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Formation of polymeric films containing supersaturated levonorgestrel contraceptive drug by transdermal metered dose aerosol

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Accepted 23 August, 2013

Due to the benefits of transdermal sprays, formulation of levonorgestrel as a transdermal metered dose aerosol was studied in this work. Simultaneously, super-saturation was evaluated as an effective method to increase the transfer of active ingredients to the skin. By using anti-nucleant polymers and solvent evaporation after spraying, we could form films containing supersaturated drug after spraying the formulation and inhibiting crystallization. Film formation and visual properties of films were investigated. The kinetics of weight loss and super saturation after film formation was calculated and finally, the rate of drug release from cellulose acetate membrane was assessed. In the formulations containing higher amounts of polyvinyl pyrrolidone (PVP) and 4 mg of levonorgestrel, 84.31% of drug was released during 4 h. While, in formulation with 2 mg drug, the maximum 95.79% of the drug was released during that time. According to these results, this kind of transdermal metered dose aerosol could be suggested as a suitable form for delivery of levonorgestrel through the skin.

Key words: Transdermal delivery, levonorgestrel, topical aerosol, supersaturation.

INTRODUCTION

Transdermal delivery is generally considered as a noninvasive and acceptable route of drug administration that provides continuous penetration of drugs through the intact skin (Fan et al., 2008; Chen et al., 2010). In this drug delivery approach, number of physical, chemical and biochemical methods have been suggested to improve the transportation of drug through the skin. All these attempts are aimed to reduce the barrier properties of stratum corneum or increase the diffusion properties of the drug (Raghavan et al., 2000; Wokovich et al., 2006).

Enhancing permeation into the skin via supersaturation of the drug has previously been shown to improve the efficiency of topical drug release (Raghavan et al., 2001a; Valenta and Auner, 2004; Moser et al., 2001). Supersaturation is achieved when a compound is solubilised at a concentration which is greater than the saturated equilibrium solubility. The increased concentration of drug in a vehicle above saturation leads to a greater thermodynamic activity which proportionally increases the rate at which the drug can pass through the skin (Reid et al., 2008; Jones et al., 2009). It has been shown that metered dose aerosol (MDA) formulations can be applied for formation of topical films by solvent evaporation to induce supersaturation of therapeutic agents (Lulla et al., 2004). Supersaturation in these transient systems is driven by evaporation of the propellant during dose actuation and disappearance of the co-solvent once the dose has reached the skin. Although these
these types of systems are considered to have high levels of organic solvents, due to their highly volatile nature and short time spent on the surface of the skin, they are not thought to pose an irritation risk (Jones et al., 2009).

Transdermal delivery of estrogens and progestogens for contraception and hormone replacement therapy has been evaluated in numerous researches (Agrawal and Pruthi, 2011; Burkmann, 2007; Raynaud, 2005). The use of long acting implants and patches are currently two conventional methods that are available in the pharmaceutical markets for this indication (Chrisman et al., 2006; Mansour et al., 2011; Toole et al., 2002; Caruso, 2003). In this way, application of topical metered dose aerosols to provide greater transdermal dosing flexibility and overcome some of the dosing limitations of current transdermal delivery dosage forms has been proposed in the recent years (Fraser et al., 2007).

The aim of this study was to investigate topical formulations that enhance release of levonorgestrel as a contraceptive drug from polymeric films via transient supersaturation and to determine the degree of supersaturation for this system after application. Levonorgestrel is a synthetic derivative of the progesterone hormone. It is thought to prevent pregnancy in three different ways, depending on the stage of the menstrual cycle at which unprotected intercourse occurs: it suppresses ovulation, inhibits the fertilization of any egg already released, and may also cause changes to the endometrium to prevent a fertilized egg implanting (Schindler et al., 2003). Metered dose aerosols that contained levonorgestrel, hydroxyfluoroalkane as propellant, ethanol as volatile cosolvent and a film forming polymer was developed to deliver drug. Efficacy of poly vinyl pyrrolidone (PVP) and hydroxy propyl methyl cellulose (HPMC) to form films containing supersaturated drug were compared and attempts were made to determine the differential scanning (DS) by testing drug release from the formulations through regenerated cellulose membranes.

**MATERIALS AND METHODS**

Levonorgestrel was kindly gifted by Iran hormone Co. (Iran). Absolute ethanol (99.7 to 100%) was purchased from Bidestan (Iran). HPMC (methocel E5) was from Colorcon (England) and PVP K30 was purchased from Fluka (Switzerland). 1,1,1,2-tetrafluoroethane (HFA 134a) propellant was kindly donated by INEOS Flur (UK). Tween 80 was supplied by Merck (Germany). Regenerated cellulose membrane (RCM) (2000 Da molecular weight cut-off) was purchased from Spectrum (USA).

**Formulation and preparation of metered dose aerosol**

A solution was prepared by mixing film former polymer (PVP or HPMC), the drug substance and pure ethanol and was left to stir overnight to allow the polymer to solvate. An aliquot of the solution was dispensed into an aerosol container (Bespak Europe Ltd, UK). A 63 µl valve was immediately crimped onto each container and the canister was filled with HFA 134a through the valve using a 3,4-methylenedioxymethylamphetamine (MDA) filler (Pamasol Willi Mader AG, Switzerland) until the desired weight was obtained. The compositions of a series of formulations are shown in Table 1. The concentration of levonorgestrel was adjusted to 33 µg per puff. The concentration of pure ethanol was fixed to a maximum of 10.0% (w/w), to minimize the effect on the vapor pressure and consequently on the release velocity of the liquefied propellant throughout the orifice. To evaluate film forming of polymers, sprays were applied onto a plastic plate and the films were characterized after evaporation of solvents.

**Optical microscopy**

The morphology of produced films was studied with an optical microscope (Euromex, Netherlands) equipped with a camera system (Sony, Japan). The samples were prepared by spraying the formulations into glass plates and were observed by 40× lens and then took photographs by the camera.

**Measurement of evaporation rate and degree of saturation**

Thirty actuations from every metered dose aerosol were applied to an aluminum stage on an analytical balance (Sartorius, Germany) and monitored for weight loss after application. Weight of the formulation was plotted against time and the rate of solvent evaporation was calculated using a line of best fit over at least three time points. The final weight of the film was compared to the weight at a set time point to calculate the weight of the remaining ethanol at that time, and this was used to determine the concentration of drug, as described previously by Jones et al. (2009). By comparing this value with the saturated solubility of the drug in ethanol, degree of saturation was obtained using equation 1:

$$DS = \frac{WD_{App}}{WF_{t} - WF_{Final}}$$

Where $WD_{App}$ (mg) was the weight of the drug applied, $WF_{t}$ (g) was the weight of the formulation at the time point t, and $WF_{Final}$ (g) was the final weight of the formulation after 4 h. This gave a concentration (mg of drug/g of solvent) at time t, which was then divided by the saturated solubility concentration of the drug in the solvent, $C_{SS}$ (mg/g). If the concentration at time t was greater than the saturated solubility, then the formulation was classified as supersaturated. The degree of saturation was plotted against time to assess degree of saturation kinetics over the time of the experiment.

**Drug release studies**

The in vitro drug release was evaluated using United States Pharmacopeia (USP) 23 dissolution test apparatus 5 (paddle over disk). It was performed using a dissolution tester (Erweka, Switzerland) and the dissolution medium comprised 500 ml
Table 1. Composition of formulations for preparation of transdermal metered dose aerosols.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Polymer type</th>
<th>Amount of polymer (%)</th>
<th>Amount of drug (mg)</th>
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<tbody>
<tr>
<td>F1</td>
<td>PVP K30</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>F2</td>
<td>PVP K30</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>F3</td>
<td>PVP K30</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>F4</td>
<td>PVP K30</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>F5</td>
<td>PVP K30</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>F6</td>
<td>PVP K30</td>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>F7</td>
<td>PVP K30</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>F8</td>
<td>PVP K30</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>F9</td>
<td>HPMC</td>
<td>0.10</td>
<td>1</td>
</tr>
<tr>
<td>F10</td>
<td>HPMC</td>
<td>0.10</td>
<td>1.5</td>
</tr>
<tr>
<td>F11</td>
<td>HPMC</td>
<td>0.10</td>
<td>2</td>
</tr>
<tr>
<td>F12</td>
<td>HPMC</td>
<td>0.10</td>
<td>2.5</td>
</tr>
<tr>
<td>F13</td>
<td>HPMC</td>
<td>0.20</td>
<td>1</td>
</tr>
<tr>
<td>F14</td>
<td>HPMC</td>
<td>0.20</td>
<td>1.5</td>
</tr>
<tr>
<td>F15</td>
<td>HPMC</td>
<td>0.20</td>
<td>2</td>
</tr>
<tr>
<td>F16</td>
<td>HPMC</td>
<td>0.20</td>
<td>2.5</td>
</tr>
</tbody>
</table>

of 5 microgram poly sorbate 80/g water maintained at a temperature of 35.0°C and a paddle rotation speed of 50 rev/min. For sample preparation, firstly, the synthetic cellulose membrane was soaked in deionized water. Then 5 puffs of aerosol were applied to the disk surface and the film surface was covered with membrane and was sealed. The disk containing the sample was submerged into the dissolution medium. Five milliliters of sample were collected at predetermined time intervals over 4 h. The drug concentration was measured by an ultra violet (UV) spectrophotometer (Spekol, Germany) according to USP monograph for levonorgestrel.

Differential scanning calorimetry (DSC)

Thermal behavior of raw materials and the selected films containing active substance were studied quantitatively and qualitatively by differential scanning calorimetry (DSC 204 F1, Netzsch, Germany). The samples (7 to 12 mg) were accurately weighed into standard aluminum pans and sealed. Thermograms were recorded during heating and cooling runs at a scan rate of 10°C min⁻¹ between 25 and 300°C.

RESULTS AND DISCUSSION

Supersaturation of a drug in a topical formulation is one approach by which skin penetration enhancement can be achieved without the use of exogenous chemical enhancers and expensive complicated technologies. In a supersaturated state, the saturation solubility of the compound in its formulation exceeds the equilibrium solubility and the driving force for diffusion is elevated and therefore a higher flux across the skin membrane can be achieved. However, such a system must remain physically stable, and minimal crystallization of the drug should occur during the permeation process. The addition of anti-nucleant polymers to supersaturated formulations prevents crystal nucleation or growth and thus maintains the elevated thermodynamic activity. Also, anti-nucleant polymers can extend the time of supersaturated solution stability and also facilitate the generation of higher levels of supersaturation. Addition of PVP and HPMC to supersaturated solutions has been reported to be effective in crystal growth inhibition of drugs (Megrab et al., 1995).

In the present study, antinucleant effects of these two polymers were investigated for formation of topical films containing supersaturated levonorgestrel. For this purpose, primarily, formulations containing levonorgestrel, film-forming polymer and ethanol were prepared and behavior of each component within the solution was assessed by visual solubility experiments. It was found that all formulations resulted in the formation of clear solutions after stirring for 24 h. However, formulations which contained relatively higher concentrations of drug or polymer than the amounts mentioned in the table did not form transparent solutions.

After addition of propellants to the solutions and preparation of metered dose aerosols, it was found that the formulations F11 to F16 containing higher levels of HPMC was not stable and the drug and polymer were precipitated in the cans. Appearance of other films was evaluated following actuation of spray onto a transparent plastic plate. Also, optical microscopy was applied for detailed evaluation of film integrity. The results showed
that all of the formulations could produce tough films with smooth surfaces. However, some fine air bubbles could be detected in all films under microscope as presented in Figure 1. In the case of F11, which contains HPMC films, some agglomerations were detected that could be attributed to the formation of drug nuclei after supersaturation.

The weight loss profiles of selected metered dose aerosols after application were plotted as a function of time to explore supersaturation kinetics. As shown in Figure 2, there were three obvious gradients in the weight loss profiles of PVP formulations. One immediately post dose application, a second after approximately 2 min and the third after 10 min. These three regions in the evaporation profile have previously been defined as Hydrofluoro-alkane loss (first region) followed by co-solvent loss (second region) and finally hardening of the film (no weight loss in the third region) (Jones et al., 2009; Stein and Myrdal, 2006). The trend of weight loss is relatively similar in the formulations containing PVP and the difference between graphs may be related to the different weights of solid materials in the formulations. However, there is a moderate decrease in the rate of weight loss with increase of polymer in the formulations. This relatively slower rate of weight loss from F1 to F8 would be attributed to the proportion of ethanol to polymer. Ethanol molecules must diffuse through the polymer to reach the surface of the film and escape. Increasing the viscosity of a solution via the addition of PVP will reduce the molecular diffusion and possibly lead to ethanol depletion at the air–liquid interface and thus a reduction in evaporation rate (Aronson et al., 2004). Weight losses in the HPMC films were much faster than PVP films and the slope of graphs is very sharp (Figure 3). In these formulations, second phase of weight loss profile could not be detected and it seems that ethanol evaporation occurs in a short time. It could be related to the low concentrations of HPMC in the formulations and lower affinity of ethanol to this polymer (Kim et al., 2006).

Monitoring the evaporation of solvents enabled theoretical calculation of the degree of drug supersaturation in the films over time (Figures 4 and 5). The saturation kinetics was different as a consequence of the altered evaporation profiles and film compositions after loss of the HFA. Formulation containing PVP were supersaturated after dose actuation and during 60 min of study; while the HPMC formulations were highly supersaturated during 10 min after actuation.

Anti-nucleant polymers such as PVP and HPMC are thought to prevent crystallization through an increase in solution viscosity which slows molecular diffusion and prevents seed nucleation (Raghavan et al., 2001b). However, a chemical interaction between PVP and the drug caused by the adsorption and the orientation of the polymer at the solid/liquid interface of the crystal as it forms makes the antinucleant capability of a polymer more efficient (Megrab et al., 1995; Sekikawa et al., 1978). Although some drug nuclei were observed in the films generated by the HPMC during the time course of the experiments, the drug in the films did not necessarily return to saturated drug concentrations immediately. The quantity of drug remaining in solution is dependent upon the rate at which the drug recrystallizes and thus the potential for enhanced drug release could still exist. Although
it was critical for HPMC formulations, melting endotherm of drug was not observed in DSC thermo-grams (Figure 6). It was attributed to the difference between crystalline structures of unprocessed sample of levonorgestrel and drug nuclei in the film. Observing such an endotherm was not expected for PVP films and it was not detected.

The profiles of drug release from the films confirmed these findings and it was deduced that release of drug...
Figure 5. Theoretical degrees of saturation drug (DS) after dose delivery from selected metered dose aerosols containing different weight ratios of HPMC.

Figure 6. DSC thermograms of raw materials and selected films containing PVP and HPMC and levonorgestrel.
from PVP films was drastically higher than both release from HPMC films and dissolution of unprocessed drug (Figure 7). In the formulations containing higher amounts of PVP and 2.5 mg of levonorgestrel (F_8), 84.31% of drug was released during 4 h while in formulation with 2 mg drug (F_7), the maximum 95.79% of the drug was released during that time. In other word, PVP could play its role as an antinucleant polymer for supersaturation of levonorgestrel, and it could probably control the release of drug by a combination of physical properties and supersaturation kinetics.

**Conclusion**

Transdermal metered dose aerosol could be considered as an efficient dosage form for delivery of supersaturated levonorgestrel as a potent contraceptive. This drug delivery system was specifically formulated such that the solution sprayed onto the skin was readily taken into the skin, and rapidly evaporates from the surface, thereby leaving the surface of the skin dry within less than 1 min of application. Furthermore, utilization of PVP as antinucleant resulted in the formation of satisfactory films capable to provide supersaturation of drug and improvement of drug release.

**ACKNOWLEDGMENTS**

This study was funded and supported by Tehran University of Medical Sciences (TUMS). The authors would like to thank Dr. Khosrow Malek Khosravi, Mohsen Nabi Meybodi, Shahryar Abashzadeh and Araz Sabzevari for their help and technical support.

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Antihyperglycemic and antioxidant potential of oil from Arachis hypogaea L. in streptozotocin-nicotinamide induced diabetic rats

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In this investigation, antihyperglycemic and antioxidant potential of oil of seeds of Arachis hypogaea (AHO) in streptozotocin-nicotinamide (STZ) induced type 2 diabetic rats was observed along with gas chromatography flame ionization detector (GC-FID) analysis. AHO was orally administered to diabetic rats to study its effect in both acute and chronic antihyperglycemic study. The body weight, oral glucose tolerance test and biochemical parameters: glucose level, insulin level, liver glycogen content, glycosylated hemoglobin and antioxidant parameters were estimated for all treated groups and compared against diabetic control group. GC-FID analysis showed the presence of major constituents as oleic acid (48.4500%), linoleic acid (32.6355%), palmitic acid (12.6988%) and arachidic acid (4.0814%). AHO (500 and 1000 mg/kg) and glibenclamide (0.6 mg/kg) in respective groups of diabetic animals administered for 28 days reduced the blood glucose level in streptozotocin-nicotinamide induced diabetic rats. There was significant increase in body weight, liver glycogen content, plasma insulin level and decrease in the blood glucose and glycosylated hemoglobin in test groups as compared to control group. In vivo antioxidant studies on STZ-nicotinamide induced diabetic rats revealed decreased malondialdehyde (MDA) and increased reduced glutathione (GSH). Thus the investigation results that oil of seeds of A. hypogaea have significant antihyperglycemic and antioxidant activity.

Key words: Seeds, Streptozotocin, Essential oil, MDA.

INTRODUCTION

Nowadays, herbal remedies have become the popular source of medicines due to lesser adverse reactions (Kumar et al., 2012) and various other reasons. There are thousands of plants used over centuries for the treatment of various diseases. Species of the genus Arachis is one of the important medicinal plants used in various systems of medicine (Velisek et al., 1995). Peanut (Arachis hypogaea L.) is one of the world’s most important legumes which is grown primarily for its high quality edible oil and protein. It is an important legume Africa (SA) (Reddy et al., 2003). Peanut is an annual herbaceous plant growing 30 to 50 cm tall (Sharma et al., 2000). Diabetes is growing with a high speed in India and has become a capital of the world which is affecting all age groups of people (Mohan et al., 2007). There were an estimated 40 million persons with
diabetes in India in 2007 and this number is predicted to rise to almost 70 million people by 2025 according to Diabetes Atlas published by the International Diabetes Federation (IDF) (Kumar et al., 2012). The country with the largest number of diabetic people will be India by 2030.

Due to these sheer numbers, the economic burden due to diabetes in India is amongst the highest in the world (Sicree et al., 2006). Diabetes is of mainly three types: Type I, type II, and Gestational. Type II diabetes is the most common type, accounting for 90 to 95% of all diabetic cases. So the main concern for management of this type of diabetes is very essential. Some studies have suggested that essential oils may be useful in the treatment of type II diabetes mellitus and various oils have been used as therapeutic agents for years without any significant adverse health effects (Pandey et al., 2011). Peanuts contain monounsaturated and polyunsaturated fats that keep the heart healthy. A good level of both monounsaturated and polyunsaturated fats results in lowering blood cholesterol levels, and thereby reducing the risk of coronary heart diseases. It is used as a high source of plant protein. The antioxidant polyphenols, primarily a compound called p-coumaric acid and oleic acid not only protect the heart but inhibit the growth of free radicals, keeping infection at bay. It is also a rich source of minerals like magnesium, phosphorus, potassium, zinc, calcium, sodium, etc.; thus ensuring a healthy heart and ensuring minimized risk of mineral deficient diseases (Yen et al., 2005).

The seed oil of peanut has been widely used in the food industries from centuries. As far as we know, the effect of oil on antihyperglycemic and antioxidant potential in diabetic animal models has not been investigated scientifically till date. In light of these findings, we carried out this study for the evaluation of antihyperglycemic and antioxidant potential of oil of seeds of A. hypogaea.

MATERIALS AND METHODS

Drugs and chemicals

The chemicals and reagents used in the study were glibenclamide (Torrent Pharmaceutical, Ahmadabad), streptozotocin, heparin (SRL, India), ethylenediaminetetraacetic acid (EDTA) (Hi-media Lab. Pvt Ltd., Mumbai, India), Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid); DTNB), sodium sulphate, methanol, pyridine, anthrone, thiourea, benzoic acid, sodium chloride (SD Fine Chem Ltd., Mumbai, India). All the chemicals used in the study were of analytical grade.

Isolation of oil

The dried seeds of A. hypogaea were purchased from oil and seed section of Chaudhary Charan Singh Haryana Agriculture University, Hisar, India. The seeds were crushed and oil was extracted with the help of Clevenger apparatus using hydrodistillation (with water) technique using 4 kg of seeds. The percentage yield of light yellow colored oil was found to be 42% w/v.

Experimental animals

Healthy albino wistar rats (150 to 250 g) were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (Haryana). The rats were housed in polycarbonate cages (size: 29 × 22 × 14 cm) under standard laboratory conditions (25 ± 3°C; 35 to 60% humidity) with alternating light and dark cycle of 12 h each, and were fed fed with a standard rat pellet diet (Hindustan Lever Ltd, Mumbai, India) and water ad libitum. The experimental protocol was approved by Institutional Animals Ethics Committee (IAEC), and animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (Registration No. 0436).

Gas chromatography flame ionization detector (GC-FID) analysis

Methyl ester analysis was performed on NUCON (Nucon Engineers, New Delhi Company, model no-5700) gas chromatograph using column (10% FFAP). The carrier gas used was nitrogen at a flow rate of 30 ml/min. The oven temperature was kept at 60°C for 1 min programmed to 220°C at a rate of 6°C/min and kept at 200°C for 3 min. Hydrogen flow rate was 30 ml/min, air flow rate was 300 ml/min. The injection volume was 1 µl. Detector used was flame ionization detector and detector temperature was 220°C.

Induction of diabetes

Type II diabetes mellitus (NIDDM) was induced in overnight fasted animals by a single intraperitoneal injection of 50 mg/kg STZ in 0.1 M citrate buffer (pH: 4.5) in a volume of 1 ml/kg body weight 15 min after the i.p. administration of 110 mg/kg nicotinamide. Diabetes was developed and stabilized over a period of 7 days. Diabetes was confirmed by the elevated blood glucose levels determined at 72 h and on 7th day after injection. Only rats confirmed with permanent NIDDM (Glucose level above 250 mg/dl) were used in the study. Blood was collected by intraocular route (Marudamuthu et al., 2008).

Experimental design

Rats were divided into the following groups comprising six rats in each group after the induction and confirmation of diabetes.

For acute antihyperglycemic model

The diabetes induced rats were used for the acute antihyperglycemic study which was carried out for a period of 4 h to check whether the oil have some effect or not. Five groups of animals were taken and labeled as Group 1: Normal rats; Group 2: Diabetic control; Group 3: Diabetic animals were administered glibenclamide (0.6 mg/kg p.o); Group 4: Diabetic animal were administered orally 500 mg/kg of AHO; Group 5: Diabetic animal were administered orally 1000 mg/kg of AHO.
For chronic antihyperglycemic model

In the chronic antihyperglycemic models the study was carried out for 28 days to study the various parameters of the diabetes to confirm the antihyperglycemic activity of AHO in streptozotocin induced diabetes in rats. The chronic study was performed in the same pattern of animal groups as in the acute chronic antihyperglycemic model except the time period. The animal groups prepared were as Group 1: Normal rats; Group 2: Diabetic control; Group 3: Diabetic animals administered glibenclamide (0.6 mg/kg p.o); Group 4: Diabetic animal administered orally 500 mg/kg of AHO; Group 5: Diabetic animal administered orally 1000 mg/kg of AHO.

Blood sample collection

The 24 h fasted animals were sacrificed by cervical decapitation on 28th day of treatment. The trunk was cut with the scissors and blood was collected in heparinized tubes. The plasma was obtained by centrifugation at 5,000 rpm for 5 min for the determination of biochemical parameters; glucose, insulin, cholesterol etc.

Estimation of plasma glucose and cholesterol

Plasma cholesterol and glucose level were measured by commercial supplied biological kit Erba Glucose Kit (GOD-POD Method) and Erba Cholesterol Kit (CHOD-PAP Method), respectively using Auto-analyser (Chem 5 Plus-V2, Erba Mannhein, Germany) in plasma sample prepared as above. Glucose and cholesterol values were expressed as mg/dl blood sample.

Estimation of glycosylated hemoglobin (Hb1Ac)

Glycosylated hemoglobin was measured using commercial supplied biological kit (Erba Diagnostic) in plasma sample prepared as above using Chem 5 Plus-V2 Auto-analyser (Erba Mannhein Germany). Values were expressed as the percentage of total hemoglobin.

Estimation of liver glycogen content

Liver glycogen estimation was performed as described by Morris et al. (1948). Immediately after excision from the animal, 1 g of the liver was dropped into a previously weighed test tube containing 3 ml of 30% potassium hydroxide solution. The weight of the liver sample was determined. The tissue was then digested by heating the tube for 20 min in boiling water bath, and following this the digest was cooled, transferred quantitatively to a 50 ml volumetric flask, and diluted to the mark with water. The contents of the flask were then thoroughly mixed and a measured portion was then further diluted with water in a second volumetric flask so as to yield a solution of glycogen of 3 to 30 μg/ml. Five ml aliquots of the final dilution were then pipetted into Evelyn tube and the determination with anthrone was carried out. The amount of glycogen in the aliquot used was then calculated using the following equation:

$$\mu g \text{ of glycogen in aliquot} = 100 \frac{U}{1.11 S}$$

U is the optical density of unknown solution. S is the optical density of the 100 μg glucose and 1.11 is the factor determined by for the conversion of the glucose to the glycogen (Selfter et al., 1950).

In vivo antioxidant activity

Estimation of MDA level

Malondialdehyde (MDA), an index of free radical generation/lipid peroxidation, was determined as described (Okhawa et al., 1979). Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid added to 0.2 ml of blood plasma. The mixture was made up to 4 ml with distilled water and heated at 95°C for 60 min. After cooling the contents under running tap water, 5 ml of n-butanol and pyridine (15:1 v/v) and 1 ml of distilled water was added. The contents were centrifuged at about 3,000 rpm for 10 min. The organic layer was separated out and its absorbance was measured at 532 nm using a double beam UV-Visible spectrophotometer (Systronics 2203, Bangalore, India) against an appropriate blank. MDA values were calculated using the extinction coefficient of MDA-thiobarbituric acid complex 1.56 × 10^5 L/mmol cm and expressed as nmol/ml.

Estimation of reduced glutathione level

The liver samples (200 mg) were homogenized in 8 ml of 0.02 M EDTA in an ice bath. The homogenates were kept in the ice bath until used. Aliquots of 5 ml of the homogenates were mixed in 15 ml test tubes with 4.0 ml distilled water and 1 ml of 50% trichloroacetic acid (TCA). The tubes were centrifuged for 15 min at approximately 3,000 rpm, 2 ml of supernatant was mixed with 4 ml of 0.4 M Tris buffer pH 8.9, 0.1 ml Ellman's reagent [5,5-dithiobis-(2-nitro-benzoic acid)] (DTNB) added and the sample shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results are expressed as μmol GSH/g tissue.

Statistical analysis

The data for various biochemical parameters were evaluated by use of one-way analysis of variance (ANOVA), followed by Dunnett’s test using the software Sigma-Stat 3 (Version 3). In all the tests, the criterion for statistical significance was p < 0.05.

RESULTS

Components of AHO

GC-FID analysis showed the presence of stearic acid (1.2%), oleic acid (48.45%), linoleic acid (32.63%), behenic acid (0.93%), palmitic acid (12.69%), and arachidic acid (4.08%) (Table 1 and Figure 1).

Oral glucose tolerance test

The effect of AHO on plasma glucose level after glucose feeding of 2 g/kg body weight orally to the STZ diabetic rats is expressed in Table 2. The blood glucose level rose to a maximum in 60 min after glucose loading. The oil (500 and 1000 mg/kg body weight) treated groups
Effect of AHO on STZ diabetic rats in chronic study

In chronic study, administration of AHO at the dose of 500 mg/kg body weight to STZ diabetic rats for 28 days showed a fall in plasma glucose level from 355 to 194 mg/dl on 28th day when compared to 0 day values. AHO at the dose of 1000 mg/kg body weight showed a significant ($p < 0.01$) fall in plasma glucose level from 365 to 156 mg/dl on 28th day (Table 4).

Effect of AHO on body weight

An increase in the body weight of normal rats was observed whereas the weight of diabetic control rats decreased from day 1 to day 28. AHO at the dose of 500 and 1000 mg/kg body weight, respectively groups when administered to diabetic rats showed a significant increase in body weight as compared to the diabetic control group ($p < 0.01$) (Table 5).

Effect of AHO on insulin level

Table 5 shows the level of plasma insulin in the control and experimental groups of rats. Diabetic rats showed a significant decrease in plasma insulin compared with normal rats. Following oral administration of AHO, plasma insulin level increased when compared to control rats (Table 6).
Table 2. Effect of A. hypogaea oil in oral glucose tolerance test (OGTT).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Mean blood glucose concentration (mg/dl) ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 min                30 min                60 min                90 min                120 min</td>
</tr>
<tr>
<td>Normal</td>
<td>----</td>
<td>80±2.6               87±2.8               90±3.8               86±2.5               83±2.7</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>----</td>
<td>290±4.6              390±5.3              413.2±4.3            360±2.7              331±2.8</td>
</tr>
<tr>
<td>AHO</td>
<td>500 mg/kg</td>
<td>277±4.8              299±3.9              344±7.2              265±7.6**            256±4.9**</td>
</tr>
<tr>
<td>AHO</td>
<td>1000 mg/kg</td>
<td>288 ± 6.5            321±5.5              388±4.9              260±3.8**            232±7.3**</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test **p < 0.01 vs. diabetic control; AHO: Oil of seeds of Arachis hypogaea.

Table 3. Effect of Arachis hypogaea oil in STZ induced diabetic rats in acute antihyperglycemic study.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Mean blood glucose concentration (mg/dl) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h                           1/2 h                          1 h                           2 h                           4 h</td>
</tr>
<tr>
<td>Normal</td>
<td>--</td>
<td>76±4.2                        80±3.2                        77±2.5                        82±4.1                        79±5.3</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>340.5±10.2                    342±11.3                     346±7.6                      341.0±6.7                     332.0±7.2</td>
</tr>
<tr>
<td>AHO</td>
<td>500 mg/kg p.o</td>
<td>342±8.7                       323.1±6.4                    299±5.6**                    284±4.4**                    277±6.6**</td>
</tr>
<tr>
<td>AHO</td>
<td>1000 mg/kg p.o</td>
<td>334±8.2                       310±4.6*                     290±3.2**                    260±3.5**                    265±4.5**</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S.E.M.; n = 6 in each group. One way ANOVA followed by Dunnett's test *p < 0.05; **p < 0.01 vs. diabetic control; AHO: Oil of seeds of Arachis hypogaea.

Table 4. Effect of Arachis hypogaea oil in STZ induced diabetic rats in chronic antihyperglycemic study.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Mean blood glucose concentration (mg/dl) ± S.E.M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0th day                          7th day                         14th day                        21st day                        28th day</td>
</tr>
<tr>
<td>Normal</td>
<td>--</td>
<td>80±4.2                          79±3.2                         82±2.5                          85.5±4.1                         78±2.1</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>380±7.3                         379±7.6                         384±6.7                         416±7.2                         410±5.4</td>
</tr>
<tr>
<td>AHO</td>
<td>500 mg/kg p.o</td>
<td>355±9.4                         277±9.8**                      256±4.2**                       235±7.2**                       194±7.8**</td>
</tr>
<tr>
<td>AHO</td>
<td>1000 mg/kg p.o</td>
<td>365±8.4                         256±7.8**                      234±4.8**                       201±5.4**                       156±6.6**</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test **p < 0.01 vs. diabetic control; AHO: Oil of seeds of Arachis hypogaea.

Table 5. Effect of Arachis hypogaea oil on body weight in diabetic rats

<table>
<thead>
<tr>
<th>S/No</th>
<th>Treatment</th>
<th>Dose</th>
<th>Initial body weight (g)</th>
<th>Final body weight (g)</th>
<th>Change in weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>--</td>
<td>220±1.1</td>
<td>240±1.5</td>
<td>+20</td>
</tr>
<tr>
<td>2</td>
<td>Diabetic control</td>
<td>--</td>
<td>215±1.8</td>
<td>194±2.0</td>
<td>-21*</td>
</tr>
<tr>
<td>3</td>
<td>AHO</td>
<td>500 mg/kg p.o</td>
<td>250±2.2</td>
<td>260±1.0</td>
<td>+10**</td>
</tr>
<tr>
<td>4</td>
<td>AHO</td>
<td>1000 mg/kg p.o</td>
<td>240±1.3</td>
<td>260±1.4</td>
<td>+20**</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S.E.M.; n = 6 in each group. One way ANOVA followed by Dunnett's test *p<0.01 vs. normal; **p < 0.01 vs. diabetic control; AHO: Oil of seeds of Arachis hypogaea.

Effect of AHO on glycosylated hemoglobin (HbA1c)

The effect of AHO on HbA1c in STZ diabetic rats is shown in Table 6. The level of glycosylated hemoglobin significantly increased (p < 0.01) in diabetic rats as compared to normal control group. The diabetic rats
when treated with AHO for 28 days showed a significant (p < 0.01) decreased level of glycosylated Hb as compared to untreated diabetic group (Table 6).

**Effect of AHO on hepatic glycogen content**

The hepatic glycogen content in diabetic rats decreased sharply as compared to control animal (Table 6). After chronic administration of AHO to diabetic rats, a significant increased (p < 0.01) liver glycogen content as compared to diabetic control group was observed.

**Effect of AHO on lipid profile**

Table 7 shows the level of lipids in normal and tested animals. There was a significant decrease in the level of HDL-cholesterol and a significant increase in the levels of total cholesterol and triglycerides in diabetic rats when compared to normal rats. The administration of AHO reversed the level of lipids significantly (p < 0.05 and p < 0.01).

**Effect of AHO on in vivo antioxidant parameters**

The data depicted in Table 8 shows the effect of oil on plasma malondialdehyde and reduced glutathione level. Plasma MDA level was found to be significantly higher in STZ diabetic rats compared to normal rats. The oil at dose 1000 mg/kg body weight p.o significantly reduced the level of MDA in diabetic rats. Plasma GSH level was found to be significantly lowered in STZ diabetic rats as compared to normal rats. The chronic administration of AHO at 1000 mg/kg body weight significantly increased the level of glutathione in diabetic rats.

---

**Table 6. Effect of Arachis hypogaea oil on glycosylated hemoglobin (HbA1c), hepatic glycogen and insulin in the study.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>HbA1c (% of Hb)</th>
<th>Hepatic glycogen (mg/g wt of tissue)</th>
<th>Insulin (micro U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>--</td>
<td>6±1.4</td>
<td>74±6.6</td>
<td>14±2.1</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>--</td>
<td>11.3±2.4</td>
<td>27±4.5</td>
<td>7.9±1.1</td>
</tr>
<tr>
<td>AHO 500 mg/kg</td>
<td></td>
<td>9±1.1</td>
<td>50±2.3</td>
<td>10±1.2</td>
</tr>
<tr>
<td>AHO 1000 mg/kg</td>
<td></td>
<td>7.3±1.3**</td>
<td>66±3.2**</td>
<td>12.1±2.5*</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S.E.M; n = 6 in each group. One way ANOVA followed by Dunnett’s test *p<0.01 vs. normal; *p < 0.05; **p < 0.01 vs. diabetic control; AHO: Oil of seeds of *Arachis hypogaea*.

**Table 7. Effect of Arachis hypogaea oil on lipid profile.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>HDL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>--</td>
<td>85±1.5</td>
<td>16±2.5</td>
<td>66±1.9</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>--</td>
<td>232±2.4^a</td>
<td>43±3.1^a</td>
<td>37.4±1.2^a</td>
</tr>
<tr>
<td>AHO 500 mg/kg</td>
<td></td>
<td>180±2.2^a</td>
<td>32±1.1**</td>
<td>46±2.1*</td>
</tr>
<tr>
<td>AHO 1000 mg/kg</td>
<td></td>
<td>106±2.3**</td>
<td>21±1.0**</td>
<td>58±1.2*</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S.E.M; n = 6 in each group. One way ANOVA followed by Dunnett’s test *p<0.01 vs. normal; *p < 0.05; **p < 0.01 vs. diabetic control; AHO: Oil of seeds of *Arachis hypogaea*.

**Table 8. Effect of Arachis hypogaea oil on antioxidant parameters (MDA and GSH).**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>MDA (nmol/dl)</th>
<th>GSH (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>--</td>
<td>2.8±0.2</td>
<td>41.2±2.8</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>--</td>
<td>5.4±0.4^a</td>
<td>14±1.15^a</td>
</tr>
<tr>
<td>AHO 500 mg/kg</td>
<td></td>
<td>3.6±0.6</td>
<td>22±1.4</td>
</tr>
<tr>
<td>AHO 1000 mg/kg</td>
<td></td>
<td>3.0±0.1**</td>
<td>34±1.2**</td>
</tr>
</tbody>
</table>

Values are presented as mean ± S.E.M; n = 6 in each group. One way ANOVA followed by Dunnett’s test *p<0.01 vs. normal; **p < 0.01 vs. diabetic control; AHO: Oil of seeds of *Arachis hypogaea*.
DISCUSSION

The aim of this study was to evaluate the antidiabetic and antioxidant potential of the AHO in STZ induced diabetic rats. Diabetes mellitus causes a disturbance in the uptake of glucose as well as glucose metabolism. A dose of STZ as low as 50 mg/kg produces an incomplete destruction of pancreatic beta cells and the rats become permanently diabetic (Aybar et al., 2001). After treatment with a low dose of STZ, many beta cells survive and regeneration is also possible (Gomes et al., 2001). Hyperglycemia generates high levels of free radicals by autoxidation of glucose and protein glycation, and oxidative stress has been reported to be a causative factor of cardiovascular complications in STZ-induced diabetes mellitus (Okutan et al., 2005).

Hyperglycemia is associated with the generation of reactive oxygen species (ROS) causing oxidative damage particularly to heart, kidney, eyes, nerves, liver, small and large vessels and gastrointestinal system (Tunali et al., 2006). The increased levels of plasma glucose in STZ-induced diabetic rats were lowered by AHO administration. The plasma glucose lowering activity was compared with glibenclamide, a standard hypoglycemic drug that stimulates insulin secretion from pancreatic beta cells (Tian et al., 1998). From the results of the present study, it appears that still insulin producing cells are functioning and the stimulation of insulin release could be responsible for most of the metabolic effects. It may be suggested that the mechanism of action of AHO is similar to glibenclamide. The glucose lowering activity of AHO may be related to both pancreatic (enhancement of insulin secretion) and extra pancreatic (peripheral utilization of glucose) mechanisms.

An increase in the level of glycosylated hemoglobin (HbA1c) in the diabetic control group of rats is due to the presence of large amount of blood glucose which reacts with hemoglobin to form glycosylated hemoglobin (Chattopadhyay, 1999). Oxidative stress increases due to the activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C. If diabetes is persistent for long time, the glycosylated hemoglobin is found to increase (Sheela and Augusti, 1992). The level of HbA1C was decreased after the administration of AHO 1000 mg/kg as compared to diabetic control group (**p < 0.01).

In STZ induced diabetes mellitus, the loss of body weight is caused by increase in muscle wasting and catabolism of fat and proteins (Chakravarti et al., 1981). Due to insulin deficiency, protein content is decreased in muscular tissue by proteolysis (Swanson-Flatt et al., 1990). A decrease in body weight was registered in case of STZ diabetic control group rats while in tested groups the weight loss was reversed. Fatty acid mobilisation from adipose tissue is sensitive to insulin. Insulin’s most potent action is the suppression of adipose tissue lipolysis (Campbell et al., 1992). A rise in plasma insulin concentration of only 5 IU/ml inhibits lipolysis by 50%, whereas a reduction in basal insulin levels result in a marked acceleration of lipolysis (Bonadonna et al., 1990). We demonstrated that AHO increased plasma insulin concentrations in diabetic rats. Insulin levels higher than those of the control group may result in inhibition of lipolysis and decreased plasma triglyceride and cholesterol levels. Some studies suggest that the antihyperglycemic action of traditional antidiabetic plant extracts may be due in part to decreased glucose absorption in vivo (Gallagher et al., 2003). This mechanistic explanation may also apply to the actions of AHO in lowering the triglyceride and cholesterol level. The conversion of glucose to glycogen in the liver cells is dependent on the extracellular glucose concentration and on the availability of insulin which stimulates glycogen synthesis over a wide range of glucose concentration (Sheela and Augusti, 1992). Diabetes reduces activity of glycogen synthase thereby affecting the glycogen storage and synthesis in rat liver and skeletal muscle (Kumar et al., 2011). Oral administration of AHO 1000 mg/kg body weight significantly increased hepatic glycogen levels in STZ diabetic rats possibly because of the reactivation of the glycogen synthase system as a result of increased insulin secretion. The antioxidant action of the oil may be due to the involvement of polyphenols like p-coumaric acid and oleic acid (Yen et al., 2005).

Conclusion

The present study showed that oral administration of AHO has potential antidiabetic and antioxidant effect in STZ induced diabetic rats. The potent antioxidant activity may be responsible for the antihyperglycemic effects. This investigation reveals the potential of AHO for use as a natural oral agent with antihyperglycemic and antioxidant effects.

ACKNOWLEDGEMENT

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REFERENCES


Formulation and evaluation of aceclofenac ophthalmic gel

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Department of Pharmacy, Sagar Institute of Technology and Management, Barabanki, UP, India.

Accepted 29 April, 2013

This study deals with ophthalmic gel of aceclofenac which is potent non-steroidal antiinflammatory drugs (NSAIDs) and was formulated using polymers hydroxy propyl methyl cellulose (HPMC) (15) cps with methyl cellulose (40) cps and HPMC (15) cps with Carbopol (940). The gels (HP with MC) and (HP with CB), were sterilized and assessed for various parameters like clarity, pH, physical appearance, physical stability, viscosity and uniformity of drug content. The release rate from the aforementioned formulation within a period of 9 h were almost HP with MC batch H4 (84.66%) and for HP with CB batch C1 (80.46%); both ophthalmic gels obeyed zero order kinetics for drug release. The ocular irritation was carried out on male albino rabbits and found no redness, no inflammation and no increases tear. Both formulations in gel were found to be more stable at ambient, refrigerator and incubated temperature. The stability of the gels was evidenced by the degradation rate constant. Ophthalmic gel formulated by HP with MC and HP with CB gels proves to be viable alternative to conventional eye drops as it offers longer precorneal residence time and excellent ocular tolerance.

Key words: Carbopol 940 (CB), ophthalmic, gel, hydroxy propyl (HP), methyl cellulose (MC).

INTRODUCTION

Ophthalmic drug delivery is one of the most interesting and challenging factors facing the pharmaceutical scientist (Shivanand et al., 2008). Existing ocular drug delivery systems are fairly primitive and inefficient, due to low bioavailability of eye drops viscous liquid and semisolid preparations were tried as alternative therapeutic system. The bioavailability of these medicines can be increased by increasing the viscosity of the preparation up to gel like consistency using various polymer like Carbopol-940, sodium carboxy methyl cellulose (SCMC), hydroxy propyl methyl cellulose (HPMC), etc. The use of such polymeric vehicles proved to enhance the ocular bioavailability or the therapeutic efficacy of applied drugs, prolong the drug duration and reduce the patient non compliance problem (Naresh et al., 2008).

However, in the recent years, there has been explosion of interest in the polymer based delivery systems. By utilization of the principles of sustained release with semi synthetic polymers as embodied by ophthalmic gel offers an attractive approach to the problem of prolonging pre-corneal drug residence times (Quinones et al., 2008). The use of gel as a delivery system can increase the residence time of drugs in ocular cul-de-sac and consequently enhance bioavailability. Gel delivery systems have several advantages such as the ease of administration, none greasy, patient compliance, high residence time in eye and better drug release (Sankar et al., 2005). Aceclofenac is a novel, multiple action non-steroidal anti-inflammatory drug that is currently approved in many countries for the treatment of ophthalmic disease. Aceclofenac reduces pain and inflammation by inhibiting the enzyme cyclooxygenase II, which is involved in the production of prostaglandins, the pain and inflammatory mediator (Debnath et al., 2009).

*Corresponding author. E-mail: pza_19jan@rediffmail.com. Tel: +91-9307905233, 9450463956
authors in favor of sustained action of drug by semi
synthetic polymers, HPMC (15) cps, methyl cellulose (40)
cps and HPMC (15) cps, Carbopol (940) as gel delivery
system. The objective of this study was to develop
ophthalmic gels of aceclofenac using HPMC (15) cps,
methyl cellulose (40) cps, HPMC (15) cps, and Carbopol
(940) as mucoadhesives polymers, and evaluate it with
the aim to provide sustained local action in the eye and
improve patient compliance.

FORMULATION METHOD

Polymer hydration method

The polymer was taken in a 250 ml beaker and water was added.
This was allowed to soak for about 1 h and to this, the required
amount of drug and other additives was added. The stirring was
continued to get a homogenous dispersion of the drug in the gel.
The gel was buffered at a pH of 7.2 to 0.05 and was sterilized by
ultraviolet (UV) radiation for 30 min, aseptically filled in sterile
plastic container, and was labeled (Shivanand et al., 2008).

Experimental design (Taguchi method)

According to several engineering and pharmaceutical scientists,
Taguchi method is a designer tool to predict the optimized formu-
lations by considering the results from experimental formulations.
The results obtained by Taguchi method mainly depends on many
factors (variables) and levels.

Taguchi method detects the optimized formulations by using the
tool, that is, orthogonal array (OA). OA is the matrix of numbers
arranged in columns and rows. The Taguchi method quantifies the
present variations by signal to noise (S/N) ratio. These S/N ratios
are used to measure the effect of factors (variables) on performing
experimental formulations. According to closeness of the average
response of optimized formulations, the S/N ratio determines the
result from experimental formulations as type of characteristics:
smaller is better, nominal is best and larger is better.

This design, 2² Taguchi orthogonal array, requires four experi-
mental formulations with two parameters (concentration of polymer
A (HPMC with methyl cellulose) and concentration of polymer B
(HPMC with Carbopol 940), at two levels (high and low) of each.
Interactions were neglected (Dobrzanski et al., 2007). There are
two S/N ratios of common interest for optimization of static
problems:

Smaller-the-better

\[ n = -10 \log_{10}[\text{mean of sum of squares of measured data}] \]

This is usually the chosen S/N ratio for all factors (variables) on
performing formulations, like "concentration of polymers", etc., for
which the ideal value is zero. The generic form of S/N ratio then
becomes,

\[ n = -10 \log_{10}[\text{mean of sum of squares of \{measured - ideal\}}] \]

Larger-the-better

\[ n = -10 \log_{10}[\text{mean of sum squares of reciprocal of measured data}] \]

This case has been converted to smaller-the-better by taking the
reciprocals of measured data and then taking the S/N ratio as in the
smaller-the-better case.

The results obtained by Taguchi method mainly depends on
many factors (variables) and levels. Taguchi method detects the
optimized formulations by using the tool, that is, OA. The best
optimized batch for both ophthalmic gels was obtained by analyzing
factors (variables) and levels for viscosity, drug content and
cumulative drug release as calculated in Tables 1 to 13 and Figures
1 to 6.

Here, 2² Taguchi designs is applied where 2 lev-

Table 1. Level of process parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>Concentration of polymers</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Taguchi L₄ (2⁴) orthogonal array for HPMC:methyl cellulose ophthalmic gel.

<table>
<thead>
<tr>
<th>No. of formulation</th>
<th>Variables</th>
<th>HPMC</th>
<th>Methyl cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>+1</td>
<td>+1</td>
</tr>
</tbody>
</table>

Table 3. Taguchi L₄ (2⁴) orthogonal array for HPMC:Carbopol 940 ophthalmic gel.

<table>
<thead>
<tr>
<th>No. of formulations</th>
<th>Variables</th>
<th>HPMC</th>
<th>Carbopol 940</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>-1</td>
<td>+1</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>+1</td>
<td>+1</td>
</tr>
</tbody>
</table>
Table 4. Experimental data (optimization of viscosity) for HPMC: MC Ophthalmic Gel.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Observed viscosity</th>
<th>Mean</th>
<th>S.D</th>
<th>Log of S.D</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11000</td>
<td>11150</td>
<td>10875</td>
<td>11008.33</td>
<td>137.689</td>
</tr>
<tr>
<td>2</td>
<td>12000</td>
<td>12360</td>
<td>11840</td>
<td>12066.6</td>
<td>266.33</td>
</tr>
<tr>
<td>3</td>
<td>12000</td>
<td>11630</td>
<td>11540</td>
<td>11723.33</td>
<td>243.789</td>
</tr>
<tr>
<td>4</td>
<td>11650</td>
<td>11320</td>
<td>12000</td>
<td>11656.66</td>
<td>340.049</td>
</tr>
</tbody>
</table>

S.D: Standard deviation; MC: methyl cellulose.

Table 5. Experimental data (optimization of drug content) for HPMC: MC ophthalmic gel.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Observed drug content</th>
<th>Mean</th>
<th>S.D</th>
<th>Log of S.D</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94.20</td>
<td>94.8</td>
<td>95.6</td>
<td>94.8</td>
<td>0.7023</td>
</tr>
<tr>
<td>2</td>
<td>95.3</td>
<td>94.1</td>
<td>95.9</td>
<td>95.1</td>
<td>0.7483</td>
</tr>
<tr>
<td>3</td>
<td>95.1</td>
<td>94.7</td>
<td>94.3</td>
<td>94.7</td>
<td>0.4000</td>
</tr>
<tr>
<td>4</td>
<td>95.7</td>
<td>95.3</td>
<td>95.2</td>
<td>95.4</td>
<td>0.2645</td>
</tr>
</tbody>
</table>

S.D: Standard deviation; MC: methyl cellulose.

Table 6. Experimental data (optimization of cumulative drug release) for HPMC: MC ophthalmic gel.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Observed cumulative drug release</th>
<th>Mean</th>
<th>S.D</th>
<th>Log of S.D</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74.40</td>
<td>74.72</td>
<td>75.05</td>
<td>74.72</td>
<td>0.325</td>
</tr>
<tr>
<td>2</td>
<td>80.72</td>
<td>79.91</td>
<td>80.02</td>
<td>80.21</td>
<td>0.439</td>
</tr>
<tr>
<td>3</td>
<td>76.94</td>
<td>75.91</td>
<td>76.04</td>
<td>76.29</td>
<td>0.560</td>
</tr>
<tr>
<td>4</td>
<td>84.21</td>
<td>84.75</td>
<td>85.04</td>
<td>84.66</td>
<td>0.421</td>
</tr>
</tbody>
</table>

S.D: Standard deviation; MC: methyl cellulose.

Table 7. Experimental data (optimization of viscosity) for HPMC: Carbopol 940 ophthalmic gel.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Observed viscosity</th>
<th>Mean</th>
<th>S.D</th>
<th>Log of S.D</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8000</td>
<td>8260</td>
<td>7929</td>
<td>8063</td>
<td>174.261</td>
</tr>
<tr>
<td>2</td>
<td>7680</td>
<td>7150</td>
<td>8070</td>
<td>7633.3</td>
<td>461.77</td>
</tr>
<tr>
<td>3</td>
<td>7000</td>
<td>7650</td>
<td>7830</td>
<td>7493.3</td>
<td>436.61</td>
</tr>
<tr>
<td>4</td>
<td>8430</td>
<td>8850</td>
<td>8010</td>
<td>8430</td>
<td>420</td>
</tr>
</tbody>
</table>

S.D: Standard deviation; MC: methyl cellulose.

Table 8. Experimental data (optimization of drug content) for HPMC: Carbopol 940 ophthalmic gel.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Observed drug content</th>
<th>Mean</th>
<th>S.D</th>
<th>Log of S.D</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94.3</td>
<td>94.1</td>
<td>93.9</td>
<td>94.1</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>93.0</td>
<td>93.4</td>
<td>92.6</td>
<td>93.0</td>
<td>0.9451</td>
</tr>
<tr>
<td>3</td>
<td>93.6</td>
<td>93.1</td>
<td>92.8</td>
<td>93.1</td>
<td>0.4041</td>
</tr>
<tr>
<td>4</td>
<td>92.1</td>
<td>93.1</td>
<td>92.8</td>
<td>92.6</td>
<td>0.5131</td>
</tr>
</tbody>
</table>

S.D: Standard deviation.
**Table 9.** Experimental data (optimization of cumulative drug release) for HPMC: Carbopol 940 ophthalmic gel.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Observed cumulative drug release</th>
<th>Mean</th>
<th>S.D</th>
<th>Log of S.D</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75.08</td>
<td>74.93</td>
<td>75.05</td>
<td>-0.993</td>
<td>-30</td>
</tr>
<tr>
<td>2</td>
<td>81.48</td>
<td>80.68</td>
<td>81.97</td>
<td>-0.061</td>
<td>-28.86</td>
</tr>
<tr>
<td>3</td>
<td>85.62</td>
<td>85.03</td>
<td>86.26</td>
<td>-0.211</td>
<td>-27.95</td>
</tr>
<tr>
<td>4</td>
<td>71.63</td>
<td>70.81</td>
<td>71.98</td>
<td>0.600</td>
<td>-26.98</td>
</tr>
</tbody>
</table>

S.D: Standard deviation. MC: Methyl cellulose.

**Table 10.** Summary of analyses of factor effects for HPMC:MC ophthalmic gel.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Mean</th>
<th>Log(s)</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+1</td>
<td>+2</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>+1</td>
<td>+2</td>
<td>0</td>
</tr>
<tr>
<td>Drug content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+1</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>B</td>
<td>+1</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>In vitro cumulative drug release</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+2</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>B</td>
<td>+1</td>
<td>+2</td>
<td>+2</td>
</tr>
</tbody>
</table>

**Table 11.** Summary of analyses of factor effects for HPMC: Carbopol 940 ophthalmic gel.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Mean</th>
<th>Log(s)</th>
<th>S/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+2</td>
<td>+2</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>+2</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>Drug content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+2</td>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td>B</td>
<td>+2</td>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td>In vitro cumulative drug release</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>B</td>
<td>+1</td>
<td>+2</td>
<td>+2</td>
</tr>
</tbody>
</table>

**Table 12.** Final optimized parameters values for HPMC:MC ophthalmic gel.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Optimized level</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+1 (High conc. of HPMC)</td>
</tr>
<tr>
<td>B</td>
<td>+1 (High conc. of Carbopol 940)</td>
</tr>
</tbody>
</table>

**Table 13.** Final optimized parameters values for HPMC:Carbopol 940 ophthalmic gel.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Optimized level</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-1 (Low conc. of HPMC)</td>
</tr>
<tr>
<td>B</td>
<td>+1 (High conc. of Carbopol 940)</td>
</tr>
</tbody>
</table>

**Figure 1.** Cumulative drug release graphs HPMC and methyl cellulose gel. (A) Estimated factor effect; (B) Estimated factor effect on logs.

---

The optimized batch was calculated by counting A and B for each parameter of both gels (Table 10 to 13).
Evaluation of aceclofenac ophthalmic gels

**Determination of pH**

Accurately 2.5 g of gel was weighed and dispersed in 25 ml of purified water. The purified water was used because it has aqueous solubility and it also match with the pH of lachrymal fluid. The pH of the gel was measured using glass electrode pH meter (Pandey et al., 2010).

**Determination of viscosity**

Viscosity of the gel was determined using a Brookfield Viscometer, Spindle No 7 (Brookfield Engineering Labs., USA). All the formulated gels were sheared at 6 rpm for 5 min. The shear stress was recorded for each formulation (Vijayendra et al., 2005).

**Determination of mucoadhesives strength**

Mucoadhesives strength of 1% aqueous solution of optimized batch (H4 and C1) was studied with QTS-25 Texture Analyzer (Brookfield Engineering Labs., USA). Freshly excised goat by using goat conjunctival mucosa, was attached to the upper probe of the instrument, and drop of 1% gel solution was kept below that. The upper probe was then lowered at a speed of 10 mm/min to touch the surface of the solution. A force of 100 g was applied for 25 s, respectively, to ensure intimate contact between the membrane and the gel. The surface area of exposed mucous membrane was 1.13 cm² (Shyamoshree and Bandyopadhyay, 2010). The studies were

---

**Figure 2.** Drug Content graphs HPMC and methyl cellulose gel. (A) Estimated factor effect; (B) Estimated factor effect on logs.

**Figure 3.** Viscosity graphs HPMC and methyl cellulose gel. (A) Estimated factor effect; (B) Estimated factor effect on logs.

**Figure 4.** Cumulative drug release HPMC and Carbopol 940 gel. (A) Estimated factor effect; (B) Estimated factor effect on logs.
also conducted for HPMC, methyl cellulose and Carbopol 940, and results were compared.

**Determination of drug content**

Drug content was determined by dissolving accurate weighed quantity of gel in phosphate buffer (7.2), because the drug is completely soluble on that pH. After suitable dilutions, the absorbance was recorded by UV Vis spectrophotometer at 273.6 nm. Drug content was determined using slope of the standard curve previously plotted (Pandey et al., 2010).

**Clarity testing (IP 2007)**

Clarity test was done against dark and white background board apparatus, for the presence of foreign particles.

**In-vitro release study**

In vitro release test was performed with Franz diffusion cell (KC cell) by using egg membrane for the study. The membrane was separated by dipping the egg in concentrated HCl solution; with properly rotating the egg, the membrane was obtained by dissolving the CaCO3 coat of egg. The semi permeable egg membrane was tied on one end of open ended cylinder (diameter 1.6 cm2) which acts as donor compartment. One gram of the gel was placed outside the semi permeable egg membrane which acts as corneal epithelium. The entire surfaces of the membrane were in contact with the receptor compartment containing 25 ml of phosphate buffer pH 7.2. The receptor compartment was continuously stirred (50 rpm) using magnetic stirrer at 37°C. The study was carried out for 9 h. The sample was withdrawn at predetermined time intervals and the same volume was replaced with fresh buffer medium. The absorbance of the withdrawn sample was measured after suitable dilutions at 273.6 nm to estimate aceclofenac. The experiment was carried out in triplicate and average values were reported (Balasubramaniam et al., 2008).

**Drug release kinetics**

The drug release data was plotted using various kinetics, such as, zero order and first order. Higuchi’s kinetics and Korsmeyer’s equation were used to evaluate the drug release mechanism. The obtained data of present study for zero order equation were studied from in vitro drug release and were plotted as cumulative amount of drug release versus time (Jain, 2001), (Lachman; 1991)

**Stability study**

Chemical and physical stabilities of optimized formulations were assessed under various storage conditions, namely room temperature (RT), 5±1 and 40±1°C and 75% RH as per ICH guideline (Jain, 2007).

**In-vivo studies**

**Ocular irritation test**

The potential ocular irritancy effects of the formulations were...
evaluated by observing for any redness, inflammation or increase in tear production. Local irritation test was performed to provide estimation of human ocular response to the tested products (Griffith et al., 1980). Both formulations were tested on 2 albino male rabbits. The treatment was performed by single instillation (0.02 g) of gel under tests into the conjunctival sac of left eye every 24 h for 3 days. Plain gel base was instilled into the right eye. Both eyes of the rabbit under test were examined for any signs of irritation before treatment and up to 10 h after instillation (Shankar et al., 2005).

RESULTS AND DISCUSSION

The pH

The pH of the optimized formulations for batch H₄ was 7.2 and batch C₁ was 7.2. So this would not produce any irritation after administration (Optimization according to Taguchi design) (Tables 14 and 16).

Viscosity

The viscosity of various formulated aceclofenac ophthalmic gels by (HPMC, methyl cellulose) and (HPMC, Carbopol 940) is shown in Tables 14 and 16. Viscosities were determined for optimized batch H₄ as 11656.66 cps and batch C₁ as 8063 cps. All gels were found to have non-Newtonian type of flow. This was due to the concentration of polymer. When the concentration of polymers increased, the viscosity may also increase the interaction between polymers form gel (Tables 14 and 16).

Mucoadhesive strength

The mucoadhesives strength of optimized formulations provides intimate contact of gel to the ocular cul-de-sac and improves sustained action of drug. The mucoadhesives strength was determined by texture analyzer using goat conjunctival mucosa. The applied force by probe was 100 g and residence time was 25 s. As per observations obtained from Table 3, batch H₄ (HPMC, methyl cellulose gel) and batch C₁ (HPMC, Carbopol 940 gel) show H₄ (14.49) and C₁ (13.83) according to Taguchi design (Tables 14 and 16).

Drug content

The drug content values of all batches from both ophthalmic gels were found to be in range between 95 and 99% (Tables 14 and 16).

Clarity testing

All formulations were found to be off white and clear. Due to the presence of foreign particles formulation leads to ocular irritation. So, the preparations are free from foreign particle so that it improves ocular tolerance.

In-vitro release study

The results of the in vitro release study from different gels by the KC cell (25 mm), are as shown in Figure 1. In vitro release for both gel formulations showed a linear relationship between cumulative percentage release versus time. In H₄ batch of HPMC, MC showed 84.66% drug release. In C₁ batch of HPMC, Carbopol 940 showed 80.46% drug release, through KC cell within a period of 9 h. Drug release obeys zero order kinetics (Figure 2). The in vitro release order of different ophthalmic gel formulations was expressed in the decreasing order after 9 h. The difference in release rate could be due to viscosity and solubility of aceclofenac in HPMC with MC and HPMC with Carbopol 940. As given in (Tables 15 and 17 and Figures 7 and 8).

Drug release kinetics

The release profiles of optimized formulation were best described by a model that represents systems where drug diffusion occurs through a polymeric structure or network,

\[
\frac{M_t}{M_\infty} = k_t^n (r^2=0.942) \text{ and } (0.902)
\]

where \( M_t/M_\infty \) is the fractional release of the drug, \( t \) is the release time, \( k \) is a constant, and \( n \) is the release constant, indicative of the mechanism of drug release.

From Table 8, it was clear that optimized formulations and zero order release kinetics H₄ have \( r^2=0.942 \) and C₁ have \( r^2=0.902 \) (Tables 20 and 21).

Stability studies

Stability studies of optimized formulations were carried out on the basis of ICH guidelines and the observed values of K (Stability constant), \( t_{1/2} \) (half life), and \( T_{10\%} \) (shelf-life) of optimized formulation after different storage conditions are shown in Tables 18 and 19.
Table 15. *In vitro* drug release of optimized batch H4.

<table>
<thead>
<tr>
<th>Optimized Batch code H4</th>
<th>0</th>
<th>0.08</th>
<th>0.25</th>
<th>0.50</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>16.81±0.571</td>
<td>20.40±0.260</td>
<td>26.91±0.779</td>
<td>32.54±0.282</td>
<td>36.47±0.25</td>
<td>49.82±0.170</td>
<td>61.96±0.100</td>
<td>79.41±0.176</td>
<td>84.66±0.439</td>
</tr>
</tbody>
</table>

Table 16. HPMC:Carbopol 940 aceclofenac ophthalmic gel: physico-chemical parameters of the optimized formulations.

<table>
<thead>
<tr>
<th>Optimized batch: C1</th>
<th>pH</th>
<th>Viscosity</th>
<th>Drug content</th>
<th>Mucoadhesive strength</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.2</td>
<td>8063 cps</td>
<td>94.1</td>
<td>13.83</td>
</tr>
</tbody>
</table>

Table 17. *In vitro* drug release of optimized batch C1.

<table>
<thead>
<tr>
<th>Optimized Batch code H4</th>
<th>0</th>
<th>0.08</th>
<th>0.25</th>
<th>0.50</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>17.04±0.280</td>
<td>21.32±0.195</td>
<td>25.89±0.096</td>
<td>29.91±0.508</td>
<td>34.75±0.091</td>
<td>43.86±0.136</td>
<td>52.62±0.220</td>
<td>67.91±0.136</td>
<td>80.46±1.15</td>
</tr>
</tbody>
</table>

Table 18. Shelf-life of optimized formulation batch F4.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Parameter</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>K (day⁻¹)</td>
<td>3.65 × 10⁻⁴</td>
</tr>
<tr>
<td>2</td>
<td>t½ (days)</td>
<td>1874.53</td>
</tr>
<tr>
<td>3</td>
<td>T_{10%} (days)</td>
<td>284.93</td>
</tr>
</tbody>
</table>

Table 19. Shelf-life of optimized formulation batch C1.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Parameter</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>K (day⁻¹)</td>
<td>3.5 × 10⁻⁴</td>
</tr>
<tr>
<td>2</td>
<td>t½ (days)</td>
<td>1943.75</td>
</tr>
<tr>
<td>3</td>
<td>T_{10%} (days)</td>
<td>295.45</td>
</tr>
</tbody>
</table>
Figure 7. Normal rabbit eye after 24 h of gel administration

Figure 8. HPMC:Carbopol and HPMC:Methyl cellulose gel in-vitro drug release studies of various formulations.
Table 20. Diffusion kinetics parameters of optimized formulation H4.

<table>
<thead>
<tr>
<th>Batch code</th>
<th>Higuchi equation</th>
<th>Korsmeyer’s Peppas equation</th>
<th>First order equation</th>
<th>Zero order</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>R²</td>
<td>N</td>
<td>R²</td>
</tr>
<tr>
<td>H4</td>
<td>24.24</td>
<td>0.97</td>
<td>0.404</td>
<td>0.250</td>
</tr>
</tbody>
</table>

N- Release exponent, R- Correlation coefficient.

Table 21. Diffusion kinetics parameters of optimized formulation C1.

<table>
<thead>
<tr>
<th>Batch code</th>
<th>Higuchi equation</th>
<th>Korsmeyer’s Peppas equation</th>
<th>First order equation</th>
<th>Zero order</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>R²</td>
<td>N</td>
<td>R²</td>
</tr>
<tr>
<td>C1</td>
<td>17.57</td>
<td>0.934</td>
<td>0.321</td>
<td>0.207</td>
</tr>
</tbody>
</table>

N- Release exponent, R- Correlation coefficient

**In-vivo studies**

*Ocular irritation test*

None of the optimized formulation showed any sign of redness, inflammation and increase in tear production, after comparison with placebo formulations.

**Conclusion**

According to graphs and tables delivered by ophthalmic gels. It is concluded that both aceclofenac ophthalmic gel prepared with HPMC:methyl cellulose and HPMC:Carbopol 940, are viable alternative of conventional eye drops, because it proves to increased contact time, frequency of administration and excellent ocular tolerance; so, in all ways it improves patient compliance.

**REFERENCES**


Full Length Research Paper

Evaluation of spermicidal and antiandrogenic activities of aqueous extract of *Tinospora cordifolia* (Willd.) stem

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Department of Pharmacology, University College of Pharmacy, Mahatma Gandhi University, Cheruvandoor, Ettumanoor P. O., Kottayam, Kerala, India.

Accepted 14 August, 2013

Control of population is very important in these years. A wide variety of synthetic contraceptive agents are available, but these cannot be used continuously due to their side effects. Thus the present study was undertaken to evaluate the antifertility effect of aqueous extract of *Tinospora cordifolia* stem studied in male Albino Wistar rats. Male rats were orally administered with aqueous extract of *T. cordifolia* stem (100 mg/kg of body weight/day, for 60 days) and effect of treatment on reproductive organ weights: fertility potential and sperm count were evaluated. The treatment caused decrease in weight of testis, epididymis and prostate. The treatment caused a significant reduction in average litter size, sperm count, number of viable and motile sperm. The administration of aqueous extract of *T. cordifolia* led to alteration in both the histoarchitecture of testis and epididymis. The results revealed that the aqueous extract of *T. cordifolia* stem has spermicidal and antiandrogenic activity.

**Key words:** *Tinospora cordifolia*, antifertility, antiandrogenic, spermicidal activity

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. World Health Organization suggested that effective, locally available plants be used as substitutes for drugs. Since the population explosion is a leading cause of poverty and pollution in developing countries, they created a population control programme, which includes studies of traditional medical practices. Fertility control is an issue of global and national public health concern. Current methods of contraceptive result in an unacceptable rate of unintended pregnancies. Approximately 50% of all pregnancies are unintended at conception; 50% occur in the 94% of sexually active couples who reported using some method of contraception (Henshaw, 1998). The only male-specific contraceptive methods currently available are withdrawal, condoms, and vasectomy. As concerns regarding side effects and inconvenience of these existing methods, which prevent their universal acceptance of the development of additional male methods of fertility control that can provide tremendous social and public health benefits.

*Tinospora cordifolia* known as Amrita (Guduchi) in Sanskrit, is widely used in folk and Ayurvedic systems of medicine. The term Amrita is attributed to its ability to impart youthfulness, vitality and longevity to the consumer. The large numbers of compounds have been isolated from the aerial parts and roots of *T. cordifolia*. Guduchi is widely used in Ayurvedic system of medicine “Rasayanas” for the immune system and the body resistance against infections. In modern medicine *T. cordifolia* used for the treatment of general weakness, fever, dyspepsia, dysentery, gonorrhoea, urinary diseases, viral hepatitis and anaemia more recently, the immunomodulatory properties, antineoplastic activities have been reported.

*Corresponding author. E-mail: ittiyavirah@gmail.com. Tel: 09446883809*
MATERIALS AND METHODS

Collection of plant material

The fresh plant was collected from University College of Pharmacy, Cheruvandoor campus, Ettumanoor during the month of February. The same were botanically identified, confirmed and authenticated by Mr. Jobi Paul, Department of Environmental Science, MG University, Athirampuzha. The voucher number is 1104.

Preparation of extract

The fresh stems of *T. cordifolia* were washed with water and cut into small pieces, air dried for 10 days and the dried materials were powdered and subjected for extraction procedure. *T. cordifolia* stem powder was immersed in aqueous solution in a 5000 ml flat bottom flask and was cold extracted for 7 days with occasional shaking and warming. At the end of the 7th day, the clear filtrate was obtained by filtering through a Buchner funnel. The filtrate was further concentrated by vacuum distillation, cooled, transferred into a Petri dish and dried in an oven at 60°C for a period of 5 min. Finally, the aqueous extract was kept in desiccators for 15 days to remove the excessive moisture and was used for further studies (Kokate, 2000).

Animals

Healthy adult Wistar albino rats, weighing about 150 to 220 g obtained from the registered animal house of University College of Pharmacy, Cheruvandoor campus, MG University, Kottayam were used for the study. The study was approved by the Institutional Animal Ethical Committee, University College of Pharmacy, Cheruvandoor Campus [014/MPH/UCP/CVR/12].

Experimental design

The daily dose of the plant extract was freshly dissolved in normal saline and orally administered to each experimental animal every morning for 60 days. Each group contained 6 animals each.

For anti-fertility activity

Group I: Control rat received normal saline and Group II: Test received 100 mg/kg of aqueous extract of *T. cordifolia* stem.

For anti-androgenic activity

Group I: Control rat received normal saline, Group II: Test received 100 mg/kg of aqueous extract of *T. cordifolia* stem and Group III: Standard received 3 mg/kg of Flutamide.

Antifertility activity

After the termination of experiment all the male rats in each group were paired individually with two fertile females. Success of mating was confirmed by the presence of sperm in vaginal smear of mated rats. The average litter size was recorded (Revathy et al., 2010).

Antiandrogenic activity

The saline, test compound, standard are dissolved in sesame oil.

They received 0.9% saline, 100 mg/kg of *T. cordifolia* aqueous extract, and 3 mg/kg (Tinwell et al., 2007) of Flutamide, respectively. The treatment continued for 60 days, and on the 60th day, the animals are sacrificed. The weight of sex organs was compared with the weight of control group (Ravinthranath et al., 1996).

Estimation of sperm motility and count

The spermatozoa were obtained by making small cuts in caudate epididymis and vas deferens placed in 1 ml of modified Krebs Ringer bicarbonate buffer (pH 7.4). The sperm suspension was evaluated for sperm count, percent motility. The sperm count was determined under a Neubauer haemocytometer.

Statistical analysis

Statistical analysis was performed by using ‘Graph pad prism 6’ software by one way analysis of variance (ANOVA) followed by Dunnet’s multiple comparison tests. All data were expressed as Mean±standard error of mean (SEM), P<0.05 was considered as statistically significant.

RESULTS

The percentage yield of the extract was calculated to be 6.6% w/w.

Fertility test

Control as well as aqueous extract treated rats mated with female rats. All the female rats that mated with the control male rats delivered litters, whereas only two of these 12 female rats that mated with test male rats delivered litters.

Effect of treatment on weight of testis

Weight of testis in test group was significantly reduced when compared with the control group, weight reduction was more marked in standard, that is, Flutamide treated group (Figure 1).

Effect on weight of epididymis

Weight of epididymis in test group was significantly reduced when compared with the control group, weight reduction was more marked in standard, that is, flutamide treated group (Figure 2).

Effect on weight of prostate

Weight of prostate (mg) in test group was significantly

Revised on 30th July 2020
Effect of treatment on sperm count

The sperm count in the treated group was significantly reduced when compared with the control. The reduction was more marked in standard treated group (Table 1).

Effect of treatment on sperm motility

The sperm count in the treated group was significantly reduced when compared with the control. The reduction was more marked in standard treated group (Table 2).

Effect of treatment on histology of testis

Effect of treatment caused degenerative changes in histology of testis and epididymis (Figures 4 to 7).

The study of cross sections of controls epididymis revealed that the majority of the tubules of ductus epididymis were compactly arranged, but both in the test and standard groups, degeneration of tubules were seen, and the degeneration more marked in standard group.

DISCUSSION

The decrease in fertility potential that was reported in male rats treated with aqueous extract has been attributed to decreased sperm count, impairment in sperm motility and viability. The treated group shows significant reduction in litter size and most of the rats do not produce any litters. The findings of the present study indicated that the aqueous extract of *T. cordifolia* could significantly alter the fertility potential of male rats. The anti-fertility activity was evaluated on the basis of the effect of extract on litter size, spermatogenesis, and effect on organ weight and histomorphological examination. The group which received aqueous extract for 60 days showed marked decrease in the epididymal sperm count. The decrease in the sperm may be due to the hormonal activity of the extract. Spermatogenesis, an sequential process of transformation of primary spermatogonia through a series of stages into the round spermatids which involves cell division through mitosis as well as meiosis (Krester et al., 1994).

The sperm have two principal attributes, motility and fertilizing ability which is prerequisite for fertilization; any negative impact on motility would seriously affect the fertilizing ability (Akbarsha et al., 2000). The sperm sample contains more than 20% of abnormal spermatozoa consider to be more infertile. Motility of the sperm is due to flagellar beat which in turn is dependent on microtubular apparatus of the flagella (Eddy et al., 2004). Sperms while leaving testis are not motile but show motility during their epididymal transit. The epididymis contribute to initiate motility by providing unique

### Table 1. Effect of treatment on sperm count (Mean±SEM).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of sperm in 10^6/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.17±3.51</td>
</tr>
<tr>
<td>Test</td>
<td>14.17±2.85</td>
</tr>
<tr>
<td>Standard</td>
<td>9.33±2.35</td>
</tr>
</tbody>
</table>

### Table 2. Effect of treatment on sperm motility (Mean±SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of motile sperms in 10^6/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.83±1.80</td>
</tr>
<tr>
<td>Test</td>
<td>3.5±0.90</td>
</tr>
<tr>
<td>Standard</td>
<td>2.02±0.32</td>
</tr>
</tbody>
</table>

reduced when compared with the control group, weight reduction was more marked in standard, that is, Flutamide treated group (Figure 3).
**Figure 4.** Effect of treatment on histology of three segments of epididymis.

**Figure 5.** Histology of caput-epididymis.

**Figure 6.** Histology of cauda-epididymis.

**Figure 7.** Histology of corpus-epididymis.
microenvironment along the length for the sperm to resist and secreting protein and some important compound which in one way or other are concerned with the initiation of sperm motility. The changes essentially involve addition of new proteins, removal of existing proteins and modification of existing proteins. Particularly, much is known about the sperm motility initiation protein, acrosomal stabilization protein, etc (Revathy et al., 2010). Such maturation, leading to initiation of motility, depends upon the modifications sperm undergo with reference to the surface proteins and small molecular weight compounds (Guyton and Hall, 2006). Hence, the sperm collected from the cauda epididymis was not at all motile, thus clearly indicating the interaction of treatment with the maturation of sperm in epididymis, a large portion of existing sperms was unviable and several of the sperm had undergone structural abnormalities. Therefore, from this point of view, is due to aqueous extract of T. cordifolia.

The decrease in sperm count due to inhibition of spermatogenesis is confirmed by histomorphological examination of the testis and epididymis. Histopathological examination showed greater reduction in the testicular spermatozoa than epididymal sperma-tozoa. This might be due to the marked decrease in the diameter of the seminiferous tubules, accounting for the gross decrease in the testicular size. This indicates that the extract may have effect on pituitary gonadotropin.

The weight reduction of sex organs in treated group indicating a significant anti-androgenic activity. For the normal growth of accessory sex organs androgen is a must. The reduced sperm count, decreased sperm motility and viability also associated with this anti-androgenic activity. It has been shown that androgens are essential for survival and motility of spermatozoa in the rat epididymis, cauda region appears to be the most favorable site. It is likely that any contraceptive agent that affects sperm motility would influence spermatozoa indirectly through disruption of epididymal epithelial cell function or act directly on the spermatozoa by affecting their enzymes. A significant increase in the abnormal sperm count and inhibition of sperm motility in treated rats suggests that this extract target is within the internal milieu of the epididymis or alterations in the epididymal epithelium in the cauda epididymis. The comparison of body weight also reveals a significant effect of the treatment on the hormonal milieu. The body weight before the treatment is almost similar in both of control, test and standard groups. After treatment there was a significant reduction in the body weight of both standard and test treated groups. This weight reduction was more marked in standard anti-androgen (Flutamide) treated group, and this reduced body weight in extract treated group shows a similar antiandrogenic activity of aqueous extract.

The antifertility activity of the extract is significant, the activity maybe due to the presence of phenolics and flavonoids. The present study revealed that the administration of aqueous extract of T. cordifolia in a dose of 100 mg/kg body weight caused a marked reduction in litter size, sperm count, sperm motility and sperm viability. Treatment group shows a significant reduction in body weight, testis, epididymis and prostate weights. These effects of treatment on the sex organs clearly suggest the antiandrogenic activity of the extract. The histology of organs confirmed the antiandrogenic activity. Hence, it is concluded that this impairment may have caused the effects produced by the treatment on the spermatogenesis which in turn results in decreased sperm count. Hence, the study revealed that treatment of aqueous extract of T. cordifolia (100 mg/kg) for 60 days significantly reduced fertility profile of male rats. Thus, further studies are needed to isolate these potential constituents of the T. cordifolia for specific male antifertility activity.

ACKNOWLEDGEMENT

The authors wish to use this opportunity to express their deep sense of gratitude and indebtedness to Dr. Sibi P. I., M Pharm, Ph.D., Assistant Professor, Head, Department of Pharmacology, University College of Pharmacy, Kottayam for his inspiring guidance, timely invaluable suggestions, co-operation and help during the course of the project.

REFERENCES

Anticonvulsant activity and neurotoxicity of the enantiomers of DL-HEPP

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Accepted 11 September, 2013

DL-3-hydroxy-3-phenylpentanamide (DL-HEPP) is an anticonvulsant with a broad profile of activity. In order to study if there exists differences in biological activity between its enantiomers, we resolved the racemate from the (-) brucine and (-) 1-phenylethylamine salts of the acids. The optically active acids were then esterified with diazomethane and reacted with ammonia to give (+)-3-hydroxy-3-phenylpentanamide ((+)-HEPP) and (-)-HEPP. The enantiomeric purity of the amides was determined using proton magnetic resonance in the presence of europium tris-[3-(trifluoromethylhydroxymethylene)-(+)camphorate] and chiral high performance liquid chromatography. Optical purity of the amides was greater than 99% enantiomeric excess, impurities were not detected. Pharmacologically, DL-HEPP and its enantiomers have a similar significant anticonvulsant activity at peak drug effect against pentylenetetrazol-induced seizures, but a variation in time between the enantiomers was found with the anticonvulsant activity. In the rotarod ataxia test, the neurotoxicity of the enantiomers of DL-HEPP was also similar. The therapeutical indices of DL-HEPP and its enantiomers against the seizures induced by pentylenetrazol were better than valproate, an antiepileptic widely used in clinics.

Key words: Anticonvulsants, DL-3-hydroxy-3-phenylpentanamide enantiomers, DL-HEPP, resolution, (-) HEPP, (+) HEPP, valproate.

INTRODUCTION

Epilepsy is a brain disorder that is characterized by recurrent seizures that affects 1% of the population worldwide (McNamara, 1999). Despite the antiepileptic drugs (AEDs) available, at present, 30% of patients with epilepsy continue to have seizures and even among those considered controlled, many unpleasant side effects are still endured (Dichter, 1994). There is clearly a need for more and better AEDs.

The compounds DL-2-hydroxy-2-phenylbutyramide (1, DL-HEPA), DL-3-hydroxy-3-phenylpentanamide (2, DL-HEPP) and DL-4-hydroxy-4-phenylhexanamide (3, DL-HEPB) have a broad profile of anticonvulsant activity (Figure 1). They protect mice against seizures induced by pentylentetrazol, maximal electroshock, bicuculline, 4-aminopyridine, thiosemicarbazide (Meza-Toledo et al., 1990) and they also protected cats and rats against...
hippocampal “kindling” (Solís et al., 1979, 1996). From this series, compound 2 possesses the lowest toxicity (Chamorro et al., 1994). Compound 2 also protects against the γ-aminobutyric acid (GABA) withdrawal syndrome, a model of focal epilepsy, which has shown an extraordinary resistance to classic antiepileptics including diazepam, one of the most effective agent for treating status epilepticus (BraiIowsky et al., 1992). Additionally, it also produces a significant decrease of focal spike activity in the genetic absence epilepsy rats of the Strasbourg model (GAERS) (BraiIowsky et al., 1992).

The profile of anticonvulsant activity of the homologous series of phenyl alcohol amides suggests that they are promising anticonvulsant drugs against epilepsy of the absence type (Carvajal-Sandoval et al., 1998; Gómez-Martínez, 2007; Meza-Toledo et al., 2008) and they are currently undergoing preclinical development. The pharmacokinetic behavior of compound 2 has been tested in animals and healthy volunteers after the oral administration of single and multiple doses (Gómez and Lehmann, 1995a, b; González-Esquível et al., 1998, 2004; García et al., 2003). These studies showed that compound 2 has a rapid absorption, a long half-life, low protein binding and clinically adverse effects have been minor, so compound 2 shows great promise as a useful antiepileptic in drug therapy. However, further clinical investigation in humans is necessary to determine its use in clinical practice.

In order to continue with the pharmacological evaluation of DL-HEPP it is necessary to resolve racemate in order to study if there exist differences in biological activity between its enantiomers. In this paper, we report the enantioselective synthesis of 3-hydroxy-3-phenylpentanamide (HEPP) and the anticonvulsant activity and neurotoxicity of its enantiomers.

### METHODOLOGY

The melting points were determined with a Mettler-Toledo apparatus FP-62 model. Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum GX 2000 FT-IR spectrophotometer with attenuated total reflectance (ATR). The IR absorption frequencies are reported in cm⁻¹. The ¹H and ¹³C NMR spectra were obtained in a Varian VNMR-500 spectrometer, at 500 MHz (125.787 MHz for ¹³C). The samples were dissolved in CDCl₃ using tetramethylsilane (TMS) as internal reference. High-resolution mass spectra (HRMS) data were obtained in a JEOL GCMate II spectrometer in electron impact (EI, 70 eV) mode. Optical rotations were measured on a Perkin Elmer 341 polarimeter equipped with a 1 dm cell at 589 nm (sodium-D-line). Analytical chromatography was performed with a modular high-performance liquid chromatography (HPLC) Beckman System Gold equipped with a 166 variable wavelength detector, a 128 pump and an injector. A Chiracel column OJ (250 × 4.1 mm) packed with cellulose tris(4-methylbenzoate) (10 µm particle diameter) was used. Rotarod tests were performed on a Rotarod (M) 85052-4 Series. Maximal electroshock test was determined using a constant current electroshock unit Ugo Basile model 7801.

### Chemistry

Compounds 5a, 5b, 6a, 6b, 7a and 7b were synthesized according to Figure 2. The DL-(+)-hydroxyester, compound 4, was hydrolyzed to give the free hydroxy acid DL-(+)-3-hydroxy-3-phenylpentanoic acid, compound 5, and resolved by means of its (+)-brucine and (−)-1-phenylethylamine salts. The (+) and (−) acids were then esterified with diazomethane to produce the hydroxyesters 6a and 6b which were reacted with ammonia to give the optically active isomers of HEPP (Figure 2). The addition of (−)-brucine to the racemic solution of 3-hydroxy-3-phenyl-pentanoic acid 5 afforded the diastereomeric insoluble salt of 5a that was collected by simple vacuum filtration, this salt was composed of (−)-brucine and the (+) enantiomer. The water soluble salt of 5b was composed of (−)-brucine and the (−) enantiomer. Both complexes were hydrolyzed separately with diluted HCl to produce 5a and 5b. The partially resolved acid 5b was treated with (−)-1-phenylethylamine to give 5b. The optically liberated acids 5a and 5b were reacted with diazomethane to form the corresponding (+) and (−) methyl esters 6a and 6b. Aminolysis of 6a and 6b formed the beta-hydroxyamides 7a and 7b which exhibited [δ]D25° = +35.02° and -34.97° (c = 3, ethanol), respectively.

**DL-(±)-Ethyl 3-hydroxy-3-phenylpentanoate (compound 4)**

The compound 4 was synthesized as described previously (Bartrop et al., 1956). IR and NMR spectra were found to be identical with the ones described (Fukuzawa and Hirai, 1993).

**DL-(±)-3-Hydroxy-3-phenylpentanoic acid (compound 5)**

A solution of 44.4 g (200 mmol) of compound 4 containing 2 N KOH solution in 130 ml anhydrous methanol was stirred at room temperature for 7 h. After saponification, the methanol was evaporated at reduced pressure. The residue was taken up with 500 ml water, extracted with diethyl ether (100 ml × 3), and the organic phase was discarded. The aqueous phase was cooled and acidified (pH 2.5) with 35 ml 6 N HCl and extracted with diethyl ether (100 ml × 4). The combined ether extracts were washed with H₂O (30 ml × 2), saturated NaCl solution (30 ml × 2), and dried over Na₂SO₄ and concentrated in vacuo. The precipitate was recrystallized from water to afford 37.5 g (96.6 %) compound 5 as a white solid [melting point: 123 to 124°C (mp 121°C)] (Maroni-Barnaud et al., 1966).
(+)-3-Hydroxy-3-phenylpentanoic acid (compound 5a)

To a stirred solution of 24.7 g (127.3 mmol) of compound 5, 300 ml ethyl acetate at 60°C was added 52.57 g (119.6 mmol) of (-)-brucine. The solution was heated under reflux for 10 min, cooled at -20°C for 24 h and concentrated. The residue was treated with 50 ml hexane and the solid was filtered off and crystallized from 90 ml ethyl acetate. The brucine salt of compound 5a was filtered off, and the mother liquors were evaporated to dryness and the solid was crystallized several times from ethanol to give 2.6 g (99.5 mmol) of partially resolved compound 5b which was treated with 31 ml 5% HCl in 350 ml diethyl ether. The ethereal layer was separated and compound 5b was extracted with acetone (150 ml x 2). The combined ether and acetone extracts were concentrated and the solid was crystallized from water to give 8.3 g (42.8 mmol) of combined ethereal and acetone extracts. The ethereal layer was separated and the mother liquors were evaporated to dryness to obtain 26.9 g of compound 5a. (Denmark et al., 2005)

(-)-3-Hydroxy-3-phenylpentanoic acid (compound 5b)

5.84 g (30.1 mmol) of compound 5b partially resolved was mixed with 30 ml ethanol, 3.64 g (30.1 mmol) of (-)-1-phenylethylamine and heated at 40°C for 5 min. The mixture was concentrated to dryness and the solid was crystallized several times from ethanol to give the 1-phenylethylamine salt of compound 5b (6 g) as white crystals, mp 174 to 175°C which was treated with 5 ml 40% NaOH, 24 ml water and 48 ml benzene. The aqueous layer was washed with benzene (22 ml x 4), cooled and the pH of the solution was adjusted to 1 with 3.7 ml 37% HCl. The precipitate was filtered off and the solid was crystallized from water to give 2.0 g (34.2%) of compound 5b as a white solid [mp 92 to 93°C; [α]D20 = +21.7° (c = 3.0, ethanol)] (Mp 98°C; [α]D20 = +22° (ethanol)] (Mitsui and Kudo, 1965).

(+)-Methyl 3-hydroxy-3-phenylpentanoate (compound 6a)

To a stirred solution of 4.5 g (23.2 mmol) of compound 5a in 15 ml diethyl ether was added a solution of 1 g (23.8 mmol) of diazometane in 90 ml diethyl ether. The reaction mixture was stirred for 10 min liberating nitrogen. Then, it was concentrated at reduced pressure to obtain 4.8 g (99.5%) of compound 5a. The residue was treated with 5 ml 20% HCl in 230 ml water and 48 ml benzene, the precipitate was filtered off and the solid was crystallized from benzene to give 3.5 g (99.5%) of compound 5a. The solid was crystallized from water to give 8 g (32.4%) of compound 5a [Mp 92 to 93°C; [α]D20 = +21.7° (c = 3.0, ethanol)] (Mp 98°C; [α]D20 = +22° (ethanol)] (Mitsui and Kudo, 1965).

(-)-Methyl 3-hydroxy-3-phenylpentanoic acid (compound 6b)

This compound was obtained from compound 5b following a similar procedure to the preparation of compound 6a. Yield 98.02%, [α]D30 = -2.2° (c = 3, ethanol) ([α]D20 = 0.57° (c = 1.03, ethanol)] (Denmark et al., 2005). NMR spectra were found to be identical with the ones described (Adachi and Harada, 2008).

(+)-3-Hydroxy-3-phenylpentanamide (compound 7a)

A mixture containing 4.8 g (23 mmol) of compound 6a, 15 ml ethanol and 15 ml 28% aqueous ammonia was cooled at 0°C and saturated with ammonia gas. The flask was closed with a rubber stopper and held at room temperature for 19 days. Then, the mixture was cooled, and it was extracted with diethyl ether (15 ml x 4), dried over Na2SO4, filtered and concentrated in vacuo. The residue was treated with 10 ml benzene, the precipitate was filtered off and the solid was crystallized from benzene to give 3 g (67.3%) of compound 7a as a white solid (mp 51 to 52°C; [α]D20 = +35.02° (c = 3, ethanol); IR (ATR): ν = 1656, 3186, 3334 cm⁻¹; 1H-NMR (500 MHz, CDCl3): δ = 0.73 (t, 3 H, J = 3 Hz), 1.76 (dq, 1 H, J = 6.5 Hz, J = 13 Hz), 1.82 (dq, 1 H, J = 6.5 Hz, J = 13 Hz), 2.65 (d, 1 H, J = 15 Hz), 2.70 (d, 1 H, J = 15 Hz), 5.11 (br, 1 H), 5.70 (br, 1 H), 5.80 (br, 1 H), 7.20 (tt, 1 H, J = 9 Hz, J = 15 Hz), 7.23 (tt, 2 H, J = 9 Hz, J = 15 Hz), 7.34...

Figure 2. Synthesis of the enantiomers of HEPP.
The method described preceding Europium tris (polyethyleneglycol-3:15) at a flow rate of 0.9 ml/min; 1% confidence. This agrees with intraperitoneally dissolved in water. All the compounds were administered to pentylenetetrazol (PTZ) and maximal electroshock (MES) models.

The anticonvulsant activity group consisted of 7 animals. Determination of the enantiomeric purity using Chiralpak AD-H (5 µm) column to resolve the racemate. Figure 3A showed the chromatographic resolution of DL-HEPP exhibiting a

(-)-3-Hydroxy-3-phenylpentanamide (compound 7b)

Compound 7b was obtained from compound 6b, following a similar procedure to the preparation of compound 7a. Yield 56.4%.Mp 51 to 52°C; [α]D20 = -34.97° (c = 2.98, ethanol). IR, 1H-NMR and 13C-NMR spectra and HRMS data were identical to compound 7a.

DL-(±)-3-hydroxy-3-phenylpentanamide (compound 2)

Compound 2 was synthesized as described previously (Meza-Toledo et al., 1990) [mp 101 to 102°C (mp 101 to 102°C (Meza-Toledo et al., 1990)].

Chromatographic determination of the enantiomeric purity

The enantiomeric purity of compounds 7a and 7b was determined by chiral HPLC using a Chiralpak AD-H column (4.1 x 250 mm), eluting with n-hexane:2-propanol (85:15) at a flow rate of 0.9 ml/min; detection was at 221 nm. 20 µl of each enantiomer dissolved in n-hexane:2-propanol (85:15) (400 ng/ml) was injected into the column and the enantiomeric excess was determined.

Determination of the enantiomeric purity using Europium tris-[3-(trifluoromethylhydroxymethylene)-(+)camphorate [Eu[TFHCam-d]]

To establish the enantiomeric purity by 1H-NMR (500 MHz), Eu[TFHCam-d] [0.017 g, 0.02 mmol] was added either to the racemic compound 2 (0.019 g, 0.01 mmol) or their enantiomers 7a and 7b (0.019 g, 0.01 mmol) dissolved in 0.5 ml CDCl3. 1H-NMR spectra were performed. Chemical shift differences (Δδ) were calculated by subtracting the low field signal to the high field signal of both enantiomers resolved in the spectrum.

Pharmacology

Animals and treatment

Male albino Swiss Webster mice (Birmex, Mexico City) weighing 25 to 30 g were housed in groups of 5, at room temperature (20 to 24°C), with tap water and food (pellet type Lab Rodent Diet 5008; PMI Nutrition International, Brentwood, MO, USA) ad libitum, with a 12-h light-dark cycle (light on: 6.00 a.m.). Mice were used in the mouse anticonvulsant and rotorod tests. The experiments were carried out according to the National Institutes of Health animal care and use guidelines, and were approved by our scientific research committee. Each treatment group and vehicle control group consisted of 7 to 10 animals.

Anticonvulsant activity

The anticonvulsant activity (Meza-Toledo et al., 1990) of DL-HEPP and its enantiomers 7a and 7b was evaluated using pentylenetetrazol (PTZ) and maximal electroshock (MES) models. Compounds DL-HEPP, 7a and 7b were dissolved in a 10% polyethylene glycol-400 solution; sodium valproate and PTZ were dissolved in water. All the compounds were administered intraperitoneally (i.p.). The convulsant dose of PTZ inducing seizures and death in 100% of mice was determined and used in the pharmacological test. The time of peak drug effect (TPE) was evaluated for each anticonvulsant before determining the dose-response curves. PTZ was administered i.p. at 80 mg/kg, to four groups of 7 to 10 mice, and suppression of clonic seizures and death was considered the end point. In the MES test, seizures were induced by application of an electrical current across the brain via earclip electrodes. Shocks were delivered at constant current of 20 mA with a frequency of 100 Hz, a pulse width of 0.4 ms and a duration of 0.2 s. Compounds DL-HEPP, 7a, 7b and sodium valproate were tested at TPE. The dose at which the hind limb tonic seizure was blocked in 50% of the animals (ED50 value) was determined by probit analysis. ED50, TD50 and 95% confidence intervals were calculated by the method described previously (Litchfield and Wilcoxon, 1949).

Effects of time

Groups of 10 mice were dosed i.p with DL-HEPP, 7a and 7b, 100 mg/kg, and protection against convulsions and death produced by pentylenetetrazol, 80 mg/kg, i.p. was evaluated at different times.

Neurotoxic effects

Separate groups of mice were trained to stay on a rotarod that rotated at 10 rpm. The drum diameter was 2.54 cm. Four groups of 7 to 10 trained mice were dosed with the test compound or drug vehicle (10% polyethylene glycol-400 solution) and were tested at TPE to measure the effect of the drug on motor performance (Meza-Toledo et al., 1990). Animals which fell off before 120 s were considered ataxic. The dose at which 50% of the animals fell off the rotarod (TD50) was determined by probit analysis (Litchfield and Wilcoxon, 1949).

Protective index

It was calculated by dividing the TD50 value by the respective ED50 values as determined in either PTZ or MES tests. The protective index is considered to be an index representing the margin of safety and tolerability between ED50 and TD50 values (Litchfield and Nolting, 1991).

RESULTS

Chemistry

Melting point of compound 5, 123 to 124°C, and those of their enantiomers 5a and 5b, 92 to 93°C, were different. Similarly, the melting point of DL-HEPP, 101 to 102°C, decreased to 51 to 52°C in the enantiomers 7a and 7b. Examination of the 1H and 13C-NMR spectra of DL-HEPP and their enantiomers 7a and 7b in a CDCl3 solution showed identical chemical shifts. This agrees with literature (Nógrádi, 1981), where racemates because of their different crystal structure have melting points which may be different from those of the pure enantiomers. In order to study the enantiomeric purity of compounds 7a and 7b they were analyzed by using a chiral HPLC column to resolve the racemate. Figure 3A showed the chromatographic resolution of DL-HEPP exhibiting a
retention time for 7a (12.08 min) and 7b (7.16 min). Figure 3b and c showed the chromatogram of pure enantiomers 7b and 7a, impurities were not detected and they have at least 99% ee.

The 1H-NMR spectrum of DL-HEPP showed an AB coupled system for diasterotopic protons H-2 centered at 2.77 ppm; H-4 was observed as an overlapped system ABX3 at 1.82 ppm (Figure 4a). The addition of Europium tris-[3-(trifluoromethylhydroxymethylene)-(+)-camphorate] (Eu[TFH-cam-d]) to racemic 7a/7b split the methylene H-2 signals of isomer 7a at 3.20 ppm and for the isomer 7b at 3.08 ppm (Figure 4b). Protons H-4 for (-) isomer were shifted at 2.18 ppm, same protons for (+) isomer were located at 2.10 ppm.

Figure 4c showed the proton NMR spectrum of isomer (+)-7a pure. The addition of 0.01 mmol of Eu[TFH-cam-d] to these compounds shifted protons H-2 to 3.08 ppm; methylene H-4 displayed two different signals, one at 2.15 ppm and other at 2.0 ppm for each diastereomeric proton. Due pseudo-contact, interaction between Eu[TFH-cam-d] and enantiomers (-) and (+) are different, isomer (-)-7b pure in Figure 4d displayed an AB coupled system for protons H-2 at 2.95 and 2.82 ppm, respectively, nevertheless, the diasterotopic methylene H-4 remained as a multiplet centered at 1.92 ppm.

**Pharmacology**

The anticonvulsant activity and neurotoxicity of DL-HEPP, 7a, 7b and sodium valproate after intraperitoneal administration is shown in Table 1. The compound DL-HEPP has been previously shown to be endowed with anticonvulsant activity in several animal seizure tests but individual evaluation of each of these enantiomers 7a and 7b was still lacking. From the data of Table 1, it can be seen that not only DL-HEPP but also each of its enantiomers exhibit interesting anticonvulsant protections in these seizure tests that are as potent as valproate, a reference antiepileptic drug widely used in human clinics. With respect to the PTZ test, compounds DL-HEPP, 7a and 7b showed a similar significant anticonvulsant activity (ED50: 55, 61 and 50 mg/kg, respectively) and sodium valproate was the least potent (ED50: 120 mg/kg) (Table 1). Sodium valproate exhibited a 50% protection by the MES test at a dose of 237 mg/kg whereas the anticonvulsant activity of DL-HEPP and its enantiomers (+) 7a and (-) 7b was something different (ED50: 138, 168 and 108 mg/kg, respectively).

The anticonvulsant activity of (+) HEPP declined rapidly after the first two hours exhibiting 10% protection while (-) HEPP showed the highest activity (90% protection) and
Figure 4. $^1$H-NMR spectra (500 MHz): a) DL-HEPP; b) DL-HEPP added with 0.02 mmol Eu[TFH-cam-d]; c) (+) HEPP added with 0.01 mmol Eu[TFH-cam-d]; d) (-) HEPP added with 0.01 mmol of Eu[TFH-cam-d].

Figure 5. Anticonvulsant activity in time of DL-HEPP and its enantiomers against pentylenetetrazol induced seizures in mice. DL-HEPP showed an intermediate activity (50% protection) (Figure 5). At 4 h after administration, the anticonvulsant activities of DL-HEPP and (-) 7b decreased to 20 and 70% protection, respectively, at this
time compound (+) HEPP had no protection. At 6 h after injection the anticonvulsant activity of DL-HEPP and (-) 7b fell to 10 and 20% protection, respectively (Figure 5). There was no difference in neurotoxicity between DL-HEPP and its enantiomers (+) 7a and (-) 7b (TD$_{50}$: 212, 223 and 207 mg/kg, respectively). In this test, sodium valproate was the least toxic (TD$_{50}$: 380 mg/kg) (Table 1).

Compounds DL-HEPP, (+) 7a and (-) 7b showed protective indices (PI: 3.85, 3.66 and 4.14, respectively) that are better than sodium valproate (PI = 3.16) against PTZ induced seizures in mice (Table 1). However, in the MES test sodium valproate (PI = 1.6) had similar protective indices as those of DL-HEPP, (+) 7a and (-) 7b (PI: 1.54, 1.32 and 1.91, respectively).

**DISCUSSION**

Synthesis of optically pure 5a has been reported previously (Mitsui and Kudo, 1965) and the absolute configuration was determined to be S [α]$_D^{20}$ = +22° (ethanol). Since none of the reactions to produce 7a from 5a affected directly the chiral carbon atom and there is no acidic protons to promote racemization, the absolute configuration of 6a and 7a was deduced to be S by comparing the optical rotation obtained for 5a [α]$_D^{20}$ = +21.7° (c = 3.0, ethanol). Denmark et al. (2005) reported a specific rotation of +16° for the corresponding carboxylic acid 5a produced from the hydrolysis of 6a. Compounds 6a and 6b has been synthesized from catalytic enantioselective aldol reaction of propiophenone (Denmark et al., 2005; Adachi and Harada, 2008; Oisaki et al., 2006).

Oizaki et al. (2006) reported a specific rotation of +1.64° (77% enantiomeric excess) for the (+) hydroxyester 6a. Adachi et al. (2008) reported a specific rotation of -0.97° for the (-) hydroxyester 6b. For compounds 6a and 6b we reported specific rotations of +2.2° and -2.2°, respectively. Compounds 7a and 7b have not been reported previously. From the higher optical rotation, values for compounds 6a and 6b respect those published previously, and considering that compounds 7a and 7b have optical purities greater than 99% enantiomeric excess, it is assumed that compounds 6a and 6b reported in this paper have at least 99% enantiomeric excess. In line with previously published methods for the preparation of (+) and (-) hydroxyesters (Denmark et al., 2005; Adachi and Harada, 2008; Oisaki et al., 2006), the advantages in the reported method were the higher optical purities obtained.

While the anticonvulsant activities of DL-HEPP and its enantiomers were similar in the PTZ assay at peak drug effect, in the MES model of epilepsy (Willow and Catterall, 1982; McNamara, 2011) it is reported that hydroxyphenylamides such as DL-2-(3-chlorophenyl)-2-hydroxynonamide and its (-) and (+) enantiomers blocked sodium channels with inhibitory concentration 50 values of: 1.81, 1.88 and 2.61 mM respectively (Davis et al., 2012). As DL-HEPP and its enantiomers are hydroxyphenylamides it may be possible that they also block sodium channels. This could explain their effect in the MES test.

The rapid onset of the anticonvulsant effect suggests that DL-HEPP and its enantiomers readily penetrate the blood-brain barrier. This finding agrees well with the low serum protein binding of HEPP as previously published (Gómez et al., 1995a). The strong direct relationship between the concentrations of HEPP in plasma and/or brain and the anticonvulsant effect demonstrated that the parent compound is responsible for the anticonvulsant action (Gómez et al., 1995a). When DL-HEPP was administered with diphenylhydantoin to rabbits, plasma HEPP levels decreased. This result suggested a pharmacokinetic interaction between diphenylhydantoin and HEPP, probably on the drug-metabolizing enzyme system in the liver (Medina et al., 1998). As phenytoin acts as an enzyme inducer of microsomal P450, it is probable that DL-HEPP and its enantiomers might be metabolized by cytochrome P450, perhaps of the same genetic subfamily on which phenytoin acts as enzyme inducer. However, it will be necessary to perform HEPP

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**Table 1. Anticonvulsant activity and neurotoxicity of DL-HEPP and its enantiomers.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED$_{50}$ (mg/kg)</th>
<th>TD$_{50}$ (mg/kg)</th>
<th>Protective index$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTZ$^c$</td>
<td>Rotarod ataxia</td>
<td>PTZ</td>
</tr>
<tr>
<td>DL-HEPP</td>
<td>55 (51-60)$^a$</td>
<td>212 (209-216)</td>
<td>3.85</td>
</tr>
<tr>
<td>(+) HEPP</td>
<td>61 (55-68)</td>
<td>223 (212-234)</td>
<td>3.66</td>
</tr>
<tr>
<td>(-) HEPP</td>
<td>50 (43-58)</td>
<td>207 (203-210)</td>
<td>4.14</td>
</tr>
<tr>
<td>Valproate</td>
<td>120 (110-132)</td>
<td>380 (357-404)</td>
<td>3.16</td>
</tr>
</tbody>
</table>

$^a$Time of test: 30 min post-dosing to peak drug effect. $^b$TD$_{50}$/ED$_{50}$ Seizures induced by: ($^c$) pentylenetetrazol and maximal electroshock. $^d$95% confidence interval. $^e$Time of test: 30 (TD$_{50}$) and 45 (PTZ and MES) min post-dosing.
metabolism studies with the racemate and its enantiomers in order to determine the mechanisms involved in the biotransformation of this drug. The variation in the anticonvulsant activity over time between the enantiomers (+) 7a and (-) 7b could be due to differences in their metabolism or distribution. Further studies to explore it are warranted.

The rotarod ataxia test was used to evaluate the neurotoxicity. In this test, the neurotoxicity of DL-HEPP, (+) 7a and (-) 7b was similar. The mechanism underlying the anticonvulsant activity of DL-HEPP and its homologues DL-HEPA and DL-HEPB is not known. They protect against seizures induced by bicuculline, a GABA receptor antagonist (Pérez de la Mora and Tapia, 1973; Tapia et al., 1979; Meza-Toledo et al., 1990). DL-HEPP also reversed GABA mediated inhibition of electrically and potassium chloride evoked exogenous [3H]-GABA release from rat substantia nigra slices without having any effect on evoked release in the absence of GABA. DL-HEPP also counteracted the inhibition in electrically evoked release of [3H]-GABA produced by the GABA receptor antagonists picrotoxin and bicuculline. DL-HEPP might be acting as a modulator at the GABA receptor complex (Meza-Toledo and Bowery, 2008).

In support of this idea, it has been reported that DL-HEPP displaces [3H]-flunitrazepam and [35S]-t renbutylbicyclicphosphorothionate from benzodiazepine and picrotoxin sites on GABA receptor complex in rat brain crude synaptic membranes (Chávez and Martínez, 1996). It is published that the hydroxybencenamide DL-3,3,3-trifluoro-2-hydroxy-2-phenyl-propionamide (Choudhury-Mukherjee et al., 2003) enhanced GABA current evoked by GABA (10 μM) in rat hippocampal neurons (Choudhury-Mukherjee et al., 2003). It is probable that DL-HEPP and its enantiomers may also modulate GABA current evoked by GABA in neurons. The enantioselective synthesis of HEPP will help us to elucidate the mechanism of action underlying the anticonvulsant action of HEPP.

Conclusion
At the time of peak drug effect (30 min) there was no differences either in the anticonvulsant activity against pentylenetetrazol induced seizures or in neurotoxicity between DL-HEPP and its enantiomers, which suggests that the chiral separation of DL-HEPP and its homologues is not necessary for further preclinical studies.

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Full Length Research Paper

Antiosteoporotic effect of *Coelatura aegyptiaca* shell powder on ovariectomized rats

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The efficacy of *Coelatura aegyptiaca* shell powder (CES, 500 mg/kg BW) was evaluated as a calcium supplement in ovariectomized (OVX) rats for ten weeks of treatment. The biochemical components and the antioxidant properties of the shell powder were determined. The bone mineral density (BMD), bone mineral content (BMC), calcium (Ca) and phosphorus (P) contents in serum and bone, serum total alkaline phosphatase (TALP) and bone alkaline phosphatase (BALP), serum calcitonin and parathyroid (PTH) hormones were determined. Furthermore, some of the oxidative stress markers [malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant capacity (TAC)] were estimated in bone. The current study revealed that CES contained 19.38% Ca, 0.315% P as well as some of antioxidant amino acids which have a potent antioxidant activity against 1,1-diphenylpicrylhydrazyl (DPPH) free radical. Administration of CES to OVX rats increased BMD, BMC, tibial Ca and P contents and BALP activity, as compared to OVX rats. An ameliorative effect was recorded in the levels of calcitonin, PTH, MDA, SOD, GPx and TAC subsequent to CES administration to OVX rats. This ameliorative effect of CES powder against osteoporosis may be attributed to its antioxidant efficacy and/or to its Ca content. In conclusion, CES may have the potential to develop a clinically useful anti-osteoporotic agent, since its effect was comparable with alendronate (6.5 mg/kg BW/week).

Key words: Ovariectomized rats, *Coelatura aegyptiaca* shell, calcium supplement, 1,1-diphenylpicrylhydrazyl (DPPH), oxidative stress markers.

INTRODUCTION

Calcium is the most abundant mineral in the organism and contributes approximately 1 to 2% to the adult human body weight. A dynamic balance exists between calcium in the extracellular medium and that found in bone, and about 500 mg of this mineral enter and depart daily from the bones (Pérez Llamas et al., 2010). Osteoporosis is the most common skeletal disorder and is considered a risk of fracture (Yalin et al., 2012). It is a systemic metabolic bone disease which presents reduction in bone density together with abnormal structure (Kanis et al., 2008). Osteoporosis may appear in aged men and women due to negative calcium balance (Heaney, 1996). This case may result from a decrease in intestinal calcium absorption, insufficient dietary calcium intake, as well as increased urinary calcium loss associated with estrogen deficiency during menopause (Kaplan and Hirsch, 2004). Dietary risk factors for bone fractures play a special role in the prevention of osteoporosis. Numerous studies indicate that adequate calcium intake improves bone mineral density (Peterlik et al., 2013).

Ovariectomized rats are used specifically as the animal model to observe the effects of osteoporosis since the...
biological mechanisms related with bone resorption in ovariectomized rats highly resemble the post-menopausal bone loss in women (Frost and Jee, 1992). The acute effects of menopause are modeled by ovariectomy which, like natural menopause, stimulates bone resorption by increasing osteoclast formation (Weitzmann and Pacifici, 2006). One important component of the osteoclasts activity is the generation of free radicals which contribute to the process of bone degradation and resorption (Fraser et al., 1996). Ovariectomy alters the generation of reactive oxygen species (ROS) and the antioxidant defense capacity of the cell (Lean et al., 2005), leading to an accumulation of ROS. Furthermore, oxidative stress (OxS) has been linked with osteoporosis (Sánchez-Rodríguez et al., 2007).

It was reported that accumulation of ROS in cellular components is the major factor causing molecular injury which ultimately leads to cell aging and other age-related degenerative diseases (Stagos et al., 2012). Basu et al. (2001) revealed that the osteoporosis could be eliminated when oxidative stress is prevented. Unfortunately, the current pharmacological therapy used for treatment of osteoporosis have serious adverse effects besides its inability to restore lost bone mass (Shirke et al., 2008). Bisphosphonates administration is widely used for management of osteoporosis. However, therapy with bisphosphonates is associated with deleterious effects such as esophageal cancer and osteonecrosis of the jaw (Kuehn, 2009). The predicted increase in the incidence of osteoporosis in women justifies a search for opportunities to prevent bone fractures (Wadolowska et al., 2013). Consequently, there has been a vast increase in the use of natural products that could provide all of the benefits but none of the risks of prescription drugs (Kupferer et al., 2009). Many therapeutic strategies tend to focus on calcium supplements for prevention and treatment of osteoporosis instead of hormone replacement therapy (Wallace et al., 2004; Shaker et al., 2005). Therefore, the present study aims to introduce a new natural calcium source derived from Egyptian freshwater bivalve Coelatura aegyptiaca as an anti-osteoporotic agent. So, the present investigation aims to evaluate the efficacy of C. aegyptiaca shell powder (CES) as a new alternative anti-osteoporotic agent as compared with alendronate, the widely used anti-osteoporotic drug. Furthermore, since osteoporosis positively correlated with OxS, the present study extends to evaluate the antioxidant capacity of the CES powder.

MATERIALS AND METHODS

Chemicals and reagents

1,1-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fosamax (Alendronate) was purchased from Global Napi Pharmaceuticals (New Jersey, USA). Kits of biochemical analyses were purchased from Biodiagnostic Company (Egypt), calcitonin kit was purchased from DRG International, Inc. (New Jersey, USA) and parathyroid hormone kit was purchased from Immunotech A Beckman Coulter Company (Marseille, France). Any other chemicals were of analytical grade.

Preparation of C. aegyptiaca shells (CES) powder

C. aegyptiaca belong to Unionoidae and are widely distributed along the River Nile from Assiut to Damietta (Moloukhia and Sleem, 2011). Freshwater mussel C. aegyptiaca was collected from the Nile River at Giza governorate, Egypt. The meat was removed and the CES was prepared according to Han et al. (2007). The prepared CES powder was stored in a desiccator to protect it from light and moisture until used.

Determination of shell contents

For determination of inorganic calcium (Ca) and phosphorus (P) contents, small amounts (100 mg) of prepared CES powder were dissolved in hydrochloric acid. The inorganic Ca and P contents of CES powder were determined using biodiagnostic kits. For amino acids determination, the CES powder was analyzed by high-performance liquid chromatography (HPLC); Beckman 6300 amino acid analyzer (Radwan et al., 2007).

In vitro antioxidant assay using DPPH

The DPPH free radical scavenging assay was carried out for evaluation of antioxidant activity according to the method adopted by Brand et al. (1995). The following concentrations of the CES powder and ascorbic acid (standard reference) were prepared in methanol 10, 20, 30, 40, 50, 60, 70 and 80 mg/ml. Each tube contained 2 ml DPPH (0.1 mM in methanol), a certain concentration of CES or ascorbic acid, and the final volume was adjusted to 4 ml with methanol. The solution was shaken and incubated at 37°C for 30 min. The control solution (containing DPPH only) underwent the same processes. All measurements were performed in triplicate and methanol was used as a blank. The decrease in absorbance (Abs) was measured at λ = 517 nm. The radical scavenging activity was calculated from the following equation:

\[
% \text{ of radical scavenging activity} = \left[ \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \right] \times 100
\]

Animals

Adult female Wistar albino rats (Rattus norvegicus) weighing 150 to170 g were obtained from the animal house of the National Research Center (NRC), Egypt. Rats were housed in polypropylene cages in air-conditioned room at a temperature of 20 ± 2°C and under natural day and night cycle. They were fed commercial food pellets and drinking water ad libitum. The rats were kept for a week before the commencement of the study for acclimatization.

Acute toxicity test

Acute oral toxicity test was done according to the organization for economic cooperation and development (OECD) based on acute oral toxicity up and down procedure 425 guideline (OECD, 2001). Two groups each of five healthy female rats were selected randomly and fasted overnight. The first group was administered, via gavage, CES powder suspension at a limit dose of 5000 mg/kg body weight. The second group (control group) was given an equal volume of distilled water. All animals were observed at 0, 30 min, 1, 2, 4, 6 h and thereafter every day for 14 days to check for mortality and abnormal clinical manifestation. The rats were sacrificed after...
14 days and their liver and kidney were excised and fixed in 10% formalin for 24 h. They were processed and stained with hematoxylin and eosin dyes for histopathological examination. The median effective dose (ED50) of aforementioned shell powder was selected based on LD50 obtained from acute toxicity study.

**Surgical procedure of ovariectomy**

Bilateral ovariectomy was performed. All surgical procedures were performed by the same surgeon for all groups. Following anesthesia with diethyl ether, a 1.5 cm skin incision was made and the ovaries were removed completely. Thereafter, the ventral muscle and skin were sutured and povidone iodine (betadine) was applied in this area to disinfect the skin (Parhizkar et al., 2008). Finally, the wounded area was powdered with erythromycin (Figure 1A to L).

**Experimental design**

Rats were randomly assigned into two main groups, Group I served as sham group that was administered distilled water. The sham rats were anesthetized, laparatomorized, and sutured without removing their ovaries (negative control, n = 14). Group II served as ovariectomized group (OVX, n = 42). After one week of recovery from surgery, the second group was subdivided into 3 subgroups (14 rats/each subgroup). Subgroup 1 treated with vehicle (distilled water, p.o. daily for 10 weeks). Subgroup 2 treated with CES powder (500 mg/kg BW, p.o. daily for 10 weeks as suspension). Subgroup 3 treated weekly with alendronate, via gavage, as a positive control with a dose of 6.5 mg/kg BW/week (Rosenberg et al., 2007). At the end of experiment, one half of the animals (n = 7) were sacrificed and blood samples were collected in centrifuge tubes without anti-coagulant, then centrifuged at 3000 rpm for 20 min and the collected sera were stored until use. The two femurs were dissected out and the surrounding tissues were removed. The right femur was frozen for scanning electron microscopy. The left femur was grinded and about 100 mg was taken and homogenized with 2 ml of 0.1 M Tris-HCl buffer (pH 7.2). The homogenate was centrifuged at 3000 rpm for 30 min at 4°C and the supernatant was used for biochemical and oxidative stress marker estimations (Ramajayam et al., 2007). All animal procedures were conducted according to the “Guide for the Care and Use of Laboratory Animals” and were approved by the local Cairo University animal research ethics committee.

**Measurements of bone mineral content (BMC) and bone mineral density (BMD)**

At the end of experiment, one half of the experimental rats (n = 7) were anesthetized and the BMD and BMC of the left tibia were estimated using dual energy X-ray absorptiometry (DEXA, Norland X 46, Version 3.9.6) instrument equipped with dedicated software for small animal measurements (Figure 2). BMC (expressed in grams) was divided by the area of the site that was scanned to obtain BMD (expressed in grams per centimeter squared) (Jo and Choi, 2008).

**Determination of tibial calcium and phosphorus contents**

The right tibia was dried overnight at 60°C for the determination of dry weight and then ashed at 700°C for 14 h. After weighing, the ashes were dissolved in conc. HCl followed by dilution with deionized water and then used for measurement of calcium and phosphorus levels by a colorimetric method using biodiagnostic kits (Teófilo et al., 2003).

Scanning electron microscopy (SEM)

The distal part of the right femur was trimmed in a coronal plane. They were treated with 5% sodium hypochlorite solution (commercial bleach) for 4 h to expose the epiphyseal and metaphyseal trabecular bone. The bones were then dehydrated in ethanol, dried, mounted on stubs and coated with gold using a sputter coater (Miller and Bowman, 1998). The bones were observed by a JEOL, JSM-5200 scanning electron microscope.

**Biochemical analyses**

The appropriate clinical kits (Biodiagnostic, Egypt) were used for the determination of several parameters. Calcium and phosphorus contents in serum and tibial bone were measured according to Gindler and King (1972) and El-Merzabani et al. (1977), respectively. Serum total alkaline phosphatase (TALP) and bone alkaline phosphatase (BALP) were determined colorimetrically according to the method described by Belfield and Goldberg (1971). TALP and BALP were estimated on the day of collection as ALP has a half-life of 1 to 2 days (Swaminathan, 2001). Parathyroid hormone (PTH) was estimated using IRMA PTH kit (A11930, Beckman Coulter Company) and calcitonin was determined by DRG ELISA kit according to Tiegä et al. (1986). Bone supernatants were used for estimation of malondialdehyde (MDA) according to Ohkawa et al. (1979), glutathione peroxidase (GPx) according to Paglia and Valentine (1967), superoxide dismutase (SOD) according to Nishikimi et al. (1972) and total antioxidant capacity (TAC) according to Koracevic et al. (2001).

**Statistical analysis**

Statistical analyses were carried out using statistical package for social sciences (SPSS) v.15 software. All data were expressed as means ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan post hoc test for comparison between two groups using SPSS software. P values of less than 0.05 were considered statistically significant.

**RESULTS**

**Chemical composition of CES**

The present study revealed that the calcium and phosphorus contents of CES powder were 193.8 and 3.15 mg/g shell powder, respectively. Table 1 shows that the CES powder containing various amino acids.

**DPPH free radical-scavenging activity**

Figure 3 shows that CES powder exhibited a great antioxidant activity where it scavenged above 60% DPPH free radical at the lowest concentration (10 mg/ml). The CES powder exhibited highly antioxidant effect at 70 mg/ml at this concentration CES exhibited 85.47% inhibition in comparison with ascorbic acid (the reference standard) which showed 93.38% inhibition at the same concentration.
**Figure 1. Ovariectomy procedure in rat.** (A) Anesthetized rat is laid on operating table from the back. (B) Shaving site indicated by black star. (C) Skin incision point approximately 1.5 cm is made in the shaving area, indicated by arrow. (D) Ovary and uterine horn are exposed, the ovary was indicated by arrow. (E) The left ovary was removed, indicated by arrow. (F) The right ovary was removed, indicated by arrow. (G) Complete bilateral ovariectomy. (H-L) Muscle, peritoneal cavity and skin were sutured and covered by antiseptic and antibiotic.

**Acute toxicity study**

The oral administration of CES powder (5000 mg/kg BW) caused neither mortality nor signs of clinical abnormality. At necropsy, no gross pathological observation was found in the target organs as liver and kidney (Figure 4). The LD$_{50}$ of CES powder was found to be more than 5000 mg/kg BW and cannot be categorized under the globally harmonized system (GHS) category.

**BMD and BMC of the tibia**

The tibial BMC and BMD were decreased significantly (P < 0.05) in the OVX group, as compared to the sham group. Treatment with CES powder (500 mg/kg BW) or alendronate (6.5 mg/kg BW/week) significantly alleviated the decreasing effect of ovariectomy on BMD (Table 2). On the other hand, treatment with CES powder or alendronate did not cause any significant difference in
Figure 2. Representative photo showing bone mineral density of tibia by DEXA.

Concentration (mg/ml)

Figure 3. Anti-free radical activity of CES determined by DPPH assay.

Calcium and phosphorus contents

Ovariectomy was found to cause a significant loss (P < 0.05) in tibial and serum calcium contents when compared to sham group (Table 3). Meanwhile, CES powder supplementation or alendronate treatments were found to help in restoring the tibial calcium level significantly (P < 0.05). On the other hand, a non-significance increase was observed in the levels of serum calcium in CES powder and in alendronate groups (Table 3). Bone and serum phosphorus contents were decreased significantly
Figure 4. Effect of CES at a limit dose (5000 mg/kg b.wt).
(A) Liver section of the control group showing the normal liver architecture with distinct hepatic cells, sinusoidal spaces, central vein, well preserved cytoplasm and prominent nuclei. (B) No obvious change in liver tissue between control and CES groups was observed. (C) Kidney section of the control group showing the normal histological structure of the glomerulus and the Bowman's capsule of Malpighian corpuscles and normal appearance of the tissue. (D) No change was observed in the cortical region of the kidney between the control and CES groups. Bowman's capsule (BC), central vein (CV), glomerulus (G), hepatocyte (H), kupffer cell (K), sinusoid (S) (100×, H&E).

Figure 5. Scanning electron micrographs of the femoral distal metaphyses in rats. (A) Sham group, (B) OVX group, (C) CES group and (D) Alendronate group. As compared with the OVX group, the trabecular bones of the femoral distal metaphysis reappear in the CES and alendronate groups. Scale bar = 500 µm.

Table 1. Amino acid constituents of CES powder.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mg/g shell powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>0.28298</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.60133</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.72873</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.41405</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.25844</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.25125</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.18246</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.48985</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.69051</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.79625</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.40131</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.23824</td>
</tr>
<tr>
<td>Isoleucine and leucine</td>
<td>0.82036</td>
</tr>
<tr>
<td>Proline</td>
<td>0.5217</td>
</tr>
<tr>
<td>Serine</td>
<td>0.95178</td>
</tr>
<tr>
<td>Threonoine</td>
<td>0.79533</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.888</td>
</tr>
<tr>
<td>Valine</td>
<td>0.512</td>
</tr>
</tbody>
</table>

Table 2. Effect of CES on BMD and BMC of the left tibia of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>BMD (g/cm²)</th>
<th>BMC (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.112±0.010abc</td>
<td>0.289±0.034a</td>
</tr>
<tr>
<td>OVX</td>
<td>0.082±0.002bc</td>
<td>0.122±0.007b</td>
</tr>
<tr>
<td>OVX+CES</td>
<td>0.102±0.004a</td>
<td>0.143±0.018c</td>
</tr>
<tr>
<td>OVX+Alendronate</td>
<td>0.122±0.002c</td>
<td>0.112±0.008b</td>
</tr>
</tbody>
</table>

All values are means ± SE (n = 7). Values with different superscript letters are significantly different (P < 0.05).

administration caused significant rise (P < 0.05) only in serum phosphorus content, as compared to OVX rat group (Table 3).

Scanning electron microscopic analysis

The connectivity of trabecular bone in the metaphysis exhibited greater connection in sham control group (Figure 5A). However, OVX caused a decrease in femoral trabeculae, appearance of porous and erosive bone as well as disintegration in bone architecture (Figure 5B). CES and alendronate treatments suppressed this decrease and the morphology of the metaphysis remained nearly normal (Figure 5C and D).

Alkaline phosphatase activity

Statistically, a non-significant difference in serum TALP concentration among the different experimental groups

(P < 0.05) in OVX rats, as compared to sham group. These contents were increased significantly (P < 0.05) subsequent to CES powder supplementation, as compared to OVX rats (Table 3). Meanwhile, alendronate...
Table 3. Effect of CES on calcium (Ca) and phosphorus (P) contents in serum and bone of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Ca (mg/dl)</th>
<th>Bone Ca (mg/g ash)</th>
<th>Serum P (mg/dl)</th>
<th>Bone P (mg/g ash)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>9.89±0.697</td>
<td>84.95±3.5</td>
<td>5.24±0.258</td>
<td>50.3±2.38</td>
</tr>
<tr>
<td>OVX</td>
<td>5.44±0.59</td>
<td>46.38±3.12</td>
<td>2.8±0.331</td>
<td>33.16±0.86</td>
</tr>
<tr>
<td>OVX+CES</td>
<td>7.21±0.697</td>
<td>90.4±2.7</td>
<td>4.298±0.369</td>
<td>51.76±2.85</td>
</tr>
<tr>
<td>OVX+Alendronate</td>
<td>6.815±0.281</td>
<td>57.64±1.27</td>
<td>4.524±0.292</td>
<td>37.57±1.84</td>
</tr>
</tbody>
</table>

All values are means ± SE (n = 7). Values with different superscript letters are significantly different (P < 0.05).

Table 4. Effect of CES on ALP activity of serum (TALP) and bone (BALP) of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum TALP (IU/L)</th>
<th>BALP (IU/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>100.315±7.383</td>
<td>24.9±2.134</td>
</tr>
<tr>
<td>OVX</td>
<td>120.078±10.147</td>
<td>6.7±0.976</td>
</tr>
<tr>
<td>OVX+CES</td>
<td>110.506±12.628</td>
<td>23.07±1.755</td>
</tr>
<tr>
<td>OVX+Alendronate</td>
<td>99.636±10.293</td>
<td>13.15±0.907</td>
</tr>
</tbody>
</table>

All values are means ± SE (n = 7). Values with different superscript letters are significantly different (P < 0.05).

Table 5. Effect of CES on serum calcitonin and parathyroid hormone (PTH) levels of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Calcitonin (pg/ml)</th>
<th>PTH (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>39.32±1.498</td>
<td>25.96±0.8135</td>
</tr>
<tr>
<td>OVX</td>
<td>32.27±0.655</td>
<td>36.73±2.0153</td>
</tr>
<tr>
<td>OVX+CES</td>
<td>37.66±0.516</td>
<td>25.47±1.271</td>
</tr>
<tr>
<td>OVX+Alendronate</td>
<td>34.78±1.239</td>
<td>26.86±0.564</td>
</tr>
</tbody>
</table>

All values are means ± SE (n = 7). Values with different superscript letters are significantly different (P < 0.05).

was recorded (Table 4). However, ovariectomy induced a significant decrease (P < 0.05) in the BALP activity, as compared to sham rats. CES powder and alendronate treatment increased the BALP activity significantly (P < 0.05), as compared to OVX rats.

Serum calcitonin and PTH levels

Serum calcitonin level of OVX rats was decreased significantly (P < 0.05), in comparison with rats of the sham group. Concerning serum PTH, OVX rats showed a significant increase (P < 0.05) in their concentration, as compared with the sham group. Interestingly, administration of CES powder restored significantly (P < 0.05) the serum calcitonin and PTH levels near to its normal value (Table 5). On the other hand, administration of alendronate drug caused a significant decrease (P < 0.05) only in the PTH level, as compared with OVX group (Table 5).

Bone oxidative stress markers

Table 6 shows a significant increase (P < 0.05) in the level of bone MDA in OVX rats, as compared to rats of the sham group. Administration of CES powder or alendronate ameliorated the abnormal effects caused by ovariectomy, since a significant decrease (P < 0.05) in MDA levels were recorded. GPx and SOD activities were significantly decreased (P < 0.05) in OVX rats in comparison with sham group. Interestingly, CES powder or alendronate treatment increase GPx and SOD activities significantly (P < 0.05), as compared to OVX rats (Table 6). TAC was significantly decreased (P < 0.05) in the OVX rats, as compared to the sham rats. Meanwhile, CES powder supplementation caused pronounced increase in TAC level, as compared to OVX rats. However, treatment with alendronate caused a non-significant change concerning serum TAC level of rats, as compared to OVX rats (Table 6).

DISCUSSION

Osteoporosis is a metabolic bone disease which results from a disturbance in the normal bone remodeling, tilting the balance to bone resorption over formation. Low calcium diet leads to a decrease in calcium absorption and this may be the most important mechanism involved in bone loss in aging humans (Alevizaki et al., 1973). Therefore, calcium supplements are used for aiding treatment of osteoporosis and fracture prevention (Nordin, 1997).

Han et al. (2007) worked on Ostreae testa shell powder that contain inorganic calcium and proved that this powder prevents bone loss in OVX mice. The present work recorded that one of the main constituent of CES powder is calcium where it contains about 19.38% of inorganic calcium. Moreover, the current study revealed that CES powder contained several amino acids (cysteine, glutamic acid, glycine and methionine). It was
reported that these amino acids reduce lipid peroxidation, protect membrane damage and restore the changes of the glutathione system (Slyshenkov et al., 2002). Furthermore, the CES powder showed a unique scavenging activity by reacting with DPPH and therefore it could be considered as one of the natural antioxidants. The present acute toxicity study revealed that the LD₅₀ of CES powder is in excess of 5,000 mg/kg. This finding represents an indication of CES powder safety. On the other hand, alendronate as many drugs has several adverse effects (Kuehn, 2009).

Measurement of BMD of bone provided good predictable information about efficacy of anti-osteoporotic agents (Diez, 2002). The current study manifested that OVX significantly decreased the BMD and the BMC of the total tibia when compared with the control group and this indicated that tibial bone loss had occurred. Picherit et al. (2000) reported that the decrease in BMD and BMC may attribute to increased bone turnover and/or disturbance in calcium homeostasis in ovariectomized rats. In addition, Sontakke and Tare (2002) reported that an increase in superoxide formation by the osteoclasts is often accompanied with active bone resorption and loss of bone density. The present study demonstrated that calcium supplementation when combined with ovariectomy had reverse the influence on tibial bone loss and this finding is in consistent with the observation of Zhang et al. (2006) who worked on Lepidium meyenii. CES powder may increase the BMD as it contains the major mineral elements such as calcium and phosphorus which are necessary to bone building. Also, CES powder could reduce bone loss by stimulating the antioxidant defense system and enhance the antioxidant status, this explanation is supported by the view of Pasco et al. (2006).

Calcium was found to be a significant predictor of BMD change (Macdonald et al., 2004). The ongoing study found that ovariectomy led to a significant hypocalcemia in the serum and bone of rats and this result was found to be in consistent with Zhang et al. (2006). Again, Burali et al. (2010) reported that ovariectomy resulted in an impaired calcium balance which could also have contributed to demineralization and eventually development of postmenopausal osteoporosis. Katz and Weinerman (2010) added that osteoclast cells secrete both hydrogen ions (resorb the mineral component) and proteases (digest the protein matrix) and these explanations interpret the decreased levels of Ca, P and ALP in bone in the current study. The decreased levels of Ca and P caused by ovariectomy were ameliorated in CES group better than alendronate group and this is in agreement with Prabhakara and Lakshmana (2003) who worked on herbomineral preparation (OST-6). This improvement may be due to an increased calcium uptake from the CES powder administration. The present finding of bone Ca and P contents support the observations concerning the BMD and BMC of the different experimental groups.

ALP is considered as one of key enzymes for bone calcification which provides an index of bone formation (Squadrito et al., 2002). In fact, the total ALP (TALP) level in serum is less sensitive as a bone formation marker, since the present levels of serum TALP showed an insignificant difference between the different experimental groups. Whyte (1994) reported that bone ALP, which is produced by immature osteoblasts, play an essential role in the initiation of bone mineralization and is a more specific marker of bone formation. Concerning bone ALP (BALP), the present result showed that OVX decreased the activity of ALP in bone tissue and this was found in consistent with Raisz (1997). He mentioned that a characteristic decline in the BALP associated with postmenopausal women is due to disturbance in osteoblast function. The decreased level of BALP was ameliorated by CES powder treatment better than alendronate treatment. This may indicate a positive effect on osteoblast differentiation, particularly bone mineralization (Mori-Okamoto et al., 2004) and a higher osteoblast activity account for bone formation which in turn prevents osteoporosis (Omara et al., 2009).

Consequently, the current study demonstrated the beneficial effect of CES powder on osteoblastic cells and bone formation. Furthermore, the increased level of BALP in CES treated group may be attributed to the increased levels of Ca and P contents in bone (Buckwalter and Cooper, 1987). Debernard et al. (1986) reported that BALP could act as a plasma membrane transporter for inorganic phosphate, or an extracellular calcium-binding protein that stimulates calcium phosphate precipitation and orients mineral deposition into osteoid. BALP may also be involved in the mineralization process by hydrolyzing
organic phosphates to release free inorganic phosphorus at sites of mineralization (Mori-Okamoto et al., 2004).

The present results revealed a significant elevation in PTH level and a significant reduction in calcitonin level in OVX rats and these results are consistent with Shalaby et al. (2011). Riggs and Melton (1986) attribute the hyperparathyroidism in osteoporotic rats to calcium deficiency and exacerbated by estrogen deficiency. The principal role of PTH lies in its responsibility for the minute-to-minute regulation of calcium levels in the blood and extracellular fluid. Furthermore, the PTH and calcitonin levels were reversed in CES treated group, as compared to OVX group. CES powder may exert its effect by increasing calcitonin level, which plays a major role in Ca homeostasis by inhibiting osteoclast-mediated bone resorption through the regulation of both the number and activity of osteoclasts (Karsdal et al., 2008). The DEXA measurements together with increased levels of bone forming markers indicate that the incorporation of minerals into bone matrix was enhanced in CES supplement. This indicates that the shell powder could be effective for bone formation.

Recently, various evidences were linked bone loss to ROS (Yalin et al., 2012). MDA is one of the main products of lipid oxidation and it has been widely used as an index of lipid peroxidation (Suttnar et al., 2001). The current investigation showed a relation between osteoporosis and ROS generation, since the OVX rats showed a significant elevation of MDA level and significant decrease of GPx, SOD and TAC activities. These results are in agreement with Sontakke and Tare (2002) and Ozogcmen et al. (2007). Yalin et al. (2005) illustrated that SOD, CAT and GPx are the major enzymatic antioxidants involved in the protection against O$_2^{-}$ and H$_2$O$_2$. Interestingly, these effects were reversed in rats treated with CES powder. In contrast, alendronate induces oxidative stress in the liver of ovariectomized rats (Yalin et al., 2010). In addition, the present results indicate that non-significant change was recorded in TAC level after alendronate treatment in comparison to OVX group. The protective effect that appears after the treatment of OVX rats with CES powder may be due to osteoblast activation which produces antioxidants such as GPx to protect against oxidative damage and this expectation was supported by Dreher et al. (1998). Furthermore, the protective efficacy of CES powder may be due to its antioxidant activity. Basu et al. (2001) reported that antioxidants scavenge free radicals and have been shown to inhibit bone resorption and stimulate bone formation.

The scanning electron microscope (SEM) images of femur add a confirmatory note to the findings of all biochemical analysis. Again, the high rate of bone turnover was well corrected by CES powder, suggesting that CES may have protective action against ovarian hormone insufficiency-related bone resorption, this is in agree with the explanation of Burali et al. (2010).

Conclusion

CES powder is considered an attractive candidate for developing a potential therapeutic agent and a cheap alternative for postmenopausal osteoporosis treatment. However, much detailed investigation has to be undertaken in this regard to validate the use of CES powder in postmenopausal osteoporotic women condition.

ACKNOWLEDGEMENT

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REFERENCES


Full Length Research Paper

Potentially inappropriate prescribing of Thai older adults in an internal medicine outpatient clinic of a tertiary care hospital

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Potentially inappropriate medications (PIMs) are a common problem in older adults and are associated with negative outcomes. The objectives of this study were to evaluate the prevalence of and the factors associated with the use of PIMs by elderly patients in an outpatient setting of the tertiary care hospital. A retrospective medical record audit was randomly reviewed in 308 elderly patients in 2010. Beers criteria (2003) and the screening tool of older persons’ potentially inappropriate prescriptions (STOPP) were used to identify PIMs. The results showed that the median number of medicines per patient was 5.6 (inter-quartile ranges 3.5 to 7). Prevalence of PIMs determined by Beers criteria and STOPP was 19.2 and 31.5%. The only factor that exhibited associations was the higher number of prescription medications based on STOPP criteria (odds ratio 1.2, 95% confidence interval 1.1 to 1.4), p<0.05. PIMs are highly prevalent among older adults in the tertiary care setting and are associated with greater number of medications based on STOPP. Beers criteria are a less sensitive tool than STOPP to detect PIMs for Thai older adults.

Key words: Beers criteria, screening tool of older persons’ potentially inappropriate prescriptions (STOPP) criteria, Thai older adults, medication errors, inappropriate drug use.

INTRODUCTION

Older adults are likely to have co-morbidities that require multiple medications. Potentially inappropriate medications (PIMs) are defined as medications which take more risks than benefits, medications with clinically significant drug-drug or drug-disease interactions and the possible omission of potentially useful medications (Chen et al., 2012). PIMs are a common problem in older adults and are associated with negative outcomes including a significant risk of adverse drug events, increased healthcare costs, and hospitalization that increases morbidity and mortality up to 100,000 deaths per year in the US (Wehling, 2011). Polypharmacy, a practice that is the use of more medication than is clinically indicated or warranted, has an increasing trend in this population (Michocki, 2001). The US reported that persons at the age of 65 and above, in approximately 44 and 57% of men and women take 5 or more drugs, and 12% take 10 or more drugs. There is evidence that the number of concomitant medications of 5 or more is associated with different percentages of adverse outcomes such as frailty 6.5, disability 5.5, mortality 4.5, and falls 4.5 (Gnjidic et al., 2012). Although PIMs are prevalent in older adults, many can be preventable which consequently decreases poor outcomes (Page et al., 2010).

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**Screening tools to detect PIMs** have been formulated to help physicians and pharmacists including Beers criteria and screening tool of older persons’ potentially inappropriate prescriptions (STOPP). Beers criteria were originated in 1991 and latest updated in 2012 (American Geriatrics Society, 2012). It was designed to identify PIMs of older adults in primary care, secondary care, and nursing homes. It detected inappropriate medication use in the US and European countries at about 21.3 to 28.8% and 9.8 to 38%. A recent systematic review reports that the prevalence of PIMs varies from 11.5 to 62.5% among community-dwelling elderly in which the majority of the studies were conducted in the US using Beers criteria (Guaraldo et al., 2011). The STOPP was developed in 2008 to decrease the limitations of the Beers criteria. It provides good inter-rater reliability with a kappa-coefficient of 0.75 and 0.68 (Fick et al., 2003; Gallagher et al., 2008; Ryan et al., 2009). The advantages of the STOPP consist of greater inter-rater reliability, inclusion of both American and European drugs, organization and structure based physiological systems, and a short time to complete (about 3 min) (Page et al., 2010). It has been found that STOPP is more sensitive than Beers criteria to detect PIMs in European countries (Miguel et al., 2010; O’Mahony et al., 2010). For Asian countries, the prevalence of PIMs based on Beers criteria in ambulatory care visits and emergency department visits of Taiwan were 19.1 and 19.3% (Chen et al., 2009; Lai et al., 2009). In the nursing home setting of Malaysia, the reported prevalence of PIMs was 32.7 and 23.7% based on Beers criteria and STOPP (Chen et al., 2012). These results indicated that STOPP is less sensitive than Beers criteria to detect PIMs. Factors associated with PIMs can be classified as patient, physician, and visit characteristics. These factors are varied in different studies. The factors associated with patient characteristics are female sex, low educational level, advanced age, black skin color, and longer stays in nursing home. For physician characteristics, significant factors associated with PIMs are male sex, older age, family medicine/general practice, and the factors related to visit characteristics are greater numbers of drug prescribed, primary care setting, and use of medications supplied by the government (in Brazil) (Chen et al., 2012, 2009; Guaraldo et al., 2011; Lai et al., 2009; Oliveira et al., 2012). Common medications associated with PIMs are short-acting nifedipine, methydopa, first-generation antihistamines, muscle relaxants/antispasmodics, and long-acting benzodiazepines.

In Thailand, PIMs used among Thai elderly have not been studied widely. Using a Delphi technique with the three-round survey of 16 geriatric medicine experts to determine high-risk medication use found that about 80% of practices embraced the use of high-risk drugs with potential adverse reactions, drug-disease interactions, and drug-drug interactions. The most common groups of high-risk medications were for the central nervous, musculoskeletal and cardiovascular systems (Winit-Watjana et al., 2008). Application of Beers criteria and STOPP in this population has not been studied broadly. Therefore, the primary objective of the present study was to evaluate the prevalence of PIMs using Beers (2003) criteria and STOPP in the geriatric outpatient care of the internal medicine department. The secondary objective was to identify factors-associated with PIMs.

**MATERIALS AND METHODS**

**Study participants**

A retrospective medical record audit was carried out for all patients of the age of 65 years of age or older, who attended the Internal Medicine Outpatient Clinic of Srinagarind Hospital Medical School between January 2010 and December 2010 and who had at least consecutive three-month visits and took at least one daily medication. The exclusion criterion was being a terminally-ill patient defined as patient who is diagnosed with disease(s) that cannot be cured or adequately treated and that is reasonably expected to result in the death within a short period of time such as advance cancer and advance dementia. Because this patient was more likely to have higher rates of inappropriate medications, they were not being representative of independently living community-based elderly patients.

**Instrument**

The instruments used in this study were Beers criteria, STOPP, and Charlson Co-morbidity Index (CCI). The Beers criteria version 2003 was used to identify PIMs in this study. The Beers criteria are explicit and composed of two comprehensive lists of medications to be avoided in older people both independent of diagnosis and considering diagnosis. Many of the criteria, however, are controversial (Fick et al., 2003). STOPP consists of 65 clinically significant criteria based on physiological systems for potentially inappropriate prescribing in older people (Fick et al., 2003; Gallagher et al., 2008; Ryan et al., 2009). Each criterion is accompanied by a brief reason for the inappropriate prescribing. CCI was developed in 1987. It is a weighted index to predict 1-year patient mortality. It is correlated with disability, readmission, and length of stay outcomes using comorbidity data from hospital chart reviews. The final CCI score is the sum of 19 predefined comorbidities that were assigned weights of 1, 2, 3, or 6. These weights were based on the magnitude of the adjusted relative risks associated with each comorbidity in a Cox proportional hazards regression model and incorporate increasing age as an independent risk factor. This tool was used to quantify the chronic illness status of the older persons (Hall et al., 2004; Needham et al., 2005; Ryan et al., 2009).

**Procedure**

The patient demographic data including medical histories, current diagnoses and current medications were recorded by a physician. The CCI using an electronic application would be calculated and recorded for each patient. Beers criteria (2003) and STOPP were applied to their clinical datasheets. All recorded disease states and medical conditions were coded to facilitate data analysis. Disease codes were assigned so that each disease was given a unique number from 1 to 308 and was then grouped according to the principal physiological system affected.

**Statistical analyses**

Demographic data variables which included baseline characteristics including patient characteristics (e.g. CCI, number of prescribed medications) and physician characteristics (that is, age group, sex
and specialty) were divided into dichotomous or polytomous variables. All variables were summarized using descriptive statistic presentations in percentages, means and standard deviations. If the distribution of these data was not a normal distribution, then medians, and inter-quartile ranges were used instead. The prevalence of PIMs was defined as having at least one PIM based on explicit criteria. Multiple logistic regression analysis was used to determine the strength and direction of the association between PIMs and the possible predictors. The results are presented as odds ratios, and 95% confidence intervals (CIs). A test result with p<0.05 was considered statistically significant. All analyses were undertaken using STATA version10 (StataCorp, College Station, TX).

Sample size

Sample size calculations were based on the estimated prevalence of potentially inappropriate prescriptions using Beers criteria and STOPP from literature reviews (Fick et al., 2003; Lai et al., 2009; Ryan et al., 2009). The estimation of a population proportion with a specified absolute precision formula was used to calculate this (Chirawatkul, 2008). A sample size of at least 270 participants was sufficient to achieve this at the significance level of 0.05.

Ethics approval was provided by Ethics Committee of the Faculty of Medicine, Khon Kaen University as instituted by the Helsinki Declaration.

RESULTS

Demographics

Descriptive demographics of the 308 study subjects are shown in Table 1. Male and female numbers were equal. Median age was 72.8 years and median number of prescribed drugs was about 6. The educational level of study subject could be identified in 70% of subjects and the majority of them were lowly educated (< 6 years of education). Regarding comorbidity, the average CCI was 4.7±3.5. The top 5 common diagnoses were hypertension, diabetes, dyslipidemia, cerebrovascular diseases and musculoskeletal conditions. Focusing on physician characteristics, most were male sex, 40 years old or younger and staff specialists in internal medicine. The median numbers of medications prescribed per age category are as shown in Figure 1. There was no statistical significance of the numbers in each age group (p>0.05).

Potentially inappropriate medications (PIMs) determined by Beers criteria and STOPP

Prevalence of PIMs determined by Beers criteria and STOPP was 19.2 and 31.5%. There was a statistically significant difference between both criteria with prevalence rate ratios (PRR) of 6.6, 95% confidence intervals (CI) of 4.9 to 8.8, p<0.05. Majority of the subjects were prescribed with 1 PIM (10.1 and 16.7% for Beers criteria and STOPP, respectively). The median number of PIMs prescribed using Beers criteria was 1 (inter-quartile range (IQR); 1 to 2) and for STOPP was 2 (IQR: 1 to 3). The common medications associated with PIMs are shown in Table 2. According to Beers criteria, calcium channel blockers, anticholinergics and tricyclic antidepressants (50%) were the common medications associated with PIMs based on considering diagnosis whereas amitriptyline group, chlorzoxazone-amitriptyline and perphenazine-amitriptyline (27.5%) were the most common medication prescribed independent of diagnosis. Followed by use of short-acting benzodiazepines over than recommended doses (25%). Regarding PIMs identified by STOPP, about one-fifth of all PIMs were medications that adversely affect falling patients (that is, benzodiazepines) and thus were the leading medications.

Predictors of potentially inappropriate medications

Multiple logistic analyses showed that only a higher number of prescription medications increased the risk of PIMs based on STOPP with an odds ratio (OR) of 1.2 (95% CI: 1.1 to 1.4), p<0.05 and subjects with hypertension showed a decrease in risk of PIMs identified by both Beers (OR: 0.2; 95% CI: 0.1 to 0.6) and STOPP (OR: 0.3, 95% CI: 0.1 to 0.6), p<0.05 after adjusted with the number of drugs, age of patient, patient’s gender, patient’s educational level, comorbidity of subjects, CCI, physician’s age, physician’s specialty and physician’s gender.

DISCUSSION

This study confirms the high prevalence of PIMs among older adults (Guaraldo et al., 2011; Lai et al., 2009; Ryan et al., 2009). In this study, approximately 20 to 30% of older adult who attended internal medicine outpatient setting had at least 1 PIM. Comparing these results to prior studies, the figures varied from 16.3 to 62.5% (Guaraldo et al., 2011). This can be explained by the diversity in the severity of disease in the study subjects. The study included subjects with underlying medical illnesses who attended internal medicine outpatient clinic in a tertiary care setting, so the disease severity is likely to be higher than primary care setting, confirming the high mean CCI (4.7±3.5). STOPP was more sensitive than Beers criteria to identify PIMs in this study, supporting previous reports (Miguel et al., 2010; O’Mahony et al., 2010). This is however the reverse of the results of some studies e.g. study in nursing homes of Malaysia. This may also be explained by the differences in drug availability and prescribing practices (Chen et al., 2012).

Considering PIMs using Beers criteria and STOPP, anticholinergics in particular tricyclic antidepressants, benzodiazepines (long term use of long-acting group or short-acting group with higher than usual doses), NSAIDs and aspirin (ASA), and muscle relaxants and antispasmodics...
showed the high proportion using both criteria. As compared to prior studies, the common medications related to PIMs were diverse according to study setting. Nevertheless, benzodiazepines and tricyclic antidepressants associated with PIMs in this report is similar to that of other reports (Buck et al., 2009; Chen et al., 2012; Lai et al., 2009). Other common prescriptions related to PIMs that were not identified in this study were short-acting nifedipine and fluoxetine (Buck et al., 2009; Oliveira et al., 2012). The possible explanation is the inclusion criteria of this study; short-acting nifedipine was usually prescribed in patients with particular conditions e.g. systemic scleroderma with Raynaud phenomenon which commonly occurs in a younger age group, but the study subjects in the older age group usually have atherosclerotic-related diseases such as hypertension, diabetes and dyslipidemia. Therefore, long-acting calcium channel blockers were the majority of prescriptions for hypertensive treatment. This study reviewed prescriptions only from the internal medicine outpatient setting, so fluoxetine which is usually prescribed by a psychologist cannot be identified as medication-related to PIMs. The high proportion of PIMs in this study may reflect a lack of understanding the prescribing medication principles in the elderly and public health policy that limits the use of elderly-friendly medications. Therefore, physicians do not have many choices for drug prescription. For example, amitriptyline is a common medication prescribed for neuralgia. Other safer drugs are spared in case of amitriptyline failure or having an adverse effect from this medication.

Regarding factors associated with PIMs, this study can identify only that a higher number of prescription medications predicts PIMs using STOPP which is similar to prior studies (Buck et al., 2009; Chen et al., 2012; Guaraldo et al., 2011; Lai et al., 2009; Oliveira et al.,

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### Table 1. Patient demographics and physician characteristics.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Total (N=308)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>157 (51%)</td>
</tr>
<tr>
<td>Age (years); median (IQR)</td>
<td>78 (68,77)</td>
</tr>
<tr>
<td>Age range</td>
<td>65-95</td>
</tr>
</tbody>
</table>

**Educational level**

<table>
<thead>
<tr>
<th>Level</th>
<th>Total (N=308)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No data</td>
<td>54 (30.52%)</td>
</tr>
<tr>
<td>≤ 6 years of education</td>
<td>133 (43.18%)</td>
</tr>
<tr>
<td>7-12 years of education</td>
<td>44 (14.29%)</td>
</tr>
<tr>
<td>&gt;12 years of education</td>
<td>37 (12.01%)</td>
</tr>
<tr>
<td>Median number of prescribing drugs (IQR)</td>
<td>5.6 (3.5,7)</td>
</tr>
<tr>
<td>Mean CCI (±SD)</td>
<td>4.7 (3.5)</td>
</tr>
</tbody>
</table>

**Top 5 common diseases**

- Hypertension: 137 (44.5%)
- Diabetes: 85 (27.7%)
- Dyslipidemia: 56 (18.2%)
- Cerebrovascular diseases: 46 (14.9%)
- Musculoskeletal conditions: 43 (14%)

**Physician characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (N=306, unavailable=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male physician</td>
<td>211 (69%)</td>
</tr>
</tbody>
</table>

**Physician age group**

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total (N=306, unavailable=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 40 years</td>
<td>130 (42.5%)</td>
</tr>
<tr>
<td>41-50 years</td>
<td>78 (25.5%)</td>
</tr>
<tr>
<td>&gt; 50 years or over</td>
<td>98 (32%)</td>
</tr>
</tbody>
</table>

**Physician position**

<table>
<thead>
<tr>
<th>Position</th>
<th>Total (N=306, unavailable=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resident and fellow</td>
<td>114 (37.2%)</td>
</tr>
<tr>
<td>Staff</td>
<td>192 (62.8%)</td>
</tr>
</tbody>
</table>

IQR: Inter-quartile range, N: total number of subjects, CCI: Charlson Co-morbidity Index, SD: standard deviation.
Table 2. Lists of common medications associated with inappropriate prescriptions.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Top 5 common medication</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Considering diagnosis</td>
<td>Calcium channel blockers, anticholinergics and tricyclic antidepressants</td>
<td>50</td>
</tr>
<tr>
<td>GU/DU</td>
<td>NSAIDs and aspirin</td>
<td>25</td>
</tr>
<tr>
<td>Blood clotting disorders/receiving anticoagulant therapy</td>
<td>NSAIDs, aspirin, dipyridamole, ticlopidine and clopidogrel</td>
<td>25</td>
</tr>
<tr>
<td>Cognitive impairment</td>
<td>Barbiturates, anticholinergics, antispasmodics, muscle relaxants and CNS stimulants</td>
<td>25</td>
</tr>
<tr>
<td>Depression</td>
<td>Long-term benzodiazepine use, sympatholytic agents: methylpoda, reserpine and quanethidine</td>
<td>25</td>
</tr>
<tr>
<td>Beers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Independent diagnosis</td>
<td>Amitriptyline, chlordiazepoxide-amitriptyline and perphenazine-amitriptyline</td>
<td>27.5</td>
</tr>
<tr>
<td>Doses of short-acting benzodiazepines: doses &gt; lorazepam 3 mg; oxazepam 60 mg; alprazolam 2 mg; temazepam 15 mg; triazolan 0.25 mg</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Anticholinergics and antihistamines: chlorpheniramine, diphenhydramine, hydroxyzine, cyproheptadine, promethazine, triplelenamine and dextchlorpheniramine</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Muscle relaxants and antispasmodics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td>Short-acting dipyridamole</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>Drugs that adversely affect fallers</td>
<td>Benzodiazepines</td>
<td></td>
</tr>
<tr>
<td>Endocrine system</td>
<td>Glibenclamide or chlorpropamide with type 2 DM</td>
<td>8.24</td>
</tr>
<tr>
<td>Central nervous system and psychotropic drugs</td>
<td>Long-term, long-acting benzodiazepine</td>
<td>8.24</td>
</tr>
<tr>
<td>Cardiovascular system</td>
<td>ASA at dose &gt; 150 mg/day</td>
<td>7.06</td>
</tr>
<tr>
<td>Cardiovascular system</td>
<td>ASA with no history of CAD, CVD or PVD</td>
<td>7.06</td>
</tr>
<tr>
<td>Gastrointestinal system</td>
<td>PPI for PUD at full therapeutic dosage for &gt; 8 weeks</td>
<td>3.53</td>
</tr>
</tbody>
</table>


2012). Beers criteria are likely less sensitive in this study because many medications documented in Beers criteria are not widely used or available in this setting, for example drugs such as propoxyphene and combination products, trimethobenzamide and reserpine. Other predictive factors associated with PIMs such as female sex, low educational level, advanced age, and physician characteristics could not be identified. Hypertension showed decreased odds ratios of PIMs in both Beers criteria and STOPP, similar to one report (Chen et al., 2012). The results cannot conclude that hypertension is a protective factor associated with PIMs. A possible reason is the hypertension guidelines are worldwide and recommend prescribed antihypertensive medications that are rather safe for the elderly. There were a number of hypertensive subjects in this study.

This study represents pattern of prescribing medication regarding geriatric pharmacotherapy in an outpatient setting of a tertiary care hospital. Nonetheless, it is not necessary that the prescription of PIMs will cause adverse events in older adults. Because there have been controversies about applying Beers criteria and STOPP in different settings and that these criteria focus on explicit criteria, they may not be useful in specific medical conditions; individual assessment remains the key factor in the consideration of prescription (Chen et al., 2012; Fick et al., 2003). These criteria can be a good clinical tool to help physicians and pharmacists considering possible
medication-related adverse effects and for reduction of drug-related costs, overall healthcare costs, adverse drug event-related hospitalizations, and improving care in older adults (Chen et al., 2012; Fick et al., 2003). Encouraging physicians and pharmacists to use a screening tool for PIMs as one of geriatric assessments would be worthwhile. The STOPP criteria are likely more sensitive to the outpatient setting among Thai older adults. Further research to study about the benefits of STOPP and Beers criteria is required in the area of negative outcomes, e.g. hospitalization rates, emergency visit rates and healthcare costs and focusing on different settings, e.g. community, hospital and institutional care, among Thai older adults. Additionally, it is essential to develop a new medication reviewing tool that is suitable for Thai older adults. An effective approach can lead to improve the appropriateness of prescribing in the ambulatory care setting and decreases adverse outcomes related to PIMs.

There were several limitations in this study. Firstly, data were collected retrospectively; some information was unavailable in all subjects such as educational level and over-the-counter drugs, and some conditions such as constipation might not be documented in medical records even though the subjects had that condition. Therefore, the prevalence of PIMs might be underestimated. Secondly, this study was conducted prior to updated Beers criteria in 2012 ("American Geriatrics Society Updated Beers Criteria for potentially Inappropriate Medication Use in Older Adults", 2012). Therefore, identification of PIMs in this study is based on Beers criteria version from 2003. Finally, this study reviewed medication prescribing in the internal medicine outpatient clinic only. Thus the actual proportion of PIMs might be higher if the patients took medication from other sources.

**Conclusions**

Potentially inappropriate medications are of a high prevalence in a geriatric ambulatory setting of a tertiary care hospital. STOPP is a more sensitive tool than Beers criteria for Thai older adults to detect PIMs. A higher number of medications are associated with greater numbers of PIMs based on STOPP. Healthcare professionals, especially physicians and clinical pharmacists play an important role by reviewing medications at every visit based on explicit criteria. Further research is required to study possible negative outcomes, diverse settings, and to develop a new medication reviewing tool that is appropriate for Thai older adults.

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A prospective study of drug consumption and wastage during anaesthesia in a tertiary hospital

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The aim of this study was to estimate the amount of anesthetic drug consumption and wastage during elective surgical procedures in a tertiary hospital. This is a prospective observational study. Using convenient sampling, data was generated on all the drugs provided to the patients included in the study. The amount of drugs administered was documented, and the leftover in syringes/ampoules and unopened anaesthetic drugs at the end of each procedure over a three month period. The following drugs that were most wasted due to not being used after loading into syringe were atracurium 37.78%, morphine 26.33%, pethidine 23.33%, propofol 15.2%, pancuronium 15.2%, and diazepam 10%. The drugs which accounted for more than 50% of the cost of waste were propofol 36.5% and 0.5% for bupivacaine 27.8%. The amount of drugs left in the penetrated vial was most abundant with lignocaine with adrenaline 95%, followed by plain lignocaine 93.3% and ketamine 47.91%. Wastage of anaesthetic drugs does occur. Efforts should be made to improve efficiency as regards drug prescription and administration by anaesthetists.

Key words: Anaesthetic agents, unused, syringe, ampoule, drug cost, wastage.

INTRODUCTION

Drugs for anaesthesia are packaged in specific amounts in ampoules or vials. These drugs can only be withdrawn after the ampoules are broken or after the rubber stopper is penetrated by the hypodermic needle. Thus they need to be used within a specific period. Following an outbreak of Staphylococcus aureus infection among patients booked for electroconvulsive therapy (ECT) using propofol based anaesthesia, an audit was instituted. This showed a higher risk for such infection among patients administered propofol that had a greater time between preparation and administration as well as breaches in infection control protocol (Kuehnert et al., 1997). Thus, due to concerns regarding infection control and contamination, partially used ampoules/syringes or loaded but unused syringes are usually discarded either after the end of a procedure or a specific time. Such wasted drugs have been shown to contribute significantly to the cost of intraoperative anaesthesia care (Weinger, 2001). In the hospital, each patient provides his/her drugs. It is thus possible to determine the amount of drugs dispensed to patients. Therefore, to calculate waste, it is necessary to know how much of each drug dispensed is actually administered to patients and the leftover. For this study, drug consumption was defined as the volume of dispensed drugs that were administered to the patient, while drug wastage was defined as the disposal of unused or partially used ampoules/vials/syringes containing these drugs. The aims of this study were to assess anaesthetic drug consumption, measure volume/percentage and cost of anaesthetic drug wastage.
and suggest ways of minimizing drug wastage.

MATERIALS AND METHODS

This prospective observational study was conducted in the operating room of a tertiary-care hospital during the first quarter of the year (January to March, 2013) for a wide range of procedures (urological, general surgical, obstetrics/gynaecological, neurosurgical, orthopaedics, plastics, ear, nose and throat (ENT; excluding cardiac/transplant surgeries). In the hospital, anaesthesia care is provided by consultants and resident anaesthetists in at least six theatre suites daily on a regular basis. To ensure that the study procedure did not affect the practice of the anaesthetists, they were not informed about the study. The choice of anaesthetic technique and drugs were left to the discretion of the anaesthetists performing the procedure. They were interviewed about the drugs used and leftover at the end of each procedure, but were shielded from knowing it was for research.

Elective surgeries conducted under general and regional anaesthesia in patients aged greater than 18 years were included in the study, while surgeries performed under local anaesthesia and emergencies were excluded. Inhalational agents were also not considered in this study. The cases were selected based on convenience; the data were collected whenever the authors were rostered for duty for elective procedures. The drugs were taken out on a case by case basis as each patient procures his/her own drugs.

An assistant (anaesthesia registrar) was enrolled to aid in data collection. The anaesthetists conducting the procedure chose the technique, drugs, and undertook drug preparation and administration. At the end of each procedure, the assistants would collect data for drug wastage. The amount of drugs issued and the unopened ampoules were documented. The volumes of drugs administered to the patients were noted from the anaesthesia chart. Drug wastage was considered as the amount of drugs left used in the syringes and ampoules at the end of the surgery. The drugs left in the syringes were discarded as waste. Unopened ampoules were kept for use at a later date.

The type of drug and amount per ampoule for each elective case were tabulated. The data generated was used to calculate the percentage drug wastage per drug.

An estimation of the consumption and wastage of the drugs was made in percentages. A cost estimation of used and wasted drugs was made human system is designed to produce waste. Anaesthesia is not exempted from waste generation. It has been noted that a routine operation in a hospital often produces more waste than a family of 4 might produce in an entire week (Roy, 2009).

A major reason cited for this is the need for sterile surgical supplies and equipment. This in turn creates the need for extra packaging and or the use of disposables. Potential sources of anaesthesia-related waste include items such as syringes, drug vials, intravenous cannula, spinal epidural needles, blood bags and drugs. For this study, the anaesthesia-related drug waste was of concern. This waste occurs when drugs are loaded in syringes but are either partially used or unused but discarded at the end of the surgical procedure. This waste is of concern in anaesthesia, because it represents a hidden source of waste of healthcare funds and inadvertently increases the cost of anaesthesia care (Gillerman et al., 2000; Smith, 2003). Furthermore, some of these accounted for 13.3%, and suxamethonium accounted for 7.3%.

The drugs which accounted for maximum wastage due to not being used after loading into syringe (in relation to the volume prescribed) were atracurium 37.78%, morphine 26.33%, pethidine 23.33%, propofol 15.2%, pancuronium 15.2%, and diazepam 10%.

The amount of drugs left in the penetrated vial was most abundant with lignocaine with adrenaline 95%, followed by plain lignocaine 93.3% and ketamine 47.91%.

Some drugs were provided but some of the ampoules were not opened. These were in descending order adrenalinine and aminophylline 100% each, acetaminophen 75%, diazepam 60%, oxytocin 28.5%, ranitidine 27.3%, ephedrine 24.4%, pancuronium 23.4%, propofol 20.95%, ketamine 17.3%, glycopyrrolate 17.1%, and atropine 11.4%.

Considering the cost, the most wasted drug was propofol ₦22,960 followed by 0.5% bupivacaine ₦17,512. A lot of drugs were not wasted, that is, every ampoule that was broken or every loaded syringe was completely administered. These were neostigmine, ergotamine, oxytocin, glycopyrrolate, atropine, etc (Tables 1 and 2).

The total amount of money wasted through drugs being loaded in syringes but not used was ₦62,886. The drugs which accounted for more than 50% of the cost of waste were propofol 36.5% and heavy bupivacaine 27.8%, while atracurium accounted for 6.1% of the total cost of waste, pancuronium 9.7%, ketamine 7.8%, plain lignocaine 4.5%, and lignocaine with adrenaline 4.8%.

DISCUSSION

Every natural or man-made human system is designed to produce waste. Anaesthesia is not exempted from waste generation. It has been noted that a routine operation in a hospital often produces more waste than a family of 4 might produce in an entire week (Roy, 2009).

A major reason cited for this is the need for sterile surgical supplies and equipment. This in turn creates the need for extra packaging and or the use of disposable equipment. Potential sources of anesthesia-related waste include items such as syringes, drug vials, intravenous cannula, spinal epidural needles, blood bags and drugs. For this study, the anesthesia-related drug waste was of concern. This waste occurs when drugs are loaded in syringes but are either partially used or unused but discarded at the end of the surgical procedure. This waste is of concern in anaesthesia, because it represents a hidden source of waste of healthcare funds and inadvertently increases the cost of anaesthesia care (Gillerman et al., 2000; Smith, 2003). Furthermore, some of these
Table 1. Showing available drugs and frequency of use.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount prescribed</th>
<th>Amount used</th>
<th>Used (%)</th>
<th>Amount leftover</th>
<th>Leftover (%)</th>
<th>Unopened ampoules</th>
<th>Unopened (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propofol</td>
<td>191</td>
<td>122.30</td>
<td>64.03</td>
<td>28.70</td>
<td>15.02</td>
<td>40.00</td>
<td>20.95</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>11</td>
<td>8.00</td>
<td>72.7</td>
<td>0.00</td>
<td>0</td>
<td>3.00</td>
<td>27.3</td>
</tr>
<tr>
<td>Suxamethonium</td>
<td>84</td>
<td>75.10</td>
<td>89.40</td>
<td>0.90</td>
<td>1.07</td>
<td>8.00</td>
<td>9.53</td>
</tr>
<tr>
<td>Ergotamine</td>
<td>5</td>
<td>5.00</td>
<td>100</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Glycopyrrolate</td>
<td>35</td>
<td>29.00</td>
<td>82.86</td>
<td>0.00</td>
<td>0</td>
<td>6.00</td>
<td>17.14</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>42</td>
<td>30.00</td>
<td>71.43</td>
<td>0.00</td>
<td>0</td>
<td>12.00</td>
<td>28.57</td>
</tr>
<tr>
<td>Atropine</td>
<td>244</td>
<td>216.00</td>
<td>88.52</td>
<td>0.00</td>
<td>0</td>
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<td>11.48</td>
</tr>
<tr>
<td>Aminophylline</td>
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<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td>0</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>Pancuronium</td>
<td>152</td>
<td>100.75</td>
<td>66.28</td>
<td>15.25</td>
<td>10.04</td>
<td>36.00</td>
<td>23.68</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>3</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td>0</td>
<td>3.00</td>
<td>100</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>73</td>
<td>68.00</td>
<td>93.15</td>
<td>0.00</td>
<td>0</td>
<td>5.00</td>
<td>6.85</td>
</tr>
<tr>
<td>Ephedrine</td>
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<td>41.00</td>
<td>74.55</td>
<td>0.00</td>
<td>0</td>
<td>14.00</td>
<td>24.45</td>
</tr>
<tr>
<td>Atracurium</td>
<td>9</td>
<td>5.60</td>
<td>62.22</td>
<td>3.40</td>
<td>37.78</td>
<td>0.00</td>
<td>0</td>
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<tr>
<td>Metoclopropamide</td>
<td>13</td>
<td>12.00</td>
<td>92.31</td>
<td>0.00</td>
<td>0</td>
<td>1.00</td>
<td>7.69</td>
</tr>
<tr>
<td>Ketamine</td>
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<td>7.98</td>
<td>34.70</td>
<td>*11.02</td>
<td>*47.91</td>
<td>4.00</td>
<td>17.39</td>
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<td>0</td>
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<td>75</td>
</tr>
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<td>Pethidine</td>
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<td>2.80</td>
<td>23.33</td>
<td>1.00</td>
<td>8.33</td>
</tr>
<tr>
<td>Ketorolac</td>
<td>25</td>
<td>20.00</td>
<td>80</td>
<td>0.00</td>
<td>0</td>
<td>5.00</td>
<td>20</td>
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<td>Tramadol</td>
<td>59</td>
<td>43.80</td>
<td>74.24</td>
<td>1.20</td>
<td>2.03</td>
<td>14.00</td>
<td>23.73</td>
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<tr>
<td>Diazepam</td>
<td>5</td>
<td>1.50</td>
<td>30</td>
<td>0.50</td>
<td>10</td>
<td>3.00</td>
<td>60</td>
</tr>
<tr>
<td>Morphine</td>
<td>15</td>
<td>11.05</td>
<td>73.67</td>
<td>3.95</td>
<td>26.33</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>3</td>
<td>3.00</td>
<td>100</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Plain lignocaine</td>
<td>9</td>
<td>0.60</td>
<td>6.67</td>
<td>*8.40</td>
<td>*93.33</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Lignocaine with adrenaline</td>
<td>9</td>
<td>0.45</td>
<td>5</td>
<td>*8.55</td>
<td>*95</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Isobaric bupivacaine</td>
<td>10</td>
<td>10.00</td>
<td>100</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>0.5% bupivacaine</td>
<td>52</td>
<td>32.10</td>
<td>61.73</td>
<td>*19.90</td>
<td>*38.27</td>
<td>0.00</td>
<td>0</td>
</tr>
</tbody>
</table>

*Volume left in vials/ampoules.

Drugs are expensive adding to the concern on wastage (Tohans, 1995).

For this study, we used the method of directly recording drugs supplied, administered and left over for individual procedures. This is similar to the method undertaken by Hannah Dee (2012) but differed from that of Weinger (2001) who considered the drugs remaining at the end of a standard workday; and Gillerman and Browning (2000) who used indirect approaches to measure waste. Mankez (2012) collected his data by analyzing waste bins at the end of each procedure. However, due to safety concerns arising from lack of appropriate safety wear, we did not use this. This may result in some underestimation especially during regional blocks when used materials (needles and used syringes for infiltration) are discarded in the sharp bin immediately after instituting the block. Also, we were unable to identify incidences of broken ampoules as they may not have been noted. Also, some anaesthetists may dispose syringes immediately or quickly at the end of a procedure to prevent misuse and mix-ups when starting a fresh case.

From our study, propofol was the most wasted by volume as was expected based on other findings. Our study showed wastage of the equivalent of 28.7 ampoules which is about 287 ml by volume. However, based on the ratio in percentage of drug provided but wasted, the drugs which accounted for maximum wastage due to not being used after loading into syringe were atracurium 37.78%, morphine 26.33%, pethidine 23.33%, propofol 15.2%, pancuronium 10.4%, and diazepam 10%. The reasons for maximal wastage with atracurium could be the use of inhalational agents which are known to prolong or add to muscle relaxant effect, because they themselves induce some degree of muscle relaxation. It was found out that pethidine and morphine were wasted most because some of the withdrawn drugs were added to local anaesthetics used for spinal blocks.

Suxamethonium is used to facilitate endotracheal intubation. It showed wastage of less than 2% while ephedrine used for its sympathomimetic effects was never wasted.

Our findings were similar to other studies as regards...
ult to time surgeries to ensure immediate rendering and 51%
found the wastage cost of rocuronium (72%) to be higher than propofol (13%). However, wastage due to discarded propofol accounted for 18 to 20% of total intraoperative costs in various studies (Stuttner et al., 1999; Rosenberg et al., 1994; Tohans, 1995). Roy et al. (2009) in their study, found 33% wastage of suxamethonium and 51% wastage of propofol.

In the health system of the country, because of low health insurance coverage (Onwujekwe et al., 2013), most patients provide all the drugs needed for surgery mostly on the basis out of pocket payment. Thus, there is no pool of drugs from which the anaesthetists can collect the drugs for use at a later date for the same patient. Also, for safety concerns, anaesthetists should ensure that sterile needles are used if multi-spike vials are provided and that the time limit for infection control is strictly adhered to.

Our study also revealed a huge leftover amount of

<table>
<thead>
<tr>
<th>Drug</th>
<th>Unit cost</th>
<th>Total cost</th>
<th>Cost of used drugs</th>
<th>Cost of wasted drugs</th>
<th>Cost of unused drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propofol</td>
<td>800</td>
<td>152800</td>
<td>97840</td>
<td>22960</td>
<td>32000</td>
</tr>
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<td>425</td>
<td>4675</td>
<td>3400</td>
<td>0</td>
<td>1275</td>
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<td>22260</td>
<td>19901.5</td>
<td>238.5</td>
<td>2120</td>
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<td>225</td>
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<td>0</td>
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<tr>
<td>Glycopyrrolate</td>
<td>90</td>
<td>3150</td>
<td>2610</td>
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<td>540</td>
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<tr>
<td>Oxytocin</td>
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<td>2700</td>
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<td>1080</td>
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<td>0</td>
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<td>0</td>
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<td>1375</td>
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<tr>
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<td>Atracurium</td>
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<tr>
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<td>3511.2</td>
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<td>1760</td>
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<td>Acetaminophen</td>
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<td>20</td>
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<td>60</td>
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<tr>
<td>Pethidine</td>
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<td>120</td>
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<td>2500</td>
<td>2000</td>
<td>0</td>
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</tr>
<tr>
<td>Tramadol</td>
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<td>4130</td>
<td>3066</td>
<td>84</td>
<td>980</td>
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<tr>
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<td>370</td>
<td>1850</td>
<td>555</td>
<td>185</td>
<td>1110</td>
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<tr>
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<td>2431</td>
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<tr>
<td>Dexamethasone</td>
<td>20</td>
<td>60</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plain lignocaine</td>
<td>340</td>
<td>3060</td>
<td>204</td>
<td>*2856</td>
<td>0</td>
</tr>
<tr>
<td>Lignocaine with adrenaline</td>
<td>350</td>
<td>3150</td>
<td>157.5</td>
<td>*2992.5</td>
<td>0</td>
</tr>
<tr>
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<td>880</td>
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<td>880</td>
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<td>0</td>
</tr>
<tr>
<td>0.5% Bupivacaine</td>
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<td>45760</td>
<td>28248</td>
<td>*17512</td>
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<tr>
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<td>191</td>
<td>800</td>
<td>-</td>
<td>122.30</td>
<td>28.70</td>
</tr>
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</table>
certain drugs in their vials. These were ketamine, lignocaine with or without adrenaline. This was probably due to the volume of drugs contained in the vials. Ketamine is not used frequently in modern times as the sole anaesthetic agent except when indicated. It may thus be prudent to provide the drug in smaller volumes. The same logic would also be applicable to lignocaine prescribed for regional blocks to minimize wastage.

Reducing drug wastage is important. Hawkes et al. (1994) have suggested that efforts to minimize drug wastage by raising cost awareness may be useful. Using cheaper drugs have been advocated by Lubarsky et al. (1997). In their study they found using cheaper drugs albeit with less favorable pharmacologic profiles, reduced drug expenditure and had no adverse effect on patient outcome. Riley (1997) however did not agree with this practice. He opined that drug costs should not be taken in isolation but that other costs should be considered. These include cost of surgeons’, nurses’, anaesthetists’ time especially where there is an increase in emergence time, as well as patient suffering and rare complications.

CONCLUSION AND RECOMMENDATION

Drug wastage does occur in our environment. The implicated drugs are propofol, atracurium and lignocaine and bupivacaine. Attempts should be made to reduce wastage. Regular system audit, creating awareness through posters placed in the theatres or changing rooms and lectures may help reduce wastage. It may also be beneficial if pre-packaged drug syringes are provided especially for lignocaine and ketamine. These can be saved if not opened.

There is also a need to study the cost preferences of patients and other costs in view of Riley’s line of thought.

REFERENCES


Substandard rifampicin based anti-tuberculosis drugs common in Ugandan drug market

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Tuberculosis (TB) is an important curable infectious disease in Uganda; its treatment however is facing a challenge of increasing drug resistance. Although rifampicin containing fixed-dose combination (R-FDC) drugs are a mainstay in TB treatment, about one fifth is reportedly substandard in the global drug market. The aim of this study was to determine the quality of R-FDC and rifampicin single formulation anti-TB drugs in Kampala city, Uganda. Eight private and five public pharmacies were randomly selected, the drug samples were purchased and in the latter case obtained free of charge. Drug quality was assessed by visual inspection, weight uniformity, dissolution, and assay. Fifteen batches of anti-TB drugs were collected; 13 R-FDC and two rifampicin single dose formulations. One batch of R-FDC collected from a public pharmacy was not assayed as it had passed its expiry date by the time of analysis. Of the samples analyzed, six batches of R-FDC and two of rifampicin single dose formulations were purchased from private pharmacies. The other six batches of R-FDC were obtained from public pharmacies. Ten samples (10/14: 71.4%) were not in the National Drug Register (NDR), eight of which were R-FDC and two rifampicin single dose formulations. Of the R-FDC drugs, four samples (4/12: 33.3%) failed the assay test and were all not in the NDR. All the R-FDC drug samples passed the dissolution, visual inspection and weight uniformity tests. All rifampicin single dose formulations passed assay, visual, and weight uniformity tests and only one failed the dissolution test. Unregistered and sub-standard rifampicin anti-TB drugs are common in drug outlets on the Ugandan drug market. These substandard drugs pose a risk to patients such as treatment failure that leads to increased cost of treatment and possibly loss of lives.

Key words: Rifampicin, fixed-dose combination, post-market quality, drug quality, assay.

INTRODUCTION

Tuberculosis (TB) is the world’s leading curable infectious killer disease (World Health Organization (WHO), 2008). The WHO estimated global TB prevalence as 14.4 million with the incidence of 363/100,000 population and 355/100,000 population in Africa and Uganda, respectively (WHO, 2008). Uganda being one of the most highly TB burdened countries of the world is faced with the challenge of drug resistance to TB treatment. In a previous study by Bertzel (1999), the reported rate of Multi-Drug Resistant TB (MDR-TB) in Uganda was slightly over 4% in
in patients with a history of prior treatment and about 1% in patients with no history of prior treatment. A number of factors contribute to development of resistance to TB medications. Among these factors is use of substandard medications in TB treatment and lack of patient supervision (Pecoul, 1999; Panchagnula, 2004).

Rifampicin (3-[4-methyl-1-piperazinyl] iminomethyl rifamycin SV) is a semi synthetic macro cyclic antibiotic derived from Streptomyces mediterranei (Martindale, 2002). It is one of the most effective anti-tuberculosis agents available and is bactericidal for both intra- and extra-cellular mycobacteria (Chambers, 2001).

In spite of the increased use of fixed-dose combination (FDC) drugs in TB treatment, initiatives by global organizations such as WHO to combat TB using such drugs are challenged due to the reported presence of poor quality FDC anti-TB drugs on the global drug market (Pillai et al., 1999). A study by Laserson (2001) showed that 21% of rifampicin containing FDC anti-TB drugs is substandard in the global drug market.

In a study done in Nigeria by Taylor et al. (2001), 33% of rifampicin single-drug formulations supplied by Nigerian pharmacies were substandard. The quality of drugs in many less developed countries is inadequate (Behrens et al., 2002). The market quality of rifampicin containing FDC anti-TB drugs has raised great concern, especially the poor bioavailability of rifampicin, and its instability (Pillai et al., 1999). This therefore calls for routine quality assessment of TB medications in the drug market, in the current study; we evaluated the quality of rifampicin containing FDC and single formulation anti-TB drugs sampled from pharmacies in Kampala city Uganda.

MATERIALS AND METHODS

Chemicals, reagents and anti-TB drugs

Rifampicin containing FDC anti-TB drugs registered in Uganda were obtained from the Human drug register of National Drug Authority (NDA). The rifampicin standard was a donation from the National Drug Quality Control Laboratory (NDQCL) of NDA. Acetonitrile (HPLC-grade) and methanol (pro analytical grade), were obtained from Scharau Chemie, Spain. Potassium dihydrogen orthophosphate, sodium hydroxide and orthophosphoric acid were obtained from Merck gmbH (Darmstadt, Germany). All other chemicals were of analytical grade and were used without purification.

Study design and sample collection

This was a cross-sectional study. The study drugs were purchased from registered private dispensing pharmacies and obtained free of charge from National Tuberculosis and Leprosy Program (NTLP-TB) treatment centers in Kampala city, Uganda. The drug outlets, private pharmacies and government TB treatment centers were randomly selected. The study was approved by Makerere University, Faculty of Medicine Research Ethics Committee and the Uganda National Council for Science and Technology. The number of drug outlets from which the drugs were sampled was calculated according to World Health Organization (1999). Operational guide for National tuberculosis control program (Hogerzell, 1997). For a country wide drug survey, the minimum number of private drug outlets from which drugs are to be collected should be 20 and 40 for government centers.

\[ S_a = P \times 20 \]

where \( S_a \) is the number of the private pharmacies to be sampled in the city and \( P = n_1/n \), where, \( n_1 \) is the number of private pharmacies in the city (328) and \( n \) is the number of private pharmacies in the country (620); 20, is the minimum number of private pharmacies to be selected (WHO, 1999) was adopted.

\[ S_a = P \times 40 \]

where \( S_a \) is the number of public pharmacies to be sampled in the city, and \( P = n_1/n \), where \( n_1 \) is the number of public pharmacies in the city (36) and \( n \) is the number of public pharmacies in the country (113); 40, is the minimum number of public outlets to be selected (WHO, 1999) was also adopted.

The drugs were collected from eleven (11) private pharmacies and thirteen (13) public pharmacies at NTLP-TB treatment centers which were selected using simple random sampling method.

Instrumentation and chromatographic conditions

The chromatographic analysis was carried out using a Schimadzu LC system (Hitachi, Japan) equipped with a photometric detector, a standard flow cell, a quaternary pump with a degasser, an auto sampler and column oven with Class-VP Ver. 6.1 software. The analytical column was an ODS1 (4.6 × 250 mm, 5 μm, Waters, Ireland). The mobile phase consisted of 40% acetonitrile in sodium phosphate buffer. The buffer was made by weighing and dissolving 1.4 g of anhydrous dibasic sodium phosphate in 1 liter of distilled water. The pH was then adjusted to 6.8 using ortho-phosphoric acid 85%. The absorbance was monitored at 238 nm and elution was carried out at room temperature using a flow rate of 1.00 ml per min. Run time was set at 12 min.

Sample preparation and analytical procedure

Twenty tablets or capsules were randomly picked from each sample and individually weighed. All the tablets were finely powdered together using a mortar and pestle. For capsules, all contents would be emptied into the mortar and thoroughly mixed. An amount of the powder equivalent to 25 mg of rifampicin was weighed in each case and dissolved in 50 ml of the mobile phase. The mixture was sonicated for 10 min and the resultant solution was filtered. Two milliliters of the filtrate was separately transferred and diluted in two 25 ml volumetric flasks with mobile phase. Ten microliters from each of the diluted solutions were injected into the chromatographic system. Ten microliters (10 μl) of the freshly prepared standard solution was injected in duplicate on each day of analysis. Fresh stock solutions of the samples and standards were prepared in a dark room. The drug samples were also analyzed in duplicate. Drug assays were performed in the department of Pharmacology and Therapeutics, Makerere University College of Health Sciences.

Standards and calibration procedure

Rifampicin standard was prepared by weighing 25.8 mg, then dissolving it in mobile phase using 50 ml volumetric flask. The mixture
mixture was sonicated for ten (10) min and the resultant solution was filtered. 2 ml of the filtrate was diluted with mobile phase in a 25 ml volumetric flask. A calibration curve with four calibration points was generated on each day of analysis and a regression equation with the slope, intercept and correlation coefficient ($r^2$) was generated using Microsoft excel program. The curve was used for calculating the drug concentrations.

**Weight uniformity**

**Tablets**

Twenty (n = 20) tablets were randomly selected from the different blister or strip packs of one sample and individually weighed using the analytical balance. This procedure was repeated for all the samples that were of tablet formulation.

**Capsules**

An intact capsule was randomly selected from the blister packs of the sample and weighed using the analytical balance. The capsule was then opened without losing any part of the shell and all the content emptied. The shells were then separately weighed. The weight of the content was the difference in the weight of the shell and the intact capsule. This was repeated for another nineteen randomly selected capsules of the same sample. This procedure was repeated for all the samples that were of capsule formulation.

**Drug dissolution**

Six vessels of the electro lab tablet tester United States Pharmacopeia (USP) (XXIII) were filled with 900 ml of 0.1 M HCl solution and allowed to equilibrate at 37± 0.5°C. Rifampicin FDC tablets or capsules of each sample were each placed in six baskets. The baskets were then fixed on six separate metallic rods corresponding to the six vessels. The metallic rods with the baskets were then lowered into the vessels until the baskets were fully submerged in the dilution medium and then rotated at 100 rpm for 45 min. After 45 min, the metallic rods automatically stopped rotating and 25 ml of the medium in each vessel withdrawn from the middle of the vessel and filtered. The filtrate was left to equilibrate to room temperature for 10 min. Dilution of the drug solutions (filtrate) for absorbance reading was done in accordance with Lambert beer’s law. The dilutions of the filtrate were made in 50 ml volumetric flasks. The required volume of the filtrate was transferred to the flask using a pipette, then 10.0 ml of potassium phosphate buffer was added and the flask filled to the mark with 0.1 M HCl (Table 1). The absorbance of the solution was measured using a spectrophotometer at a wavelength of 475 nm. Absorbance of a corresponding concentration of the rifampicin standard prepared at the same time was also measured and percentage drug (rifampicin) dissolution calculated using the formulae.

**Preparation of rifampicin standard for dissolution test**

Rifampicin standard (16.7 mg) was weighed and transferred into a 50 ml volumetric flask. The powder was dissolved in 10 ml of 0.1 M HCl, sufficient amount of 0.1 M HCl was added up to the mark. The solution was then sonicated for 45 min. The resultant solution was filtered, discarding the first 5 ml of the filtrate. The filtrate was left to equilibrate to room temperature for 10 min. A quantity of the filtrate was transferred using a pipette and diluted in a 50 ml volumetric flask using 10.0 ml of potassium phosphate buffer and 0.1 M HCl (Table 1). The dissolution test was done in Kampala Pharmaceutical Industries (1996) Uganda.

**Formulae used in calculations**

The percentage (%) drug content of the samples was calculated using a formula as per British Pharmacopeia (BP) 27 (2009) and USP 32 (2009) specifications:

$$\text{Percentage} \% = \frac{AUC \times \text{Concentration of sample}}{AUC \times \text{Concentration of standard} \times \text{Potency of standard} \times 100}$$

Where AUC: area under the curve, potency of standard = 100%.

The percentage rifampicin dissolution in the samples was calculated using a formula as per USP 32 (2009):

$$\% \text{dissolution} = \frac{\text{Absorbance (sample)} \times \text{Concentration (standard)} \times \text{Potency of std} \times 100}{\text{Absorbance (standard)} \times \text{Concentration (sample)}}$$

Where absorbance of the standard = 0.455 nm (for dilution of 150/900 × 10/50) and 0.563 nm (for dilution of 450/900 × 2.5/50 and 600/900 × 10/50 × 10/50). Potency of (Std) standard = 100%.

**Statistical analysis**

Drug concentrations obtained from the assay were calculated from the calibration curve in Microsoft excel. Assay, weight uniformity and dissolution data was analyzed using Microsoft excel. The mean, relative standard deviation and proportions were then generated. The sample passed the weight uniformity test if not more than two weights of the twenty individual capsules/tablets had more than 5.0% relative standard deviation (BP, 2009; USP 32, 2009). A sample passed the dissolution test if not less than 80.0% of the drug (rifampicin) dissolved after 45 min (BP, 2009; USP 32, 2009). A sample passed the assay if the content as percentage of the label claim was within the range of 90 to 110% (BP and USP, 2009).

**RESULTS**

The samples were purposively purchased from 8 instead of 11 private pharmacies since the batches already collected from previous pharmacies were being encountered in subsequent pharmacies. The samples were also collected from 5 NTLP-TB treatment centers instead of 13 because at the time of sample collection there was a drug stock out in most NTLP-TB treatment centers in Kampala city. These were the only National TB treatment centers in the city that had drugs at the time and were located in each of the five administrative divisions of Kampala city.

Fifty tablets or capsules per batch were purchased from private pharmacies or obtained free of charge from the NTLP-TB treatment centers. All the drugs were collected in black polythene bags and kept in clean dry drawers at
Table 1. Dilutions of rifampicin standard and quantities of the drug sample solutions used in the dissolution test.

<table>
<thead>
<tr>
<th>Rifampicin standard weight taken (16.7 mg)</th>
<th>Drug dilutions made based on rifampicin label claim</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.7 mg x 4mls</td>
<td>150 mg: 450 x 2.5mls, 50 x 50</td>
</tr>
<tr>
<td></td>
<td>450 mg: 900 x 50</td>
</tr>
<tr>
<td></td>
<td>600 mg: 900 x 50</td>
</tr>
<tr>
<td></td>
<td>10mls x 50</td>
</tr>
<tr>
<td></td>
<td>10mls x 50</td>
</tr>
</tbody>
</table>

Table 2. Details of the rifampicin single-dose and fixed dose combination formulation anti-tuberculosis drugs collected.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample code</th>
<th>Registered in NDR</th>
<th>Stated APIs</th>
<th>Label Amount of APIs (mg)</th>
<th>Pharmacopeia standard label</th>
<th>Manufactured date</th>
<th>Expiry date</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A02T</td>
<td>Yes</td>
<td>R/H</td>
<td>150/100</td>
<td>B.P</td>
<td>08/2008</td>
<td>07/2011</td>
<td>Asia</td>
</tr>
<tr>
<td>2</td>
<td>A03T</td>
<td>No</td>
<td>R/H</td>
<td>150/100</td>
<td>B.P</td>
<td>06/2007</td>
<td>05/2010</td>
<td>E. Africa</td>
</tr>
<tr>
<td>3</td>
<td>A04T</td>
<td>Yes</td>
<td>R/H</td>
<td>150/100</td>
<td>B.P</td>
<td>08/2007</td>
<td>07/2010</td>
<td>Asia</td>
</tr>
<tr>
<td>4</td>
<td>A05T</td>
<td>Yes</td>
<td>R/H</td>
<td>150/100</td>
<td>B.P</td>
<td>11/2007</td>
<td>10/2010</td>
<td>Asia</td>
</tr>
<tr>
<td>5</td>
<td>A06T</td>
<td>No</td>
<td>R/H/Z/E</td>
<td>150/75/400/275</td>
<td>B.P</td>
<td>10/2006</td>
<td>09/2009</td>
<td>Asia</td>
</tr>
<tr>
<td>7</td>
<td>B09T</td>
<td>No</td>
<td>R/H</td>
<td>600/300</td>
<td>U.S.P</td>
<td>03/2008</td>
<td>02/2010</td>
<td>Asia</td>
</tr>
<tr>
<td>8</td>
<td>B10C</td>
<td>Yes</td>
<td>R/H</td>
<td>450/300</td>
<td>U.S.P</td>
<td>07/2007</td>
<td>06/2010</td>
<td>Asia</td>
</tr>
<tr>
<td>9</td>
<td>B11T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
<td>R/H/Z/E</td>
<td>150/75/400/275</td>
<td>U.S.P</td>
<td>07/2008</td>
<td>06/2010</td>
<td>Asia</td>
</tr>
<tr>
<td>10</td>
<td>B12T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
<td>R/H</td>
<td>150/75/275</td>
<td>U.S.P</td>
<td>05/2007</td>
<td>04/2009</td>
<td>Asia</td>
</tr>
<tr>
<td>11</td>
<td>B13T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
<td>R/H/Z/E</td>
<td>150/75/400/275</td>
<td>U.S.P</td>
<td>07/2008</td>
<td>06/2010</td>
<td>E. Africa</td>
</tr>
<tr>
<td>12</td>
<td>B14T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
<td>R/H/Z/E</td>
<td>150/75/400/275</td>
<td>U.S.P</td>
<td>07/2008</td>
<td>06/2010</td>
<td>E. Africa</td>
</tr>
<tr>
<td>13</td>
<td>B15T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes</td>
<td>R/H/E</td>
<td>150/75/400/275</td>
<td>U.S.P</td>
<td>04/2007</td>
<td>03/2009</td>
<td>Asia</td>
</tr>
<tr>
<td>14</td>
<td>AS01C</td>
<td>No</td>
<td>R</td>
<td>150</td>
<td>B.P</td>
<td>04/2008</td>
<td>03/2011</td>
<td>E. Africa</td>
</tr>
<tr>
<td>15</td>
<td>AS08C</td>
<td>No</td>
<td>R</td>
<td>300</td>
<td>B.P</td>
<td>08/2008</td>
<td>07/2011</td>
<td>Asia</td>
</tr>
</tbody>
</table>

A: Samples purchased from the private pharmacies in Kampala. B: Samples donated by the National Tuberculosis and Leprosy Treatment Program tuberculosis treatment centers in Kampala. T - Tablets, C - Capsules, R - Rifampicin, H - Isoniazid, Z - Pyrazinamide, E - Ethambutol, B.P - British Pharmacopeia, U.S.P - United States Pharmacopeia, APIs - Active Pharmaceutical Ingredients, Mg-Miligrams NDR – National Drug Register. *These were drug samples labeled ‘Ministry of Health’ of a neighboring country. **This drug sample was not analyzed since it had passed its expiry date by the time of analysis.

Room temperature in the laboratory in the department of pharmacology and therapeutics until analysis. Each batch of the rifampicin containing FDC anti-TB drugs in the market was collected only once from either the private pharmacies or NTLP-TB treatment centers in Kampala. A total of 15 rifampicin containing formulations were sampled. Two samples were single formulation rifampicin capsules obtained from private pharmacies, one originated from East Africa and the other from Asia. Thirteen samples were FDC containing rifampicin, comprising of two-drug (6), three-drug (2) and four-drug (5) formulations, of which only 12 FDC formulations were analyzed as one had reached expiry before analysis could be done. Most of the FDC drug samples (9/12, 75.0%) analyzed in this study were manufactured from Asia with only a few (3/12, 25.0%) manufactured from East Africa. Of the collected drug samples, 10/15 (66.7%) were not found in the National Drug Register (NDR), comprising eight FDC and the two single formulations (Table 2). All the samples however passed visual inspection and weight uniformity tests (Table 3).

Content and dissolution test results

Four (4/12; 33%) R-FDC anti-TB drug samples failed the assay test. All the samples that failed quality test were not in the NDR. One of the samples that failed the assay
Table 3: Weight uniformity test results of the rifampicin containing fixed dose combination tablet and capsule formulations.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample code</th>
<th>Mean weights (n=20)/g</th>
<th>Standard deviation ($\times 10^{-2}$)</th>
<th>Percentage RSD</th>
<th>Verdict</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A02Tc</td>
<td>0.5</td>
<td>0.6</td>
<td>1.2</td>
<td>Pass</td>
</tr>
<tr>
<td>2</td>
<td>A03Tc</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>Pass</td>
</tr>
<tr>
<td>3</td>
<td>A04Tc</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>Pass</td>
</tr>
<tr>
<td>4</td>
<td>A05Tc</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>Pass</td>
</tr>
<tr>
<td>5</td>
<td>A06Ts</td>
<td>1.1</td>
<td>1.5</td>
<td>1.4</td>
<td>Pass</td>
</tr>
<tr>
<td>6</td>
<td>A07Ts</td>
<td>1.2</td>
<td>1.5</td>
<td>1.3</td>
<td>Pass</td>
</tr>
<tr>
<td>7</td>
<td>B09Tc</td>
<td>1.4</td>
<td>6.5</td>
<td>4.6</td>
<td>Pass</td>
</tr>
<tr>
<td>8</td>
<td>B10Cc</td>
<td>0.8</td>
<td>1.2</td>
<td>1.5</td>
<td>Pass</td>
</tr>
<tr>
<td>9</td>
<td>B11Ts</td>
<td>1.1</td>
<td>0.9</td>
<td>0.8</td>
<td>Pass</td>
</tr>
<tr>
<td>10</td>
<td>B12Td</td>
<td>0.8</td>
<td>0.7</td>
<td>0.9</td>
<td>Pass</td>
</tr>
<tr>
<td>11</td>
<td>B13Tc</td>
<td>1.1</td>
<td>0.6</td>
<td>0.5</td>
<td>Pass</td>
</tr>
<tr>
<td>12</td>
<td>B14Ta</td>
<td>1.1</td>
<td>0.9</td>
<td>0.8</td>
<td>Pass</td>
</tr>
</tbody>
</table>

A - Samples purchased from private pharmacies, B - Samples donated by the National Tuberculosis and Leprosy Treatment Program tuberculosis treatment centers in Kampala. T - Tablet, C - Capsule, S - Single dose capsules, g - grams, RSD - Relative Standard Deviation (Sample passes the weight uniformity test if the percentage RSD is less than 5%).

Table 4. Content test results of rifampicin analysis from the fixed dose combination formulation samples.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sample code</th>
<th>Rifampicin label claim (mg)</th>
<th>Assayed rifampicin content (mg)</th>
<th>Content as % label claim</th>
<th>Verdict</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A02Tc</td>
<td>150</td>
<td>165.0</td>
<td>110.0</td>
<td>Pass</td>
</tr>
<tr>
<td>2</td>
<td>A03Tc</td>
<td>150</td>
<td>169.5</td>
<td>113.0</td>
<td>Fail</td>
</tr>
<tr>
<td>3</td>
<td>A04Tc</td>
<td>150</td>
<td>151.4</td>
<td>100.9</td>
<td>Pass</td>
</tr>
<tr>
<td>4</td>
<td>A05Tc</td>
<td>150</td>
<td>140.9</td>
<td>93.9</td>
<td>Pass</td>
</tr>
<tr>
<td>5</td>
<td>A06Ts</td>
<td>150</td>
<td>124.8</td>
<td>83.2</td>
<td>Fail</td>
</tr>
<tr>
<td>6</td>
<td>A07Ts</td>
<td>150</td>
<td>118.4</td>
<td>78.9</td>
<td>Fail</td>
</tr>
<tr>
<td>7</td>
<td>B09Tc</td>
<td>600</td>
<td>619.4</td>
<td>102.7</td>
<td>Pass</td>
</tr>
<tr>
<td>8</td>
<td>B10Cc</td>
<td>450</td>
<td>462.2</td>
<td>102.7</td>
<td>Pass</td>
</tr>
<tr>
<td>9</td>
<td>B11Ts</td>
<td>150</td>
<td>111.5</td>
<td>74.3</td>
<td>Fail</td>
</tr>
<tr>
<td>10</td>
<td>B12Td</td>
<td>150</td>
<td>155.4</td>
<td>103.6</td>
<td>Pass</td>
</tr>
<tr>
<td>11</td>
<td>B13Tc</td>
<td>150</td>
<td>156.0</td>
<td>104.0</td>
<td>Pass</td>
</tr>
<tr>
<td>12</td>
<td>B14Ta</td>
<td>150</td>
<td>158.9</td>
<td>105.9</td>
<td>Pass</td>
</tr>
</tbody>
</table>

A - Samples purchased from private pharmacies in Kampala District, B - Samples donated by the National Tuberculosis and Leprosy Treatment Program tuberculosis treatment centers in Kampala. T - Tablet, C - Capsule, S - Single dose capsules, g - grams, RSD - Relative Standard Deviation (Sample passes the weight uniformity test if the percentage RSD is less than 5%).

Of the samples which failed, three were four-drug FDC samples of Asian origin purchased from private pharmacies, while the fourth was a two-drug FDC sample manufactured from a neighboring East African country. Three of the samples that failed the test had rifampicin content below the lower limit of 90.0%, while the fourth had more than the upper limit of 110.0% (Table 4). Of the R-FDC, the dissolution test was only performed on the two-drug FDC samples and all samples passed the test. The single formulation rifampicin capsules, one which was of East...
Table 5. Percentage dissolution of rifampicin single-drug capsules.

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Sample code</th>
<th>Stated active ingredients</th>
<th>Percentage (%) dissolution</th>
<th>Pass/Fail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lowest value</td>
<td>Median value</td>
</tr>
<tr>
<td>1</td>
<td>AS01C</td>
<td>R (150 mg)</td>
<td>71.6</td>
<td>74.6</td>
</tr>
<tr>
<td>2</td>
<td>AS08C</td>
<td>R (300 mg)</td>
<td>83.7</td>
<td>86.4</td>
</tr>
</tbody>
</table>


Figure 1. Calibration curve. $R^2$: slope of the curve. Scale: vertical; 01 small square is equivalent to 100000 volts, horizontal; 01 small square is equivalent to 0.004 mg/ml of rifampicin.

African origin, failed the dissolution test.

**Rifampicin single-formulation capsules**

Two samples of rifampicin single-drug capsules (150 and 300 mg) were tested for percentage rifampicin dissolution. A dissolution test result of one sample was less than 80.0%; hence it failed the test as per BP specifications (Table 5).

**Chromatograms for rifampicin standard and the different R-FDC anti-tuberculosis drugs**

Figures 2, 3, 4, and 5 show the chromatograms of the standard and some of the various R-FDC anti-tuberculosis drugs that were analyzed in this study. The peak that was consistently eluting between 3 to 4 min could not be identified in this study since our interest was rifampicin which eluted between 5 to 6 min.

**DISCUSSION**

The findings from this study show that most rifampicin containing FDC anti-TB drugs in Kampala are mainly manufactured in Asia and they constitute the majority of drugs not found in the NDR. This finding should be of concern to the National drug regulatory body since it clearly shows that these drugs are able to find their way to the Ugandan drug market by-passing the regulatory system. The risks these unregistered drugs pose cannot be understated since they also constituted the drug samples that were declared of poor quality. The proportion of poor quality FDC anti-TB drugs (33.3%) in this study surpassed what had been found in the world market, 21% in a study by Laserson (2001) and in a Nigerian study by Taylor et al. (2001).

One of the R-FDC samples that failed the quality test was obtained from the NTLP-TB treatment center in Kampala; however it was a donation to Uganda from the National government of a neighboring East African country. This brings into focus the dilemma of drug donations (Hogerzeil, 1997). The decision to allow these donated drugs to be circulated through the National TB treatment centers could have been on the premise that the donating country had proof of their quality. This however is no excuse as to why the drugs were not tested for quality before their distribution to the treatment centers.
Figure 2. Rifampicin standard chromatogram.

Figure 3. Two drug R-FDC (B09T) chromatogram.
The use of single formulation rifampicin is discouraged by World Health Organization due to the risks it poses such as unexpected exposure of TB pathogens to sub-therapeutic drug concentrations thus leading to resistance (WHO, 1999). The fact that they are readily available on sale in the private pharmacies in Kampala is an indicator that they are in use probably for other indications other than tuberculosis. Furthermore, they were not registered and one of them failed the quality test. A lot of concern should be raised, not only for the regulating authorities but also for the TB patients and the general population. The possible drug pressure from use of these single formulations in other conditions would directly contribute to emergence and acceleration of resistance to rifampicin, since there are already reports of multidrug resistant tuberculosis in Uganda (Bertzel, 1999). The unsuspecting tuberculosis patients who take these drugs for other indications could be unduly exposing the TB causative organisms to sub-therapeutic rifampicin concentration as a result of taking these substandard drugs.

The findings of this study define the state of the drug market in the country as Kampala is the major centre for drug importation and distribution to other parts of the country. The possibility of the porous nature of the boarders may have contributed to the free entry of unregistered and substandard drugs into the Ugandan drug market, since all the drugs that failed the quality tests were those that were not on the NDR. This should raise concern for the population and the policy makers. However the fact that all the drug formulations passed the visual inspection test is an indication that these were probably genuine products, but the poor quality formulations raise issues of the manufacturing processes.

The WHO emphasizes that anti-TB drugs should not be sold in the private sector due to their lack of patient supervision (Panchagnula, 2004). However, during the sample collection, the study team was alarmed to note that anti-TB drugs could be purchased from the private pharmacies without a prescription, a practice that undermines any efforts to regulate the use of these drugs exclusively in patients diagnosed with tuberculosis. Substandard anti-TB drugs coupled with lack of patient supervision in the private sector are a twin challenge which should raise specific attention from drug regulatory bodies both locally and internationally if the risk and spread of resistance development is to be checked.

The study findings show that four-drug R-FDCs were more likely to have substandard rifampicin content than the other R-FDC drugs. The more the number of drugs incorporated in a fixed dose combination, the higher is the likelihood of compromised quality, a finding similar to what other researchers reported from the global drug market (Bhutani, 2004; Singh, 2003). Previous studies have attributed the frequent occurrence of substandard rifampicin content in four-drug and three-drug FDCs to
the presence of ethambutol in the formulations, which creates an environment that causes rifampicin to be degraded in the presence of isoniazid (Singh, 2001; Sankar, 2003). However, bad formulations due to poor manufacturing practice are more likely to be the reason for the poor quality products found in this study. This is supported by the findings that some two-drug FDC and single formulation rifampicin products also failed the quality test. While the low rifampicin content in the FDC drugs pose risk of resistance development in patients and in the general population (Panchagnula, 2004; Long, 1979), it would however be most tragic if the single formulation of rifampicin was still being used for treatment of patients with tuberculosis in the private sector. This is because of lack of patient monitoring in the private sector exposing them to risks of inadequate treatment (Blomberg, 2001).

CONCLUSION AND RECOMMENDATION

On the Ugandan drug market, unregistered and sub-standard R-FDC anti-TB drugs were found from both the private sector and national TB treatment centers.

The drug regulatory body needs to intensify quality inspection of anti-tuberculosis drugs at the entry points into the country (border posts) since non-registered drugs constituted the highest failures in the current quality assessment study.

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