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Columbamine</td>
<td>89.9</td>
<td>86.5</td>
<td>NC</td>
<td>94.5</td>
<td>NC</td>
<td>66.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxoberberine</td>
<td>41.6</td>
<td>85.9</td>
<td>NC</td>
<td>87.9</td>
<td>NC</td>
<td>65.2</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

% D control, Percentage of DMSO control; NC, not calculated.

ACKNOWLEDGMENTS

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REFERENCES


The production of counterfeit drugs is a broad and under reported problem particularly affecting poorer countries. It is an important cause of unnecessary mortality and morbidity, and loss of public confidence in medicines and health structures. Empirical observations show that there may be more counterfeit than genuine drugs in circulation. This article discusses the prevalence of counterfeit drugs in Nigeria. It highlights factors contributing to the preponderance of counterfeit pharmaceuticals and discusses strategies that may influence policy to combat the menace of counterfeit drugs. Major factors contributing to the prevalence of counterfeit drugs in Nigeria include ineffective enforcement of existing laws, non-professionals in drug business, loose control systems, high cost of genuine drugs, greed, ignorance, corruption, illegal drug importation, chaotic drug distribution network, demand exceeding supply amongst many others. Counterfeit drugs pose great threats to the attainment of the millennium development goals 4, 5 and 6 which hopes for a reduction in infant mortality, improved maternal health and combating human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), malaria and other diseases. Due to the complexity of the counterfeit drug problem, no single technique can eliminate the public health threat posed by counterfeit pharmaceuticals. A multi-disciplinary and cross-functional approach will help combat the prevalence of counterfeit drugs in Nigeria.

Key words: Counterfeit drugs, public health, Nigeria.
The products could include incorrect ingredients, may misstate the amount of the active ingredients, or are manufactured under circumstances that lack quality control. Counterfeit drugs in Nigeria include preparations without active ingredients, toxic preparations, expired drugs that are relabelled, drugs issued without complete manufacturing information and drugs that are unregistered with the National Agency for Food and Drug Administration and Control (NAFDAC). Current estimate suggests that 10% of prescription drugs sold worldwide are counterfeits, fake or contaminated, and in parts of Africa and Asia, the figures exceed 50% (Newton et al., 2001; Cockburn, 2002).

Counterfeit pharmaceuticals remain one of the world’s fastest growing industries. Recent trends suggest an increase in counterfeit drug sale to over $70 billion in 2010, an increase of over 90% from 2005. A report by Pfizer, a global pharmaceutical firm, on counterfeit drugs states that profits from counterfeiting today surpasses gains made from heroin and cocaine (PGS, 2007). While the issue of counterfeit drugs has long been treated as an illicit case of intellectual property infringement, the view has often masked what is in fact a public health crisis. In light of this, this article aims to discuss the prevalence of counterfeit drugs in Nigeria and highlights strategies which may influence policy to help eliminate the public health threat posed by counterfeit pharmaceuticals.

Scope of the problem

The counterfeiting of all manner of products is on the rise globally. In Nigeria today, there is counterfeiting of documents, currency, software and electronics, amongst many others. However, no other product has the capacity to harm, as much as kill its consumers, as do illicit pharmaceuticals. The era 1985 to 2000 heralded the regime of counterfeit drugs, unlicensed drug vendors, illegal pharmacy stores and hospitals (Erhun et al., 2001). The menace of counterfeit drugs became prevalent in the last two decades and the present situation is alarming in the West-African sub-region, including Nigeria. Empirical observations show that there may be more counterfeit than genuine drugs in circulation (Osibo, 1998). A worrisome aspect of the counterfeit drug hazard is that the effects of consuming such drugs go unnoticed, except in cases where it results in mass deaths. The effects of counterfeit drugs on patients are difficult to quantify and are mostly hidden in public health statistics. There are no reliable data on the mortality and morbidity resulting from the consumption of counterfeit drugs in Nigeria (Erhun et al., 2001). Most data on the epidemiology of counterfeit drugs are kept secret by the pharmaceutical industry and by governmental agencies. The estimate of 192,000 patients killed by fake drugs in China in 2001 gives an indication of the magnitude of the problem (Cockburn et al., 2005).

Over the past two decades, Nigeria struggled to reduce the production and trafficking of counterfeit drugs without adequate infrastructure or political will to properly enforce legislation and standards (Garuba et al., 2009). The high trends of mortalities and morbidities prompted the public and the Pharmaceutical Society of Nigeria (PSN) to pressure the government to take incisive steps towards controlling the prevalence of counterfeit and substandard drugs in Nigeria. The government responded by promulgating the counterfeit and fake drug (miscellaneous provisions) decree No. 21 of 1998 which prohibited the sale and distribution of counterfeit, adulterated, banned, and fake drugs or poisons in open markets and without a license of registration. Additionally, NAFDAC was established in 1993 to help create a fake drug free environment with the intent of ensuring effective registration of good quality drugs (NAFDAC Consumer Safety, 2003). However, in 2001, under the leadership of Dora Akunyili as the new director general of NAFDAC, the agency underwent intense restructuring and reforms with the aim of revitalizing NAFDAC’s mandate to “safeguard the health of the nation”. As a result, drug failure rates fell to roughly 16% in 2006 from 2002 and the circulation of counterfeit drugs was reported to have been reduced by over 80% to what it was in 2001 (NAFDAC News, 2006).

Prior to these reforms, the prevalence of counterfeit drugs had a prominent and destructive impact on those who used them unknowingly (Garuba et al., 2009). In 1947, fourteen children were reported dead after being administered chloroquine phosphate injections and in 1990, 109 children died after being administered fake paracetamol (Aluko, 1994). In 1995, the Nigerian supply of 88,000 Pasteur Merieux and SmithKline Beechammenengitis vaccines to Niger during an epidemic resulted in about 2,500 deaths after vaccination (Attaran et al., 2011). Despite NAFDAC’s reported successes, counterfeit pharmaceuticals still remain prevalent. In 2004, three Nigerian hospitals reported cases of adverse reactions from the use of contaminated infusions produced by four Nigerian companies (Akunyili, 2005). It was established that the infusions were heavily contaminated with microorganisms and 147 of the 149 brands of screened water for injection were found to be unsterile. In November 2008, 34 Nigerian children, aged 4 months to 3 years died and more than 50 were hospitalised with severe kidney damage after taking the drug “My Pikin” (“my child” in local pidgin), a teething mixture containing paracetamol (Bonati, 2009). The outbreak was due to the
use of diethylene glycol (DEG) as a solvent for the paracetamol. DEG was present because of inadvertent or deliberate substitution of propylene glycol, a less toxic compound than DEG, widely used in the pharmaceutical industry.

**METHODOLOGY**

In getting materials for this paper, electronic databases were searched for articles published in English between 2005 and 2011. The electronic databases searched included Pubmed central, Cumulative index to nursing and allied health literature (CINAHL), Cochrane, Medline, Embase, Web of science and Google scholar. Keywords used were: counterfeit drugs, public health, Nigeria. Titles and abstracts were screened and full text papers were retrieved for studies considered relevant and for studies that contained insufficient information to allow judgment of relevance. The full text papers and papers considered relevant were assessed against the inclusion criteria. Seven papers from Medline, three papers from Pubmed, nine papers from Web of science and nine papers from Google scholar were finally selected in writing this paper. Evidence from the selected papers suggests that anti-infective agents, particularly antibiotics and anti-parasitic agents are the most counterfeited products in developing countries. However, there have been reports of fake antiretrovirals in sub-Saharan Africa (Ahmed, 2004). Drug counterfeiting is not just prevalent in developing countries; it is a global problem. There have been reports of drug counterfeiting even in the developed countries. Counterfeit anti-ulcer (ranitidine) and anti-impotence (tadalafil) drugs have been reported in the United Kingdom in 1994 and 2004, respectively (Gibson, 2004); sub-standard thyroxine has also been reported in the United States (Dong et al., 1997). Counterfeit anti-cancer and anti-allergic drugs are equally predominate in the Western world (Newton et al., 2006).

**HEALTH AND ECONOMIC CONSEQUENCES OF DRUG COUNTERFEITING**

The problem of counterfeit drugs have embarrassed the Nigerian healthcare providers and denied the confidence of the public on the nation’s healthcare delivery system. The result of fake drug proliferation has led to treatment failures, organ dysfunction or damage, worsening of chronic disease conditions and death of many Nigerians. Even when patients are treated with genuine drugs, no response is seen due to resistance caused by previous intake of fake drugs (Akunyili, 2005). Counterfeit drugs pose great threats to the attainment of the millennium development goals 4, 5 and 6 which hopes for a reduction in infant mortality, improved maternal health and combating HIV/AIDS, malaria and other diseases (WHO, 2012). It denies the Nigerian people the right to safe, effective and quality medicines. Counterfeit drugs rob the country of valued man power resources and economic benefits. Laxity of ineffective judicial system and widespread corruption are reasons behind the easy production and sale of fake drugs in Nigeria (Chiwendu, 2008). It enables counterfeit drug producers sell their products cheap to vendors who in turn sell to the consumers. The major factors facilitating the preponderance of fake drugs in Nigeria have been reported to include: the ineffective enforcement of existing laws, non-professionals in drug business, loose control systems, high cost of genuine drugs, greed, ignorance, corruption, illegal drug importation, chaotic drug distribution network, demand exceeding supply amongst many others (Chiwendu, 2008; Erhun et al., 2001).

**THE NIGERIAN PHARMACEUTICAL MARKET**

There is a large market for drugs in Nigeria with over 130 existing pharmaceutical manufacturers (Erhun et al., 2001). Despite the enormous numbers of these pharmaceutical industries, only 60 are in active manufacturing. This is against the installed capacity of the industry to produce between 50 and 75% of the nation’s drug needs. With the production capacity below 30%, much of the nation’s drugs are imported (Okoli, 2000), with a bulk of the import coming from Asia. Drug counterfeiters see Nigeria as a good base for their criminal but lucrative trade. Bate and Boateng (2007) reports that India and China are the market leaders in pharmaceutical manufacturing and the biggest culprits of drug counterfeiting globally. Much of the global outsourcing is contracted to firms in Asia, both for manufacturing and increasingly, for services. A statistics by the European commission described India as the source of 75% of counterfeit drugs (Chika et al., 2011). It is therefore not surprising that most of the counterfeit drugs in Nigeria originate from India (Raufu, 2003). However, this is not to suggest that the problem is limited to Asia. In many cases, the goods are only misbranded in places far from the production site.

**AVAILABILITY OF COUNTERFEIT DRUGS**

The loose control system in the Nigerian economy has contributed to the circulation of fake and counterfeit drugs in the country. A major function of NAFDAC is the regulation and control of imported products. This is done by having inspectors at various airports and seaports. Registration of pharmaceuticals is a criterion that must be passed before any drug is released into the Nigerian market. A condition for registration is the analysis and testing of the drug to ensure quality and safety. Unfortunately, the forensic laboratory, which is the major public laboratory for the purpose of quality control analysis, is not adequately equipped to cope with the volume of requests, particularly for analysis of imported...
drugs (Chiwendu, 2008). These loose control systems are exploited by counterfeiters to manufacture, import and distribute fake and adulterated products. There is therefore a need for government to provide funds for the agency to enable the purchase of equipment necessary for testing and analysis of all drugs, both imported and locally manufactured, in the bid to assure the quality and safety of drugs in the Nigerian market. This has however been put in motion with the deployment of the handheld spectrometers which allows the inspection and authentication of products at the point of sale (Roger and Aparna, 2011).

Various laws regulate and control the manufacture, sale and distribution of drugs in Nigeria. Sadly, empirical data shows that the situation is far from adequate (Erhun et al., 2001). The weakest point in Nigeria’s drug regulation is in the area of implementation and enforcement. Some Nigerian drug laws conflict each other resulting in a legal framework that deter offenders, thus making it difficult to try offenders. This encourages drug counterfeiters to continue with their criminal acts. A review of the law is therefore essential to help ensure stability in the legislation and regulations guarding drug laws in Nigeria. An important short term strategy for fighting counterfeit drugs is that pharmaceutical companies focus more on developing better technologies for protecting the identity of their genuine products (Chika et al., 2011). In 2006, the WHO launched the International Medical Products Anti-Counterfeiting Task Force (IMPACT) to assist countries strengthen their detection and enforcement systems and work with industries to develop secure measures as high-tech pharmaceutical packaging. Pharmaceutical companies should develop complex labels and holograms which are difficult for counterfeiters to imitate.

The penalties for drug offenders are not commensurate with the severity of the crime. Currently, the maximum punishment for contravening the decree on fake drugs in Nigeria is N500,000 (US $3,000) or 3 months to 5 years jail term upon conviction (Akunyili, 2007). Stiffer penalties would help sharpen the attitudes of fake drug dealers (Ratanawijitrasin and Wondemagegnehu, 2002). It would make the practice harder and less lucrative for drug counterfeiters. The present director general of NAFDAC, Dr Paul Orhi has advocated for the passage of a new bill, which he hopes will be made into law. The new law seeks life jail term and confiscation of assets upon conviction and compensation of victims, where fake drug is found to be the proximate cause of injury (Odiegwu, 2011). Drug counterfeiting is a grievous crime comparable to murder, hence use of lenient punishment is inadequate. Harris et al. (2009) however argue that use of extremely harsh punishment such as life jail term may be associated with an increased risk of drug counterfeiting being hijacked by organised criminals.

Health care professionals are in a good position to assist the government in fighting the problem of counterfeit drugs. This is most useful in countries that lack the resources needed to combat this crime. The presence of non-professionals in the pharmaceutical business is a contributing factor to the availability of counterfeit drugs in Nigeria. These non-professionals are less capable of identifying fake drugs and are more out to make profit than seek the general wellbeing of the community. Health professionals may have a high index of suspicion on the possibility of counterfeit drugs in cases of treatment failures or unusual side effects (Chika et al., 2011). They can educate themselves and patients on ways of identifying fake drugs using visual security tools which may include the size and shape of tablets, the quality of the print and the examination of holograms. Cases of suspected drug counterfeiting should be reported to the appropriate authority. Sadly, a survey by Odili et al. (2006) of 69 pharmacists in Lagos, Nigeria revealed that of the 42 (61%) respondents who have come across at least an incidence involving fake drugs, only 13 (31%) bothered to report the case to the appropriate authority. This finding reveals the lack of interest of healthcare professionals on the problem. It is essential for health personnel to contribute to the fight against the menace of counterfeit drugs in Nigeria. Appropriate authorities should monitor non professionals such as patent medicine vendors in the pharmaceutical business to help eliminate the prevalence of counterfeit drugs in Nigeria.

The high cost of drugs allows for the proliferation of counterfeit drugs in Nigeria and poses a major challenge to public health. Most genuine drugs are expensive and counterfeiters take advantage to supply cheap fake drugs to consumers, especially those who cannot afford the high priced good quality version in the legal sector (Chiwendu, 2008). The high cost of drugs have made access to medicines very difficult (Lambo, 2006). Majority of Nigerians cannot afford good medicines. 70% of the Nigerian population live below the poverty line (Central Intelligence Agency (CIA), 2011). The low input in local manufacture of drugs has also contributed to the high cost of drugs. Most raw materials are imported and equally attract high tariffs. Devaluation of the Nigerian currency is equally a contributing factor. The high prices makes drug unaffordable. People opt for cheaper drugs which are counterfeits in many cases. Local manufacture of drugs should be encouraged and there should be a reduction in drug importation. Importation of counterfeits across national boundaries is part of this increasingly complex problem (Finlay, 2011). Heavy tax should be
placed on drug importation to discourage importers; however, Chike et al. (2009) suggest that decreasing taxes and tariffs placed on genuine drugs may help reduce the problem by decreasing the cost of the drug reaching the consumers.

The drug distribution network in Nigeria consist of chaotic open markets that act as major source of purchase to pharmacy stores, hospitals, wholesalers, retailers, medicine stores and pharmaceutical manufacturers. Most importers supply drugs to open drug markets because they make more profit from there. The lack of strict monitoring and regulatory mechanisms allows for easy access to legitimate channels of distribution, making counterfeiting an appealing source of illicit revenue (Finlay, 2011). There is poor accountability to the disposal of medicines which complicates the work of the drug regulatory agency, NAFDAC (WHO, 2005). Monitoring of the supply chain at every stage of distribution is essential to ensure continued supply of good quality drugs in the Nigerian drug market. This should be integrated into the duties and activities of NAFDAC. Inter-agency involvement from within government and enhanced cooperation between governments, as well as improved partnerships with legitimate private pharmaceutical and supply chain industry actors will be required to reduce the chaotic drug network in the country.

Greed, ignorance and corruption are other factors contributing to the prevalence of fake drugs in Nigeria. Corruption and greed is seen from the drug regulating authorities and the drug importers and manufacturers. The effectiveness of regulatory bodies is negatively affected by the high level of official manipulations and corruption in the Nigerian healthcare system. It is common knowledge that the law enforcement agency are paid off to look the other side while the business of fake drugs flourishes. Corruption and conflict of interest are the driving forces behind poor drug regulation, which directly encourages drug counterfeiting (WHO, 2007). Beyond the widening public health challenge posed by this growing and increasingly lucrative crime, evidence suggests that counterfeit and fake drugs are also providing material support to criminals and terrorist organizations working to undermine national security (Finlay, 2011). Shutting down these fake drug markets, producers, traffickers, and illicit tradesmen must be a top public health priority.

Conclusion

Due to the complexity of the drug production and distribution system, there is no single technique that can eliminate the public health threat posed by fake pharmaceuticals. As such, a layered strategy is fundamental, involving a wide array of inter-agency actors from within and outside the government; enhanced cooperation between international bodies and improved partnership with legitimate private supply chain to help reduce the prevalence of fake drugs in Nigeria. Immediate action would include: Increased awareness on the counterfeiting of drugs to the public; reduction in the importation of drugs and increased local production of drugs which would make drugs cheaper and readily available; increased tax for drug importers to discourage importation; legal gap analysis and review of laws regulating manufacture, sale and distribution of drugs; passing into law stiffer penalties for drug offenders consistent with the magnitude of crime; strict monitoring of premises involved in sale of drugs; monitoring of the supply chain at each stage of drug distribution; development of a transparent and verifiable chain of custody from point of production to point of sale; enhanced early authentication procedure in validation of manufacturing sites and formal registration/validation of all importers from a public health perspective; and enhanced enforcement to inhibit the growth of counterfeiters.

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Must Communicate the Dangers. PLoS Med 2(4):100
UPCOMING CONFERENCES

International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 24 Dec 2013


1st Annual International Conference on Pharmacology and Pharmaceutical Sciences (PHARMA 2013)

18th - 19th November 2013
SINGAPORE
**Conferences and Advert**

**November 2013**
1st Annual Pharmacology and Pharmaceutical Sciences Conference (PHARMA 2013).

**December 2013**
ICPP 2013: International Conference on Pharmacy and Pharmacology
Bangkok, Thailand  December 24-25, 2013

**December 2013**
46th Annual Conference of Pharmacological Society of India
African Journal of Pharmacy and Pharmacology

Related Journals Published by Academic Journals

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