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Full Length Research Paper

Chloral hydrate-dependent decrease in peptidoglycan-induced inflammatory macrophage response is associated with lower expression of toll-like receptor 2

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The objective of this study was to investigate the effect and mechanism of action of chloral hydrate on the peptidoglycan (PGN)-induced inflammatory macrophage response. The effect of chloral hydrate on the production of TNF- α and IL-6 was investigated by murine peritoneal macrophages with PGN-stimulation. In this work, RAW264.7 cells transfected with a NF- κ B luciferase reporter plasmid stimulated by PGN were used to study the effect of chloral hydrate on NF- κ B activity. Flow cytometry and western blotting were performed to investigate the expression levels of toll-like receptor 2 on treated RAW264.7 cells. It was found out that chloral hydrate decreased the levels of IL-6 and TNF- α produced by the peritoneal macrophages stimulated with PGN. The NF- κ B activity of the RAW264.7 cells stimulated by PGN was decreased, which was related to inducing lower expression of toll-like receptor 2 and decreased toll-like receptor 2 signal transduction by chloral hydrate. Chloral hydrate decreased the PGN-induced inflammatory macrophage response associated with lower expression of toll-like receptor 2.

Key words: Chloral hydrate, peptidoglycan, inflammatory response, macrophage, toll-like receptor 2.

INTRODUCTION

Chloral hydrate is a well-known sedative and anesthetic that is used in pediatric procedures including echocardiograms and magnetic resonance imaging (Mellon et al., 2007; Sury and Fairweather, 2006; Harrison et al., 2009; Layangool et al., 2008; Liu et al., 2008) and in animal experiments (Yuksel et al., 2009; Nazam et al., 2008; Uematsu et al., 2009; Zhu et al., 2009). Recently, its safety and pharmacological mechanism in clinical practice have been emphasized (Martinbiancho et al., 2009; Bronley-DeLancey et al., 2006; Merdink et al., 2008).

In our previous work, it was demonstrated that therapeutic concentrations of chloral hydrate decreased and delayed the inflammatory response and improved the survival of mice following lipopolysaccharide/D-galactosamine-induced acute liver injury. This altered inflammatory response was associated with the inhibitory effects of chloral hydrate on NF- κ B activity and the levels of serum proinflammatory cytokines induced by lipopolysaccharide (Pan et al., 2010).

Peptidoglycan (PGN) is a common and conserved component of the cell walls of Gram-positive (G⁺) bacteria

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including *Staphylococcus aureus* and was used as a toll-like receptor 2 (TLR2)-specific ligand (Chang et al., 2007). It has been reported that PGN can be detected in the blood of 80% of the patients with serious bacterial infections (Merkel and Scofield, 2001). The mononuclear phagocyte system plays a crucial role against infection in the innate immune response, and murine macrophages are an important model to study infection. After stimulation with PGN, NF- κ B becomes activated and macrophages release large quantities of the proinflammatory cytokines IL-6 and TNF- α . Simultaneously, the level of TLR2 expression becomes upregulated (Chen et al., 2009; Lin et al., 2011). Activation of a murine macrophage cell line (RAW264.7) by PGN is mediated through the TLR2 signaling cascade, which involves the activation of a number of kinases, including p38 mitogen-associated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK)1/2 (ERK1/2), I κ B α , and Akt (Li et al., 2010; Lee et al., 2012).

In this study, the effect of chloral hydrate was investigated on the production of the proinflammatory cytokines and the activity of NF- κ B in murine peritoneal macrophages and RAW264.7 cells stimulated with PGN. The mechanisms was explored and the effects of chloral hydrate treatment on the expression of the TLR2 and TLR2 signal transduction in murine peritoneal macrophages and RAW264.7 cells stimulated with PGN was investigated.

MATERIALS AND METHODS

Reagents

PGN (*S. aureus*, strain DSM346), chloral hydrate (Cat. No. 302-17-0) and Trypsin-EDTA solution (10 \times) (Cat. No. T4174) were purchased from Sigma (St. Louis, MO, U.S.A). Iscove's Modified Dulbecco's Medium (IMDM) and fetal bovine serum (FBS) were purchased from Gibco (NY, U.S.A). The tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) ELISA kits were from R&D Systems, Inc. (Minneapolis, U.S.A). The FITC-conjugated anti-mouse TLR2 antibody (clone TL2.5), FITC-conjugated mouse IgG1 (isotype control), anti-mouse TLR2 antibody (clone TL2.5), HRP-goat anti-mouse secondary antibody and cell staining buffer were purchased from Biolegend (San Diego, CA, USA). The antibodies against p38 mitogen-associated protein kinase (MAPK), phospho-p38 MAPK, ERK1/2, phospho-ERK1/2, I κ B α , phospho-I κ B α , Akt, phospho-Akt, or β -actin were from Cell Signaling Technology (Waltham, MA, USA). The NF- κ B-luciferase, β -galactosidase reporter vectors and the dual luciferase reporter assay system were from Promega Corp. (Madison, WI, USA). The Immobilon membrane was obtained from Millipore (Watford, UK) and the ECL chemiluminescence from Amersham Life Science Ltd (Little Chalfont, UK).

The effects of chloral hydrate on proinflammatory cytokine production by murine peritoneal macrophages stimulated with PGN

The separation and cultivation of mouse peritoneal macrophages were performed as previously described (Yin et al., 2006). Briefly, abdominal cavity of mouse were doused using Dulbecco's

Modified Eagle Medium (DMEM) without serum, and the contained cells were drawn out and cultured in DMEM medium with 10% fetal bovine serum. The cells were identified by morphological observation, endocellular enzyme and phagocytosis. The institutional review board of the Affiliated Hospital of Guangdong Medical College approved the removal of the macrophages from the mice in this study. The cells were seeded at 10⁵ cells/well in 12-well plates in IMDM supplemented with 5% heat-inactivated FBS at a humidified 5% CO₂ and air incubator at 37°C. The supernatants were collected at the indicated time points after stimulation with PGN (1 μ g/ml) or PGN (1 μ g/ml) plus chloral hydrate (0.25 and 1 mg/ml) and stored at -80°C before the TNF- α and IL-6 levels were measured using an ELISA kit according to the manufacturer's instructions.

The effects of chloral hydrate on NF- κ B activity of PGN-stimulated RAW264.7 cells transfected with a NF- κ B luciferase reporter plasmid

The RAW264.7 cells were cultured in IMDM (5% FBS) and maintained at 37°C in 5% CO₂. The cells at 1.0 \times 10⁶ cells per well were seeded in 25-mm dishes. 48 h later, these cells were harvested with 0.25 g/L trypsin, and the 5 \times 10⁶ RAW 264.7 cells were transiently transfected with NF- κ B-luciferase (5 μ g) and beta-galactosidase reporter (5 μ g) vectors (for normalization of the efficiency of the transfection) in a volume of 400 μ l by electroporation at 250 V, 960 μ F of capacitance pulse. The cells were subsequently washed 1 time in IMDM and then split into 50 wells (100 μ l/well) and cultured for 24 h in IMDM (5% FBS) before stimulation with PGN (1 μ g/ml) for 6 or 12 h in the presence or absence of chloral hydrate (0.25 or 1 mg/ml). For the luciferase activity assays, the transfected RAW264.7 cells were harvested and lysed after stimulation for 6 or 12 h and subsequently, dual luciferase assays in extracts were performed with the dual luciferase reporter assay system as to the manufacturer's instructions.

The effects of chloral hydrate on PGN-induced upregulation of TLR2 expression in RAW264.7 cells

Flow cytometry was performed to investigate the levels of TLR2 expression on the RAW264.7 cells. The RAW 264.7 cells were cultured in IMDM (5% FBS) for 12 h with PGN (1 μ g/ml) in the presence or absence of chloral hydrate (0.25 mg/ml). After harvesting, the living cells were incubated with FITC-conjugated anti-mouse-TLR2 (1 μ g) for 30 min at room temperature without permeabilization. As isotype control, FITC-conjugated mouse IgG1 was used to detect nonspecific staining. The cells were washed three times with cell staining buffer. A six-parameter flow cytometer (FACScan; BD Biosciences) was used in data acquisition. The analysis of the acquired data was performed using CellQuest software (BD Bioscience Immunocytometry Systems, San Jose, CA). The mean channel fluorescence intensity (MFI) derived from the fluorescence histogram was used to study the level of TLR2 expression.

The TLR2 expression in the extracts from the RAW264.7 cells stimulated with PGN (1 μ g/ml) for 12 h in the presence or absence of chloral hydrate (0.25 mg/ml) was semi-quantitatively analyzed by western blotting using the anti-mouse TLR2 antibody (clone TL2.5). The extracts from the Raw264.7 cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The immunoblotting was performed by a standard western blotting procedure onto the Immobilon membrane. Horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody, followed by ECL chemiluminescence was used for detection of the TLR2 signal. The semi-quantitative analysis of the extracts from the RAW264.7 cells was by Band scan software 5.0 (Glyko Inc).

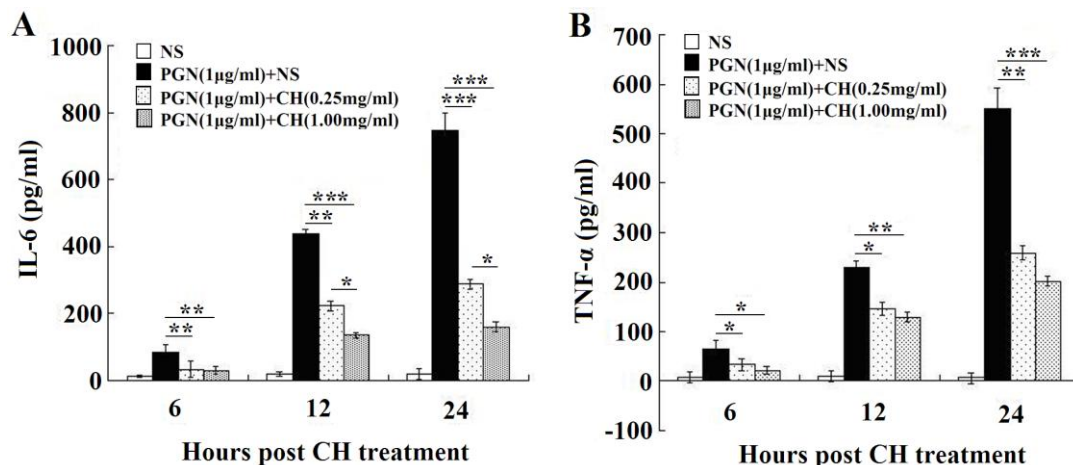


Figure 1. Chloral hydrate (CH) treatment decreased the production of IL-6 and TNF- α by peritoneal macrophages following PGN stimulation. Effects of chloral hydrate (0.25 and 1 mg/ml) on the levels of IL-6 (A) and TNF- α (B) produced by peritoneal macrophages (10^6 cell/well) after PGN stimulation for 6, 12 and 24 h. The data are expressed as the means \pm standard deviation (SD) of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

The effects of chloral hydrate on PGN-induced TLR2 signal transduction in RAW264.7 cells

The TLR2 expression in the extracts from the RAW264.7 cells stimulated with PGN (1 μ g/ml) for 12 h in the presence or absence of chloral hydrate (0.25 mg/ml) was semi quantitatively analyzed by western blotting using antibodies against MAPK, phospho-p38 MAPK, ERK 1/2, phospho-ERK1/2, IKB α , phospho-IKB α , Akt, phospho-Akt, or β -actin. Western blotting and detection were performed as described earlier. The semi-quantitative analysis of the extracts from the RAW264.7 cells was by band scan software 5.0 (Glyko, Inc).

Statistical analysis

The data were presented as the means \pm standard deviation (SD), and the statistical analysis was performed with Statistical Package for Social Science (SPSS) version 15.0 statistical software. The statistical significance between the two groups was determined by the unpaired Student's t-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Chloral hydrate-treatment decreased the levels of IL-6 and TNF- α produced by PGN-stimulated peritoneal macrophages

Production of IL-6 (Figure 1A) and TNF- α (Figure 1B) increased sharply post stimulation with PGN (1 μ g/ml) for 6, 12 and 24 h and decreased significantly post the chloral hydrate treatment (0.25 and 1 mg/ml) (all $P < 0.01$). The higher concentration of chloral hydrate (1 mg/ml) significantly decreased the production of IL-6 after stimulation with PGN (1 μ g/ml) for 12 and 24 h compared to the lower concentration of chloral hydrate (0.25 mg/ml) (all $P < 0.05$) (Figure 1A).

Chloral hydrate-treatment decreased the NF- κ B activity of PGN-stimulated RAW264.7 cells

Luciferase activities in the transfected RAW264.7 cells were measured at 6 and 12 h after PGN stimulation (Figure 2). The results were presented as a fold of induction over the values obtained in the normal saline groups. As shown in Figure 2, approximately 1.5- and 3.5-fold increases in the luciferase activity were observed after the PGN stimulation for 6 and 12 h, respectively. The effect of PGN-stimulation was significantly decreased by the chloral hydrate treatment (0.25 and 1 mg/ml) (all $P < 0.01$). A higher concentration of chloral hydrate (1 mg/ml) led to a significantly larger decrease in the luciferase activity after 12 h than did the lower concentration of chloral hydrate (0.25 mg/ml) ($P < 0.01$).

Chloral hydrate-treatment decreased the increased TLR2 expression in PGN-treated RAW264.7 cells

Based on the fact that both concentrations (0.25 and 1 mg/ml) of chloral hydrate treatment were shown to significantly decrease the inflammatory response of peritoneal macrophages and RAW264.7 cells after PGN-stimulation, the lower effective concentrations (0.25 mg/ml) to test the effect of chloral hydrate treatment on TLR2 expression in RAW264.7 cells was chosen.

By morphological observation, the cultured cells grow well (Figure 3A), the cultured cells preserved the FS and SS properties and showed a centralized and uniform cell population by flow cytometry. Also, by western, the results showed that the total number of cells almost the same based on the band of β -actin (Figure 3E), even cultured in the IMDM supplemented with 5% heat-inactivated FBS

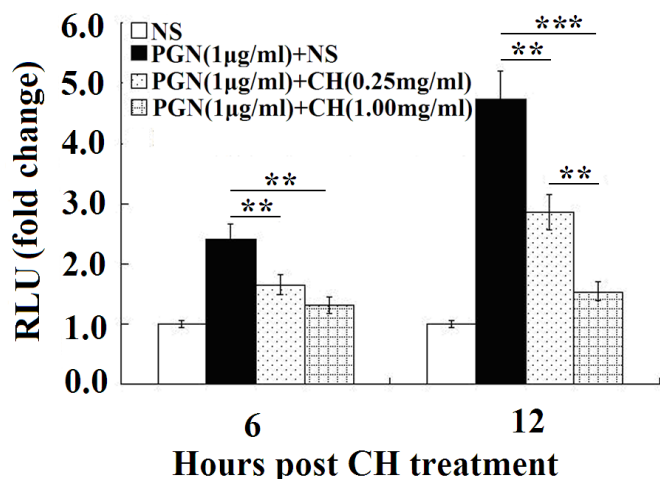


Figure 2. Chloral hydrate (CH) treatment decreased the NF- κ B activity of RAW264.7 cells during PGN stimulation. One day after the RAW264.7 cells were co-transfected with NF- κ B-luciferase- and β -galactosidase- reporter vectors by electroporation, PGN (1 μ g/ml) or PGN (1 μ g/ml) plus chloral hydrate (0.25 or 1 mg/ml) was added to the medium and incubated for 6 or 12 h. The luciferase activity of the cell extracts was measured and expressed as the folds of luciferase-induction over a saline-treated control \pm standard deviation (SD) of three independent experiments. RLU: Relative light unit), ** $P < 0.01$ and *** $P < 0.001$.

for 12 h with PGN (1 μ g/ml) in the presence or absence of chloral hydrate (0.25 mg/ml).

The levels of mean channel fluorescence intensity (MFI) of TLR2 on RAW264.7 cells stimulated by PGN (1 μ g/ml) for 12 h were significantly upregulated compared to the normal group (Figure 3C and D). Treatment with CH (0.25 mg/ml) significantly attenuated the upregulation of TLR2 stimulated by PGN ($P < 0.05$) (Figure 3C and D).

Also, semi quantitative analysis of TLR2 expression in extracts of RAW264.7 by western blotting showed a similar trend (Figure 3E and F).

Treatment of PGN-induced RAW264.7 cells with chloral hydrate reduced TLR2 signal transduction

Although chloral hydrate-treatment decreased the increased TLR2 expression in the PGN-treated RAW264.7 cells, it remains unknown whether the TLR2 related signal transduction including phospho-p38 MAPK, phospho-ERK1/2, phospho-IK β and phospho-Akt was reduced.

The effect of chloral hydrate on TLR2 signal transduction by western blotting was tested, and selected the lower effective concentrations (0.25 mg/ml) to test the effect of chloral hydrate treatment on TLR2 signal transduction in RAW264.7 cells.

After being cultured in IMDM supplemented with 5% heat-inactivated FBS for 12 h with or without PGN (1 μ g/ml) in the presence or absence of chloral hydrate

(0.25 mg/ml), the upregulation of signal transduction of TLR2 in RAW264.7 stimulated by PGN (1 μ g/ml) for 12 h was tested for significance with western blotting. The upregulation of TLR2 signal transduction stimulated by PGN was decreased after chloral hydrate (0.25 mg/ml) treatment (Figure 4A and B).

DISCUSSION

In recent years, the mechanisms of bacteria-induced acute inflammation and anti-cytokines as therapeutic agents have been re-evaluated. Most of the reported cases of sepsis-related anti-cytokine therapeutic projects were unsatisfying or disappointing, because they exhibit several drawbacks: difficulties of production, excessive costs, and a few side-effects (Hall and Muszynski, 2009; Zeni et al., 1997; Ratsimandresy et al., 2009). The development of new drugs and treatment strategies for severe sepsis and acute inflammation have recently been intensively investigated.

The discovery of the protective effect of some anesthetics and sedatives such as isoflurane, lidocaine, and bupivacaine against infection or inflammation may yield new insights into anti-inflammatory therapies (Plachinta et al., 2003; Gallos et al., 2004; Fuentes et al., 2006; de Klaver et al., 2002). As a traditional anesthetic, chloral hydrate is typically used in patients and animal models, and can be administered by mouth, injection and direct placement into the intestine/small bowel. Unlike isoflurane, an inhalation agent, must be inhaled continuously and is expensive. Most importantly, chloral hydrate can be used in many countries, but other sedatives, for example, ketamine is under strictly regulated conditions in China, also, is not approved for patients under 16 years old by the USFDA (Mellon et al., 2007).

Our studies in murine peritoneal macrophages show that chloral hydrate treatment decreased the increase of the inflammatory cytokine levels produced after PGN stimulation (Figure 1), indicating that the effect of chloral hydrate on inflammation could be attributed to the inhibition of the macrophage function. The TNF- α and IL-6 levels sharply increased at 6, 12, and 24 h after the PGN-challenge. Similar studies using PGN (10 or 25 μ g/ml) were performed by Shirasawa et al. (2004) and Wang et al. (2004), although tested 24 h after one challenge, levels of TNF- α and IL-6 also sharply increased. The treatments with different concentrations (0.25 and 1.0 mg/ml) of chloral hydrate significantly decreased the rise of inflammatory cytokines at the indicated time points (Figure 1).

PGN-induced NF- κ B-dependent gene transcription was then examined whether it is regulated by chloral hydrate. The results of the RAW264.7 cells transfected with a NF- κ B luciferase reporter vector revealed that chloral hydrate treatment reduces NF- κ B activity (Figure 2). Because NF- κ B plays a key role in the transcriptional regulation of proinflammatory cytokine expression, this result suggests

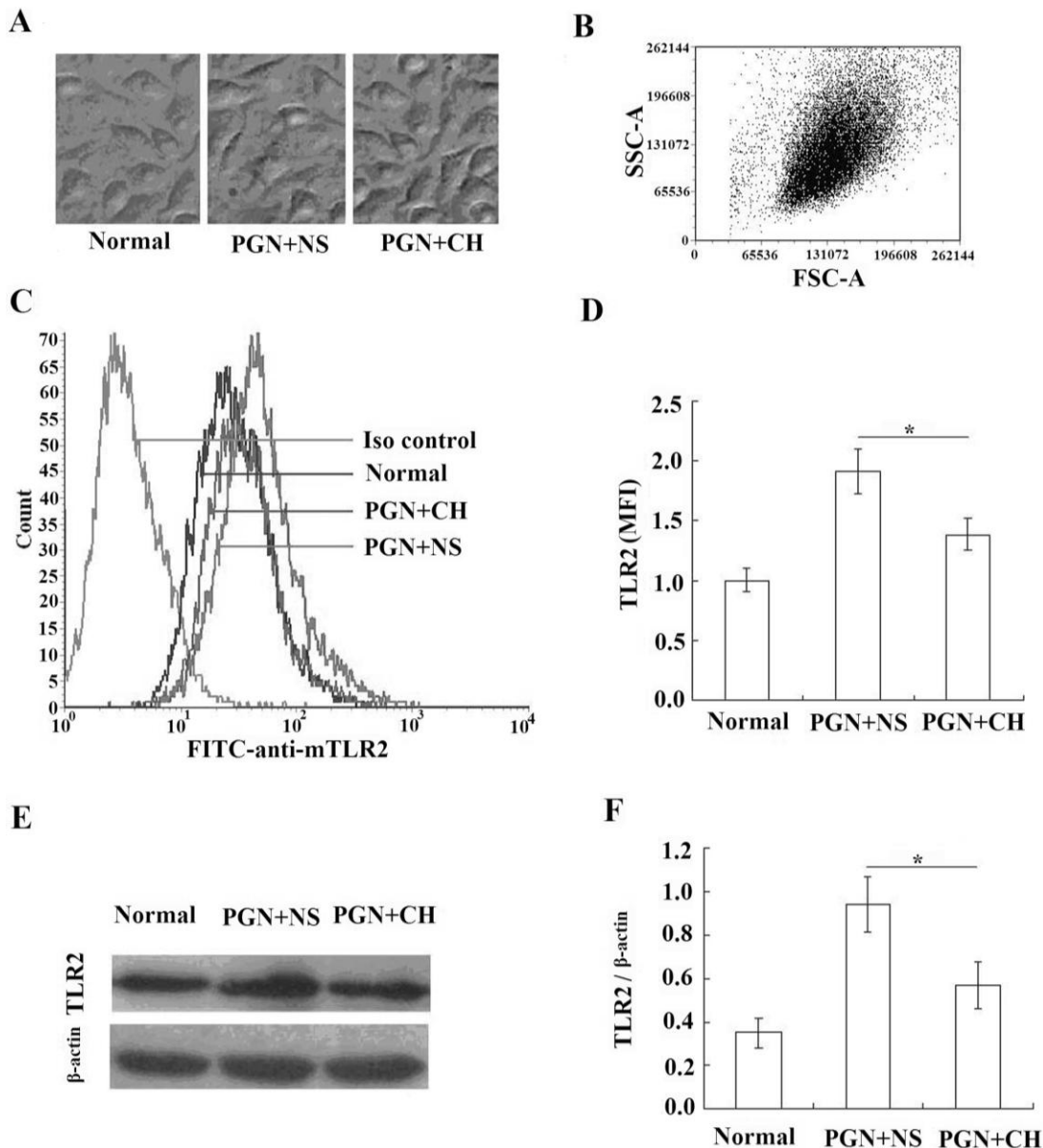


Figure 3. Chloral hydrate (CH) treatment reduced the expression of TLR2 in the PGN-stimulated RAW264.7 cells. The RAW 264.7 cells were cultured in the IMDM supplemented with 5% heat-inactivated FBS for 12 h with PGN (1 μ g/ml) in the presence or absence of chloral hydrate (0.25 mg/ml) (A). Flow cytometry was performed to investigate the expression levels of TLR2 on the treated RAW264.7 cells (B) represented as dot plots and (C) represented as histograms. The mean channel fluorescence intensity (MFI) derived from the fluorescence histogram was used to study the level of the cell surface TLR2 expression. The MFI was calculated as a ratio and recorded as the MFI of the TLR2 antibody divided by the MFI of the normal control (normal saline groups) (D). The semi-quantitative analysis of the extracts from the RAW264.7 cells by band scan and the ratio of TLR2 to β -actin was shown, (E) shows a western blot of β -actin and TLR2 of normal, PGN+NS and PGN+CH group and (F) shows a quantitation of them. The data are expressed as the mean \pm SD of three independent experiments. *P<0.05.

that chloral hydrate could affect cytokine expression by influencing the activity of NF- κ B. After 6 and 12 h of the PGN challenge, the respective increase of NF- κ B-induced luciferase activity in RAW264.7 cells was approximately 1.5- and 3.5-fold compared to the basal level. A similar study using PGN (5 μ g/ml) was performed

by Ito et al. (2005), and they observed the same effect. PGN-induced the upregulation of TLR2 expression was examined whether it is regulated by chloral hydrate. Flow cytometry was used to analyze the effect of chloral hydrate on the expression of TLR2 in the RAW264.7 cells stimulated by PGN. The expression of TLR2 in RAW264.7

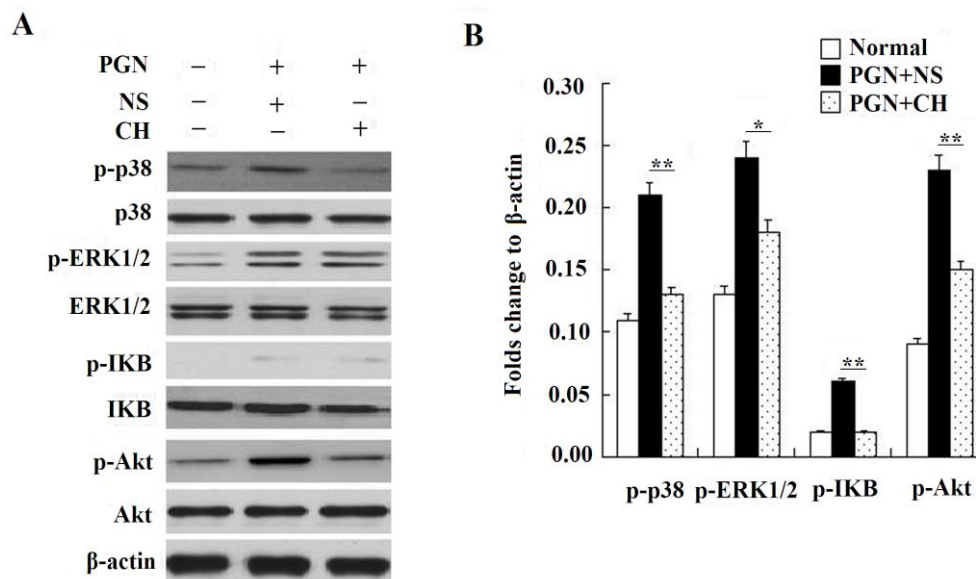


Figure 4. The RAW264.7 cells were treated with PGN (1 µg/ml) for 12 h in the presence or absence of chloral hydrate (CH) (0.25 mg/ml). The cell lysates were immunoblotted with antibodies against MAPK, phospho-p38 MAPK, ERK1/2, phospho-ERK1/2, IKB α , phospho-IKB α , Akt, phospho-Akt, or β -actin (A), and semi-quantitated to show the reduction in signal (B). The experiments were repeated 3 times with similar results.

cells is significantly upregulated after PGN stimulation. Similar results have been found by Chen et al. (2009). After the chloral hydrate treatment, the strong upregulation of the TLR2 expression in response to PGN exposure was remarkably decreased (Figure 3), which is consistent with the effect of chloral hydrate on NF- κ B activity and inflammatory cytokine production by RAW264.7 stimulated with PGN. Knowing that the strong upregulation of TLR2 expression in response to PGN exposure was remarkably diminished after the chloral hydrate treatment, the TLR2 signal transduction including p38 MAPK, ERK1/2, IKB α , and Akt was checked. In the RAW264.7 cells, PGN strongly induced the activation by phosphorylation of p38 MAPK, ERK1/2, IKB α and Akt, but this phosphorylation was decreased by the chloral hydrate treatment (Figure 4).

Our study showed for the first time that chloral hydrate decreased the upregulation of PGN-induced NF- κ B activity and TNF- α and IL-6 production by macrophages in a time- and concentration-dependent manner. This study showed that this decrease was associated with a decreased up regulation of the PGN-induced-TLR2 expression TLR2 signal transduction. Knowing the mechanisms of chloral hydrate treatment in inflammation provides opportunities to design new therapeutic strategies to reduce inflammation caused by Gram-positive (G⁺) organisms.

Conclusion

This study showed for the first time that chloral hydrate

decreased the upregulation of PGN-induced NF- κ B activity and TNF- α and IL-6 production by macrophages in a time- and concentration-dependent manner. The study showed that this decrease was associated with a decreased upregulation of the PGN-induced-TLR2 expression TLR2 signal transduction. Knowing the mechanisms of chloral hydrate treatment in inflammation provides opportunities to design new therapeutic strategies to reduce inflammation caused by Gram-positive (G⁺) organisms.

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Full Length Research Paper

A novel approach and *in-vitro* evaluation of bioactive components for the development of nutraceuticals

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The nutritional ability of various bioactive components from *Solanum melongena* pulp (SMP) and *Musa paradisiaca* pseudo stem pulp (MSP) samples were investigated. The objective of the present investigation was the evaluation and determination of the nutraceutical properties of pulp extracts obtained from these plants. The antimicrobial activity was evaluated by the agar well diffusion method. The total phenolic contents were determined by the Folin-Ciocalteu colorimetric method. The highest total phenolic content was observed in *S. melongena* pulp extract (459.72 ± 6.3 mg GAE/g of sample on dry weight basis). The highest flavonoid content was observed in SMP (36.5 ± 6.7 mg QE/g of sample on dry weight basis). The antioxidant activity was evaluated by H₂O₂ radical scavenging methods. *S. melongena* showed the best antioxidant activity of $92 \pm 0.1\%$. Among these two extracts the methanolic extracts of *S. melongena* had the best antimicrobial activity against *Staphylococcus aureus*. The extracts of both *S. melongena* and *M. paradisiaca* pseudo stem pulp showed best antimicrobial activity against *Candida albicans*. The high content of ascorbic acid was observed with *M. paradisiaca* pseudo stem pulp extract (35.6 ± 2.5 mg/g dry weight of the sample). Maximum catalase activity was found in *M. paradisiaca* pseudo stem pulp extract (3.099 ± 0.3 Units/mg of protein). The tocopherol content was also investigated. The formulated extracts of these plants may be given as the best nutritional health supplements in human diet without raising any controversial adverse effects.

Key words: *Solanum melongena*, *Musa paradisiaca* pseudo stem, antioxidant, ascorbic acid and antimicrobial activity.

INTRODUCTION

Plant products have fulfilled most of the human dietary requirements and also, they act as a protector against many diseases by means of its medicinal activity (Sonia et al., 2009). For example, the plant products such as neem, garlic and green vegetables provide us a vast source of fat-less, low calorie diet which is essential to lead a healthy life (Fatouma et al., 2010). Herbs are considered as medicinal plants and taken only for their medicinal or aromatic properties. It is estimated by the world health organization that approximately 75 to 80% of

the world's population uses plant derived medicines either in part or entirely. Growing number of American health care consumers are turning onto plant-originated medicines for reasons like, low cost, all time availability (Antonio et al., 2012) and seeking natural alternatives with fewer side effects.

The role of antioxidants is to detoxify the reactive oxygen intermediates (ROI) within the body (Edziri et al., 2012; Nisha et al., 2009). Over the past several years, antioxidants have attracted considerable interest in the

potential treatment for a wide variety of diseases including cancer (MasudaHollman et al., 1999), atherosclerosis, chronic inflammatory disease and aging (Paola Bontempo et al., 2013) and there is an increasing need to acquire them from our diet. In an attempt to study the antioxidant activity of vegetables, *Solanum melongena* (brinjal) and pseudo stem of *Musa paradisiaca* (banana) were selected (Cordeiro et al., 2004). The agricultural activity generates a large amount of residues because each plant produces only one bunch of bananas and after its harvesting, the bare pseudo stems are cut and usually left in the soil plantation to be used as organic material. These crops could and should find more rational way of utilization. These medicinal plants contain large amounts of antioxidants such as polyphenols (Djeridane et al., 2006) which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The extracts of both the plant pulps were used to study the antioxidant activity. The deep-purple skin gets its rich color and anti-aging power from nasunin like compounds which are present in *S. melongena* (Mai et al., 2012; Nadia, 2011).

Nasunin is high on the list of potent antioxidants. In this capacity, it helps to neutralize free radicals, making it an excellent anti-aging phytonutrient. Nasunin has many additional health benefits. It has antiangiogenic properties which help it to fight cancer by restricting the growth of new blood vessels (Salem et al., 2013). It also helps keep blood vessels clear and relaxed. It helps to fight the spread of cancerous cells by cutting off the blood supply they need to multiply (Matsubara et al., 2005). In this study, we have investigated the nutraceutical contents of the pulps from these two plants. Antimicrobial activities of these samples against a group of selected microorganisms were also investigated.

MATERIALS AND METHODS

Collection of samples

Fresh purple coloured moderate size Indian variety of *S. melongena* and the Indian variety of *M. paradisiaca* pseudo stem were purchased from a super market (Reliance fresh) in Chennai, Tamilnadu, India and prior to this study, these varieties were identified and authenticated by experts at Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India. The pulp of *S. melongena* and pseudo stem of *M. paradisiaca* was utilized for all the investigation.

Extraction procedure

About 200 g of *S. melongena* and pseudo stem of *M. paradisiaca* pseudo stems were cleaned and cut into pieces and ground by using mixer grinder (Preethi Chefro Model, Indian make 2013). Then the pulp was dried in a laboratory fluidized bed drier (Sherwood Model 501, England) at 45°C for 6 h (Lila et al., 2013). The dried powder sample was stored at 4°C for different studies. 50 g of *S. melongena* and pseudo stem

of *M. paradisiaca* samples were extracted with methanol using Soxhlet apparatus at 55°C for 6 h. The extracts were filtered by Whatman filter paper No.1 and concentrated at 45°C using a rotary evaporator (Stuart RE 300 model, UK make 2012) and resultant residues were collected. Finally the samples were dissolved in methanol (1:10) for radical scavenging, total phenolic content, catalase assays and antimicrobial activity.

Hydroxyl radical scavenging

The reaction mixture contained 0.1 ml of deoxyribose, 0.1 ml of FeCl₃, 0.1 ml of ethylenediaminetetraacetic acid (EDTA), 0.1 ml of H₂O₂, 0.1 ml of ascorbate, 400 µl of sample extracts in a final volume of 1 ml made up with KH₂PO₄-KOH buffer. The mixture was incubated at 37°C for 1 h. At the end of the incubation, 1 ml of 1% thiobarbituric acid (TBA) was added and heated at 95°C for 20 min to develop the color. After cooling, the thiobarbituric acid reactive substances (TBARS) formation was measured spectrophotometrically at 535 nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the sample (Aline et al., 2005). The percent TBARS production for positive control H₂O₂ was fixed at 100% and the relative percent TBARS was calculated for the extract treated groups (Marappan and Leela, 2007).

Estimation of ascorbic acid

Ascorbic acid (AA) content was determined using the method described by Omaye et al. (1979). The extract was prepared by grinding 20 g of fresh *S. melongena* skin pulp (SMP) and *M. paradisiaca* pseudo stem with 100 ml of 10% TCA. Then the mixture was centrifuged at 3500 rpm for 20 min and re-extracted twice. 0.5 ml of supernatant (clear solution without debris) was taken along with 1 ml of 2,4-dinitrophenyl hydrazine-thiourea-CuSO₄ reagent (DTC reagent). Then the mixture was incubated at 37°C for 3 h and 0.75 ml of ice-cold 65% H₂SO₄ was added, allowed to stand at 30°C for 30 min and resulting color was read at 520 nm in spectrophotometer. The AA content was determined using a standard curve prepared with AA and the results were expressed in milligram per gram dry weight (DW) (Koh et al., 2009).

Estimation of total phenolic content (TPC)

Total phenolic content was determined with the Folin-Ciocalteu reagent using the method of (Lister et al., 2001). 100 µl of sample was dissolved in 500 µl (1:10 dilution) of Folin-Ciocalteu reagent and 1000 µl of distilled water. The solutions were mixed and incubated at room temperature (37°C) for 1 min. After 1 min, 1500 µl of 20% sodium carbonate (Na₂CO₃) solution was added. The final mixture was shaken and then incubated for 2 h in the dark at room temperature (37°C). The absorbance of samples was measured at 760 nm using a Milton Roy 601 UV-Vis spectrophotometer and the results are expressed in mg of gallic acid per g (GEA) of dry weight of plant (Ephraim and Masaharu, 1997). A standard curve was plotted using gallic acid as standard. Different concentrations of gallic acid were prepared in 80% of methanol and their absorbances were recorded at 765 nm.

Estimation of tocopherol

Tocopherol content was assayed as described by Becker et al. (1980). Five hundred milligrams of sample was homogenized with 10 ml of a mixture containing petroleum ether and ethanol (2:1.6, v/v) and the extract was centrifuged at 10,000 rpm for 20 min then

the supernatant was used for estimation of tocopherol. To the 1 ml of extract, 0.2 ml of 2% 2,2-dipyridyl in ethanol was added and mixed thoroughly and kept in dark for 5 min. The resulting mixture was diluted with 4 ml of distilled water and mixed well. The resulting color in the aqueous layer was measured at 520 nm. The tocopherol content was calculated using a standard graph made with known amount of tocopherol (Filipa et al., 2011).

Estimation of flavonoids

The total flavonoid content was determined by aluminium chloride colorimetric method described by Chang et al. (2002). 0.5 ml of extract was mixed with 1.5 ml of 95% alcohol, 0.1 ml of 10% aluminium chloride hexahydrate (AlCl_3), 0.1 ml of 1 M potassium acetate (CH_3COOK) and 2.8 ml of deionized water. After incubation at room temperature (37°C) for 40 min, the absorbance of reaction mixture was measured at 415 nm against a deionized water blank on a spectrophotometer (Jenway 6305 model, UK make 2011). Quercetin was chosen as a standard. Using the seven point standard curve (0 to 50 mg/ml), the levels of total flavonoid contents in the sample were determined in triplicate, respectively. The data were expressed as milligram quercetin equivalents (QE)/g of DW.

Estimation of catalase

The activity of CAT was measured according to the method of Toshiki et al. (1995) with small modification. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH: 7.0), 0.4 ml of 15 Mm H_2O_2 and 0.04 ml of sample extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in U mg^{-1} protein ($\text{U} = 1 \text{ mM of } \text{H}_2\text{O}_2 \text{ reduction min}^{-1} \text{ mg}^{-1} \text{ protein}$). For all the enzymatic calculations protein was determined by the Bradford, using bovine serum albumin (BSA, Sigma, USA) as the standard.

Antimicrobial activity of the samples

The extracts were tested for antimicrobial activity against selected microorganisms such as *Bacillus subtilis* (MTCC 2391), *Staphylococcus aureus* (MTCC 9542), *Escherichia coli* (MTCC1677), *Candida albicans* (MTCC 7315) and *Mycobacterium smegmatis* (MTCC 991) microorganism using agar-well diffusion method (Igbiosa et al., 2009; Nabila et al., 2008). 20 ml of nutrient agar (Hi Media Pvt LTD) was poured into the Petri-dish and 8 mm well bored in the agar. 1 mg and 100 μg of each extract and pure compounds, respectively were dissolved in 200 μl of dimethylsulfoxide (DMSO) and poured into the wells. The plants were incubated for 24 h at 37°C and the zone of inhibition was measured in mm. DMSO was used as negative control, while Chloramphenicol was used as a positive control (Christian et al., 2005)..

RESULTS AND DISCUSSION

Hydroxyl radical scavenging

The present work was an attempt to study the phytochemical constituents present in fresh *S. melongena* pulp and *M. paradisiaca* pseudo stem pulp and to identify the various constituents present in their pulp extracts. Various solvents were used for extracting the samples. The antioxidant constituents present in the samples include ascorbic acid, phenolic compounds,

catalase, tocopherol and flavonoids (Lister et al., 2001). Each of these has been estimated individually followed by a total hydroxy radical scavenging activity assay. Hydroxyl radicals are known to be the most reactive of all reduced forms of dioxygen and are thought to initiate cell damage *in vivo*. The effect of the extract on hydroxyl radicals generated by Fe^{3+} ions were measured by determining the degree of deoxyribose degradation, an indicator of TBA-MDA adduct formation. As shown in the Figure 1, the purified extract exhibited a dose dependent inhibition of the hydroxyl radical scavenging activity. A maximum of more than 90% inhibition was obtained at 400 μmol of SMP extracts. The inhibition was statistically significant when compared to samples without any inhibitor. The scavenging potential was compared with known antioxidants such as curcumin and butylated hydroxyanisole (BHA). Both extracts inhibited lipid peroxidation by 92 and 86% at 400 μl concentrations, respectively. Among the various concentrations tested, the extracts showed maximum hydroxyl radical scavenging activity at 800 μmoles , which was a much lower concentration than those of standard antioxidants such as curcumin and BHA. *S. Melongena* showed higher hydroxyl radical scavenging effect of $92 \pm 0.1\%$ when compared to the *M. paradisiaca* pseudo pulp extract. *M. paradisiaca* pseudo stem showed moderate inhibition of free radicals ($86 \pm 1.4 \%$) (Figure 1).

Estimation of ascorbic acid

Among the various samples the ascorbic acid content of *Musa* pseudo stem pulp was recorded higher when compared to other extracts (Table 1). This antioxidant contents play major roles in maintaining the balance between free radical production and elimination. *M. paradisiaca* pseudo stem sample was found to contain $48.7 \pm 3.1 \text{ mg/g}$ of dw which was higher than *S. melongena* sample ($35.6 \pm 2.5 \text{ mg/g}$ of dw).

Estimation of total phenolic content

It has been recognized that the total phenolic compounds and flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. Highest total phenolic content was observed in the extract of *S. melongena* pulp was $459.72 \pm 6.3 \text{ mg GAE/g}$ dry weight when it was compared to *M. paradisiaca* pseudo stem pulp extract ($290.91 \pm 5.4 \text{ mg GAE/g}$ dry weight) (Table 1).

Estimation of tocopherol

The standard reading was used to analyze the sample readings. From Table 1, it can be seen that *S. melongena* pulp extracts had $23.5 \pm 3.6 \text{ mg/g}$ of dw

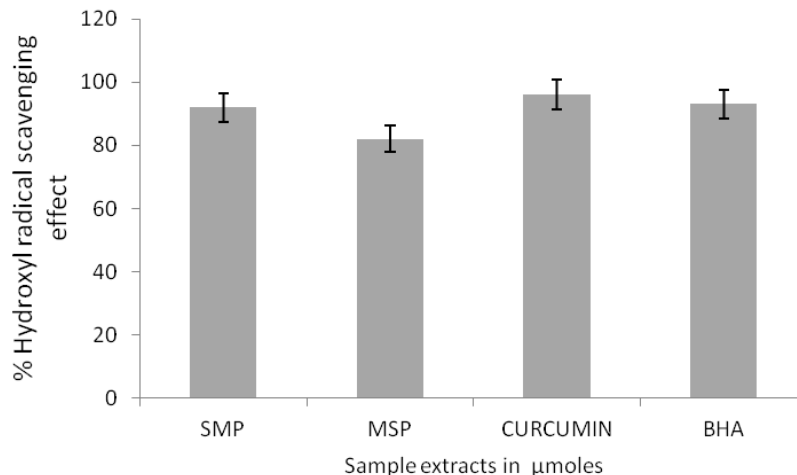


Figure 1. Effect of hydroxyl radical scavenging by deoxyribose method. Values are means \pm SD of triplicate.

Table 1. Total Ascorbic acid (mg/gm DW), phenolic content (GAE/g DW), flavonoids (QE/g DW) and tocopherol content (mg/gm DW) of the extracts.

Sample extracts	Ascorbic acid (mg/g dw)	Total phenolic content (mg GAE/g dw)	Total flavonoids (mg QE/g dw)	Tocopherol content (mg/gdw)
<i>Solanum melongena</i> pulp	35.6 \pm 2.5	459.72 \pm 6.3	36.5 \pm 6.7	23.5 \pm 3.6
<i>Musa paradisiaca</i> stem pulp	48.7 \pm 3.1	290.91 \pm 5.4	26.5 \pm 4.6	12.7 \pm 4.6

tocopherol (Vitamin E) compared to *M. paradisiaca* pseudo stem pulp (12.7 \pm 4.6 mg/g of dw).

Estimation of flavonoids

Based on this study (Margaret et al., 2009), we proposed that the potent free radical-scavenging and antioxidant activity of medicinal plant might result from its high contents of flavonoid type compounds. Flavonoids are regarded as one of the most widespread groups of natural constituents found in plants (Nabila et al., 2008). The results are shown in Table 1. All the tested samples had high flavonoid content between the two. *S. melongena* pulp showed maximum flavonoid content (36.5 \pm 6.7 mg QE/g dw) when it compared to *M. paradisiaca* pseudo stem pulp (26.5 \pm 4.6 QE/g dw).

Estimation of catalase

The higher activity of catalase enzyme in *M. paradisiaca* pseudo stem sample was recorded (3.099 \pm 0.3 U/mg of protein). The level of antioxidative response depends on the species, the development and the metabolic state of the plant, as well as the duration and intensity of the stress. CAT scavenges H₂O₂. The involvement of the antioxidative system in the regulation

of free-radical metabolism was followed by measuring changes in the antioxidant enzyme activities when plant sample experiences stress from an abiotic factor, there should be an enhancement in the production of toxic free radicals of H₂O₂, O₂⁻, ·O₂ or ·OH, which should be detoxified in terms of increased antioxidant enzyme activities (Table 2).

Antimicrobial activity of samples

The extracts of fresh *S. melongena* skin pulp and *M. paradisiaca* pseudo stem pulp were effective in inhibiting the growth of the tested strains. Both two extracts showed antifungal activity against *C. albicans*. Among the two extracts, *S. melongena* pulp extract has shown highest antibacterial activity against *S. aureus*. The observed antibacterial and antifungal activities of the extracts might also be attributed to the high quantity of poly phenols which are known to possess antimicrobial activity (Table 3).

Conclusion

Antioxidant and antimicrobial activities could be derived from compounds such as flavonoids, polyphenols and ascorbic acids. By coupling the results

Table 2. Total catalase activity of the sample.

Sample extracts (ml)	Total protein (mg)	Total catalase activity (Units)	Specific activity (Units/mg of protein)
<i>Solanum melongena</i>	5680	11700	2.06 ± 0.1
<i>Musa pseudo stemPulp</i>	5028	15580	3.099 ± 0.3

Table 3. Results of antimicrobial activity of the extracts.

Microorganism	Inhibition zone	
	<i>Solanum</i> extract	<i>Musa pseudo pulp</i>
<i>Bacillus subtilis</i>	+	+
<i>Staphylococcus aureus</i>	+	-
<i>Escherichia coli</i>	+	+
<i>Candida albicans</i>	+	+
<i>Mycobacterium smegmatis</i>	-	-

Chloramphenical was used as positive control at a concentration of 3 µg/ml. DMSO was used as negative control. +The size of inhibition is > 10 mm in diameter; - The size of inhibition is < 10 mm in diameter.

of different assays for evaluating antioxidant activity with H₂O₂ assay. Among these two extracts, *S. melongena* had the highest antimicrobial activity (Table 3). These results showed that they possess numerous nutraceutical properties as well. The present study evaluated and determined that the *in vitro* antioxidant and antimicrobial activities in terms of total phenolic content, flavonoids and H₂O₂ radical scavenging activity with the zone of inhibition. The extracts of *S. melongena* and *M. paradisiaca* pseudo stem pulp stem could effectively scavenge reactive oxygen species.

Concerning the antioxidant activity, high reducing power and inhibition of H₂O₂ activities of phenolic extract was observed. The present study confirms that the extracts of *S. melongena* and *M. paradisiaca* pseudo stem pulp stem could be a significant source of natural antioxidant with highest phenolic content that may have potent beneficial health effects. The formulated extracts of these *S. melongena* and *M. paradisiaca* pseudo stem pulp may be considered as a source of important nutraceutical compounds for human diet without raising any side effects.

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Full Length Research Paper

Determining potential drug-drug interactions between lopinavir/ritonavir and other antiretrovirals and prescribed daily doses in a section of the private health care sector in South Africa

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Lopinavir/ritonavir forms part of the antiretroviral therapy for the treatment of human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS). The aim of this non-experimental, quantitative drug-utilization study was to determine and identify potential drug-drug interactions between lopinavir/ritonavir and other antiretrovirals in general practitioners and specialists prescriptions with inappropriate prescribed daily doses. The study was performed on 49,995 (2005), 81,096 (2006) and 88,988 (2007) anti-retroviral (ARV) prescriptions claimed through a pharmacy benefit management company. Of the total 2,638 ARV general practitioners prescriptions and 472 specialist's prescriptions claimed with potential drug-drug interactions (DDIs), 505 (19.1%) were for general practitioners and 143 (30.3%) for specialists. Potential drug-drug interactions identified between lopinavir/ritonavir and other anti-retrovirals with inappropriate prescribed daily doses accounted for 88.9% (n = 449) for general practitioners and 98.6% (n = 141) for specialist's prescriptions. The highest percentage of anti-retroviral prescriptions with potential drug-drug interactions were between lopinavir/ritonavir at 1066.4 mg/264 mg and efavirenz at 600 mg average of prescribed daily doses with 61.4% (n = 276) for general practitioners and 38.3% (n = 54) for specialists, prescribed to patients between 19 and 45 years. The recommended standard adult dose for lopinavir/ritonavir is 400 mg/100 mg twice daily or 800 mg/200 mg once daily. The dose prescribed to HIV/AIDS patients in this section of the private health care sector of South Africa was therefore high. It is therefore recommended that more education be given to prescribers and dosage adjustments be done where indicated.

Key words: Drug-drug interactions, anti-retroviral drugs, prescribed daily doses, lopinavir/ritonavir, inappropriate prescribing, private health care sector, South Africa.

INTRODUCTION

The current guidelines for the use of anti-retrovirals (ARVs) do recommend the combinations of different ARV

agents, due to the fact that these combinations have led to major improvements in the management of human

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immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) in both developed and developing world (Arshad et al., 2009). ARVs have transformed the human immunodeficiency virus (HIV) infection from a fatal to a chronic illness due to their potency (Kiser et al., 2008). In spite of the so many beneficial effects of these ARVs, health care providers are faced with challenges of drug-drug interactions (DDIs) and drug-related adverse-effects (Tourret et al., 2007). Lopinavir/ritonavir (LPV/RTV) is a co-formulated boosted protease inhibitor (PI) containing lopinavir and low-dose ritonavir and forms part of the highly active antiretroviral therapy (HAART) for the treatment of HIV infection in adults and children (Oldfield and Plosker, 2006). As a PI, it is widely used for its high effectiveness in treating of both treatment-naive and -experienced HIV infected people (Chandwani and Shutter, 2008). Studies done on LPV/RTV recommended the standard twice daily dosage regimen, both in treatment-naive and -experienced children (Resino et al., 2004; Saez-Llorens et al., 2003). LPV/RTV is approved at a dose of 400 mg/100 mg every 12 h. While in treatment-naive patients the recommended dose is 800 mg/200 mg once daily, this regimen is not recommended for therapy in treatment-experienced patients (South African HIV Clinicians Society Clinical Guidelines, 2009). Its use in children below the age of 6 months, in terms of safety, efficacy and pharmacokinetics has not been established. Furthermore the once-daily dosing has not been evaluated, therefore the recommended dosage in children from 6 months to 12 months is 100 mg/25 mg to 400 mg/100 mg twice daily, and this is based upon the weight and body surface area of the child (Chandwani and Shutter, 2008). Drug-drug interactions (DDIs) involving PIs are common, for there are well known inhibitors of the 3A4 isoenzyme of cytochrome (CYP) P450 (Hughes et al., 2007). As with other PIs, lopinavir and ritonavir act as substrates for CYP 3A4 and CYP 3A5. Lopinavir is enzymatically inactivated by the cytochrome P450 3A4 isoenzyme; while ritonavir inhibits CYP 3A4 activity, resulting in the increase of plasma concentration of lopinavir and other substrates of CYP 3A4 (Cvetkovic and Goa, 2003). DDIs between LPV/RTV and other ARVs are complex, as has been demonstrated by the fact that LPV/RTV has the ability to induce its own metabolism, at the same time induce the metabolism of other drugs that are metabolized by CYP450 enzymes (Cvetkovic and Goa, 2003). For this reason LPV/RTV may have significant interactions with drugs that are inducers or inhibitors of these enzymes and more so with drugs that are substrates for the CYP3A4 and CYP3A5 (Chandwani and Shutter, 2008). One study determined the prevalence of possible DDIs between ARVs in different age groups and results reported a high prevalence of DDIs between ARVs that are inhibitors of CYP450 enzyme (Katende-Kyenda et al., 2008). The current study is relevant

because LPV/RTV is an inhibitor of the CYP450 enzyme, thus presenting potential interactions with other ARVs metabolized by the same enzyme. Therefore the aim of this study was to determine potential DDIs between LPV/RTV and other ARVs in general practitioner's (GPs) and specialist's (SPs) prescriptions with inappropriate PDDs for years 2005 to 2007.

METHODOLOGY

This was a non-experimental, retrospective quantitative, drug utilization study performed on 49,995 (2005), 81,096 (2006) and 88,988 (2007) ARV prescriptions prescribed to HIV patients. Data were obtained from a South African Pharmacy Benefit Management (PBM) company managing the medical schemes medicines benefits of the private health care sector of South Africa. Data were selected for three years from 1 January, 2005 to 31 December, 2007. The following information was obtained from the database: drug's trade name, National Pharmaceutical Product Interface (NAPPI)-code, date of refilling the prescription, prescription number, number of medicine items prescribed, days supplied, patients' gender and age, treatment date (dispensing date). Unique encrypted, physician and pharmacy numbers (randomly allocated by the PBM) were used to avoid the identification of the patient, pharmacy and physician; thus maintaining anonymity. ARV drug names were classified according to pharmacological groups as described in the monthly index of medical specialities (MIMS) (Snyman, 2009). Prescribers of ARV prescriptions were divided into the following categories:

1. General practitioners (GPs): This group includes all the medical providers who are registered with the Health Professions Council of South Africa (HPCSA) as general medical practitioner.
2. Prescribers from the following specialist (SP) areas which include *inter alia*: Anaesthesiology, cardiology, paediatrics, clinical haematology, dermatology, gastroenterology, neurology, obstetrics and gynaecology.

Potential DDIs between LPV/RTV and other ARVs were identified and classified according to a clinical significant rating described in three degrees of severity: major, moderate and minor as described by Tatro (2003). Drug interactions assigned documentation levels of established, probable, or suspected were considered to be well substantiated and to have significance ratings of 1, 2 or 3 (Tatro, 2003). These interactions were considered to have a probability of occurring, while those ratings 4 or 5 were considered as not substantiated – having documentation levels of possible or unlikely. This study focused only on DDIs with clinical significance rating of 2 being the most common interactions between ARVs. According to Tatro (2003), clinical significance 2 can be considered to have a moderate severity, with effects causing deterioration in a patient's clinical status, thus requiring additional treatment, hospitalization or an extended hospital-stay. The study evaluated potential DDIs between LPV/RTV and other ARVs in GPs and SPs prescriptions with inappropriate PDDs for 2005 to 2007. According to World Health Organization (WHO), a PDD is defined as "the average dose prescribed according to a representative sample of prescriptions" (WHO, 2003). It is important that the PDD be related to the diagnosis made for the prescribed medication. The PDD of a drug can be calculated by multiplying the number of tablets (or volume of suspension or syrup) dispensed during the treatment period and the strength per tablet (or per ml), divided by the days supplied (WHO, 2003). In this study, the reference guides used to evaluate PDDs

were according to the recommended ARV- dosing guidelines (National Department of Health and Human Service, 2007). Basic descriptive statistics, that is frequencies, the arithmetic mean (average), standard deviations were used to characterize the study sample and were calculated using the computer software Statistical Analysis System® SAS for Windows 9.1® (SAS, 2006-2007). The age groups used in this study were: Group 1: $0 \leq 12$ years; Group 2: $12 \leq 19$ years; Group 3: $19 \leq 45$ years; Group 4: $45 \leq 59$ years and Group 5: > 59 years. For the purpose of this study a drug item (medicine item) is defined according to the Medicines and Related Substances Control Act of 1965, Act 101 of 1965 as amended as “substance intended for use in the diagnosis, cure, mitigation, treatment, modification or prevention of disease, abnormal physical or mental state or the symptoms thereof in man.” In this research the words “drug items” are used interchangeably with the words “medicine items.” In the South African context, a prescription can consist of one or more medicine items (or drugs). Permission to conduct the study was granted by the PBM Company and approval was obtained from the Research and Ethics Committees of the North-West University, Potchefstroom campus, (ethical number 07M01) and the Walter Sisulu University, Mthatha campus.

RESULTS

Of 2,638 GP and 472 SP ARV prescriptions claimed, 505 (19.1%) of GP prescriptions and 143 (30.3%) of SP prescriptions had DDIs and inappropriate PDDs as shown in Table 1. Of the total number of ARV prescriptions, potential DDIs were identified between LPV/RTV and other ARVs with incorrect PDDs accounting for 88.9% ($n = 449$) for GP and 98.6% ($n = 141$) for SP prescriptions. As observed in Table 1, the percentage of ARV prescriptions with potential DDIs increased from 2005 to 2006, remained almost the same from 2006 to 2007 for GP prescriptions while there was an increase from 2005 to 2007 for SP prescriptions. There was a percentage increase in the number of ARV prescriptions with potential DDIs and incorrect PDDs from 2005 to 2007 for both GPs and SPs. The same trend also reflected in the percentage of ARV prescriptions with potential DDIs between LPV/RTV and other ARVs with incorrect PDDs. In all these cases, there were more GP prescriptions as compared to SP prescriptions. The number of prescriptions with potential DDIs between LPV/RTV and other ARVs and with incorrect PDDs according to prescriber and age group is reflected per year in Tables 2 to 4. For the three years, the highest percentage of ARV prescriptions with potential DDIs and incorrect PDDs were with LPV/RTV at an average PDD of 1066.4 mg/264 mg and efavirenz (EFV) at an average PDD of 600 mg for both GPs and SPs prescriptions. Furthermore, these regimens were prescribed to patients in age group 19 to 45 years. In these regimens, GPs prescriptions were more, accounting for 61.5% ($n = 276$) and 38.3% of SPs prescriptions ($n = 54$). The total percentage of LPV/RTV prescriptions with potential DDIs and incorrect PDDs were for SP prescriptions accounting for 98.6% ($n = 143$)

and 88.9% ($n = 505$) of GP prescriptions from 2005 to 2007 (Tables 2 to 4).

DISCUSSION

The aim of this study was to determine potential DDIs between LPV/RTV and other ARVs in GPs and SPs prescriptions with inappropriate PDDs. From the results obtained from this study, it was evident that the percentage of ARV prescriptions claimed from the PBM Company increased from 2005 to 2007. This may have resulted from an increase in the number of patients registered with medical schemes that claimed through the PBM. According to the WHO/UNAIDS press release, it was reported that in the Sub-Sahara Africa, the number of HIV-infected people who were receiving ART was steadily increasing as from year 2005 (WHO/UNAIDS, 2005), and of those, 5 million were living in South Africa (UNAIDS/WHO, 2007). It was also observed that LPV/RTV, the first co-formulated HIV-1 PI, was the most commonly prescribed and at the same time the PI with the most potential DDIs and with PDDs not according to the recommended ARV dosing. A review by Chandwani and Shutter reported that large clinical trials had demonstrated this drugs' efficacy in both treatment-naïve and experienced patients. Furthermore, the immunologic and virologic benefits of the same drug had been proven in HIV-infected adults, adolescents and children (Oldfield and Plosker, 2006).

DDIs were identified between LPV/RTV and EFV and nevirapine (NVP). As already stated LPV/RTV is a PI and EFV and NVP are non-nucleoside reverse transcriptase inhibitors (NNRTIs). According to studies performed by Seden et al. (2009), Clarke et al. (2008) and Miller et al. (2007), all PIs are predicted to have numerous DDIs because they are metabolized by the cytochrome P450 system and are also inhibitors of CYP3A4. Therefore it was not surprising that LPV/RTV interacted with EFV and NVP because non-nucleoside reverse transcriptase inhibitors (NNRTIs), like the PIs are also metabolized by the CYP450 and are also inhibitors of CYP3A4 (Malaty and Kuper, 1999). Potential DDI between LPV/RTV with EFV may result in increased or decreased concentrations of the PI. DDIs is a major concern to all health care providers especially those caring for HIV/AIDS, it is therefore recommended that multiple reminders and warnings be available whenever more than two medicines are administered. It was also evident that potential DDIs were identified between LPV/RTV at an average PDD of 800 mg/200 mg and EFV at an average PDD of 200 mg and NVP at an average PDD of 2600 mg, all prescribed to patients 12 years and younger. The safety, efficacy and pharmacokinetic profile of this drug have not been established in pediatric patients younger than 6 months

Table 1. Comparison of the number of ARV prescriptions with potential DDIs, ARV prescriptions with DDIs and inappropriate PDDs prescriptions with potential DDIs between LPV/RTV and ARVs with inappropriate PDDs according to type of prescriber and year.

Year	ARV prescriptions with potential DDIs (level 2)		ARV prescriptions with DDIs and PDDs not according to the recommended ARV dosing		ARV prescriptions with potential DDIs between LPV/RTV and other ARVs with PDDs not according to ARV dosing	
	GPs (%)	SPs (%)	GPs (%)	SPs (%)	GPs (%)	SPs (%)
2005	681 (25.8)	97 (20.6)	84 (16.6)	15 (10.5)	79 (17.6)	13 (9.2)
2006	976 (37.0)	179 (37.9)	183 (36.3)	63 (44.0)	168 (37.4)	63 (44.7)
2007	981 (37.2)	196 (41.5)	238 (47.1)	65 (45.5)	202 (45.0)	65 (46.1)
Total	2 638	472	505	143	449	141

Table 2. Number of LPV/RTV prescriptions with potential DDIs not prescribed according to recommended ARV dosing guidelines and age group for 2005

Age group (years)	Number of ARV prescriptions with DDIs (N = 84)	ARV combinations with average PDD			
		ARV medicine item	PDD (mg/mg)	ARV medicine item	PDD (mg)
General practitioners					
0≤12	8	Lopinavir/Ritonavir	800/200	Efavirenz	200
	4	Lopinavir/Ritonavir	320/80	Nevirapine	2600
19≤45	30	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	1		4500/3999		1800
	15	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
	1		1066.4/264		500
45≤59	16	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	1	Lopinavir/Ritonavir	1066.4/264	Nevirapine	500
>59	3	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
Total	79				
Specialists					
19≤45	6	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	2		1142.6/282		600
	1		3999/990		1200
	2	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
2	799.8/198		500		
Total	13				

(De Maat et al., 2003). Because the analysis was done in age groups and not individual age of a specific patient and with the limitation that the weight of the patient was not available, it was difficult to compare the PDD with the recommended doses for a specific child. Nevertheless,

the pediatric dosage prescribed in this study was high, considering that the recommended pediatric dose for LPV/RTV according to the treatment guidelines formulated by the National Department of Health South Africa in 2005 is < 15 kg + 12 mg LPV/kg and ≥ 15 kg =

Table 3. Number of LPV/RTV prescriptions with potential DDIs not prescribed according to recommended ARV dosing and age group for 2006.

Age group (years)	Number of ARV prescriptions with DDIs (N = 183)	ARV combinations with average PDD			
		ARV medicine item	PDD (mg/mg)	ARV medicine item	PDD (mg)
General practitioners					
0≤12	6	Lopinavir/Ritonavir	800/200	Efavirenz	200
	3	Lopinavir/Ritonavir	320/80	Nevirapine	2600
19≤45	101	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	26		1066.4/264		400
	1	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
	2		799/198		1600
45≤59	11	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	7	Lopinavir/Ritonavir	1066.4/264		500
>59	9	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
	2	Lopinavir/Ritonavir	1066.4/264		500
Total	168				
Age group (years)	Number of ARV prescriptions with DDIs (N = 63)	ARV combinations with average PDD			
		ARV medicine item	PDD (mg/mg)	ARV medicine item	PDD (mg)
Specialists					
19≤45	25	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	16	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
45≤59	22	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
Total	63				

10 mg LPV/kg twice daily. Therefore in this study, the PDD was high, considering that one capsule of LPV/RTV is 133.3 mg/33 mg, and the maximum dose should be 3 capsules (399.9 mg/99.9 mg) (National Department of Health South Africa, 2005). According to a study by Murphy et al. (2008), the recommended dosage for LPV/RTV in children is 100 mg/25 mg twice daily to 400 mg/100 mg, and this dose is based upon the body surface or the weight of the child. It is therefore recommended that ARV dosing for LPV/RTV be adhered to so as to avoid side effects like diarrhoea, nausea and vomiting, and metabolic derangements, including hyperlipidemia and glucose intolerance (Murphy et al., 2008). Results from this study also revealed that patients, 12 years and younger, were prescribed LPV/RTV and NVP at an average PDD of 2600 mg for years 2005 and 2006. This was a very high dose prescribed by GPs. According to the Department of Health Guidelines, the recommended pediatric dose for NVP is 10 mg/ml or 200 mg tablet as an initial dose and 4 mg/kg once daily for 14 days. Therefore this high dose could lead to adverse

effects like rash including, Stevens-Johnson syndrome, symptomatic hepatitis, including hepatic necrosis (Murphy et al., 2008). In all three years, potential DDIs were identified between LPV/RTV at an average PDDs of 1066.4 /264, 4500/3999 and 1599.6 mg/264 mg to patients 19 to 45 years. As stated in the guidelines for the Department of Health and Human Service (2007), the standard dose for LPV/RTV in adults is 400 mg/100 mg (2 tablets or 5 ml) twice daily of LPV/RTV 800 mg/200 mg (4 tablets or 10ml) once daily. Therefore in this study the PDD for LPV/RTV was high and could lead to toxic levels with adverse effects like nausea, diarrhea and vomiting (Murphy et al., 2008). It is therefore recommended that the dose be adjusted to LPV/RTV 800 mg/200 mg (4 tablets or 10 ml once a day) for treatment-naïve patients. Though in this study it was not clear whether the dosage was for treatment-naïve or -experienced HIV patients, since information about these specific patients was not given. Nevertheless, the once daily dosing for LPV/RTV is only recommended for HIV-naïve patients, not for patients receiving EFV, NVP or NFV. According to the ART

Table 4. Number of ARV prescriptions with potential DDIs not prescribed according to recommended ARV dosing guidelines and age group for 2007.

Age group (years)	Number of ARV prescriptions with DDIs (N = 238)	ARV combinations with average PDD			
		ARV medicine item	PDD (mg/mg)	ARV medicine item	PDD (mg)
General practitioners					
0≤12	9	Lopinavir/Ritonavir	640/160		350
	6	Lopinavir/Ritonavir	799.8/198	Efavirenz	200
19≤45	145	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	3	Lopinavir/Ritonavir	1599.6/264	Efavirenz	600
	8	Lopinavir/Ritonavir	1066.4/264		400
	9	Lopinavir/Ritonavir	799.8/198	Nevirapine	500
45≤59	17	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
>59	8	Lopinavir/Ritonavir	1066.4/264	Nevirapine	500
Total	202				
Age group (years)	Number of ARV prescriptions with DDIs (N = 65)	ARV combinations			
		ARV medicine item	PDD (mg/mg)	ARV medicine item	PDD (mg)
Specialists					
19≤45	23	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	16	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
45≤45	22	Lopinavir/Ritonavir	1066.4/264	Efavirenz	600
	3	Lopinavir/Ritonavir	1066.4/264	Nevirapine	400
>59	1	Lopinavir/Ritonavir	3999.9/990	Efavirenz	600
Total	65				

ART guidelines as per Department of Health and Human Sciences (2007) and National Department of Health South Africa (2005), when LPV/RTV is given with EFV or NVP, the recommended dose for treatment-experienced patients is 600 mg/150 mg. Results from this study showed that LPV/RTV was the PI commonly prescribed by both GPs and SPs for three years. It was the drug with most potential DDIs with EFV and NVP and with incorrect PDDs. This could be due to prescribing-medication errors which could result in overdosing of LPV/RTV thus leading to serious adverse effects and furthermore leading to the non-achievement of the main treatment goals for ARV therapy in HIV/AIDS patients (Purdy, 2009). It is therefore recommended that dosage adjustments be made and more so, more education be provided to both GPs and SPs in the private health care sector in South Africa on LPV/RTV recommended dose and potential DDIs, with the aim of achieving an optimal therapy to the HIV/AIDS patients.

LIMITATIONS

Some limitations to this study were the non-availability of patient clinical data to do in-depth analysis of DDIs and PDDs analysis, as well as information on treatment-naive and -experienced HIV patients, and weights for the patients.

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Full Length Research Paper

Cytolytic and antinociceptive activities of starfish *Protoreaster linckii* (Blainvilli, 1893)

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Echinoderms are exclusively marine animals. Starfish extracts have drawn attention, because of their wide spectrum of biological effects associated with antifungal, cytotoxic, hemolytic and immunomodulatory activities. The study was carried out to investigate the hemolytic and antinociceptive effect of the methanolic extract of *Protoreaster linckii* (MEPL) using male albino mice models of chemicals and thermal antinociception. The extract was injected intraperitoneally at doses of 5, 10, 15 and 20 mg/kg. The hemolytic activity was tested against human, chicken, sheep and goat blood red blood cells (RBCs). The percentage of protein was estimated in the crude extract as 4.14%. Among the ten human pathogens tested, *Proteus mirabilis* showed the most sensitive activity against 0.75 g/ml (9 mm) of sample compared with positive control (15 mm) and *Streptococcus pyogenes* showed activity at 0.75 g/ml concentration and no activities was noticed at 0.25 and 0.50 g/ml concentration. The hemolytic activity was high in human blood (128 HU). The crude extract shows dose dependent analgesic effect in male albino mice. The maximum mortality was noticed at 100% concentration, that is, 93.6±1.2 and minimum amount of mortality was noticed at 20% concentration, that is, 22.0±2.1 in brine shrimp cytotoxicity assay. The results from the present study appear to support the steroids belief in the medicinal properties of *P. linckii* against pain in which the central antinociception activity was found and it has also showed good cytolytic properties against blood RBCs.

Key words: Echinoderm, hemolytic, antinociception, mortality.

INTRODUCTION

Marine organisms represent excellent source for bioactive compounds (Bickmeyer et al., 2005). Approximately 7000 marine natural products, 25% of which are from algae, 33% from sponges, 18% from coelenterates, 24% from representatives of other invertebrate phyla such as ascidians, opisthobranchs, mollusks, echinoderms and byozoans (Anake, 2004). The phylum Echinodermata includes a diverse group of typically slow-moving and non-aggressive marine animals, including three venomous classes, namely the crown-of-thorns starfish, *Acanthaster planci* is being venomous animal for

the human envenomations (Monico et al., 2007). The huge potential of echinoderms as a far fairly untapped source of bioactive molecules is described. Examples are presented that show the usefulness of echinoderm-derived molecules for therapeutic application in selected fields of cancer research, in the control of bacterial growth as substances with new antibiotic properties, and finally in the context of technical applications such as antifouling substances.

Asteroidea, Echinoidea and Holothuroidea. Among them, the sea stars have variety of chemical constituents such as steroids, glycosides consisting of astrosaponins,

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steroids cyclic glycosides, steroid monoglycosides and diglycosides, saponins, astrosaponins and carotenoids. Moreover, the echinoderm tissue extracts have demonstrated cytotoxic (Wang et al., 2003), hemolytic (Ivanchina et al., 2006), antifungal (Chludil and Maier, 2005), antimicrobial (Haug et al., 2002), feeding deterrent (McClintock et al., 2003), antifouling (Iorizzi et al., 1995; Bryan et al., 1996; Greer et al., 2006) activities. Hence, the present investigation was made from sea star *Protoreaster linckii* demonstrated the hemolytic, cytotoxic and antinociceptive properties.

MATERIALS AND METHODS

Collection

The starfish *P. linckii* was collected from landing centre of Mandapam, Southeast coast of Tamilnadu (Ramanadhapuram district) and brought to the laboratory with seawater in fresh condition and immediately air dried for further analysis.

Extraction

Crude extract from *P. linckii* was extracted following the method of Braekman et al. (1992) with certain modifications. For methanolic extraction, starfish was dried in air for 10 days and completely dried specimen was put in methanol, covered and kept standing for 7 days. The solvent was then removed after squeezing of starfish and the extract was filtered using Whatman No. 1 filter paper (pore size 0.4 µm). The solvent was removed at low pressure using rotary evaporator (VC 100A, Lark Innovative, India) at 30°C. The resultant compound was finally dried in vacuum desiccators and stored at 4°C in a refrigerator for future use as crude methanol extracts.

Purification

A properly cleaned glass column (29×2.3 cm) was mounted vertically on to a stand. The methanol extract was dissolved in water and passed through an Amberlite-XAD-2 column, washed with water and eluted using methanol (Chludil et al., 2000). The methanol fraction was further purified using silica gel column and eluted using methanol and distilled water (2:1) and distilled water. The aqueous fraction was lyophilised for further analysis.

Biochemical assays

The percentage of protein, carbohydrate and lipid in crude extract of *P. linckii* was estimated.

Protein estimation

The concentration of protein was estimated by the method of Raymond and Howard, (1976). 200 mg of crude sample was dissolved in 1 ml of distilled water, 4 ml of biuret reagent was added and incubated at room temperature for 30 min and read at 540 nm spectrophotometrically.

Carbohydrate estimation

The concentration of carbohydrate was estimated followed by the

method of Dubois et al. (1956). 5 mg of crude sample was dissolved in 1ml of distilled water followed by 1ml of 5% phenol and 5 ml of concentrated sulphuric acid was added and incubated for 15 minutes at room temperature and measured at 490 nm spectrophotometrically.

Lipid estimation

Total percentage of lipid was estimated using the methodology of Folch et al. (1956). For this 400 mg of sample was taken in a test tube, 5 ml of chloroform: methanol (2:1) was added and incubated at room temperature for overnight. After incubation the mixture was filtered using Whatman No.1 filter paper. The filtrate was collected in a 10 ml pre-weighed beaker, which was then kept on a hot plate. The beaker with the residue at the bottom was weighed after the chloroform: phenol mixture gets evaporated and the weight of the empty beaker was subtracted from this to know the weight of the lipid present in the sample.

Antibacterial assay

Antibacterial activity was carried out by using standard disc diffusion method (Dulger and Gonuz, 2004; Parekh and Chanda, 2007; Laouer et al., 2009). The antibacterial activity of crude sample of *P. linckii* was tested against 10 human pathogens. Various concentration of crude sample dissolved in methanol (0.25, 0.50, and 0.75 g each in 1ml of solvent) and used for assay. The extracts were applied to 6 mm sterile discs in aliquots of 30 µl of solvent, allowed to dry at room temperature and placed on agar plates seeded with microorganisms. The bacteria were maintained on nutrient agar plates and incubated at 37°C for 24 h. Zones of growth inhibition were measured following incubation.

Hemolytic study

Hemolytic assay microtitre plate

The crude extract of *P. linckii* was assayed on chicken, sheep, goat and human erythrocytes followed by the method of Prasad and Venkateshvaran (1997). The chicken, goat and sheep bloods were obtained from nearby slaughter house in Parangipettai, while clinically healthy human blood was obtained from local hospital using 2.7% ethylene diamine tetra acetic acid (EDTA) solution as an anticoagulant at 5% of the blood volume and brought to the laboratory. The blood samples were centrifuged thrice at 5,000 rpm for 5 min. 1% erythrocyte suspensions was prepared for hemolytic study.

Hemolytic assay on blood agar plate

The hemolytic activity was assayed using blood agar plates by following the method of Lemes-Marques and Yano (2004). Chicken and goat blood agar plates were prepared by adding 5 ml of blood to 95 ml of sterile blood agar aseptically, with the result poured immediately onto the Petri dishes. After solidification, wells were cut into the agar plate using a corkscrew borer (8 mm diameter). Wells were loaded with 50 µl (1 mg/ml) of samples. The plates were observed for hemolysis after overnight incubation at room temperature.

Cytotoxicity using brine shrimp lethality assay

The toxicity effects of the crude extract on *Artemia salina* (brine

Table 1. Hemolytic activity of chicken and goat blood samples (Blood agar plates)

Sample	Zone of inhibition (mm)	
	Chicken	Goat
Crude	8.3±0.4	4.3±0.4
Fraction 1 (Methanol)	4.6±1.2	2.0±0.8
Fraction 2 (Aqueous)	4.6±0.4	1.6±0.9

shrimp) were determined using the methodology of Meyer et al. (1982). The extracts were dissolved in 0.01 ml of DMSO and incorporated into 5 ml of sea water (pH= 8.8 and salinity = 28%) containing ten *A. salina*. Each concentration (10, 20, 40, 60, 80 and 100 µg/ml) was tested thrice, and a control DMSO was done each time. The vials were maintained under illumination. Survivors were counted after 24 h and the percentage of deaths at each dose and control (DMSO and saline). The LC₅₀ values of brine shrimp were obtained from counts using the prohibit analysis method described by Litchfield and Wilcoxon (1949).

Antinociceptive activity

The crude extract of *P. linckii* was tested for anti-nociceptive activity by two models, namely, acetic acid induced writhing response (chemical method) and tail flick assay (thermal methods). The albino mice were employed to study the anti-nociceptive effect followed by Turner and Hebban, (1984) method.

Chemical method

Acetic acid induced writhing test

Writhing test was performed by Whittle (1964). Various amount of sample (5 mg/ml), that is, 0.125, 0.250, 0.375 and 0.50 ml was administered orally to different healthy male albino mice. After 15 min, 1 ml of 0.6% acetic acid was injected intraperitoneally. Antinociception was recorded by counting the number of writhes after the injection of acetic acid for a period of 20 min. A writhe is indicated by abdominal constriction and full extension of hind limb.

Thermal method

Tail flick test

Analgesic activity was measured according to the method described by Jansen et al. (1963) using tail flick analgesia meter INCO. During the testing period, the mice were restrained in a plastic tube, to which they had been previously adapted twice (10 min) a day for three days. The tail flick latency was recorded as the time onset of stimulation to the withdrawal of the tail. The crude extract of *P. linckii* was dissolved in DMSO at the dose of 5, 10, 15 and 20 mg/kg of body weight and then injected intraperitoneally (i.p) to mice. Mice without administration of any toxin or known painkiller was used as control while those injected i.p with paracetamol (crocin ® at 0.25 ml/170±2 g) will serve as reference standards. The mice were tested 30 min after injection. Analgesic activity was expressed as a ratio between the difference in reaction time of envenomated mice and control since analgesic potential is proportional to the difference in tail flick latency between the toxin and control.

Tail immersion test

The procedure is based on the observation that morphine like drugs selectively prolongs the reaction time of the typical tail withdrawal reflex in mice (Toma et al., 2003). The animals were treated as discussed earlier. Above 1 to 2 cm of the tail of mice was immersed in warm water kept constant at 55°C. The reaction time of the mice to deflect their tails were noted. The first reading was discarded and the reaction time was recorded as a mean of the next three readings. A latency period of 20 s was defined as complete analgesia and the measurement was then stopped to avoid injury to mice. The latent period of the tail-flick response was determined 30, 60 and 90 min after the administration of drugs.

Fourier transform infrared (FTIR) spectroscopy

Chemical characterization of sample was performed using FTIR spectroscopy (Abu et al., 1991).

RESULTS

Biochemical assays

The percentage of protein was estimated in the crude extract shown as 4.14%. The amount of lipid and carbohydrate was estimated at 3.63 and 17.5%, respectively.

Antibacterial activity

Ten human pathogens were used for the antibacterial activity of starfish extracts (Figure 1). Among the ten human pathogens tested, *Proteus mirabilis* showed the most sensitive activity against 0.75 g/ml (9 mm) of sample compared with positive control (15 mm) followed by *Klebsiella oxytoca* (8 mm), *Vibrio parahaemolyticus* (8 mm), *Salmonella paratyphi*, *Salmonella typhi* (6 mm) at 0.75 g/ml concentration. *Streptococcus pyogenes* showed activity at 0.75 g/ml concentration and zone of inhibition was noticed at 0.25 and 0.50 g/ml concentration. The one way analysis of variance (ANOVA) of antibacterial activity showed 0.05% level significance at between the groups (p>0.05).

Hemolytic activity

The hemolytic activity was high in human blood (128 HU). The activity on human blood is very high compared with other bloods (Figure 2). The hemolytic activity was further confirmed using blood agar plates with chicken and goat blood. The zone of inhibition was measured and the results were presented in Table 1.

Antinociceptive activity

Chemical method: Acetic acid induced writhing test

In writhing test, the number of writhing was decreased with increase in concentration of the extract and the results

Table 2. Writhing test of starfish extracts against male albino mice (mg/kg).

Treatment	Dose (mg/kg)	Route of injection administration	No. of writhing	Inhibition of writhing response (%)
Control	5	I.P	48	-
Aspirin	10	I.P	10	75.6
Group I	5	I.P	42	12.5
Group II	10	I.P	19	60.4
Group III	15	I.P	13	73.0
Group IV	20	I.P	11	77.1

Table 3. Tail flick analysis of starfish extracts against male albino mice (mg/kg).

Treatment	Dose (mg/kg)	Response after extract injection (s) time in min			
		30	60	90	120
Control (Saline)	5	12	10	8	7
Group I	5	9	10	7	6
Group II	10	16	18	14	7
Group III	15	20	22	16	14
Group IV	20	33	34	30	20
Standard	-	5	6	3	4

Table 4. Tail immersion test of starfish extracts against male albino mice (mg/kg).

Group/Treatment	Dose (mg/kg)	Mean reaction time before administration	Mean reaction time after administration (min)		
			30	60	90
Group I (Control)	5	3.20	-	-	-
Group II	5	3.53	6	3	4
Group III	10	3.67	11	5	3
Group IV	15	3.82	15	3	3

results were tabulated (Table 2).

Tail flick method

The heat sensitivity of the mice injected with crude extract was calculated using tail flick analgesia meter. The tail flick latency was assessed by the analgesiometer (Inco, India). No adverse effect or mortality was detected in albino mice up to 1 g/kg, i.p administration of extracts during 24 h observation period (Table 3).

Tail immersion test

The analgesic studies revealed that the methanolic extract of *P. linckii* exhibited potent analgesic (central analgesic activity) effect against thermal noxious stimuli (Vogel, 2002) and also revealed that the extract shows dose dependent analgesic effect (Table 4).

Cytotoxic assay

In the brine shrimp lethality assay, the degree of inhibition observed was directly related to the concentration of the active *P. linckii* crude extract. The concentration level was 20 to 100%. The maximum mortality was noticed at 100% concentration (93.6±1.2) and minimum amount of mortality was noticed at 20% concentration (Figure 3). The regression analysis showed the LC₅₀ value of 65.3% (Figure 4). The regression analysis showed R²=0.989.

Fourier transform infrared spectroscopy

The FTIR spectrum of methanolic extract of starfish produced by *P. linckii* (Figure 5) exhibited many peaks and absorption at 3411.36 to 2958.90 cm⁻¹ are assigned to N-H stretches (primary amine groups) and C-H stretches (alkyl group). Stretches 1216.51 to 1047.42 cm⁻¹

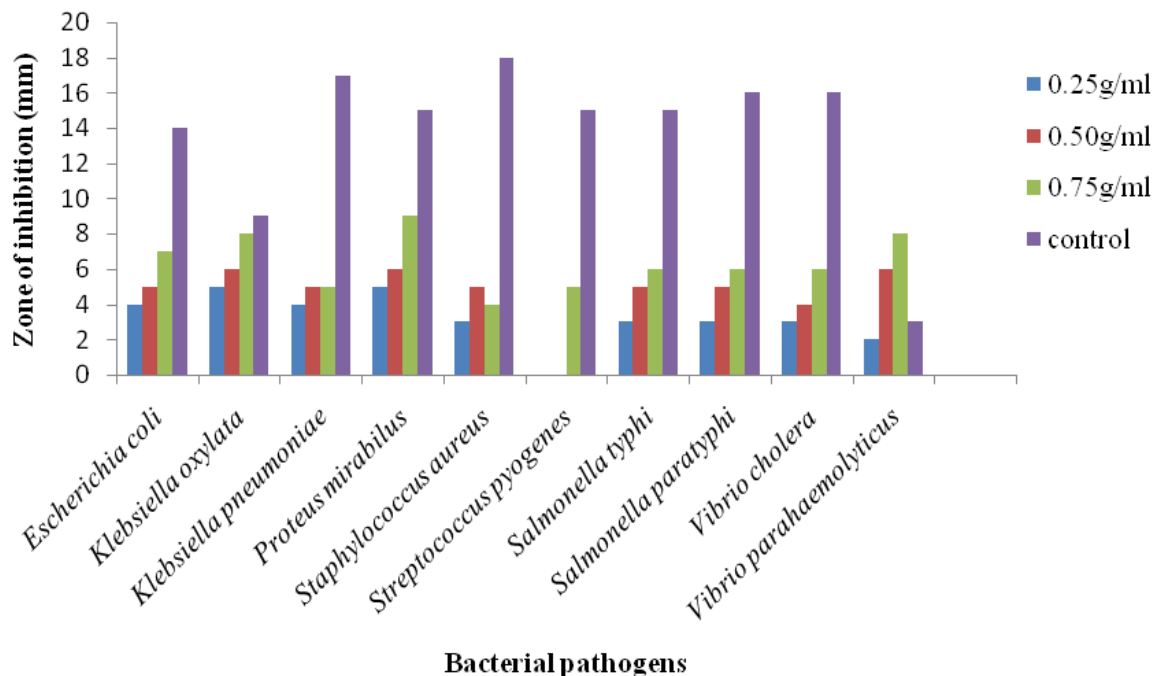


Figure 1. Antibacterial activity of starfish extracts against human pathogens.

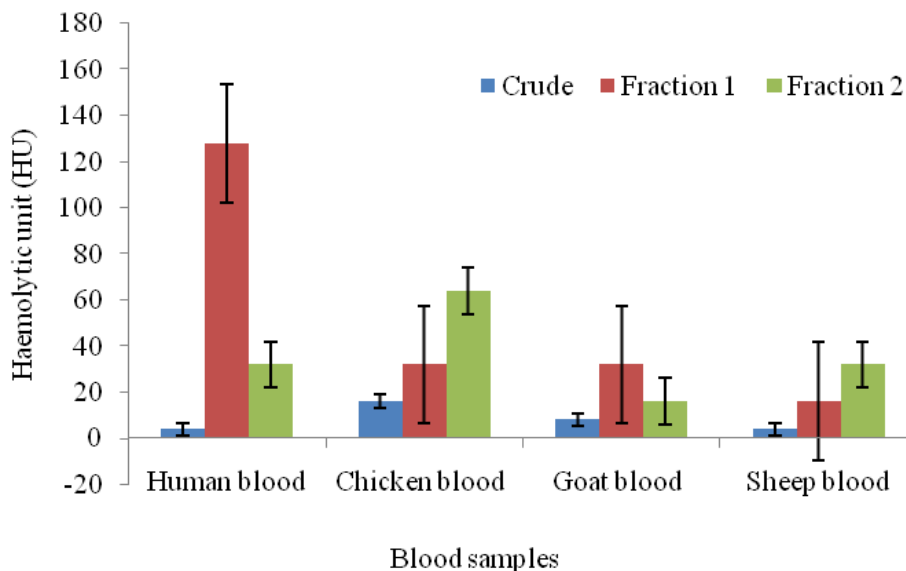


Figure 2. Hemolytic activity of starfish extracts against various blood samples.

were assigned to C-N stretch (aliphatic amine groups). The spectrum 1084 to 1044 cm^{-1} is assigned to C-O strengthening in the COH groups. This spectrum showed that the aromatic C-H stretches are to the left of 3000 cm^{-1} and the alkyl C-H stretches are right of 3000 cm^{-1} .

DISCUSSION

Echinodermata is one of the most distinct phyla among

the marine invertebrates, which contain antibacterial, antifungal, antiviral, antitumor, anticoagulant, cytotoxic, hemolytic, antithrombotic and even anti-HIV agents. In this present investigation, crude extract have been isolated from *P. linckii* using methanol solvent which is a universal solvent could extract even the basic proteins. Rio et al. (1965) demonstrated some biochemical and physiological properties from Asteroidea species, with water soluble fractions. Sunahara et al. (1987) extracted two toxins from the body extracts of *Anthopleura*

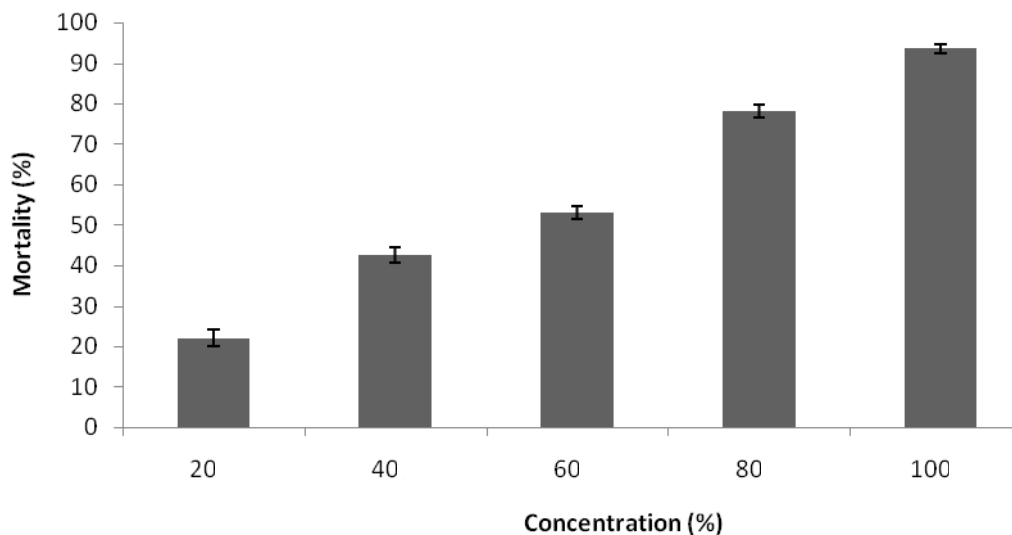


Figure 3. Cytotoxicity of *P. linckii* extract against *Artemia salina*.

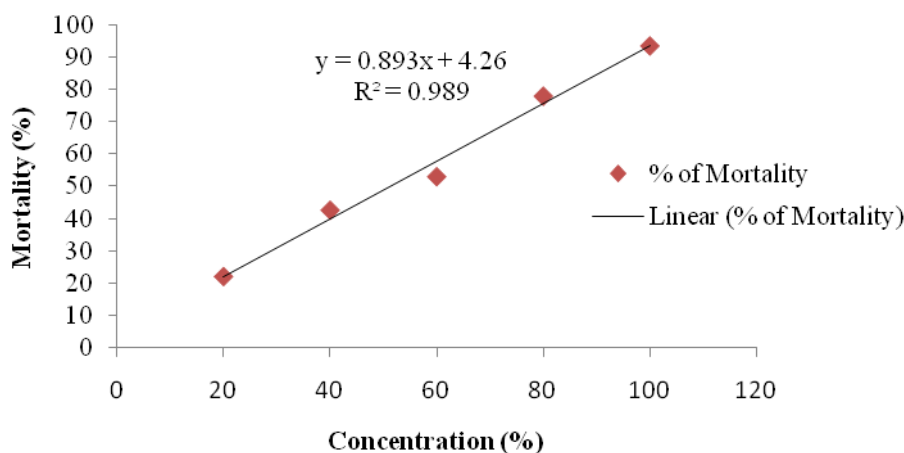


Figure 4. Regression analysis of brine shrimp lethality test.

fuscoviridis using methanol as extraction medium. Kanagarajan et al. (2008) reported the protein content of the crude toxin/fractions was 531 $\mu\text{g/ml}$, while the amount of protein in the purified fractions varied between 29 and 68 $\mu\text{g/ml}$ in *Stellaster equestris*. The present investigation also coincides with the previous study to the extraction of crude proteins (4.14, 3.63 and 17.5%) from the body of *P. linckii*. Karasudani et al. (1996) purified an anticoagulant factor from the spine venom of the crown-of-thorns starfish, *A. planci*. Antiadhesive mucintype glycoproteins were characterized from the mucus secretions of starfish *Marthasterias glacialis* and *Porania pulvillus*, and the brittle star *Ophiocoma nigrum* (Bavington et al., 2004).

The alcoholic extracts of holothurians were found to be inhibitory to human pathogens like *Aeromonas hydrophila*, *Klebsiella pneumonia*, *Pseudomonas*

aeruginosa and *Staphylococcus aureus* and the green sea urchin *Strongylocentrotus droebachiensis*, the common sea star *A. rubens* and the sea cucumber *Cucumaria frondosa* studied for antibacterial activity (Abraham et al., 2002; Haug et al., 2002). The crude extract of *Holothurians atra* was non-inhibitory to *E. coli* and *S. typhi* at low concentrations. In our study, the crude extract shows good activity against the same, but in higher concentration, that is, 0.75 g/ml and moderate activity in lower concentration, that is, 0.25 g/ml. Similar results were found by SriKumaran et al. (2011) in antimicrobial activity against crude extracts of starfishes *P. linckii* and *P. regulus*. Sri Kumaran et al. (2012) reported the antimicrobial activity of ascidian *Lissoclinum fragile* against human bacterial pathogens and biofouling bacteria.

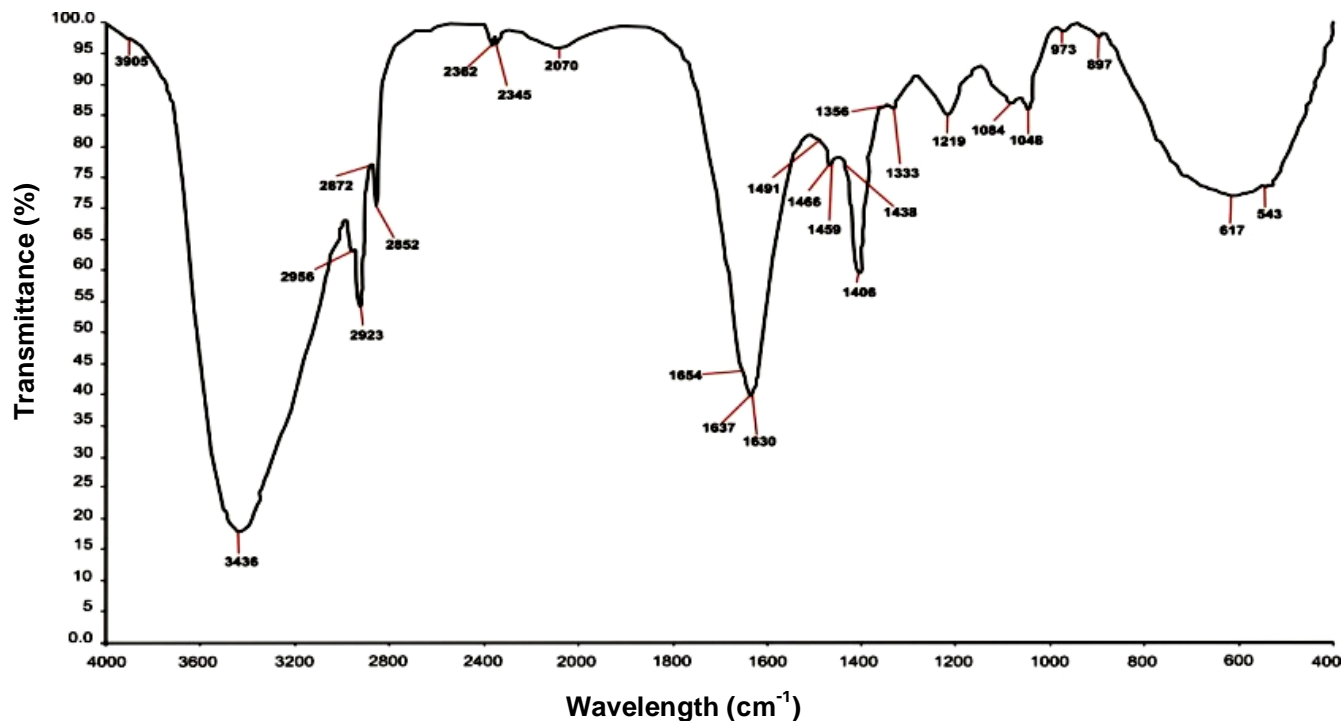


Figure 5. FTIR analysis of starfish *P. linckii* crude extract.

Several drug discovery projects have screened echinoderms for antibiotic activities. Rinehart et al. (1981) showed that the presence of antimicrobial activity in 43% of 83 unidentified species of echinoderms and 58% of 36 unidentified Caribbean species displayed antimicrobial activities. In the Northern Gulf of Mexico, 80% of 22 echinoderm species showed antimicrobial activity (Bryan et al., 1994). Body wall extracts of echinoderms displayed activity against marine bacteria, but did also inhibit settlement of marine larvae (Bryan et al., 1996). The previous studies done about the anti-microbial properties of echinoderm extracts were mainly focus on their glycosides component, particularly the saponin (triterpene glycosides) and saponins-like components that are present on the polar fraction of echinoderm samples (Ivanchina et al., 2000; Avilov et al., 2000; Maier et al., 2001). These substances exhibit anti-microbial activities against fungi, yeast and viruses, however, majority of them do not act against Gram (+) and Gram (-) bacteria. The capacity of crude extract to lyses red blood cells was found by performing hemolytic assay on microtitre plates and blood agar plates. In the present study, more haemolysis has occurred in human blood. In chicken, sheep and goat blood, moderate haemolytic activity was observed. The metabolites from echinoderms have tendency to cause cell lysis (Carte, 1996) and Rao et al. (1991) found out that echinoderms are toxic to both fish and mice. It is evident from the present study that the starfish species have potent hemolytic activity against

various red blood cells.

Shiomi et al. (1988) studied the properties of starfish venom and found out the amount of protein. In the present study, the amount of protein in crude sample was found out as 4.14%. This will be comparable to previous results. Saponins are the major compound found in starfish and it may be the reason for the pharmacological activities of starfish. The toxin from soft coral *Sarcophyton trocheliophorum* shows significant inhibitory effect on blood agar plate (Karthikayalu et al., 2010) and fish epidermal mucous shows significant hemolytic effect (Bragadeeswaran et al., 2011). The elimination of hemolytic activity by the serum is probably due to the antagonistic effects of serum proteins.

The results of anti-nociceptive assays reveal that the crude extract of *P. linckii* provides the heat tolerance capacity up to 55°C to the mice. This indicates the central nervous system (CNS) depressant activity. The result of writhing test shows that our extract has the capacity to cure stomach irritation as the writhing response decreases when the sample was injected to mice. The present result exhibited preliminary idea to study the analgesic activity of starfish as a potent drug which may be used as analgesic. Monastyrnaya et al. (2002) have reported the cytolyisin from *Radianthus* species to be thermolabile and its haemolytic activity decreased linearly with increasing temperature. Kanagarajan et al. (2008) reported that the Paw edema in mice was caused by the crude toxin and all fractions of the starfish *Stellaster*

equestris. Sudharsan et al. (2013) screened for hemolytic activity, mice bioassay, analgesic activity and neuromodulatory activity of crude methanolic extract (CME) of sea anemone *Stichodactyla mertensii*.

Recently, novel cytotoxic triterpene glycosides have been isolated from sea cucumbers *Pentamera calcigera* (Avilov et al., 2000), *Staurocucumis liouvillei* (Maier et al., 2001), *Hemoiedema spectabilis* (Chludil et al., 2002) and *Mensamaria intercedens* (Zou et al., 2003). The crude extract of starfish *P. linckii* has been exposed at the cytotoxicity of brine shrimp lethality assay at different concentration. The larval mortality has been increased with increasing concentration. The regression analysis shows that the significance between larval mortality and concentration at 0.01% level ($R^2=0.989$). The LC_{50} value of the larval mortality has been shown to be $LC_{50}=62\%$. Previously, Carballo et al. (2002) used two brine shrimp assays to identify potential cytotoxic substances useful in cancer therapy. The whole body extracts from three echinoderms (*Holothuria impatiens*, *Pseudoconus californica* and *Pharia pyramidata*) that showed a strong cytostatic (growth inhibition) and cytotoxic effect against two human cell lines, lung carcinoma A-549 and colon carcinoma HT-29. Palagiano et al. (1996) isolated up to 20 steroid glycosides from the starfish *Henricia downeyae* that caused growth inhibition in bacteria and fungi.

Conclusion

In this work, it is remarkable that the biological activity originally identified in methanolic extracts was related to single compounds whose molecular structures were even identified. The current results suggest that the crude and fractionated extract of *P. linckii* had antimicrobial, haemolytic, antinociceptive and cytotoxic activities.

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Full Length Research Paper

Ameliorative potential of rutin on streptozotocin-induced neuropathic pain in rat

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The aim of the present study is to investigate the effect of rutin, a potent antioxidant and anti-inflammatory compound on experimentally-induced diabetic neuropathy (DN) in male Wistar rats. In diabetic and normal rats, the pain-related behavior tests were performed before and after rutin (50 and 100 mg/kg/day for 6 weeks) treatments. In serum, fasting glucose, insulin, tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) levels were estimated and in sciatic nerve, thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) levels, and superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione-reductase (GR) and glutathione peroxidase (GSH-Px) activities were measured. Diabetic rats developed neuropathy which was apparent from decreased tail-flick and paw-withdrawal latencies and by following decrease in performance on Rota-Rod treadmill. Rutin treatments ameliorated the hyperalgesia, analgesia and improved motor coordination. Streptozotocin (STZ) significantly increased TBARS and decreased GSH levels in sciatic nerve where rutin treatment significantly protected those changes. In diabetic rats, SOD, CAT, GST, GSH-Px and GR activities were significantly inhibited. Rutin treatment significantly ameliorated decrease in antioxidant defense. Present results demonstrate protective effect of rutin in diabetic neuropathy through attenuation of oxidative stress and they suggest that rutin is a potential drug for the prevention of early diabetic-induced neuropathy.

Key words: Streptozotocin, rutin, oxidative stress, neuropathy, diabetes.

INTRODUCTION

World-wide, diabetic neuropathy (DN) is a major complication of diabetes mellitus. It affects about 15 to 25% in type-1 and 30 to 40% in type-2 diabetic patients, causing disabilities and a high mortality rate (Callaghan et al., 2012). Neuropathy pain is defined as a form of chronic pain that results from damage or abnormal function of central or peripheral nervous system (Abdi et al., 2004). The patients suffering from neuropathic pain frequently report sensory abnormalities such as burning

sensations, hyperalgesia, allodynia and dysesthesia (Woolf, 2004). It can also alter the patient's quality of life by interfering with emotional well-being (Galer et al., 2000). Furthermore, it is a challenge in clinical practice, because of its severity, chronicity and resistance to some classical analgesics (Gilron et al., 2006).

Experimental studies in diabetic rats models have established that DN is the outcome of a complex network of unified vascular (Cameron and Cotter 1999), metabolic

(Stevens et al., 2000) and neurotrophic (Calcutt et al., 2004) defects which conclude in electrophysiological discrepancies, abnormal sensory perception and progressive damage and loss of unmyelinated and myelinated nerve fibers (Sima et al., 2000). Diabetic neuropathies include several distinct syndromes of which symmetric sensory polyneuropathy, often associated with autonomic polyneuropathy is the most common and occurs in both type 1 and type 2 diabetes (Zochodne, 2008). Diabetic neuropathies accompanying type 1 diabetes tends to occur more predictably and to progress more rapidly, resulting in a more severe neuropathy (Sugimoto et al., 2000). Metabolic changes that may be involved in the pathogenesis of DN include polyol pathway flux, increased oxidative stress via glucose autoxidation and the subsequent formation of advanced glycation end products (AGEs), altered eicosanoid metabolism, activation of nuclear enzyme poly (ADP-ribose) polymerase and decreased antioxidant defense (Edwards et al., 2008). Oxidative stress is believed to be a biochemical trigger for sciatic nerve dysfunction and reduced endoneurial blood flow in diabetic rats (Figueroa-Romero et al., 2008; Zhrebetskaya et al., 2009). In this regard, the potential sources of reactive oxygen species (ROS) including endothelial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, nitric oxide synthase and mitochondrial respiratory chain inefficiency are more notable (Cameron and Cotter 1999). Furthermore, diabetes is linked with reduced activity of reduced glutathione (GST), glutathione peroxidase (GSH-Px), glutathione-reductase (GR), Cu-Zn superoxide dismutase and lower levels of glutathione (Yu et al., 2006; Arora et al., 2008; Cui et al., 2008). Opposite to this, diabetes causes increase in the lipid peroxidation products such as malondialdehyde (MDA) or conjugated dienes in sciatic nerves (Cunha et al., 2008). Enhanced oxidative stress consecutively activates nuclear factor kappa B (NF- κ B), which up-regulates genes such as cytokines, adhesion molecules, endothelin-1 and tissue factor (Bierhaus et al., 1998).

Although DN is traditionally considered a nonimmune disease, accumulating evidence now indicates that immunologic and inflammatory mechanisms play a significant role in its development and progression (Tuttle, 2005; Mora and Navarro, 2006). TNF- α plays a major role in the immune system and it found increased in type 1 diabetic patients with DN and also showed independent correlation between interleukins and diabetic-induced neuropathy (Goldberg, 2009). Therefore, the present study is an attempt to estimate the levels of oxidative and inflammatory biomarkers in order to establish their association with experimentally-induced type 1 diabetes in rats.

Rutin (quercetin-3-rutinosid or vitamin-P) is a flavonol glycoside, which comprises of the flavonol quercetin and the disaccharide rutinose and it is known to have

pharmacological activity such as lowering blood pressure and capillary reinforcement and also as an anti-inflammatory (Ihme et al., 1996; Lindahl and Tangesson, 1997). Moreover, rutin has inhibitory effects against membrane lipid peroxidation (Lopez-Revuelta et al., 2006) and effectively suppressed adipocyte differentiation from pre-adipocytes (Choi et al., 2006). In addition, Park et al. (2002) showed that rutin can significantly decrease the thiobarbituric acid reactive substances (TBARS) content and increase the superoxide dismutase (SOD) activity in rat plasma suggesting possible protective role in oxidative stress-mediated diseases. Recently, Hao et al. (2012) documented the preventive effect of rutin on the development of DN, which is closely related to oxidative stress and the TGF- β_1 /Smad/ECM and TGF- β_1 /CTGF/ECM signaling pathways. These results suggest that rutin can prevent the development of experimental DN in rats.

Antioxidants such as quercetin, n-acetylcysteine and α -lipoic acid have already progressed to clinical trials in DN, so we can speculate that rutin may pave its way for clinical trials in diabetic patients. Thus it is worth to experimentally examine the neuroprotective potential of rutin supplementation on the development of behavioral, biochemical and histopathological deficits in DN.

MATERIALS AND METHODS

Animals

Male Wistar albino rats, roughly the same age of 3 months, weighing 180 to 200 g were received from the Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). They were maintained under controlled conditions of temperature ($22 \pm 1^\circ\text{C}$), humidity (50 to 55%), and light (12 h light/dark cycles) and were provided with Purina chow (Grain Silos & Flour Mills Organization, Riyadh, Saudi Arabia) and water *ad libitum* (unless otherwise indicated during the experiment). All procedures including euthanasia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Diabetes induction

Diabetes was induced in overnight fasted rats by a single intraperitoneal injection of streptozotocin (SIGMA Chemicals, USA) at a dose of 65 mg/kg body weight freshly dissolved in 0.1 mol/L citrate buffer, pH 4.5 (Wang et al., 2013). Control rats as vehicle received equal volume of citrate buffer. Twenty five rats were injected with streptozotocin (STZ), from among them, 18 rats whose fasting blood glucose values were found more than 250 mg/dl after 72 h with no mortality were considered diabetic.

Experimental design

Normal healthy rats were divided in two groups (six rats in each

group): (1) control (vehicle) and (2) rutin (100 mg/kg/day, gavage), and the STZ-induced diabetic rats were randomly divided as (3) diabetic (vehicle), (4) rutin (50 mg/kg/day, gavage) treated to diabetic rats and (5) rutin (100 mg/kg/day, gavage) treated to diabetic rats. Vehicle and drug treatment were started three weeks after the diabetes induction and continued for six consecutive weeks. Behavioral assessments were under taken before and after treatments.

Mechanical hyperalgesia (Randall and Selitto method)

Paw pressure thresholds were recorded with the paw pressure analgesia meter (MK-20D Analgesy meter, Muromachi KIKAI CO. Ltd., Japan). Pressure increasing at a linear rate of 10 g/s, with a cut-off at 250 g to avoid tissue injury, was applied to the center of the hindpaw. Similar technique has been used in our earlier study (Al-Enazi, 2013). When the animals displayed pain by withdrawal of the paw, the applied paw pressure was recorded by an analgesia meter and expressed as gram. Three tests separated by at least 10 min were performed for each rat, and mean of value is used.

Tail flick test

The method described by Sugimoto et al. (2008) and in our earlier study (Al-Enazi, 2013) was used with slight modifications. Acute nociception was induced by using a tail flick apparatus (Tail Flick model DS 20 Sorrel Apelex, France). Briefly, each rat placed in a restrainer and the tail flick latency was determined by focusing the intensity controlled beam of light on the distal last 2 cm of the animal's tail and recording the time taken to remove the tail from the noxious thermal stimulus. For each animal, 2 to 3 recordings were made at an interval of 15 min; the mean value was used for statistical analysis.

Rota-rod treadmill test

Treadmill test was performed by using Rota-rod Treadmill for rats and mice (Model MK-670, Muromachi Kikai Co. Ltd., Tokyo, Japan) to evaluate motor coordination of the animals (Cartmell et al., 1991; Al-Enazi, 2013). Animals were initially trained to maintain themselves on the rotating rod for more than 2 min. A day before treatment started and at the end of the treatment, the rats were placed on rotating rod for two trails each. Animals were scored for their latency to fall (in seconds) in each trial.

Sample collections

At the end of the treatment and behavioral assessments, animals were fasted overnight, under deep anesthesia, blood samples were collected through cardiac puncture and then they were sacrificed, and sciatic nerves were rapidly removed and dipped in liquid nitrogen for a minute and was kept in deep freezer at -80°C till analysis. Blood samples were centrifuged at 3,000 rpm for 10 min and serum samples were stored at -20°C till analysis.

Serum parameters

Serum fasting glucose levels were estimated by using commercially available kits (RANDOX Laboratories Ltd., UK) and insulin levels were measured by insulin enzyme immunoassay (ELISA) kit (DRG, Germany). Serum pro-inflammatory cytokines including tumor

necrosis factor- α (TNF- α), interleukin-6 and 1 β (IL-6 and IL-1 β) concentrations were assayed by an enzyme-linked immunosorbent assay kit (ShangHai SenXiong Science and Technology Company, China). The levels were estimated by following the instruction provided by the manufacturer.

Tissue parameters

Sciatic nerves were homogenized in 50 mM phosphate buffered saline (pH 7.4) by using a glass homogenizer (Omni International, Kennesaw, GA, USA). The homogenate was centrifuged at 1000 g for 10 min at 4°C to separate nuclei and unbroken cells. The pellet was discarded and a portion of supernatant was again centrifuged at 12000 g for 20 min to obtain post-mitochondrial supernatant. In homogenate, MDA and GSH levels were estimated. In post-mitochondrial supernatant, SOD, catalase (CAT), GST, GSH-Px and GR activities were measured.

Estimation of TBARS levels

A TBARS assay kit (ZeptoMetrix) was used to measure the lipid peroxidation products, MDA equivalents. One hundred microliters of homogenate was mixed with 2.5 ml reaction buffer (provided by the kit) and heated at 95°C for 60 min. After the mixture had cooled, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The lipid peroxidation products are expressed in terms of nmoles MDA/mg protein using molar extinction coefficient of MDA-thiobarbituric chromophore (1.56×10^5 /M/cm).

Estimations of GSH levels

The concentration of GSH was measured using the method described by Sedlak and Lindsay (1968). Homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 ml of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was measured using spectrophotometer at 412 nm in 1 cm quartz cells.

Estimations of SOD activity

The activity of SOD in sciatic nerve was estimated using the method described by Kono (1978) with the aid of nitroblue tetrazolium as the indicator. Superoxide anions are generated by the oxidation of hydroxylamine hydrochloride. The reduction of nitroblue tetrazolium to blue formazon mediated by superoxide anions was measured 560 nm under aerobic conditions. Addition of superoxide dismutase inhibits the reduction of nitroblue tetrazolium and the extent of inhibition is taken as a measure of enzyme activity. The SOD activity was expressed as units/mg protein.

Estimation of CAT activity

The CAT activity was measured by the method of Aebi (1984) using hydrogen peroxide as substrate in post-mitochondrial supernatant. The hydrogen peroxide decomposition by catalase was monitored spectrophotometrically (LKB-Pharmacia, Mark II, Ireland) by following the decrease in absorbance at 240 nm. The activity of enzyme was expressed as units of decomposed/min/mg proteins by using molar extinction coefficient of H₂O₂ (71/M/cm).

Estimations of GST activity

The GST activity in sciatic nerve was measured by the method of Habig et al. (1974). The reaction mixture consisted of 0.067 mM GSH, 0.067 mM 1-chloro-2,4-dinitrobenzene (CDNB), 0.1 M phosphate buffer (pH 6.0) and 0.1 ml of post-mitochondrial supernatant in a total volume of 3 ml. Absorbance was read at 340 nm for 10 min every 30 s by an optical plate reader and the enzyme activity was calculated as mmol CDNB conjugate formed $\text{min}^{-1} \text{mg}^{-1}$ protein using a molar extinction coefficient of $9.6 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$.

Estimations of GSH-Px activity

GSH-Px activity was assessed by modified method of Flohe and Gunzler (1984). For the enzyme reaction, 0.2 ml of the post-mitochondrial supernatant was placed into a tube and mixed with 0.4 ml reduced glutathione and the mixture was put into an ice bath for 30 min. Then the mixture was centrifuged for 10 min at 3000 rpm, 0.48 ml of the supernatant was placed into a cuvette, and 2.2 ml of 0.32 M Na_2HPO_4 and 0.32 ml of 1.0 mmol/L DTNB were added for color development. The absorbance at wavelength 412 nm was measured on spectrophotometer (LKB-Pharmacia, Mark II, Ireland) after 5 min. The enzyme activity was calculated as nmol/mg protein.

Estimations of GR activity

GR activity was measured in the post mitochondrial supernatant by the method of Carlberg and Mannervik (1985). GSSG is reduced to GSH by NADPH in the presence of GR. Enzyme activity was measured by following the decrease in absorbance (oxidation of NADPH) for 3 min spectrophotometrically at 340 nm. The activity of enzyme was expressed as nmoles NADPH oxidized/min/mg protein, using molar extinction coefficient of NADPH (6.22; 106/M/cm).

Histopathological screening of sciatic nerve

A part of sciatic nerve was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 3 μm , stained with Hematoxylin and Eosin (H&E) stain and placed in slides for under light microscopic examination.

Statistical analysis

Data were expressed as means \pm standard error of mean (SEM). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test. P value of ≤ 0.05 was considered statistically significant. All statistics tests were conducted using Graph Pad Prism (version 5) software.

RESULTS

Mean body weights are significantly decreased in rats 9 weeks after the STZ injection. Rutin treatment with higher dose significantly increased the body weights compared to untreated diabetic animals (Figure 1).

Plasma fasting glucose levels significantly increased while insulin levels were decreased in STZ-induced diabetic rats. Treatments with rutin (50 and 100 mg/kg/day)

to diabetic animals for 6 consecutive weeks showed significant decrease in fasting glucose and increase in insulin levels when compared with untreated diabetic rats (Figure 2).

In paw pressure analgesia test, vehicle-treated diabetic rats exhibited significantly decreased paw withdrawal latency (PWL) compared to non diabetic animals. The diabetic group of animals treated with rutin at both doses (50 and 100 mg/kg/day) for 6 weeks significantly increased the PWL time(s) compared to untreated diabetic rats (Figure 3A).

A significant decrease in tail flick latency was observed after 9 weeks of STZ-induced diabetic rats compared to control group. This decrease in tail flick response latency was significantly reversed on treatments with rutin low and high dose, respectively (Figure 3B).

Rota-rod treadmill performance of diabetic and non-diabetic animals, before and after treatment with two doses of rutin is as shown in Figure 3C. The running performance on treadmill was significantly decreased in diabetic animals as compared to the control rats. Rutin treatments to diabetic rats significantly enhanced the performance in dose dependent manner.

Serum pro-inflammatory markers including TNF- α , IL-1 β and IL6 levels were markedly increased in diabetics as compared to control rats. Rutin treatments at both doses during six consecutive weeks in diabetic rats significantly decreased the levels of TNF- α and interleukins (Figure 4A to C respectively).

Lipid peroxidation bio-marker, TBARS levels were significantly increased in the sciatic nerves of diabetic rat as compared to control group (Figure 5A), while GSH levels were significantly decreased in same group of animals (Figure 5B). Treatments with rutin, significantly decreased the TBARS levels and significantly prevented the decrease in GSH levels in sciatic nerves of diabetic rats (Figure 5A and B).

In sciatic nerves, SOD, CAT, GST, GSH-Px and GR activities were significantly decreased in diabetic animals as compared to the control ones (Figure 6). In diabetic rats, these activities were significantly enhanced by the daily treatment with rutin during six consecutive weeks.

In control group, microscopic feature of transverse section of sciatic nerve showed benign looking with even distributed axons with its myelin sheath (Figure 7A). In transverse section of sciatic nerve of diabetic rat, partial and focal peripheral axonal loss, early regenerative process in the form of regenerating clusters of thinly myelinated axons with originally Schwann cell's basal lamina and few scattered degenerated bodies were found. Partial axonal loss also was noticed with longitudinal section of nerve (Figure 7B). In low doses rutin-treated rats, transverse section of sciatic nerve showed focal scattered peripheral axons loss, very few perineural scattered inflammatory cells infiltrate composed of small lymphocytes and also few scattered

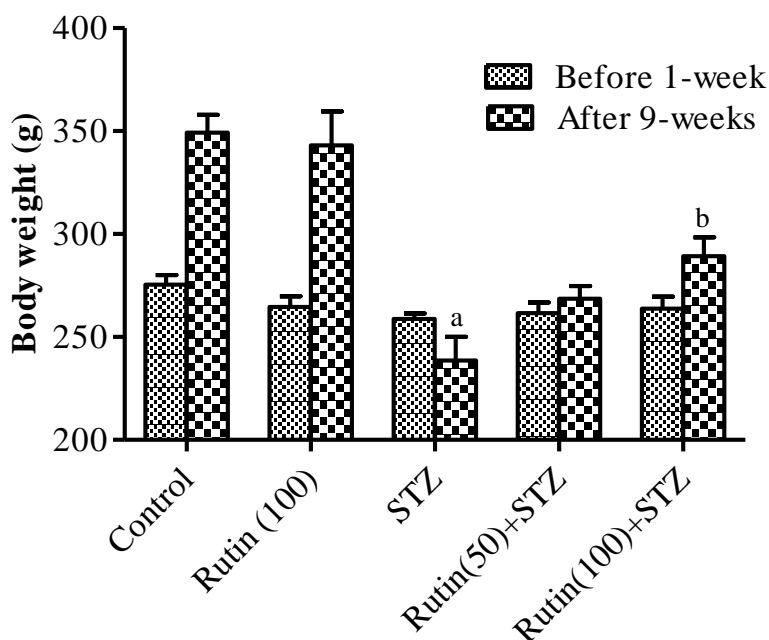


Figure 1. Effect of rutin on body weights of diabetic and non-diabetic animals at the start and at the end of the study. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. ^aSignificantly different from control group (P<0.05) and ^bSignificantly different from STZ group (P<0.05). Values are expressed as Mean ± SEM and six rats were used in each group.

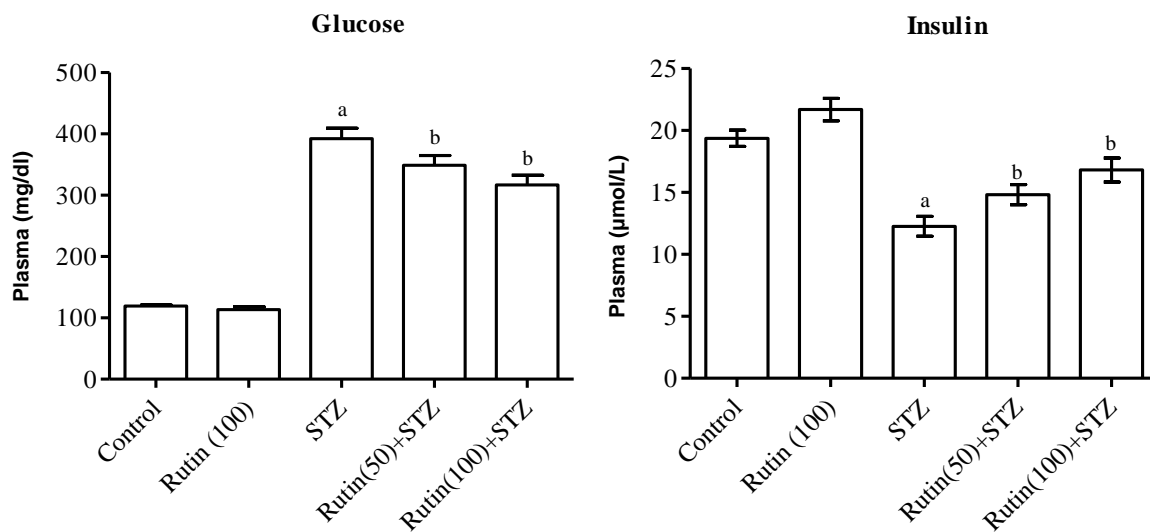


Figure 2. Effect of rutin on plasma glucose and insulin levels of diabetic and non-diabetic animals. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Significantly different from control group (P<0.05) and 'b' Significantly different from STZ group (P<0.05). Values are expressed as Mean ± SEM and six rats were used in each group.

lipoid degeneration of axons was seen (Figure 7C). However, in higher doses, rutin treatment showed benign

normal looking with even distributed axons having its myelin sheath with congested blood vessels in transverse

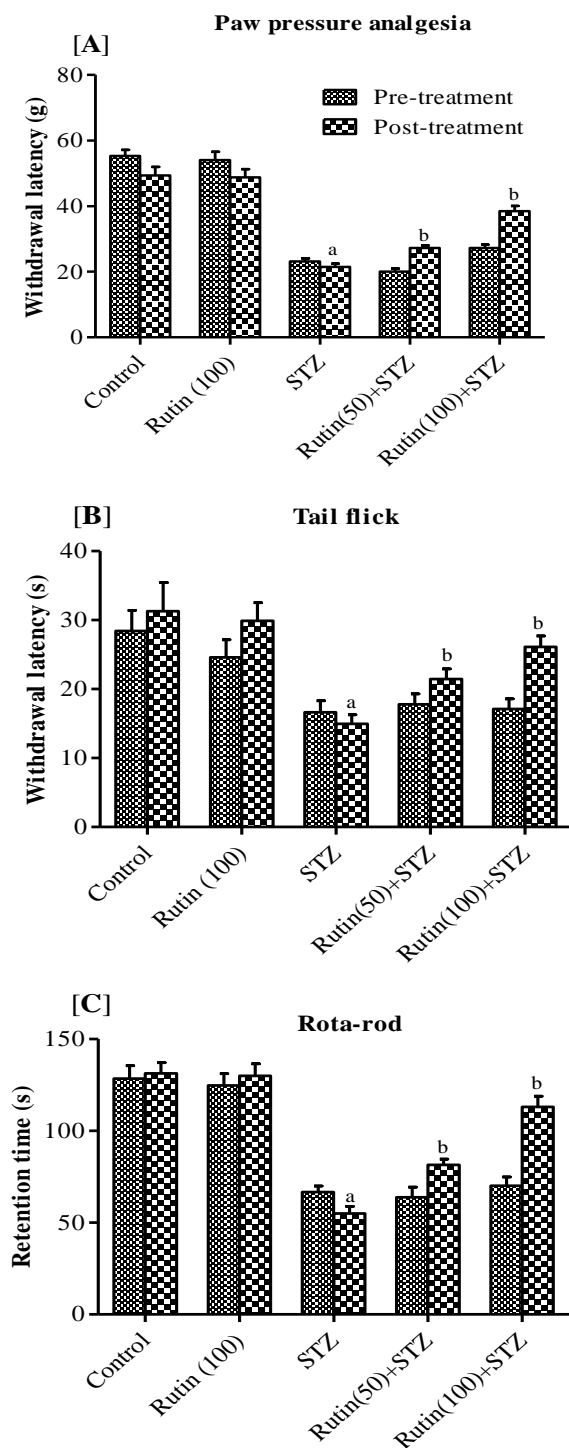


Figure 3. Effect of rutin on pain threshold in paw pressure analgesia, tail flick and Rota-rod treadmill performance of diabetic and non-diabetic animals. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied.

^aSignificantly different from control group ($P < 0.05$) and ^bSignificantly different from STZ group ($P < 0.05$). Values are expressed as Mean \pm SEM and six rats were used in each group.

section of sciatic nerve (Figure 7D).

DISCUSSION

Neuropathic pain is one of the most common complications of diabetes mellitus. Along the disease course, almost 50% of the diabetic patients develop neuropathy with symptoms including spontaneous pain, allodynia and hyperalgesia (Apfel et al., 2001). STZ-induced diabetic animals are used to model chronic neuropathic pain with hyperalgesia and allodynia that reflect symptoms observed in diabetics (Gul et al., 2000; Kamei et al., 2001). In the present study, in diabetic rats, the tail withdrawal latency was significantly shorter than that observed in control animals, indicating development of thermal hyperalgesia. This was accompanied by decreased motor coordination as assessed by performance on Rota-rod treadmill. The present results are in good agreement with the literature which frequently reports on hypersensitivity to mechanical stimulation (Chen and Levine, 2001; Dobretsov et al., 2003). A decrease of the pressure withdrawal threshold by 30 to 40% after 3 weeks of STZ-diabetes is reported by Romanovsky et al. (2004). Increased nociceptor activity and sensitivity during hyperglycemic hypoxia could be a mechanism e.g. burning pain attacks in painful neuropathy (Fuchs et al., 2010). Most of the phenolic compounds are known to have anti-inflammatory, analgesic and also have antinociceptive properties (Kamboj et al., 2010; Ramirez et al., 2010; Lee et al., 2006). This may be because of rutin treatment to diabetic rats for 6 consecutive weeks showed significant improvement in tail withdrawal latency rate as compared to untreated diabetic animals in the present study. Shen et al. (2002) and Alsaif (2009) have reported that, rutin has anti-inflammatory and analgesic properties.

Tumor necrosis factor is a cytokine that is involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction (Locksley et al., 2001). The promoter polymorphism in the TNF- α gene has been implicated in the regulation of TNF- α production and has been associated with a wide spectrum of inflammatory and infectious diseases. Furthermore, increased levels of inflammatory mediators such as proinflammatory cytokines (IL-6 and IL-1 β) have been reported in diabetic states to be a consequence of hyperglycemia (Brownlee, 2005) and these mediators have been considered to be the link between inflammation and insulin resistance (Navarro-Gonzalez and Mora-Fernandez, 2008). In present study, serum proinflammatory markers including TNF- α , IL-6 and IL-1 β are significantly increased in STZ-induced diabetic rats. Rutin treatment in diabetic rats significantly reduced such markers in the present study. This may be because rutin showed antioxidant and anti-inflammatory properties in

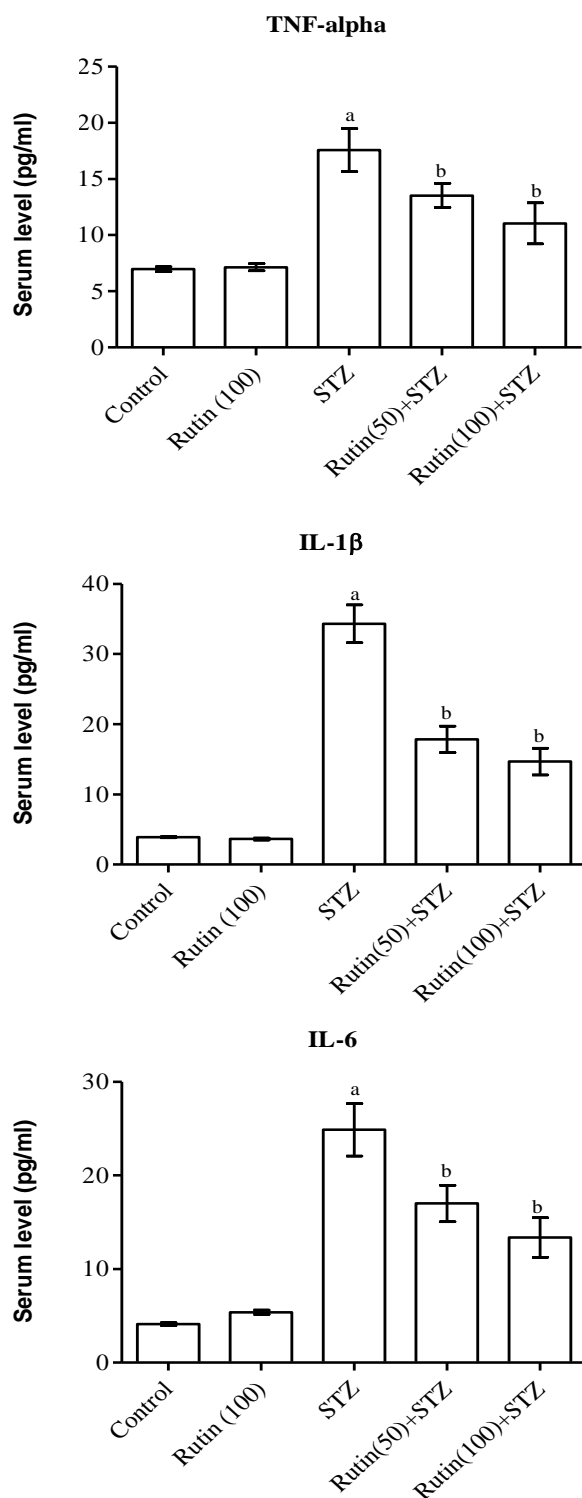


Figure 4. Effect of rutin on serum TNF- α , IL-1 β and IL-6 levels of diabetic and non-diabetic rats. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied.

^aSignificantly different from control group ($P < 0.05$) and ^bSignificantly different from STZ group ($P < 0.05$). Values are expressed as Mean \pm SEM and six rats were used in each group.

earlier studies (Abu-El-Fattah et al., 2010; Alsaif, 2009). Kwon et al. (2005) reported that, rutin ameliorates dextran sulfate sodium-induced experimental colitis in mice by following attenuation of pro-inflammatory gene expressions.

Chronic hyperglycemia induces oxidative stress by the autoxidation of monosaccharides (Bonnefont-Rousselot, 2002), which leads to production of superoxide and hydroxyl radicals. It is well known that pain transmission requires production of reactive oxygen species (Viggiano et al., 2005). We observed a significantly higher level of lipid peroxidation marker, MDA, in sciatic nerve of diabetic animals. Glutathione, a potent endogenous antioxidant is a first line of defense against free radicals. The GSH levels were significantly lowered in the sciatic nerve of diabetic animals. These observations are in agreement with previous findings showing reduction in GSH levels in diabetes (Kuzumoto et al., 2006; Arora et al., 2008). Intracellular GSH levels have been observed to decrease in brain (Kamboj et al., 2008) and sciatic nerve (Kuzumoto et al., 2006) of diabetic animals. Rutin treatment significantly reduced lipid peroxidation and re-generated intracellular GSH content in the sciatic nerve; this is probably because of its free radical scavenging activity or endogenous synthesis of GSH induced by rutin.

The results of the present study are in agreement with earlier studies wherein decreased SOD activity was observed in nerves isolated from diabetic rats (Cui et al., 2008). SOD and CAT are major antioxidant enzymes involved in protection from oxidative stress. SOD offers protection from highly reactive superoxide anions ($O_2^{\cdot-}$) and converts them to H_2O_2 (Halliwell, 1991). Hyperglycemia caused reduction in the activity of SOD in sciatic nerve of diabetic animals. Reduction in SOD activity in hyperglycemia might involve non-enzymatic glycosylation (Arai et al., 1987). Increased SOD activity after rutin administration to the diabetic animals is in accordance with reported restoration of SOD activity by rutin in hepatic cells (Kamalakkannan and Prince, 2006) and kidney (Alsaif, 2009). CAT is responsible for the catalytic decomposition of H_2O_2 to O_2 and H_2O . The decreased CAT activity in diabetes might reduce protection against free radicals. It is clear that the simultaneous reduction in the activity of both SOD and CAT makes the sciatic nerve more vulnerable to hyperglycemia-induced oxidative stress. Reports are available wherein rutin has been shown to bring about improvement in the CAT activity during diabetic-induced nephrotoxicity in rats (Alsaif, 2009). The results obtained emphasize that rutin protects the sciatic nerve from hyperglycemia induced damage by restoring the activity of both these enzymes. Glutathione reductase is an important enzyme involved in maintaining high GSH/GSSG ratios (Carlberg and Mannervik, 1985). Present data showed a significant decrease in the activity of GR in sciatic nerve of diabetic

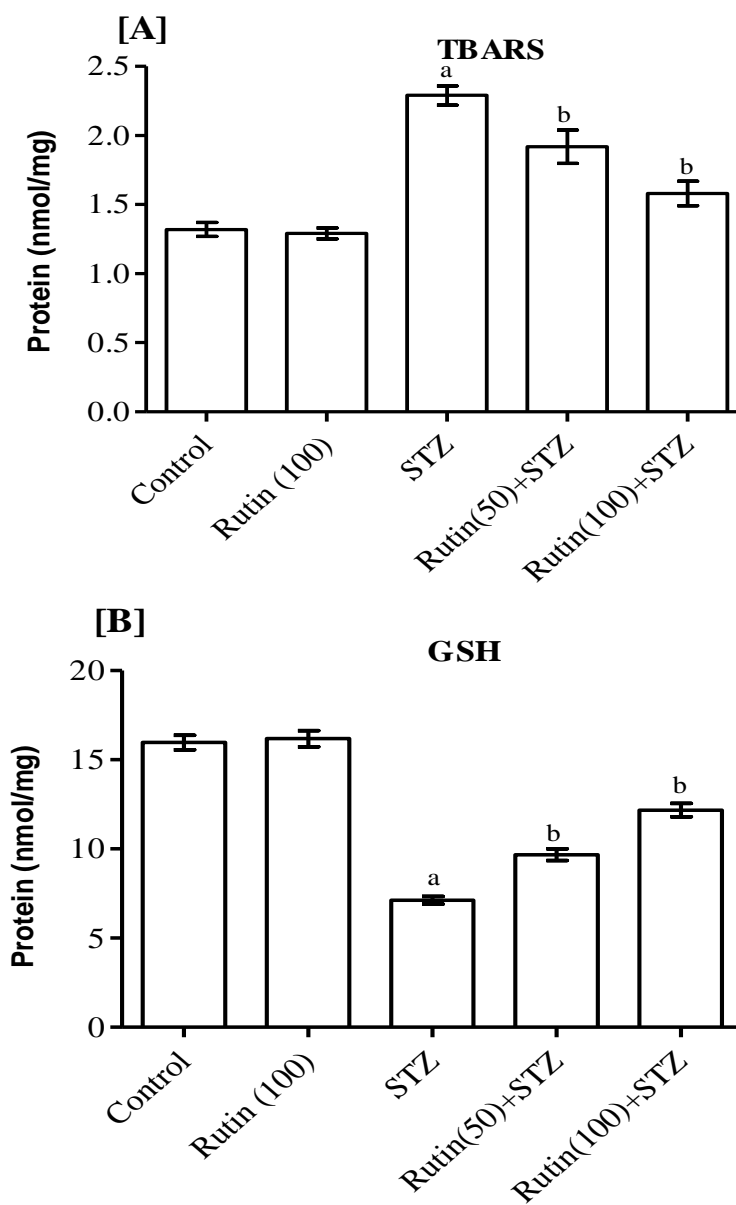


Figure 5. Effect of rutin on TBARs and GSH levels in sciatic nerve of diabetic and non-diabetic rats. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. ^aSignificantly different from control group ($P < 0.05$) and ^bSignificantly different from STZ group ($P < 0.05$). Values are expressed as Mean \pm SEM and six rats were used in each group.

animals. The results obtained from the earlier studies also showed depressed GR activity in sciatic nerve (Kamboj et al., 2010) and increase and decrease in brain and other organs of diabetic animals (Uluslu et al., 2003; Sanders et al., 2001). The reversal of GR activity by rutin treatment might result in increasing intracellular GSH levels. Previous studies have shown that rutin protects against the reproductive toxicity effects of

cyclophosphamide by increasing GR activity (Abarikwu et al., 2012). In another study, Kamalakkannan and Prince (2006) reported that, rutin treatment to diabetic rats significantly enhanced the GR activity in hepatic cells. In the present studies, the activity of GSH-Px was found to be significantly depressed in sciatic nerve of diabetic rats. This decrease in GSH-Px activity was reversed by the rutin treatment.

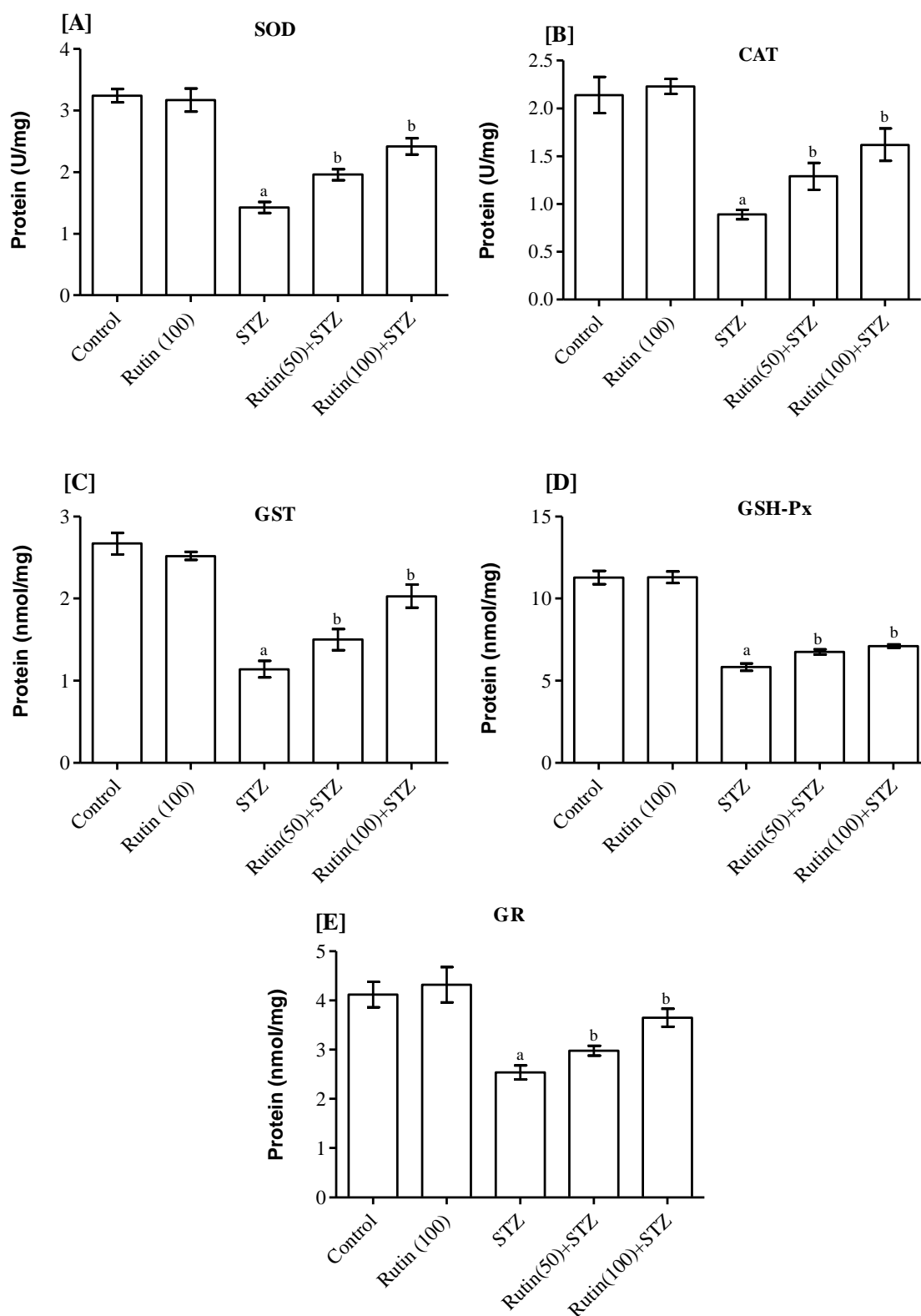


Figure 6. Effect of rutin on SOD, CAT, GST, GST-Px and GR activities in sciatic nerve of diabetic and non-diabetic rats. One-way ANOVA and Student-Newman-Keuls multiple comparisons test was applied. 'a' Significantly different from control group ($P < 0.05$) and 'b' Significantly different from STZ group ($P < 0.05$). Values are expressed as Mean \pm SEM and six rats were used in each group.

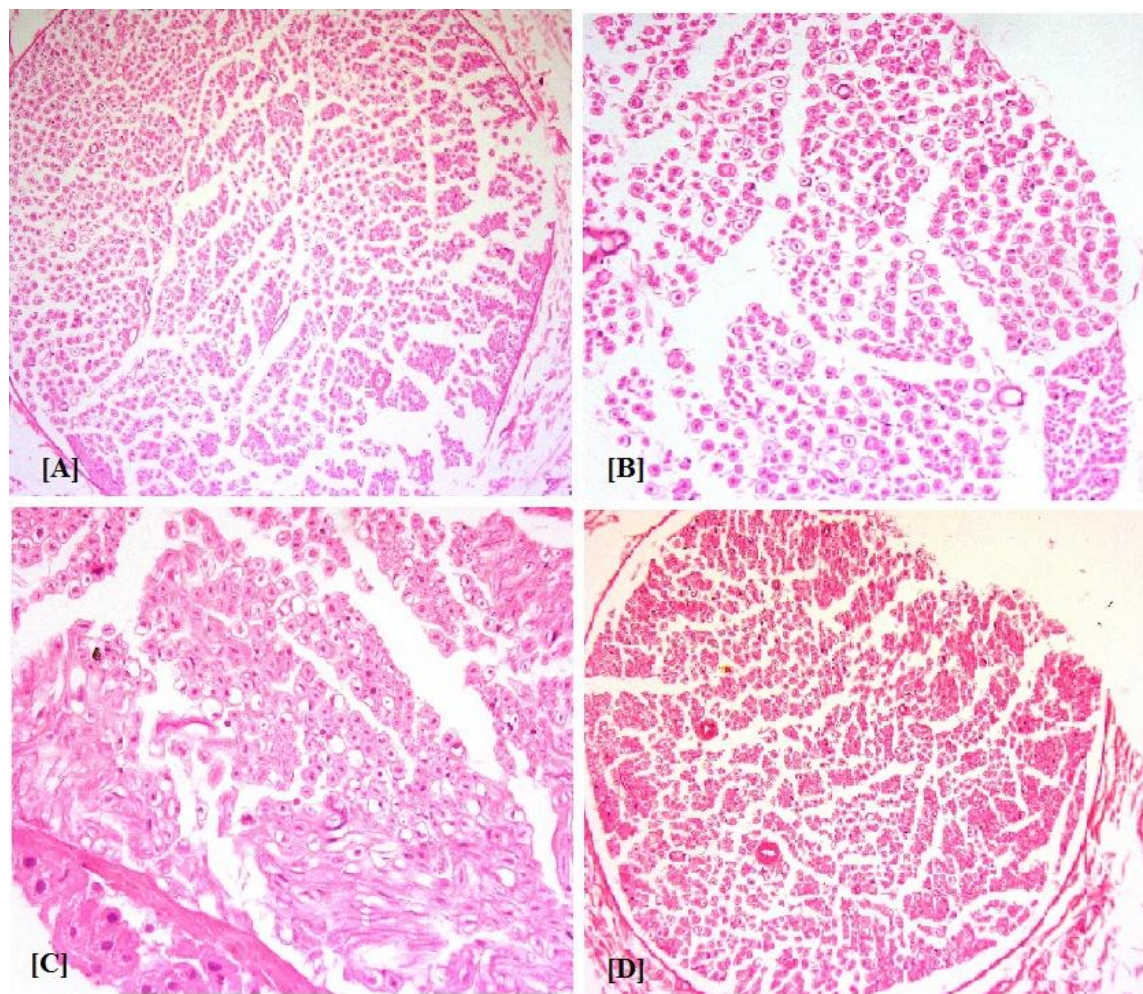


Figure 7. [A] Rat transverse section of sciatic nerve from control group looks normal, [B] in diabetic rat, severe nerve damage was seen, [C] rutin (50 mg/kg/day) treatment to diabetic rats produced mild protection in sciatic nerve, and [D] in higher dose (100 mg/kg/day) of rutin treatment to diabetic rats showing normal look similar to control.

Glutathione reductase is responsible for the regeneration of GSH, whereas GSH-Px and GST work together with GSH in the decomposition of H_2O_2 or other organic hydroperoxides. A reduction observed in sciatic nerve GR, GSH-Px and GST activity in diabetic rats might be reflection of decreased protein thiols observed in the study as -SH groups play a critical role in enzyme catalysis (Mak et al., 1996). Rutin treatment ameliorates decrease in the activity of these enzymes which might be mediated by GSH regeneration.

In conclusion, results obtained from the present study revealed that rutin ameliorates hyperglycemia-induced thermal hyperalgesia and improves the neuropathic pain by reducing oxidative stress in the nerve of diabetic rats by virtue of its antioxidative properties. Morphological assessments also show that the damage caused by streptozotocin to the sciatic nerve was also markedly reduced by the administration of rutin. Finally, these

findings suggest that rutin treatment might be beneficial in chronic diabetics exhibiting neuropathy.

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Full Length Research Paper

Antipsychotic drug prescribing to patients with dementia in a South African patient population

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The primary aim of this study was to determine the prescribing pattern of antipsychotic drugs to patients with dementia in a South African patient population. A retrospective, cross-sectional drug utilisation study was conducted on 2010 prescription data of a national community pharmacy group. A total of 1231 patients were prescribed medication for dementia. The average age of patients was 75.10 (standard deviation (SD)=10.27) years, with 56.13% of female patients. A total of 5264 anti-dementia products were prescribed at an average cost of R584.06 per product. Donepezil accounted for 45.84% of prescriptions, followed by memantine (36.51%). Differences were observed between females and males with respect to the prescribing frequency of the different active ingredients ($\chi^2=48.491$; d.f.=3; $p<0.0001$). Patients received on average 4.28 (SD=3.77) anti-dementia products over the year. Most patients (94.31%) received only one anti-dementia active ingredient during the year. Nearly a quarter of patients (23.23%) received one or more antipsychotic drugs despite “black box” warnings. Slightly more females were prescribed antipsychotic medication, with risperidone and quetiapine the most often prescribed antipsychotics. Older generation antipsychotics, such as haloperidol, were also prescribed. Despite warnings by health authorities, nearly a quarter of patients were prescribed antipsychotics. More comprehensive studies on antipsychotic use in dementia are needed to determine whether these drugs are used rationally and to ensure that the health of patients with dementia is not placed at risk.

Key words: Alzheimer’s disease, dementia, antipsychotics, prescribing patterns, drug utilisation study, risperidone.

INTRODUCTION

The 2013 World Alzheimer Report (Prince et al., 2013) estimated that the numbers of dependent older people will increase nearly threefold from 101 million in 2010 to 277 million in 2050, and in addition, that nearly half of these older people with needs for care are likely to be living with and experiencing the effects of dementia. The most accurate number remains that 36 million people worldwide live with Alzheimer’s disease or other dementias (Prince et al., 2013).

Alzheimer’s disease is the most common component of dementia (Anderson, 2013). The prevalence of Alzheimer’s disease increases with age, and is most prevalent in individuals older than 60 years. It has been shown to double every five years after the age of 65 years (Bridges-Webb and Wolk, 2003). Latin America, China and India are experiencing unprecedentedly rapid demographic aging with an increasing number of people with dementia (Prince et al., 2007). The International

10/66). Dementia Research Group's (10/66 DRG) initiative aims to establish the prevalence of dementia worldwide. The 10/66 studies indicate that two-thirds (66%) of people with dementia live in low and middle income countries, yet 10% or less of population-based research has been carried out in these regions (Prince et al., 2007).

The exact prevalence of dementia and Alzheimer's disease in South Africa is not known. World Health Organization (WHO) estimates of the burden of disease in South Africa suggest that non-communicable diseases caused 28% of the total burden of disease measured by disability-adjusted life years (DALYs) in 2004 (Mayosi et al., 2009). Cardiovascular diseases, diabetes mellitus, respiratory diseases, and cancers together contributed to 12% of the overall disease burden, and neuropsychiatric disorders (such as schizophrenia, bipolar depression, epilepsy and dementia) accounted for 6% (Mayosi et al., 2009). However, dementia rates are predicted to increase at an alarming rate in the least developed and developing regions of the world despite mortality resulting from malnutrition, poverty, war and infectious diseases (Kalaria et al., 2008). WHO projections suggest that by 2025, about three-quarters of the estimated 1.2 billion people aged 60 years and older will reside in developing countries (Kalaria et al., 2008; Active Ageing: A Policy Framework, 2002; Health Report, 2002). Thus, by 2040, if growth in the older population continues, and there are no changes in mortality or burden reduction by preventive measures, 71% or 81.1 million dementia cases will be in the developing world (Kalaria et al., 2008; Ferri et al., 2005).

Psychotropic medicine is often used to treat secondary symptoms of Alzheimer's disease such as depression, agitation and sleep disorders (Anderson, 2013). These non-cognitive symptoms may prove more bothersome to patients and families than the cognitive impairment and can cause considerable stress on both patients and caregivers. Despite the potential value of psychotropic medicines, a particular concern is the use of excess antipsychotics to control disruptive behaviour. Appropriate use of antipsychotic medication can relieve symptoms and reduce distress and can increase safety for patients and caregivers. However, their use may be associated with worsening cognitive impairment (Taylor et al., 2009), oversedation, falls, tardive dyskinesia, neuroleptic malignant syndrome, as well as with hyperlipidaemia, weight gain, diabetes mellitus, cerebrovascular accidents and death. An increased risk of especially cardiovascular symptoms, glucose intolerance, stroke and death has been associated with both the use of typical and atypical antipsychotics in some psychiatric disorders.

In April 2005, the USA Food and Drug Administration (FDA) issued an advisory and subsequent black box warning regarding the risks of atypical antipsychotic use among elderly patients with dementia (DeNoon, 2008;

Dorsey et al., 2010). The FDA warned that clinical trial results strongly suggested that newer "atypical" antipsychotics increase the risk of death in dementia patients. The FDA required these drugs to carry its strongest black box warning on their labels. More recently, in 2008, based on observational studies, the FDA warned that older antipsychotics also seem to increase dementia patients' risk of death (DeNoon, 2008). These drugs therefore, too, will carry a black box warning.

An increased mortality risk therefore exists in elderly dementia patients receiving antipsychotic agents. There is an increased risk of fatal arrhythmias associated with several older and newer (atypical) antipsychotic agents due to the prolongation of the QT interval. The older antipsychotic drugs may even be more likely than the newer atypical antipsychotics to cause troublesome "extrapyramidal" symptoms such as tics and Parkinsonism. But the drugs, many in use since the 1950s, are still being prescribed. Older drugs that carry the FDA black box warning (not all available in South Africa) include prochlorperazine, haloperidol, loxapine, thioridazine, molindone, thiothixene, pimozide, fluphenazine, trifluoperazine, chlorpromazine and perphenazine. Newer drugs that continue to carry the FDA black box warning include aripiprazole, clozapine, paliperidone, risperidone, quetiapine, olanzapine, ziprasidone, and the combination of fluoxetine and olanzapine (DeNoon, 2008) (the combination is not available in South Africa).

The overuse of antipsychotics in dementia is reported to be a shared issue in Europe and several other countries in the world (Antipsychotics in Dementia, 2012). Although the exact extent of this exposure is not sufficiently documented, the rate of exposure is reported to range from 15% in the ambulatory setting to 20 to 40% in nursing homes, which is a much higher rate than in the general population (Antipsychotics in Dementia, 2012). A 2009 study suggested that 180000 people with dementia were taking antipsychotic medication in the UK and found that the drugs resulted in 1800 additional deaths (Huybrechts et al., 2012). The study published in the British Medical Journal in February 2012 (Huybrechts et al., 2012) concluded that although causality could not be proved and the possibility of residual confounding could not be ruled out, evidence was provided about the risk of using antipsychotic drugs in older patients and it reinforced the concept that antipsychotics should not be used in the absence of a clear need.

The Medicines Control Council (MCC) in South Africa published a Medicine Safety Alert in December 2008 which was updated in June 2009 entitled: "Atypical Antipsychotics in Elderly Patients with Dementia" in which they informed prescribers of increased risks of cerebrovascular adverse events (including strokes and transient ischaemic attacks) and mortality, associated with

the use of atypical antipsychotics in elderly patients with dementia. The atypical antipsychotics to which this medicine safety alert referred to included clozapine, risperidone, olanzapine, quetiapine, ziprasidone and aripiprazole (Medicine Safety Alert: Atypical Antipsychotics in Elderly Patients with Dementia, 2009).

Risperidone is currently the only drug licenced in the UK for the behavioural and psychological symptoms of dementia (Taylor et al., 2009). It is only indicated for the short-term treatment (up to 6 weeks) of persistent aggression in patients with moderate to severe Alzheimer's dementia unresponsive to non-pharmacological approaches and when there is harm to self or others (Use of Atypical Antipsychotics in Treatment of Dementia Declined after FDA Warning, 2011). The MCC in South Africa has also subsequently deleted risperidone from the list of antipsychotic drugs not indicated for use in the elderly (Medicine Safety Alert: Atypical Antipsychotics in Elderly Patients with Dementia, 2009).

During February 2011, it was reported that, in the USA, the warning issued by the FDA regarding the use of atypical antipsychotics for the treatment of dementia resulted in a significant decline in the use of these medicines for treating dementia symptoms in elderly patients (Taylor et al., 2009; Kales et al., 2011). Extreme caution should be exercised in using these drugs, although their judicious use can have a positive impact on problematic behavioural symptoms. A dose decrease or discontinuation should be considered periodically for all dementia patients receiving antipsychotic medicine. No data are available for South Africa. The hypothesis for the study was therefore that despite warnings by health authorities, patients with dementia were still being prescribed antipsychotics. Studies on antipsychotic use in dementia are needed in South Africa to determine whether these drugs are used rationally and to ensure that the health of patients with dementia is not placed at risk. This is not only important for patients with dementia, but also for the health care system to ensure that scarce health care resources are used optimally and rationally.

The primary aim of the study was to determine the prescribing of antipsychotic drugs to patients with dementia in a South African patient population.

MATERIALS AND METHODS

A retrospective, cross-sectional drug utilisation study was conducted on prescription data for 2010 of a national community pharmacy group in South Africa. The pharmacies in this group are distributed throughout South Africa, and are located in all nine provinces of South Africa. These pharmacies are mostly located in urban areas, and therefore have the same pattern of distribution as the majority of pharmacies in South Africa.

The database contained 2,665,025 records of central nervous

system medicine for 575469 patients for the year 2010. Each medication record contained information on the age and gender of the patient, with a unique number to identify each patient, the date of the prescription, detailed information on the dispensed drug (name, package size, formulation, strength and quantity), price and various reimbursement variables.

The Anatomical Therapeutic Chemical (ATC) Classification System (ATC/DDD Index 2011, 2011), MIMS (Snyman, 2011) and the South African Medicines Formulary (Rossiter, 2012) were used to identify the medicines that were prescribed. All prescriptions for Alzheimer's disease (MIMS Category 1.10 (Snyman, 2011) or ATC Group N06D (ATC/DDD Index 2011, 2011; Rossiter, 2012)) were extracted and analysed. No diagnoses were available in the database, therefore MIMS and the ATC drug classification system were used to identify the active ingredients used most commonly for Alzheimer's disease and dementia (the active ingredients analysed included donepezil, galantamine, rivastigmine and memantine). The dementia patients in this study were therefore identified using a proxy measure, namely anti-dementia drug prescriptions since no diagnoses were available in the database. Microsoft Access[®] and Excel[®] were used to analyse the data. Descriptive statistics were calculated. Ethical approval to conduct the study was granted by the Research Ethics Committee (Human) of the Nelson Mandela Metropolitan University.

One Euro (€1.00) was equal to R9.38 (South African Rands), one US Dollar (\$1.00) was equal to R7.64 and one British Pound (£1.00) was equal to R11.48 at the time of the study (30 June 2010).

RESULTS

Age and gender distribution of patients

A total of 1231 patients were prescribed medication for dementia. The age and gender distribution of patients is as shown in Figure 1. More than half (56.13%) of the patients were females. The average age of patients was 75.10 (standard deviation (SD)=10.27) years (range: 21 to 98 years). The average age of females was 75.05 (SD=9.99) years and of males 75.16 (SD=10.63) years. Most patients (41.27%) were between 70 and 79 years of age, followed by patients between 90 and 89 years (32.58%). A small percentage (2.44%) of patients receiving anti-dementia products was younger than 50 or 90 years and above (3.49%).

Anti-dementia products prescribed

A total of 5264 anti-dementia prescriptions (products) was prescribed at a cost of R3 074 487 (average cost of R584.06 per prescription). The four active ingredients prescribed were donepezil, rivastigmine, galantamine and memantine. Patients received on average 4.28 (SD=3.77) prescriptions for anti-dementia products over the year. The AChEIs accounted for 63.49% of prescriptions and the NMDA-receptor antagonist (memantine) for 36.51%. Donepezil accounted for 45.84% of prescriptions for

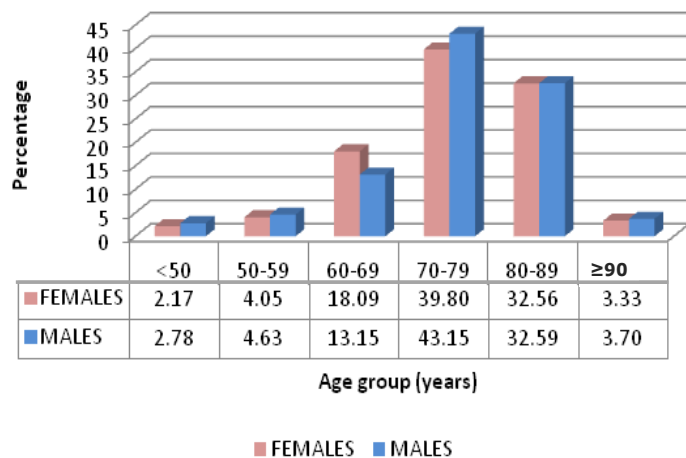


Figure 1. Age and gender distribution of patients (n=1231).

anti-dementia medicine, followed by memantine (36.51%) (Table 1). Differences were observed between females and males with respect to the prescribing frequency of the different active ingredients ($\chi^2=48.491$; d.f.=3; $p<0.0001$). Donepezil was more often prescribed to female patients and memantine to male patients.

Only one trade name product for memantine (Ebixa[®], Lundbeck) is available on the South African market. Ebixa[®] 10 mg tablets was the single most often prescribed trade name product accounting for 36.47% of trade name products prescribed for dementia. Only two prescriptions for Ebixa[®] oral drops 10 mg/g were prescribed (accounting for 0.04% of trade name products prescribed for dementia).

Most patients (94.31%) received only one active ingredient over the year, while 69 patients received two different active ingredients, and one patient three different active ingredients. The most often prescribed combination was memantine and donepezil (37 patients), followed by memantine and galantamine (21 patients).

Prescribing of antipsychotic products

A total of 1505 antipsychotic products were prescribed to 286 of the 1231 patients in the study (23.23% of patients) at a cost of R633 655. The average cost per antipsychotic product was R421.03. Slightly, more females were prescribed antipsychotic medication (24.89% females versus 21.11% males). Patients received on average 5.26 (SD=4.39) antipsychotic products over the year. Female patients received on average 4.97 (SD=4.41) antipsychotic products and male patients 5.70 (SD=4.36) products over the year. The average age of patients that prescribed psychotropic drugs was 71.69

(SD=14.44) years.

Risperidone was the most often prescribed antipsychotic (53.16%) followed by quetiapine (31.10%) (Table 2). Older generation antipsychotics, such as haloperidol, were also prescribed although at low prescribing frequencies.

The percentage prescription of anti-dementia and anti-psychotic products over the year is illustrated in Figure 2. There was a small increase in the prescribing rate of antipsychotic products, but the study was too small to make any definite conclusions from this finding.

DISCUSSION

Donepezil, rivastigmine, galantamine and memantine were the drugs prescribed in this study for the treatment of dementia. Anti-dementia drugs appear to be equally efficacious, have a similar side effect profile, their cost is approximately equivalent and there is little evidence to recommend one over the other. They appear to provide a modest enhancement of cognitive function in patients with Alzheimer's disease, but do not seem to have a significant impact on the underlying pathophysiology of Alzheimer's disease.

A total of 1231 patients were prescribed medication for dementia. This represented less than one percent of the patients in the total database. The exact reason for this apparently low percentage is not clear. It may have been that patients may have had dementia but were not prescribed anti-dementia drugs that patients with dementia may not have been diagnosed with dementia, or that patients may have been prescribed anti-dementia drugs but were not claiming the products from their medical insurance schemes and were therefore not included in the study. It is therefore possible that a potentially large proportion of dementia patients was overlooked and did not receive anti-dementia drugs. The diagnosis of dementia is also not always accurate, which can lead to a misclassification of disease. The findings of this study are therefore not necessarily generalisable to the whole of South Africa as far as the prevalence of dementia is concerned.

Further limitations of the study were that no clinical information and diagnoses were available in the database. These medicines are, however, nearly exclusively indicated for the treatment of dementia of Alzheimer's disease (Rossiter, 2012), except in the cases where it is used off-label. The possibility of off-label use is supported by the fact that a small percentage of younger patients received anti-dementia medicines. These younger patients received once-off prescriptions for dementia medicines, which was probably for off-label use. Memantine, for example, is according to the literature used off-label for various psychiatric disorders,

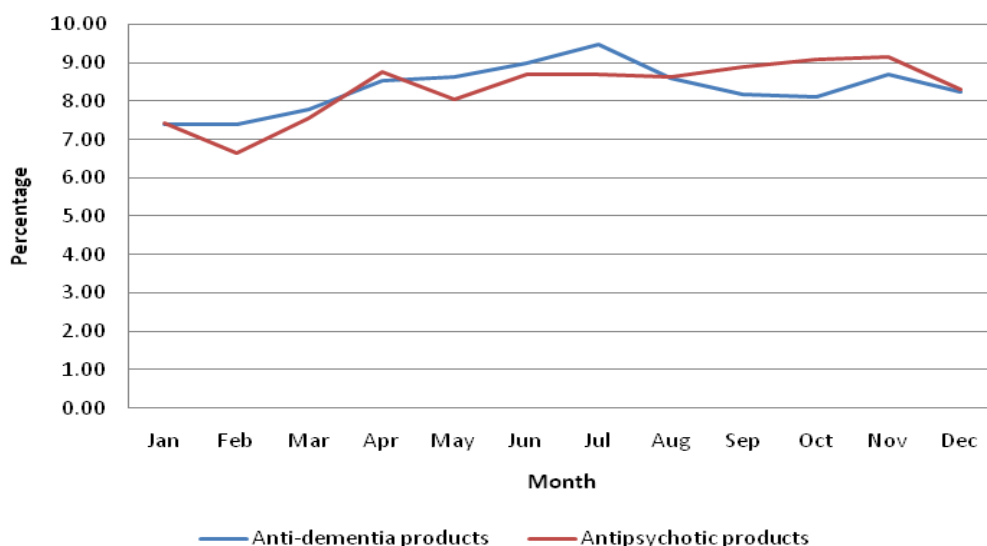


Figure 2. Percentage prescribing frequency of anti-dementia and antipsychotic products over the 12 months*. *The line diagram illustrates the monthly number of anti-dementia products expressed as a percentage of the total number of anti-dementia products (blue line) and the monthly number of antipsychotic products as a percentage of the total number of antipsychotic products (red line).

Table 1. Prescribing frequency of anti-dementia active ingredients according to gender groups (n = 5264)*.

Active ingredient	Percentage		Both genders	
	Female (n = 2969)	Male (n = 2295)	Number	%
Donepezil	49.98	40.48	2413	45.84
Memantine	33.98	39.78	1922	36.51
Galantamine	15.59	19.39	908	17.25
Rivastigmine	0.44	0.35	21	0.40
Total	100.00	100.00	5264	100.00

* $\chi^2=48.491$; d.f.=3; $p<0.0001$.

Table 2. Antipsychotic active ingredient prescribing frequency according to gender groups (n = 1505).

Active ingredient	Percentage		Both genders	
	Female (n = 855)	Male (n = 650)	Number	(%)
Risperidone	60.12	44.00	800	53.16
Quetiapine	25.73	38.15	468	31.10
Olanzapine	5.15	7.54	93	6.18
Haloperidol	2.46	2.00	34	2.26
Aripiprazole	1.52	2.92	32	2.13
Chlorpromazine	1.64	0.92	20	1.33
Amisulpiride	0.58	1.85	17	1.13
Trifluoperazine	0.58	1.54	15	1.00
Clozapine	1.17	0.46	13	0.86
Ziprasidone	0.70	0.62	10	0.66
Clothiapine	0.35	0.00	3	0.20
Total	100.00	100.00	1505	100.00

such as depression, schizophrenia, obsessive-compulsive disorder, substance abuse and binge eating disorder (Zdanys et al., 2008).

The age and gender distribution of patients given in Table 1 was similar to that of a previous South African retrospective database study on pharmacy records (Truter, 2010) conducted on 588 patients using 2008 data. In the study (Truter, 2010), the average age of patients was 75.54 years and 55.44% of patients were female (compared to 75.10 years and 56.13% in this study), and patients received on average of 4.28 (SD=3.77) prescriptions for anti-dementia products over the year.

The AChEIs accounted for 75.30% of prescriptions in this study, compared to 63.49% of prescriptions in the 2008 study (Truter, 2010). There was an increase in the prescription of the NMDA-receptor antagonist (memantine) from 24.70 to 36.51%. Donepezil accounted for 37.09% in the 2008 study compared to 45.84% in the 2010 study. There are three trade name products of donepezil available (the originator plus two generic products), whilst the other products have no generic equivalents on the South African market. The lower cost of the generic equivalents may have impacted on their increased prescription. The prescription of rivastigmine remained low.

Continuity of prescription was investigated, and it was observed that nearly a half of the patients (586 of the 1231 patients) received only one or two anti-dementia prescriptions during the year, which means that not all patients were using these products on a chronic or continual basis. Only 30.87% of the patients were prescribed six or more anti-dementia products during the year. There are various reasons why this may be the case. These products are relatively expensive and not all medical insurance schemes reimburse these products, so affordability may have played a role. It may also be because patients or caregivers may feel these products are not effective or making a big enough improvement on their quality of life.

Combination therapy with an AChEI and memantine is sometimes indicated in South Africa if the patient can afford it. The rationale for the combination therapy is based on the fact that the two drugs have different and complementary mechanisms of action. The National Institute for Clinical Excellence (NICE, 2011) guidelines did not find any additional benefit of combination therapy, although a study by Atri et al. (2013) did find that combination therapy has benefit. Generally therefore, combination therapy is not indicated by clinical guidelines although it may be used. Similar to the 2008 study (Truter, 2010) in which 5.27% of patients were prescribed more than one anti-dementia active ingredient, only 5.69% of patients in this study were prescribed a combination (the most often prescribed combination was memantine and donepezil). The cost of combination

therapy and the lack of cost-effectiveness data may be reasons why combination therapy was not utilised more in this study.

The use of antipsychotic drugs in patients with dementia has been debated extensively yet little information is available in South Africa. Nearly a quarter of patients in this study (23.23% or 286 patients) received one or more antipsychotic drugs despite the black box warning issued in 2003 by the FDA and a warning in December 2008 by the MCC that antipsychotic drugs are best avoided in patients with dementia of the Alzheimer's type. Risperidone was the most often prescribed antipsychotic in this study and is currently also the only drug licenced in the UK for the behavioural and psychological symptoms of dementia (Use of Atypical Antipsychotics in Treatment of Dementia Declined after FDA Warning, 2011). It is indicated for the short-term treatment (up to 6 weeks) of persistent aggression in patients with moderate to severe Alzheimer's dementia unresponsive to non-pharmacological approaches and when there is harm to self or others (Taylor et al., 2009). The MCC in South Africa has also deleted risperidone from the list of antipsychotic drugs not indicated for use in the elderly (Medicine Safety Alert: Atypical Antipsychotics in Elderly patients with Dementia, 2009). It is therefore positive that risperidone was the active ingredient of choice in South Africa when antipsychotics were prescribed. A low percentage (4.78%) of the antipsychotics prescribed was conventional or first-generation antipsychotics.

These results were fairly similar to other studies (Ilyas et al., 2012; Kales et al., 2011). In a study examining the trends in prescriptions and costs of drugs for mental disorders in England between 1998 and 2010, it was observed that the bulk of antipsychotic prescription in 2010 was for olanzapine, quetiapine and risperidone, which accounted for 24, 23 and 17% of antipsychotic prescription items, respectively (Ilyas et al., 2012). In this study, the same three antipsychotic active ingredients were the most commonly prescribed although in different proportions (risperidone accounted for 53.16%, quetiapine for 31.20% and olanzapine for 6.18% in this study). In a study by Kales et al. (2011) on National Veterans Affairs data in the USA, trends in antipsychotic use in outpatients with dementia between 1999 and 2007 were investigated. These investigators found that olanzapine and risperidone showed declining rates and quetiapine showed an increase during the early warning period, but rates of use for all three antipsychotics declined during the black box warning period. Interestingly, there was a small but significant increase in anticonvulsant prescriptions in the black box warning period. The current study only investigated prescribing trends in 2010, and the exact effect of the FDA and MCC warnings can therefore not be detected over time.

It was, however, of concern that for every 3.50 anti-dementia products in this study, one antipsychotic product was prescribed (a total of 5264 anti-dementia and 1505 antipsychotic products were prescribed, that is, a ratio of 3.50 anti-dementia products for every one antipsychotic product). Although the anti-dementia prescriptions and the antipsychotic prescriptions did not always coincide exactly in terms of dates dispensed or issued, there was definite overlap for many patients indicating that patients were using these medicines concurrently. The use of antipsychotics in dementia needs a case-by-case evaluation weighing up the benefits and risks in order to ensure the best possible treatment for the patient. Because of the lack of clinical information, it is difficult to determine whether the prescription of antipsychotic drugs in dementia patients in this South Africa population is reasonable or excessive. More comprehensive studies on antipsychotic use in dementia are needed to determine whether these drugs are used rationally and to ensure that the health of patients with dementia is not placed at risk.

Conclusion

The results of this study regarding the prescription patterns of anti-dementia products were generally similar to that of a previous South African study. However, the prescription of antipsychotic drugs together with products for dementia has not been investigated previously in South Africa. Despite serious warnings by health authorities, nearly a quarter of patients on medication for Alzheimer's disease were prescribed antipsychotic drugs in this study. It is difficult to make definite conclusions since the study was firstly, too small, and secondly, there are no previous studies on antipsychotic prescription in dementia in South Africa to compare the findings of this study with. Also, it was not known what percentage of patients was living in nursing homes, the severity of their condition or the stage of Alzheimer's disease, or any information on co-morbid conditions. Further investigation is needed, and it is recommended that clinical studies as well as qualitative studies be conducted where the actual symptoms of patients can be evaluated against the effectiveness of treatment with antipsychotic drugs.

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liability in regard thereto.

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Full Length Research Paper

Inhibitory effects of Neem (*Azadirachta indica* Linn.) and Bitter Kola (*Garcinia kola* Heckel) leaves on selected pathogenic bacteria

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Concoctions of *Azadirachta indica* Linn (Neem) and *Garcinia kola* Heckel (Bitter kola) are commonly used for medicinal purposes in most Nigerian rural communities, because they are believed to possess some healing properties. The antibacterial activity of both neem and bitter kola was evaluated by agar well diffusion method against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Both neem and bitter kola leaf extracts in varying concentrations showed moderate antibacterial activity against the clinical pathogens. Cold water and hot water extracts showed better activity as compared to the methanol and ethanol extracts. Gram positive bacteria (*S. aureus* and *S. pneumoniae*) were more susceptible to the plant extracts than the Gram negative pathogens (*K. pneumoniae* and *P. aeruginosa*). The MICs of the plant extracts were determined at varying effectiveness of 25 and 12.5 mg/ml. The results obtained in this study give credence to the traditional use of neem and bitter kola in the treatment of infectious diseases in Nigerian rural communities.

Key words: *Azadirachta indica* Linn., *Garcinia kola* Heckel, clinical pathogens, resistance, Nigeria.

INTRODUCTION

The growing prevalence of antibiotic resistance amongst clinically important pathogens necessitates the search for potential healing powers in herbal plants, as a way of containing the menace. The use of herbs with healing powers for the treatment of infectious diseases is as old as mankind, and this form of therapy is practiced in most cultures especially in the rural areas (Izzo et al., 2009; Iwu et al., 1999). Majority of these herbal plants contain substances which are precursors for the synthesis of conventional drugs, or substances that can be used for therapeutic purposes. According to the World Health Organization (WHO), about 80% of individuals from developing countries meet their primary health care needs

through the use of traditional medicine that incorporates one herb to another as the main therapeutic agent (WHO, 2001). Nigeria and the rest of African continent are endowed with plethora of plants with putative medicinal properties which are yet to be tapped for the development of novel antimicrobial agents. Plant parts (including leaves, stem, bark and seeds) contain a variety of phytochemical compounds that gives impetus to their extensive usage in all healing traditions; and these plants are either used alone or in combination as concoctions with other plants of similar effect (Srivastava and Shukla 2000; Sibanda and Okoh, 2008). Plants with well documented antimicrobial effect include *Azadirachta indica*

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Linn (Neem) and *Garcinia kola* Heckel (Thakurta et al., 2007; Sibanda and Okoh, 2008; Srivastava and Shukla, 2000). Though advances in medicinal chemistry and pharmaceutical sciences have helped in producing many antimicrobial agents with little or no origin from plants, plant-derived substances still holds promise to revolutionize medicine, and many herbal plants have recently become of great interest owing to their versatile applications in the treatment of a variety of infectious diseases in most parts of the world (Baris et al., 2006; Barnes et al., 2007). The high cost of conventional drugs, drug inaccessibility in the rural areas and the mounting resistance of pathogens to available drugs has further justified the need for the use of plants for therapeutic purposes in many remote areas. Plants (including *A. indica* Linn and *G. kola* Heckel) have contributed immensely in meeting the primary health care needs of people in the developing countries (Ayogu and Amadi, 2009; Esimone et al., 2010; Saseed and Khan, 2008). It is because of this that this study was designed to evaluate and update on the antibacterial efficacy of *A. indica* (Neem), and *G. kola* against selected pathogenic microorganisms.

MATERIALS AND METHODS

Clinical isolates

The test microorganisms used in the present study included *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Streptococcus pneumoniae*. These were obtained from the culture collection unit of Microbiology Laboratory Department of Federal Teaching Hospital, Abakaliki (FETHA), Ebonyi State, Nigeria. All test isolates were subcultured, purified and biochemically identified based on standard microbiological techniques (Cheesbrough, 2006).

Collection and identification of plant

Leaves of *A. indica* Linn and *G. kola* Heckel were collected from Abakaliki Metropolis, and they were identified by Prof. A. Okafor of the Applied Biology Department, Faculty of Biological Sciences, Ebonyi State University, Abakaliki, Nigeria.

Preparation of plants extract

The plants were washed with water, shade-dried, grounded to powdery form and soaked in 250 ml flask of cold water, hot water, ethanol and methanol, respectively. Fifty grams of each ground plant material was consecutively soaked in 250 ml of water (cold and hot), ethanol and methanol for 24 h in a conical flask and allowed to stand on shaker for extraction (Ayogu and Amadi, 2009).

Screening for antimicrobial activity

Twenty milliliters each of molten agar (Muller Hinton) was poured aseptically into sterile Petri dishes and then allowed to gel. The surface of the agar plates were then streaked with standardized inoculum of the test bacteria that was adjusted to 0.5 McFarland turbidity standards. Thereafter, a sterilized 6 mm cork borer was used to bore 5 holes on the agar plate(s), and 4 of the holes were

filled with equal volumes of the respective plant extracts that was diluted with 0.5% dimethyl sulfoxide (Esimone et al., 2010). Dimethyl sulfoxide was also tested on test organisms at different concentration to ensure they did not show antibacterial activity. The plates were allowed to stand for about 30 min for pre-diffusion of the plant extracts, and these were incubated at 37°C for 24 h. The inhibition zone diameters were determined after incubation (Onyeagba et al., 2004; Esimone et al., 2008).

Minimum inhibitory concentrations (MIC)

MIC was evaluated for only ethanol and methanol extracts. Varying concentration of each extract (100, 50, 25, 12.5 and 6.25 mg/ml), were prepared. 0.1 ml of each concentration was added to each 5 ml of nutrient broth containing 0.5 ml of standardized test organism of bacterial cells. The tubes were incubated aerobically at 37°C for 24 h. A tube containing no antibiotics and no plant extract was used as a positive control. The tube with least concentration of extracts without growth after incubation was taken as the MIC (NCCLS, 1990).

RESULTS

The plant extracts of bitter kola and Neem plant produced varying levels of antimicrobial activity against the test organisms including *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, *E. coli*, and *S. pneumoniae*. All the extracts of *G. kola* Heckel (Bitter kola) leaf showed antibacterial effect against the tested pathogens except for *K. pneumoniae*. The ethanol and methanol leaf extracts of bitter kola had no activity against the *P. aeruginosa* used in our study. The antibacterial activity of *A. indica* Linn leaf extracts was found to be better against tested clinical pathogens than the bitter kola leaf extracts. The test clinical pathogens were susceptible to all the extracts of Neem except for the ethanolic extract where no inhibitory activity was observed for *K. pneumoniae*. The test pathogens were successfully inhibited at MICs of 25 mg/ml and 12.5 mg/ml.

DISCUSSION

Discovering and harnessing the hidden potentials of plants with medicinal properties is the basis for unleashing novel antimicrobials for the fight against antimicrobial resistance. In the present study, the antibacterial activity of two medicinal plants (neem and bitter kola) was evaluated against clinical isolates of *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*. Table 1 shows the antibacterial properties of *G. kola* Heckel (Bitter kola) leaf extracts against pathogenic microorganisms. From this Table 1, it was observed that the cold water, hot water, ethanol and methanol leaf extracts of bitter kola were ineffective against the Gram-negative organism *K. pneumoniae*. However, *S. aureus*, *E. coli* and *S. pneumoniae* were susceptible to all extracts of *G. kola*. The largest zones of inhibition (18, 17 and 16 mm) were obtained with the cold and hot water

Table 1. Antibacterial properties of *Garcinia kola* Heckel leaf extracts (100 mg/ml) against different bacterial pathogens.

Organism	Inhibition zone diameter (mm)				Control (ampicillin, 1 mg/ml)
	Cold water	Hot water	Ethanol	Methanol	
<i>Staphylococcus aureus</i>	18	17	11	13	27
<i>Klebsiella pneumoniae</i>	NI	NI	NI	NI	23
<i>Escherichia coli</i>	18	16	09	11	28
<i>Streptococcus pneumoniae</i>	14	16	07	09	33
<i>Pseudomonas aeruginosa</i>	10	12	NI	NI	30

NI = No Inhibition.

Table 2. Antibacterial activity of *Azadirachta indica* Linn leaf extracts (100 mg/ml) against different bacterial pathogens.

Organism	Inhibition zone diameter (mm)				Control (ampicillin, 1 g/ml)
	Cold water	Hot water	Ethanol	Methanol	
<i>Staphylococcus aureus</i>	10	14	12	13	28
<i>Klebsiella pneumoniae</i>	09	10	NI	08	22
<i>Escherichia coli</i>	16	12	14	13	27
<i>Streptococcus pneumoniae</i>	17	16	11	14	32
<i>Pseudomonas aeruginosa</i>	14	16	08	07	29

NI = No Inhibition.

extracts of *G. kola* against *S. aureus*, *S. pneumoniae* and *E. coli*. *P. aeruginosa* was not susceptible to the ethanol and methanol extracts of bitter kola (Table 1). Bitter kola leaf and seed is used in most tradition as a masticatory agent, where they are chewed, and the plant is believed to provide some level of healing to dental and oral diseases (Esimone et al., 2007; Schwach-Abdellaoui et al., 2000; Sibanda and Okoh, 2008). The moderate antibacterial effect of bitter kola against clinical pathogens as obtained in our study (Table 1) is comparable to other studies conducted to elucidate the antimicrobial properties of *G. kola* leaf and seed (Ndukwe et al., 2004; Sibanda and Okoh, 2008; Esimone et al., 2007). Cold water and hot water extracts of bitter kola produced better inhibitory effect against the tested pathogenic microorganisms (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*) as against the ethanol and methanol extracts, which are both believed to be better solvents for the extraction of the bioactive constituents of medicinal plants (Ogueke et al., 2006; Onuoha and Obi, 2000). The antibacterial effect of *A. indica* Linn (neem) against the tested pathogenic microorganisms is shown in Table 2. In this study, the hot water and cold water leaf extracts of neem showed reasonable inhibitory effect, and inhibited the growth of all the test clinical pathogens to a high degree. The susceptibility of the test pathogens to the cold water and hot water extracts of neem was also observed for the ethanol and methanol extracts except for *Klebsiella* species, where no inhibitory activity was recorded for ethanol extract. The test pathogens (*E. coli*, *P. aeruginosa*,

K. pneumoniae, *S. aureus* and *S. pneumoniae*) used in our study have been implicated in a number of bacterial-related infections including tooth decay, urinary tract infections (UTIs), typhoid fever and stomach-related illnesses (Taiwo and H-X Lee, 1999; Ayogu and Amadi, 2009); and they are mostly treated in Nigerian rural communities with herbal plants believed to possess some form of healing powers (Neem and bitter kola inclusive). The antibacterial effects of the duo of Neem and bitter kola leaves as observed in our study further gives credence to the traditional use of neem and bitter kola plant parts for the treatment of some infectious diseases in this area. The level of antibacterial activity of the tested plant extracts (leaves of *A. indica* Linn and *G. kola* Heckel) against the clinical pathogens was however expected, owing to their continued use in most African rural traditions (Nigeria inclusive) to meet certain primary healthcare needs. Comparable antibacterial activity of leaf extracts of neem has been reported by Thakurta et al. (2007), Faiza et al. (2009), Taiwo and H-X Lee. (1999) and Saseed and Khan, (2008). The *in vitro* effectiveness of neem and bitter kola as depicted in our study has been attributed to some phytochemical constituents and bioactive compounds that these plants are known to possess, which justifies their usage for medicinal purposes. The MICs for the ethanol and methanol leaf extracts of neem and bitter kola are shown in Table 3. The ethanol extract of neem leaf inhibited the growth of the test clinical pathogens at a concentration of 100 and 50 mg/ml, 25 mg/ml, and 12.5 mg/ml except for *K. pneumoniae* where no inhibitory activity was recorded

Table 3. Minimum inhibitory concentration (MIC).

Extract	Conc. (mg/ml)	<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>S. pneumoniae</i>	<i>K. pneumoniae</i>
AIE	100	-	-	-	-	-
	50	+	+	+	+	-
	25	+	+	+	+	-
	12.5	+	+	+	+	-
	6.25	-	-	-	-	-
AIM	100	-	-	-	-	+
	50	-	-	-	-	+
	25	-	+	+	+	-
	12.5	-	+	+	+	-
	6.25	-	-	-	-	-
GKE	100	-	-	-	-	-
	50	-	-	-	-	-
	25	+	+	+	+	+
	12.5	+	+	+	+	+
	6.25	-	-	-	-	-
GKM	100	-	-	-	+	+
	50	-	-	-	+	+
	25	+	+	-	-	-
	12.5	+	+	-	-	-
	6.25	-	-	-	-	-

AIE: *Azadirachta indica* Linn ethanol, AIM: *A. indica* Linn methanol, GKE: *Garcinia kola* Heckel ethanol, GKM: *G. kola* Heckel methanol.

(Table 3). The MICs for the methanol extract were 25 mg/ml against *E. coli*, *S. aureus*, *P. aeruginosa*, *S. pneumoniae* and *K. pneumoniae*; and 50 mg/ml against *S. aureus*, *Pseudomonas* species, and *Streptococcus* species. The MICs for the ethanol extract of *G. kola* were 25 and 12.5 mg/ml against the tested clinical pathogens (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*). Methanol extracts of bitter kola successfully inhibited the growth of the test pathogens except for the Gram negative organism *P. aeruginosa* where no inhibitory effect was recorded (Table 3). Further research into the molecular characterization of the bioactive components of neem and bitter kola and other medicinal plants used for traditional medicine is therefore advocated as a way of identifying potent and putative constituents that will serve as a foundation for the development of new drugs that can be used to fight the mounting cases of drug resistance in the health sector. Our study further gives impetus to the health benefits of neem and bitter kola in the treatment of some bacterial related diseases in Nigerian rural communities.

Conclusion

The results of our study indicated that leaf extracts of *A. indica* Linn. (Neem) and *G. kola* Heckel (Bitter kola)

possess antibacterial activity, which give good reason for their continued usage for the treatment of some bacterial related infections in this region.

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Full Length Research Paper

Antioxidant and anticholinesterase activities of aqueous extract of *Uraria picta* (Jacq.) DC

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Numerous plants have been used in treating/managing Alzheimer's disease in folklore. Inhibition of cholinesterase enzymes is an alternative way used in treating/managing Alzheimer's disease. This study therefore sought to investigate the interaction of aqueous extract of *Uraria picta* with key enzymes (acetylcholinesterase and butyrylcholinesterase) linked with Alzheimer's disease *in vitro*. Inhibition of acetylcholinesterase and butyrylcholinesterase, the total phenolic content and radical scavenging abilities were assessed *in vitro*. The extract inhibited acetylcholinesterase and butyrylcholinesterase in a dose dependent manner. Present in the extract are phenol and flavonoids. The extract also scavenged 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS*) and hydroxyl radical (OH*) in a dose-dependent manner. Inhibition of acetylcholinesterase, butyrylcholinesterase and the exhibited antioxidant properties could make *U. picta* extract a good means in treating/managing Alzheimer's disease.

Key words: Alzheimer's disease, acetylcholinesterase, butyrylcholinesterase, *Uraria picta*, antioxidant.

INTRODUCTION

Medicinal role of herbs is an important topic in plant research. Finding natural antioxidants and anticholinesterases in plants is of great interest lately. Antioxidants have the ability to inhibit oxidative damage, prevent inflammatory conditions (Khanna et al., 2007), and prevent neurodegenerative conditions (Fusco et al., 2007). Some of the commercially available synthetic drugs used in treating neurodegenerative diseases have their side effects on prolonged use (Johnson et al., 2000; Wentrup et al., 2008; Winblad et al., 2007).

Dementia is a disease marked by gradual loss of cognitive functioning which can also incorporate losses of motor, emotion, and social functioning. It is a permanent and progressive disease that eventually renders people unable to care for themselves. Older people with dementia exist in nearly every country in the world. Dementia rates are predicted to increase at an alarming

rate in the least developed and developing regions of the world despite mortality resulting from malnutrition, poverty, war, and infectious diseases. World Health Organization (WHO) projections suggest that by 2025, about three-quarters of the estimated 1.2 billion people of ages 60 years and older will reside in developing countries (WHO, 2002). Thus, by 2040, if growth in the older population continues, and there are no changes in mortality or burden reduction by preventive measures, 71% of 81.1 million dementia cases will be in the developing world (Ferri et al., 2005). Oxidative stress is critical to the pathologies associated with brain damage and cognitive abilities (Markberry and Lovell, 2007). Although multiple factors are involved in the development of neurodegenerative diseases, dysregulation in the inflammatory network and oxidative imbalance are key components in the pathogenesis of diseases such as

Alzheimer's disease, Parkinson's disease, brain tumors, and multiple sclerosis (Marchetti and Abbracchio, 2005; Kannappan et al., 2011). The brain and nervous system are thought to be particularly vulnerable to oxidative stress due to limited antioxidant capacity (Marksberry and Lovell 2007). Present in the senile plaque of Alzheimer's disease patients are iron, ferritin, and transferrin (Tuppo and Forman, 2001; Ademosun and Oboh, 2012). Alzheimer's disease (AD) is the major cause of dementia. Cognitive decline in Alzheimer's disease patients is related to progressive cholinergic degeneration since studies have shown increased levels of cholinesterase enzymes in postmortem brain samples of Alzheimer's disease patients (Farfara et al., 2008; Snyder et al., 2001). So a promising approach to treating Alzheimer's disease patient is to enhance the level of cholinergic neurotransmitters in the brain by cholinesterase inhibitors (Scarpini et al., 2003). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are the two major cholinesterase enzymes and they play an important role in decreasing choline levels in the body.

Uraria picta (Jacq.) DC is a woody herb, single-stemmed, fibrous, 1 m high, or more and of grass savanna. It belongs to the family Leguminosae-Papilionoideae. It is common throughout the region in all states and widespread in tropical Africa, India, Asian tropics and Australia. In Nigeria, *U. picta* is locally known as 'Alupayida' (Yoruba), 'Obuodo dumbwada' (Igbo), 'kaskaifi', 'dakushe', 'wutsiyarbera', 'wutsiyarkusu' (Hausa) (Adegoke et al., 1968). The leaves of *U. picta* have a trace of alkaloid and they are considered to be antiseptic. It is also used in treating malaria (Adegoke et al., 1968). The dried powder is used in treating gonorrhea and for contractions of the uterus leading to abortion (Anislie, 1937). Studies have shown that cognitive function can be enhanced traditionally by some plants (Howes and Houghton, 2003), and *U. picta* has promising potential. However, there is dearth of information on inhibition of cholinesterase enzymes by *U. picta*. This study therefore sought to investigate the plant as a medicinal intervention in the management of Alzheimer's disease.

MATERIALS AND METHODS

Plant

U. picta leaves were sourced locally in Akure town and authentication was done in the Department of Biology, Federal University of Technology Akure, Nigeria.

Extract preparation

The aqueous extract of the medicinal plant was prepared by homogenizing 1 g of the plant in 20 ml-distilled water and the homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was subsequently freeze-dried and used for subsequent assays (Oboh et al., 2010).

Reagents

All chemicals used in this study were of analytical grade, and glass-distilled water was used.

AChE and butyrylcholinesterase (BChE) inhibition assay

Inhibition of AChE was assessed by a modified colorimetric method of Perry et al. (2000). The AChE activity was determined in a reaction mixture containing 200 μ l of a solution of AChE (0.415 U/ml in 0.1 M phosphate buffer, pH 8.0), 100 μ l of a solution of 5,5'-dithio-bis(2-nitrobenzoic) acid (3.3 mM in 0.1 M phosphate-buffered solution, pH 7.0) containing NaHCO₃ (6 mM), extract dilutions (0 to 100 μ l), and 500 μ l of phosphate buffer, pH 8.0. After incubation for 20 min at 25°C, acetylthiocholine iodide (100 μ l of 0.05 mM solution) was added as the substrate, and AChE activity was determined with an ultraviolet spectrophotometer from the absorbance changes at 412 nm for 3.0 min at 25°C.

Hundred microliters of butyrylthiocholine iodide was used as a substrate to assay butyrylcholinesterase enzyme, while all the other reagents and conditions were the same. The AChE and BChE inhibitory activity was expressed as percentage inhibition.

Determination of total phenol content

The total phenol content was determined according to the method of Singleton et al. (1999). In brief, appropriate dilution of the extract was oxidized with 2.5 ml of 10% (v/v) Folin-Ciocalteu reagent and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C, and the absorbance was measured at 765 nm in a spectrophotometer. The total phenol content was subsequently calculated as gallic acid equivalents (GAE).

Determination of total flavonoid content

The total flavonoid content was determined using a slightly modified method reported by Meda et al. (2005). In brief, 0.5 ml of appropriately diluted sample was mixed with 0.5 ml of methanol, 50 μ l of 10% AlCl₃, 50 μ l of 1 M potassium acetate, and 1.4 ml of water and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm; the total flavonoid content was subsequently calculated. The non flavonoid polyphenols were taken as the difference between the total phenol and total flavonoid content. Quercetin was used as standard.

ABTS radical scavenging ability

The ABTS* scavenging ability of the extract was determined according to the method described by Re et al. (1999). The ABTS* was generated by reacting an (7 mmol/L) ABTS aqueous solution with K₂S₂O₈ (2.45 mmol/L, final concentration) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.700 with ethanol. 0.2 ml of appropriate dilution of the extract was added to 2.0 ml ABTS solution and the absorbance was measured at 734 nm after 15 min. Trolox was used as standard and trolox equivalent antioxidant capacity (TEAC) was subsequently calculated.

Fenton reaction (degradation of deoxyribose)

The method of Halliwell and Gutteridge (1981) was used to determine the ability of the extract to prevent Fe²⁺/H₂O₂-induced

Table 1. Total phenol content, total flavonoid content, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging ability and IC₅₀ values of aqueous extract of *Uraria picta*.

Extract	Value
Phenol content (mg/100 g)	25.24 ± 1.67
Flavonoid content (mg/100 g)	19.43 ± 0.91
ABTS*scavenging ability (mmol/TEAC g)	0.233 ± 0.02
IC ₅₀ AChE (mg/ml)	39.12 ± 1.01
IC ₅₀ BChE (mg/ml)	39.02 ± 1.08

Values represent means ± standard deviation of triplicate readings.

decomposition of deoxyribose. The extract (0 to 100 µl) was added to a reaction mixture containing 120 µl of 20 mM deoxyribose, 400 µl of 0.1 M phosphate buffer, and 40 µl of 500 µM FeSO₄. The volume was made up to 800 µl with distilled water. The reaction mixture was incubated at 37°C for 30 min, and the reaction was then stopped by addition of 0.5 ml of 28% trichloroacetic acid. This was followed by addition of 0.4 ml of 0.6% thiobarbituric acid (TBA) solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm in a spectrophotometer.

Data analysis

The results of triplicate experiments were pooled and expressed as mean ± standard deviation (SD) (Zar, 1984). Statistical significance was determined by Student's t-test and IC₅₀ (inhibitory concentration) was determined using linear regression analysis.

RESULTS

The AChE inhibitory potential of *U. picta* extract was investigated and the result is shown in Figure 1; the result revealed that the extract inhibited AChE activity in a dose-dependent manner, having an inhibitory activity of 80.56% at the highest concentration (57.69) tested [IC₅₀ (inhibitory concentration) = 39.12 mg/ml] as presented in Table 1. Also, the ability of the extract to inhibit BChE activity *in vitro* was also investigated, and the result shown in Figure 2 revealed that the extract inhibited BChE in a dose-dependent manner with inhibitory activity of 77.27% at the highest concentration (57.69) tested [IC₅₀ = 39.02 mg/ml] (Table 1).

The results of the total phenol, total flavonoid content and ABTS radical scavenging ability of the extract which was reported as TEAC are shown in Table 1. The extract contained 25.24 mg/100 g total phenolic content and 19.43 mg/100 g total flavonoid content. The extract was able to scavenge ABTS radical having a scavenging ability of 0.233 mmol/TEAC g. Furthermore, the result of the hydroxyl radical (OH*) scavenging ability of the extract is as shown in Figure 3; the extract scavenged OH* in a dose-dependent manner having a scavenging ability of 84.96% at the highest concentration (71.43 mg/ml) tested.

DISCUSSION

AChE and BChE inhibition have been accepted as an effective model for treating/managing AD (Howes et al., 2003). The aqueous extract of the *U. picta* studied was able to inhibit AChE and BChE in a dose dependent manner. Cholinesterases inhibition by the *U. picta* studied could be of great importance as it is an acceptable therapeutic way in the management/treatment of neurodegenerative conditions. Also, in some forms of Alzheimer's disease, BChE variant has been shown to increase brain susceptibility, thereby making BChE inhibition of the extract an alternative approach in managing neurodegenerative conditions.

Once AChE is inhibited, acetylcholine breakdown in the brain becomes impossible. The consequent increase in the brain acetylcholine concentrations facilitates communication between nerve cells that use acetylcholine as a chemical messenger, and this may improve or stabilize the symptoms of Alzheimer's disease temporarily (Howes et al., 2003). Aqueous extract of *U. picta* was able to inhibit AChE and BChE activities in a dose-dependent manner *in vitro*. This AChE and BChE inhibition is in agreement with some earlier reports where plant phytochemicals from *Citrus medica* inhibited AChE and plants extracts of *Ginkgo biloba* and *Salvia lavandulaefolia*, showed a significant improvement in cognitive performance and memory (Mazza et al., 2006; Maruyama et al., 2006; Akhondzadeh and Abbasi 2006). This also agreed with a work reported by Oboh et al. (2010) where red and white ginger inhibited AChE activity *in vitro*. The ability of the extract to inhibit AChE and BChE could be due to the antioxidant ability of the plant.

Free radicals produced in the body can be neutralized/scavenged by the help of antioxidants (Oboh et al., 2007). Phenolic compounds can protect the body against free radicals, whose formation is associated with the normal metabolism of aerobic cells. These phenolics have the ability to remove free radicals, activate antioxidant enzymes, chelate metal catalysts, reduce α-tocopherol radicals and inhibit oxidases (Amic et al., 2003). Many plants have flavonoids and studies have conclusively shown that the majority of the antioxidant activity maybe from compounds such as flavonoids, isoflavones, flavones, anthocyanins, catechin and isocatechin rather than from vitamins C, E and β-carotene (Marin et al., 2004; Oboh et al., 2007). Antioxidant activity of flavonoid makes them useful in lowering cellular oxidative stress (Oboh et al., 2007). Also, the free radical scavenging ability of the extract was investigated using a moderately stable nitrogen-centred radical species (ABTS) (Re et al., 1999). The aqueous extract exhibited scavenging ability and this is in agreement with the flavonoid content and some reports where correlations were reported between flavonoid content and antioxidant capacity of some plant foods (Amic et al., 2003), this also agrees with previous work where methanolic extract of *U. picta* showed free radical

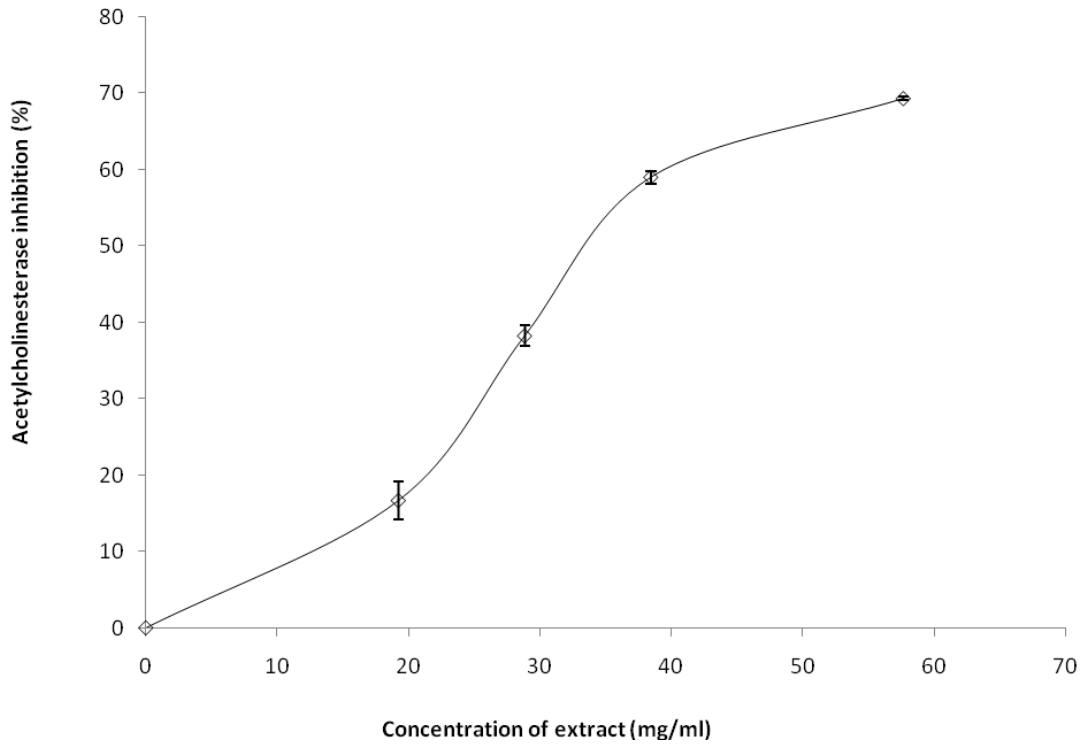


Figure 1. Acetylcholinesterase inhibitory activity of aqueous extract of *Uraria picta*.

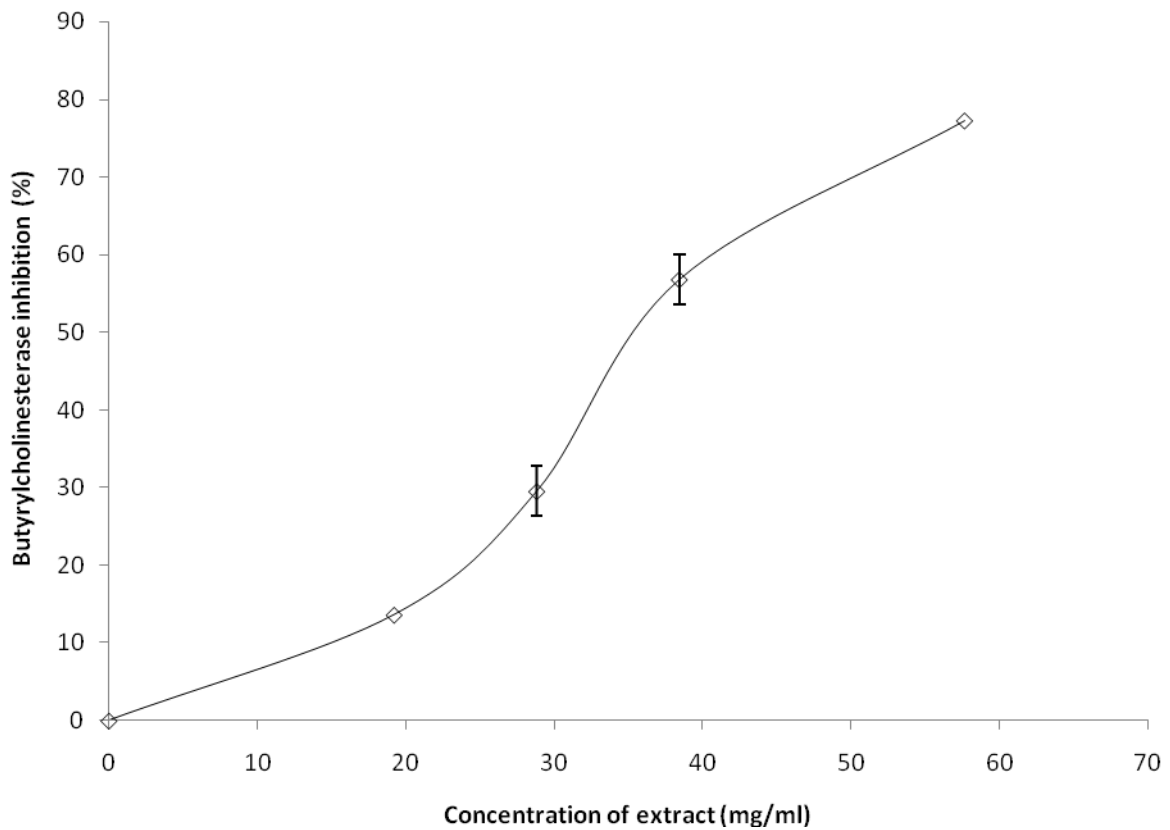


Figure 2. Butyrylcholinesterase inhibitory activity of aqueous extract of *Uraria picta*.

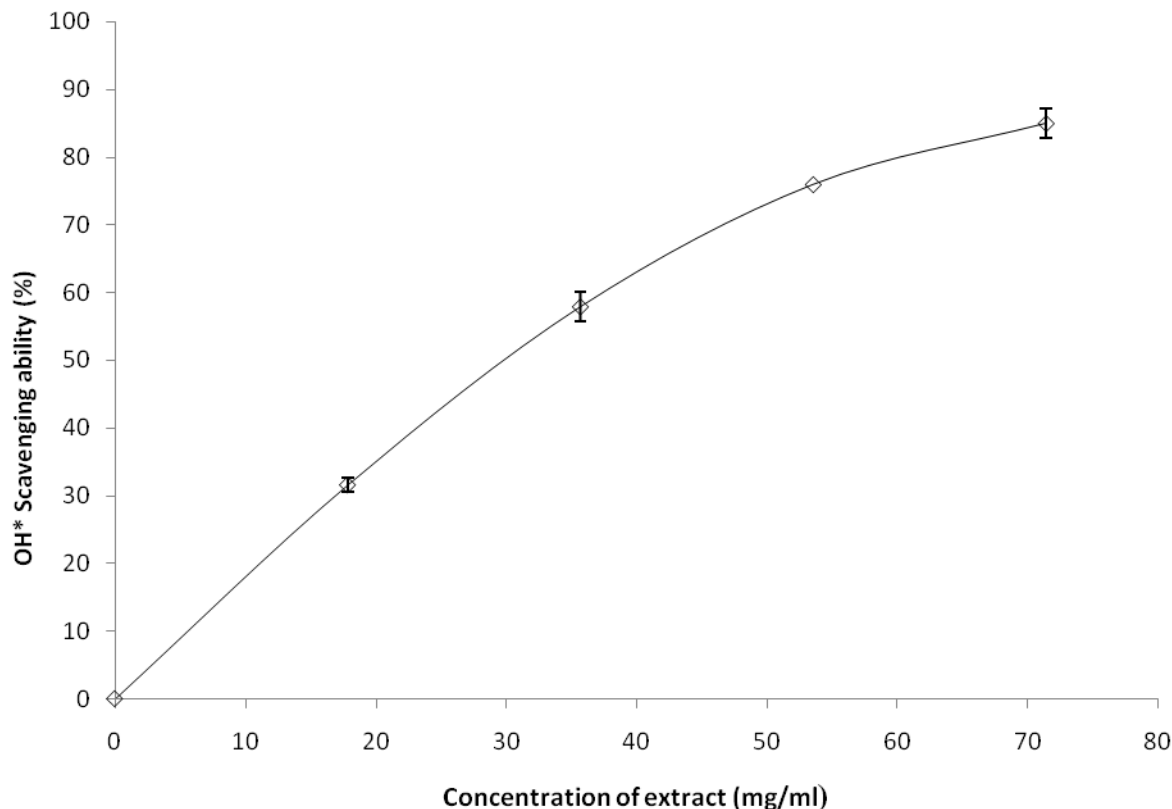


Figure 3. Hydroxyl radical (OH*) scavenging ability of aqueous extract of *Uraria picta*.

scavenging ability (Patel et al., 2011). Oxygen radicals are produced by iron in Fenton reaction. An electron is then donated to hydrogen peroxide by iron converting Fe^{2+} to Fe^{3+} , bringing about radicals. Present in the senile plaque of Alzheimer's disease patients are iron, ferritin, and transferrin (Tuppo and Forman, 2001; Ademosun and Oboh, 2012). Production of superoxide is catalyzed by a membrane-associated enzyme known as NADPH oxidase (Azumi et al., 1999). NADPH oxidase generates superoxide by transferring electrons from NADPH inside the cell across the membrane. Superoxide anion produced is a reactive free radical, which can lead to the production of hydrogen peroxide spontaneously, this will then undergo further reactions to generate reactive oxygen species, and it can also generate hydroxyl radicals. The extract, however, scavenged hydroxyl radicals in a dose-dependent manner. This result is important since oxidative damage is a pathogenesis in Alzheimer's disease and studies show that hydrogen peroxide mediates oxidative damage (Behl et al., 1994; Kim et al., 2003). Recent studies have shown that polyphenols have neuroprotection against damage caused by Fenton reaction (Heo and Lee, 2004; Ademosun and Oboh, 2012) and that they are able to cross the blood-brain barrier (Youdim et al., 2004; Abd El Mohsen et al., 2002; Ademosun and Oboh, 2012).

Conclusions

In these findings, it can be concluded that aqueous extract of *U. picta* inhibited AChE and BChE in a dose-dependent manner and exhibited radical scavenging ability due to the phytochemicals present in the extract and this could be part of the mechanism through which the extract helps in treating/managing Alzheimer's disease.

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