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A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

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The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

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Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b; Tristan, 1993,1995), (Kumasi et al., 2001)

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Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

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Research Articles

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Full Length Research Paper

Pattern of fatty acids as modulator for dietary iron overload and its influences on testicular function of experimental rats

Mohamed M. Elseweidy[1]*, Mervat E. Asker[1], Sousou I. Ali[1], HebatAllah H. Atteia[1] and Hala Soliman[2]

[1]Biochemistry department, Faculty of pharmacy, Zagazig university, Egypt.
[2]Histology department, Faculty of medicine, Zagazig university, Egypt.

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An iron enriched diet was adopted by different health authorities as an effective tool to combat iron deficiency anemia. However, dietary fat may alter absorption and utilization of iron either in human or animal models. Being a potent pro-oxidant, iron can lead to generation of reactive oxygen species, inducing oxidative damage and inflammations in different tissues. Present study aimed mainly to illustrate any alterations in testicular function of experimental rats kept on dietary iron. Long intake for 10 weeks may affect the testicular function, supplementation of dietary fat of various pattern may alter iron effect. We found a significant increase in serum iron, ferritin, nitrogen oxide (NO), TNF-α, testicular hydroxyproline, thiobarbituric acid reactive substances (TBARS) along with decrease in testosterone level in the group that received dietary iron only (control group). Iron overload induced adhesion of seminiferous tubules, disorganization of germinal epithelium, multiple collagen fibers and iron deposition were also observed. While co-supplementation of palm oil with dietary iron intake significantly decreased testosterone level, olive oil intake induced significant increase. That effect was associated with moderate alleviation of fibrosis and mild regeneration of testicular tissues. We concluded that the iron overloaded diet enhances oxidative stress and inflammation leading to decreased spermatogenesis and testosterone secretion (testicular function). Therefore, supplementation of dietary fats can modulate iron effect.

Key words: Dietary iron, lipid peroxidation indices, olive-palm oils, testicular function.

INTRODUCTION

Previous studies illustrated that dietary fat (type and amount) might affect iron absorption and utilization in animal models (Droke and Lukaski, 1996; Pabon and Lonnerdal, 2001). For example, saturated fatty acids like stearic acid can increase iron absorption and utilization as well as the liver iron content in iron deficient rats (Johnson et al., 1987; Fields and Lewis, 1999). However the reverse was true for oil rich in unsaturated fatty acids like safflower oil. In spite of inducing no increase in liver iron content, safflower oil promoted the development of iron deficiency state (Rao et al., 1983; Fields and Lewis, 1999).

In humans, a diet rich in polyunsaturated fatty acids like linoleic acid can reduce iron retention and balance as compared to highly saturated fatty acids (Lukaski et al., 2001) and in turn affectting mineral status (Milin et al., 2001). According to some researchers, olive oil may exert certain influence on iron status and utilization which may be related to certain alterations in iron absorption or fatty acid composition of cellular membranes (Shotton and

*Corresponding author. E-mail: mmElseweidy@yahoo.com.

Droke, 2004).

In this study, we studied the effect of biscuits enriched with iron (BEI) on testicular tissues in experimental rats through estimation of certain inflammatory marker, serum iron, ferritin, testosterone, nitric oxide (NO), and testicular iron, reduced glutathione (GSH), lipid peroxidation, "thiobarbituric acid reactive substances" (TBARS), and hydroxyproline (Hyp) contents. We have evaluated also the potential effect of an oil rich in saturated fatty acids (palm oil) and another rich in unsaturated fatty acid (olive oil) on these markers using the previously mentioned markers in addition to histological pattern in testicular tissues. The level of dietary iron used was BEI containing 0.3% w/w ferrous sulphate, a dietary iron level adopted by the Egyptian Ministry of Education and used in Egyptian schools and institutes to combat anemia.

MATERIALS AND METHODS

Experimental animals

Sixty male Wistar rats (180 ± 20 g, 12 weeks old) were supplied by the Egyptian Organization for Biological Products and Vaccines. The rats were subjected to controlled temperature (25 ± 2°C) and illumination (12 h light/dark) and allowed free access to a normal rat chow diet and water. This protocol was approved by the Animal Care and Use Committee of the Biochemistry department, Faculty of Pharmacy, Zagazig University.

Experimental design

One week after acclimatization, rats were randomly divided into six experimental groups. Ten rats were kept on BEI (0.3% w/w ferrous sulphate) for 10 weeks, expressed as iron overloaded group while the second group of 10 rats received iron free biscuits and served as a normal control group. The biscuits were composed of flour, starch, cane sugar, hydrogenated vegetable oil, eggs, flavours and vanillin. The third group of 10 rats were given palm oil; the fourth group of 10 rats received olive oil; the fifth group received 15 percent palm oil premixed with BEI; and the sixth and final group received 15 percent olive oil premixed with BEI for 10 weeks. Palm oil was selected since it is rich in saturated fatty acids (plam oil) while olive oil represented an oil rich in monounsaturated fatty acids (oleic acid). At the end of the study, blood samples were collected for serum separation and kept at -80°C until biochemical assays were performed. Rats were killed by decapitation; testicular tissues were removed, rinsed with cold normal saline, divided into parts and dried with filter paper. First part was quickly frozen in liquid nitrogen (-170°C) then stored at -20°C for determination of biochemical parameters. The other part was kept in 10% formalin-saline at 4°C for 1 week, subsequently dehydrated with a series of ethanol solution from 75 to 100% before embedding in paraffin. Cross sections (5 µm thick) were stained with hematoxylin and eosin (H & E) for microscopical examination and Perl´s Prussian blue stain to localize deposited iron and Mallory trichrome stain to illustrate collagen fibers and any fibrotic changes.

Analytical procedures

Serum

Serum iron was determined by colorimetrics using commercial kit (Spinreact, S.A., Spain) (Burits and Ashwood, 1999), NO was measured as nitrite (Moshage et al., 1995), TNF-α was evaluated using ELISA kit purchased from Biosource Int (Ca., USA) (Chen et al.,1998).

Testicular tissues

Determination of TBARS in testes homogenate: 0.5 G tissue was homogenized in 5 ml phosphate buffer (pH = 7.2), centrifuged at 3000g for 15 min at 4°C. The supernatant was collected and lipid peroxidative products were determined (Buege and Aust, 1978) using 1,1,3,3 tetramethoxy propane as a standard. The data were expressed as malondialdehyde equivalents (nmol MDA/g tissue).

Determination of GSH: Glutathione (GSH) was determined spectrophotometrically using Ellman's reagent according to modified method (Ahmed et al., 1991). 0.1 G of tissue was homogenized in 1 ml phosphate buffer (pH = 8) at 4°C. 0.5 ml of homogenate was mixed with 0.5 ml 10% TCA in 5 mM ethylenediaminetetraacetic acid (EDTA) sodium, mixed well and centrifuged at 2,000 g for 5 min. Supernatant was used for determination of reduced GSH.

Determination of iron content: The iron content was determined by flame atomic absorption spectrophotometer. Briefly, 0.1 g tissue was incubated with a mixture of 2 ml conc. nitric acid and 2 ml perchloric acid at room temperature for 24 h for digestion. After incubation, the mixture was filtered, diluted and absorption was measured at 248 nm (Basset et al., 1986).

Determination of hydroxyproline (Hyp): This was determined spectrophotometrically by Ehrlich reagent (Fujita et al., 2003). 0.01 g tissues was pulverized with 500 µl of 6 N HCl, incubated overnight at 120°C. 5 µl of the acid hydrolysate was mixed with 5 µl of citrate acetate buffer and 100 µl chloramines T in ELISA plate and incubated for 20 min at room temperature before addition of Ehrlich solution.

Statistical analysis

All values were expressed as mean ± standard deviation "SD". Analysis was performed using statistical package for social sciences (SPSS) program for windows version 10 (SPSS, Chicago, USA) "student t-test", the analysis of variance "one way ANOVA" was used for the comparison between groups. Pearson correlation was used to study any association between variables. P values < 0.05 were considered statistically significant.

RESULTS

Table 1 shows that rats fed with the iron enriched diet demonstrated significant increase in serum iron, ferritin, TNF-α ($P < 0.001$) and NO ($P < 0.01$), while testosterone was found to be significantly decreased ($P < 0.001$) as compared to normal control iron-free group. Administration of palm oil significantly decreased TNF-α, NO ($P < 0.001$), testosterone ($P < 0.01$) in serum. However olive oil administration significantly increased testosterone level in serum ($P < 0.05$). Table 2 showed that rats receiving palm oil premixed with iron enriched diet demonstrated significant decrease in serum testosterone,

Table 1. Effect of palm oil, olive oil intake and iron enriched diet on serum biochemical parameters

Parameters	Normal control group	Iron overloaded group	Palm oil group	Olive oil group
Iron (mg/dl)	264.6 ± 44	518 ± 90***	242.3 ± 64	207.5 ± 73
Ferritin (ng/ml)	4.83 ± 0.7	10.5 ± 1.6***	4.8 ± 1	5.2 ± 0.5
Testosterone (ng/dl)	123.6 ± 24.9	43.9 ± 9.3***	67 ± 14**	165.7 ± 24*
NO (µmol/l)	42.98 ± 3.6	55.7 ± 5**	26.1 ± 3.4***	32 ± 3.6***
TNF-α (pg/ml)	24.2 ± 4.6	66.3 ± 6.3***	21.6 ± 4.7**	23.5 ± 4.6**

*significantly different from normal at p < 0.05. **significantly different from normal at p < 0.01. ***significantly different from normal at p < 0.001, (n =10).

Table 2. Effect of iron enriched diet either in combination with palm oil or olive oil on serum biochemical parameters

Parameters	Iron overloaded group	Palm oil + iron group	Olive oil + iron group
Iron (mg/dl)	518 ± 90	609.7 ± 95	444.4 ± 66
Ferritin (ng/ml)	10.5 ± 1.6	11.6 ± 2.2	12.5 ± 1.66
Testosterone (ng/dl)	43.9 ± 9.3	28.4 ± 6.7**	80 ± 13**
NO (µmol/l)	55.7 ± 5	45.6 ± 3**	45.3 ± 3.6**
TNF-α (pg/ml)	66.3 ± 6.3	36.3 ± 7**	32.5 ± 5.5*

*significantly different from control iron group at p < 0.05. **significantly different from control iron group at p < 0.01, (n =10).

Table 3. Effect of palm oil, olive oil intake and iron enriched diet on certain testicular parameters

Parameters	Normal control group	Iron overloaded group	Palm oil group	Olive oil group
TBARS (nmol/g tissue)	669.2 ± 49.6	1439 ± 70.6 ***	515.6 ± 47.9***	466.4± 50***
GSH (nmol/g protein)	38 ± 1.9	18.6 ± 2.5**	41.7 ± 2.2**	42.7 ± 2.5**
Hyp (µg/g tissue)	131 ± 7	213.6 ± 4.3***	131.1 ± 7.4	130.6 ± 5.9
iron (µg/g tissue)	122.7 ± 7.8	265.2 ± 11***	125.7 ± 11.1	119.3 ± 7.6

*significantly different from normal at p < 0.05. **significantly different from normal at p < 0.01.*** significantly different from normal at p < 0.001, (n =10).

Table 4. Effect of iron enriched diet either in combination with palm oil or olive oil on certain testicular parameters

Parameters	Iron overloaded group	Palm oil + iron group	Olive oil + iron group
TBARS (nmol/g tissue)	1439 ± 70.6	1240 ± 81.2***	1115.2 ± 91.9***
GSH (nmol/g protein)	18.6 ± 2.5	28.1 ± 3.2*	30.2 ± 2.2**
Hyp (µg/g tissue)	213.6 ± 4.3	207.1 ± 3**	206.1 ± 2.9**
iron (µg/g tissue)	265.2 ± 11	271 ± 10.1	263.5 ± 11.8

*significantly different from control iron group at p < 0.05. **significantly different from control iron group at p < 0.01. ***significantly different from control iron group at p < 0.001, (n =10).

NO, TNF-α (P < 0.01). Administration of olive oil premixed with iron enriched diet resulted in significant decrease of NO (P < 0.01) and TNF-α in serum (P < 0.05), while testosterone level demonstrated significant increase in serum (P < 0.05) as compared to iron overloaded group.

Table 3 showed that testicular tissues of rats fed iron enriched diet demonstrated significant increase in TBARS, Hyp, iron (P < 0.001) together with marked decrease in GSH content (P < 0.01) as compared to normal control group. Administration of palm oil resulted in significant decrease of TBARS (P < 0.001) along with increased GSH (P < 0.01). Olive oil administration induced nearly similar changes in palm oil group compared to iron overloaded group. Table 4 illustrated that testicular tissues of rats fed iron enriched diet premixed with palm

Table 5. Correlation coefficient between different serum and testicular biochemical parameters

Parameters	Serum iron	Testicular iron content
Serum TNF-α	0.57*	
Serum ferritin	0.8*	
Serum testosterone	-0.72*	
Testicular TBARS		0.95*
Testicular Hyp		0.98*
Testicular GSH		-0.86*

*significantly different at $p < 0.05$.

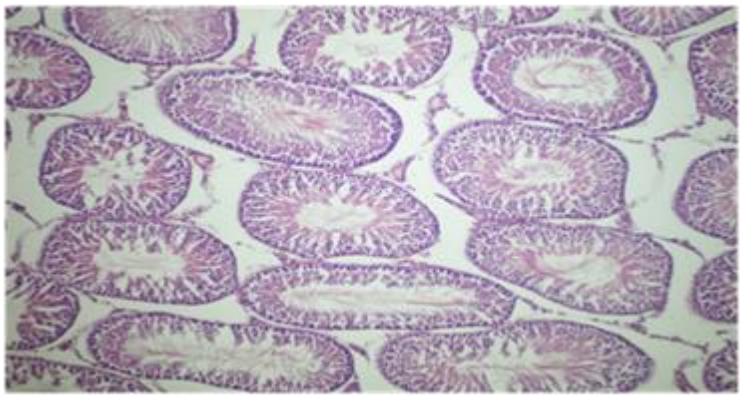

Plate 1. A photomicrograph of adult male albino rat testis (control group) showing seminiferous tubules separated by interstitial tissue (double arrow) (H&E ×100).

oil demonstrated significant decrease in TBARS ($P < 0.001$), Hyp ($P < 0.01$) while GSH showed significant increase ($P < 0.05$), while premixing of olive oil with the dietary iron have induced nearly similar effects compared to iron overloaded group. Correlations between studied biochemical parameters are illustrated in Table 5.

Histopathological study results

The iron enriched diet induced adhesion of some testicular seminiferous tubules associated with disorganized germinal epithelium, extensive areas of exudates, sloughed cells in the lumen, few sperms, multiple vacuoles within interstitial tissues and wide spaces separating spermatogenic cells (Plate 2) as compared to control group (Plate 1). Iron deposition in interstitial tissues (Plate 6) was also evident in addition to massive collagen deposition (Plate 10) in comparison with control group (Plates 5 and 9). However, tissue pattern in rats fed with olive oil in combination with iron demonstrated few exudates, narrow interstitial space, some seminiferous tubules had empty lumen, while others had many sperms (Plate 3), iron precipitation (Plate 7) and mild fibrosis (Plate 11) was also observed. Dietary intake of palm oil in combination with iron resulted in disorganized, compressed germinal epithelium of tubules, wide interstitial space, fewer sperms (Plate 4), iron deposition (Plate 8) and moderate fibrosis (Plate 12).

DISCUSSION

Present study demonstrates that rats fed BEI exhibit significant increase in serum iron, ferritin (2 fold) as compared to the normal group, in agreement with earlier reported studies (Silvana et al., 2003; Elmegeed et al., 2005; Zhao et al., 2005). Certain evidences suggested that dietary iron overload can specifically activate target genes in the liver (L ferritin and procollagen), further studies supported such suggestion (Pietrangelo et al., 1990; Valerio and petersen, 2000). Testicular iron content also showed significant increase in accordance with reported studies (Lucesoli and Fraga, 1999; Lucesoli et al., 1999). The testis represents a secondary target for iron accumulation (Galleano and Puntarulo, 1997). It can also produce its own transferrin and participate in an iron shuttle system to fulfill its requirements of iron for spermatogenesis (Sylvester and Griswold, 1993).

Present results demonstrated the pro-oxidant properties of iron in testicular tissues, in turn generation of reactive oxygen species (ROS) to be implicated latter in oxidative

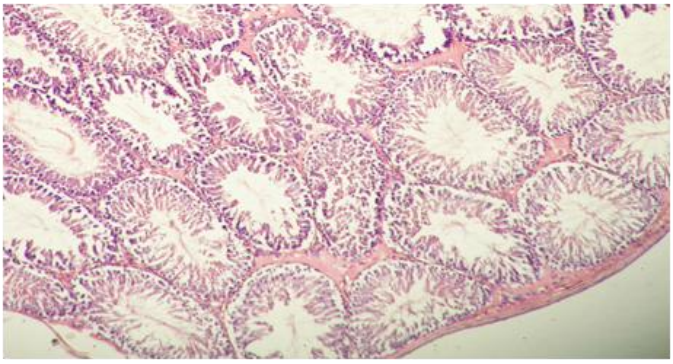

Plate 2. A photomicrograph of adult male albino rat testis of iron overload group showing adhesion of seminiferous tubules. Some tubules revealed disorganized germinal epithelium. Extensive area of exudates can be seen (H&E × 100).

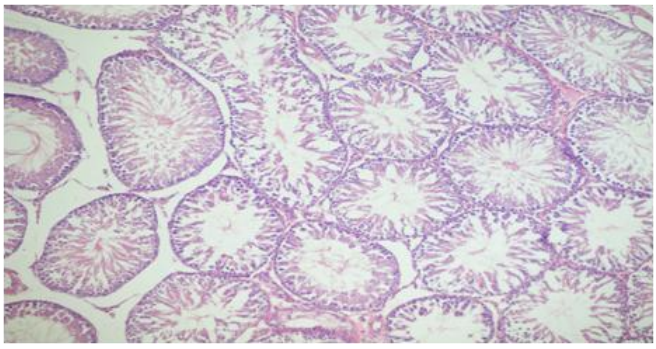

Plate 3. A photomicrograph of adult male albino rat testis of iron overload group treated with olive oil showing a few of exudates and narrow interstitial space. Some seminiferous tubules have empty lumen, while others have many sperms (s) (H&E × 100).

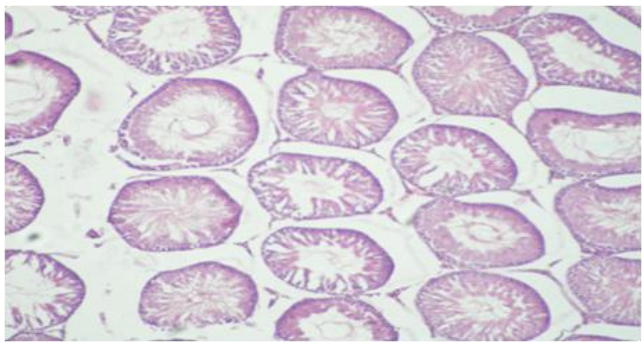

Plate 4. A photomicrograph of adult male albino rat testis of iron overload group treated with palm oil showing some tubules with disorganized germinal epithelium and wide interstitial space few sperms can be seen (H&E × 100).

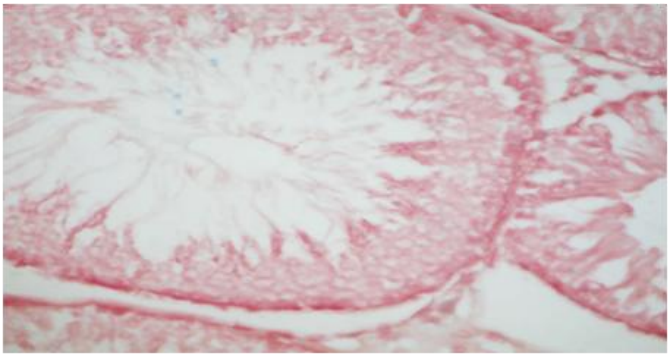

Plate 5. A photomicrograph of of control group showing negative Perl´s Prussian blue stain (Perl´s Prussian blue × 400).

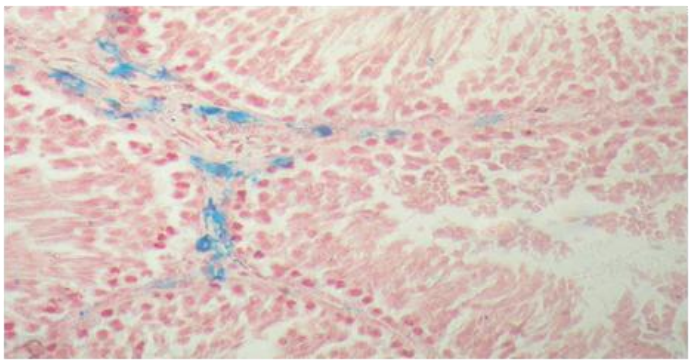

Plate 6. A photomicrograph of adult male albino rat testis (iron overload group) showing positive Perl´s prussian blue stain in interstitial tissue (Perl´s Prussian blue × 400).

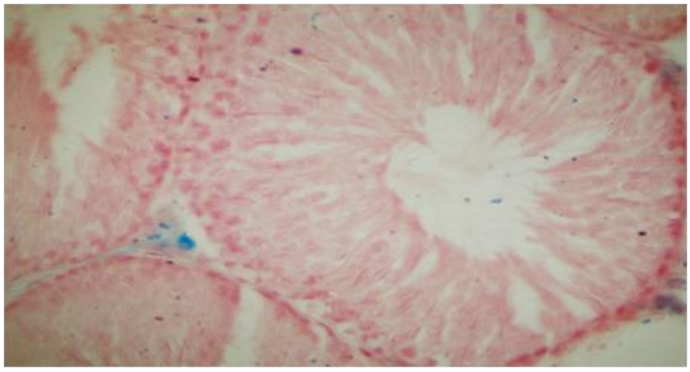

Plate 7. A photomicrograph of adult male albino rat testis of iron overload group treated with olive oil showing positive Perl´s prussian blue stain in interstitial tissue (Perl´s Prussian blue × 400).

damage of cellular components (Jagetia et al., 2004; Harandi et al., 2005). Iron is essential also for normal collagen synthesis acting as cofactor for prolyl-hydroxylase as reported before in various models of iron overload state (Poli and Parola, 1997). Consequently, intake of dietary iron significantly increased testicular hydroxyproline content, in agreement with the ealier reported study (Zhang et al., 2006). Previous reports recorded that iron overload induces moderate fibrosis in testicular interstitium and Leydig cells (Lucesoli et al.,

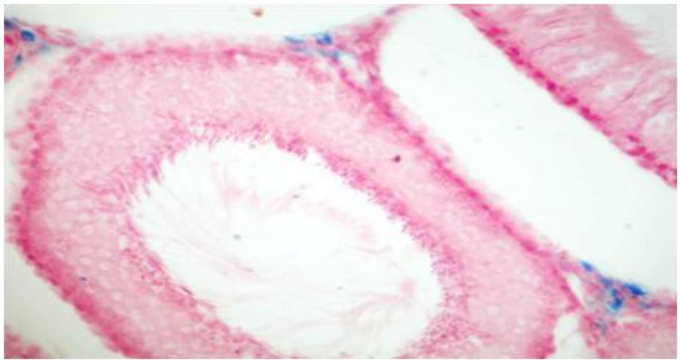

Plate 8. A photomicrograph of adult male albino rat testis of iron overload group treated with palm oil showing positive Perl´s prussian blue stain in interstitial tissue (Perl´s Prussian blue × 400).

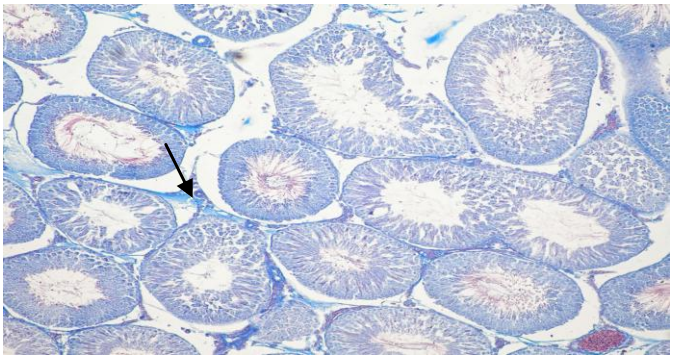

Plate 11. A photomicrograph of adult male albino rat testis of iron overload group treated with olive oil showing mild fibrosis (arrow) (Mallory trichrome × 100).

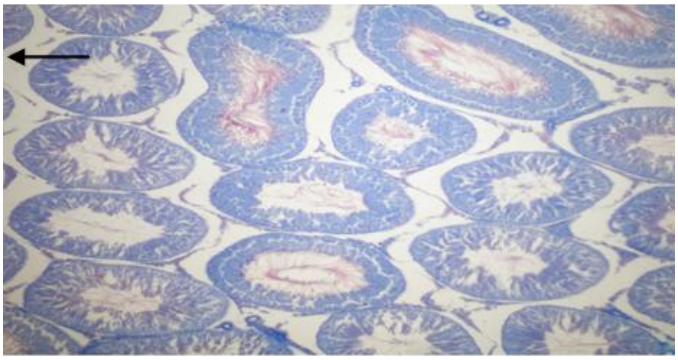

Plate 9. A photomicrograph of adult male albino rat testis (control group) showing distinctive boundary (arrows) formed of collagen fibers (Mallory trichrome × 100).

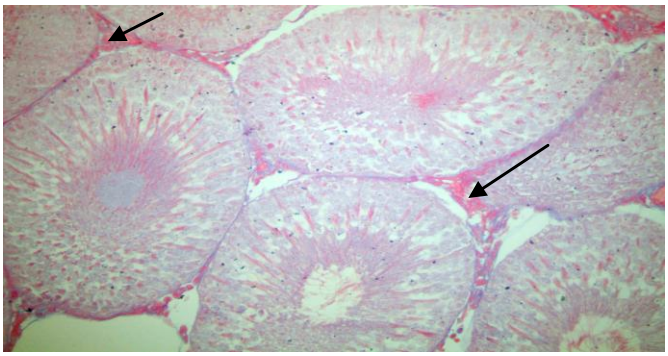

Plate 12. A photomicrograph of adult male albino rat testis of iron overload group treated with palm oil showing moderate fibrosis (arrows) (Mallory trichrome × 100).

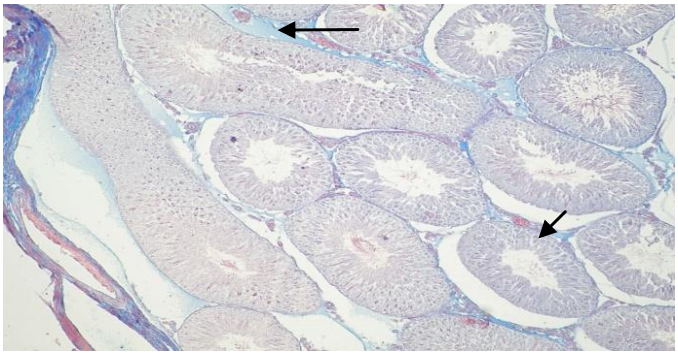

Plate 10. A photomicrograph of adult male albino rat testis (iron overload group) showing multiple collagen fibers (arrows) (Mallory trichrome × 100).

1999). TNF-α showed also significant increase in control iron group, in agreement with others (Elmegeed et al., 2005).

Generally iron can exacerbate various types of liver injury where NF-kB-driven genes are implicated. Accordingly, ferrous serves as a direct agonist to activate NF-kB, and TNF-α promoter activity and finally TNF-α protein. This was observed in cultured Kupffer cells in a redox status-dependent manner. These finding may offer a molecular basis for iron-mediated accentuation of TNF-α dependent liver injury (Hongyun et al., 2002).

In a recent study, it has been proposed that chronic iron overload enhanced proliferation and increased TNF-α mRNA in iron loaded livers. Iron therefore may act as a direct hepatic mitogen (Brown et al., 2006). Iron group also demonstrated significant increase in NO in agreement with reported studies (Elmegeed et al., 2005). Complex relationship between iron and NO was reported before (Galleano et al., 2004), indicating that iron overload can enhance iron uptake by liver Kupffer cells and NO increase via inducible nitric oxide synthase (iNOS). NO expression is also controlled by the redox-sensitive transcription factor NF-$_k$B (Kleinert et al., 2004). This led others to conclude that NO response is mediated by CIO and represents a molecular mechanism affording protection against iron toxicity (Cornejo et al., 2007).

Serum testosterone showed significant decrease in iron group as compared to normal (P < 0.01), mostly attributed to many causes. Sexual dysfunction due to

hypogonadotrophic hypogonadism represents a well recognized disturbances among male patients suffering from β-thalassaemia. This was attributed formerly to damage induced by hemosiderin deposition in pituitary gland in consequent to iron overload (Seracchioli et al., 1994). Hypogonadism was also demonstrated in hemochromatosis patients, attributed mostly to iron-induced cellular damage to the gonadotrophs. Accordingly cytotoxic effect of iron regarding gonadotrophs may take place as soon as iron overload threshold is reached (Sparacia et al., 2000).

Gupta et al. (2004) pointed out the possible involvement of oxidative stress in the suppression of steroidogenesis via substantial reduction in the mRNA of steroid acute regulatory protein as well as activities of testicular Δ^5-3β and 17- β hydroxysteroid dehydrogenases via strong affinity of divalent heavy metal for the thiol groups of these proteins and enzymes. TNF-α may be implicated also as a mediator of many diseases related with hypothalamic-pituitary-testicular (HPT) function. Van der Poll et al. (1993) demonstrated that TNF-α induced an early and transient increase in serum luteinizing hormone (LH) levels followed by transient decrease in serum testosterone levels, keeping follicle-stimulating hormone (FSH) unchanged.

Administration of olive oil or palm oil either individually or in combination with dietary iron induced no change in serum iron and ferritin levels, in agreement with previous studies (Perez-Grandos et al., 2000; Mesembe et al., 2004). Both oils however induced significant decrease in serum TNF-α level. Hydroxytyrosol (HT), a phenolic compound from virgin olive oil, was reported to block NF-kB activation (Carluccio et al., 2003; Maiuri et al., 2005) that is, release of TNF-α and IL-1β. This could modulate immune response selectively (Reimund et al., 2004). Others illustrated also that rats fed palm oil rich diet demonstrated reduced expression of Cox-2 and TNF-α (Nanji et al., 1997; Nanji et al., 2001), mostly attributed to decreased lipid peroxidation rate, TNF-α and Cox-2 levels.

Karsten et al. (1994) reported that palmitic acid can enhance the release of interferon-gamma (IFN-γ) which decreases TNF-α production. They suggested that saturated fatty acids may exhibit more potent effect than unsaturated one regarding cytokine production. Accordingly modulation of free fatty acid (FFA) ratios may be an effective means for fine tuning of the immune system. Administration of olive oil or palm oil or their combination with iron significantly reduced serum NO level. This outcome may be through blocking NF-kB activation by the phenolic constituents of olive oil (HT) (Maiuri et al., 2005) or to be mediated by oxidized low density lipoprotein (ox-LDL) by palmitic acid in palm oil (Moers and Schrezenmeir, 1997).

Serum testosterone level demonstrated significant increase in olive oil group, the reverse was true for palm oil. Lu et al. (2003) concluded that palmitic and stearic acids induced apoptosis in testicular Leydig cells through ceramide production and arachidonic acid can partly prevent such apoptotic effect. Gromadzka-Ostrowska et al. (2002) indicated that diet rich in MUFA content can stimulate testicular function in rats through stimulating 17β-HSD activity, the most important key-enzyme in the testosterone synthesis pathway in male rat gonads and androgen secretion. However, saturated fatty acids may exert inhibitory effect leading finally to opposite results.

Administration of both oils significantly reduced testicular hydroxyproline content, in agreement with reported study (Fernandez et al., 1997). Experimental studies indicated that olive oil administration significantly decreased collagen ratio and connective tissue in CCl_4 induced fibrosis and supported hepatocyte recovery regarding its ultrastructural and morphometric values (Szende et al., 1994; Fernandez et al., 2005). Palmitic acid in palm oil potentially induced hepatic stellate cells deactivation and significantly decreased collagen type I expression reducing fibrosis (Abergel et al., 2006).

Taken together, the results reported in this study suggested that chronic dietary iron overload induces testicular tissue damage, as illustrated by increased oxidative stress parameters, Hyp, TNF-α, depletion of antioxidants and decreased testosterone level. We found also that olive oil demonstrated a protective effect on testicular tissues. This was illustrated by decreased oxidative stress parameters, TNF-α, Hyp and increased testosterone level. Like olive oil, palm oil demonstrated also a protective effect on testis. Unlike olive oil, it significantly reduced serum testosterone level which makes supplementation of olive oil to be highly recommended.

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Full Length Research Paper

Sparfloxacin determination in bulk materials, pharmaceutical formulations and human serum by reverse phase HPLC method

Somia Gul[1]*, Najma Sultana[2], M. Saeed Arayne[2], Sana Shamim[1,3] and Mahwish Akhtar[1,3]

[1]Jinnah University for Women, Karachi.
[2]United Biotechnologies, Karachi-75290, Pakistan
[3]Dow University of Health Sciences.

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A simple reverse phase high-performance liquid chromatography (HPLC) method was developed and validated for the quantitative determination of sparfloxacin (SPFX) in the bulk material, pharmaceutical formulation and human serum. Purospher Start C_{18} (25 cm × 4.6 mm, 5 µm) and Discovery C_{18} (25 cm × 4.6 mm, 5 µm) columns were used. The mobile phase, methanol, water and acetonitril (60:30:10 v/v/v pH 2.70 adjusted by phosphoric acid), was delivered at a flow rate of 1.0 mL min^{-1}, eluent was monitored by ultra-violet (UV) detector at 290 nm. Gemifloxacin (GFX) was used as an internal standard. The proposed method is specific, accurate (98.11 to 102.83%), precise (intra and inter-day variations were 0.108 to 0.712% and 0.013 to 0.575%) and linearity was within the desired ranges of 2.5 to 100 µg mL^{-1} concentration having r^2 > 0.998. All the results were correlated through analysis of variance (ANOVA) and Student's *t*-test. The limit of detection (LOD) and limit of quantification (LOQ) were 0.0009 to 0.0064 and 0.0028-0.0196 µg mL^{-1}, respectively. This method is not only applicable to routine analysis of SPFX in bulk and pharmaceutical formulations but as well as on human serum samples.

Key words: Sparfloxacin, reverse phase high-performance liquid chromatography (RP-HPLC), serum, analysis of variance (ANOVA), student's *t*-test.

INTRODUCTION

The fluroquinolones are advanced class of synthetic antibiotics having a fluorine atom attached to the central ring system, typically at the 6-position or C-7 position (Nelson et al., 2007; Ivanovo and Budanov, 2006). These agents have broad antibacterial activity for the treatment of a wide variety of infectious diseases (Goodman et al., 2001). Sparfloxacin (SPFX) (Figure 1) is an orally active synthetic broad spectrum third generation quinolone, characterized by good to excellent activity against Gram positive cocci (notably *Streptococcus pneumoniae*) and anaerobes and atypical pathogens. It is also moderately active against some (*Bacteroides fragilis* group) L. monocytogenes resistant (Francis et al., 1997; Andersson and MacGowan, 2003; Barrett et al., 1991; Crumplin, 1988; Sultana et al., 2010).

Literature survey revealed that few analytical methods have been developed for estimation of SPFX in bulk and pharmaceutical dosage form. Akram M. El-Didamony developed fluorescence probe enhanced spectrofluorimetric method for the determination of sparfloxacin in tablets and biological fluids and spectrophotometric determination method of sparfloxacin in pharmaceutical

*Corresponding author. E-mail: drsomi1983@yahoo.com

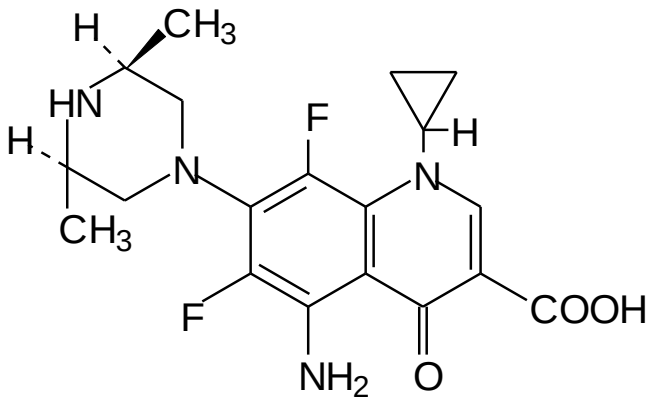

Figure 1. Structure of sparfloxacin.

preparations by ternary complex formation with Pd (II) and eosin (El-Didamony, 2007, 2010). CHO Hea-Young and colleagues developed an high-performance liquid chromatography (HPLC) method for quantitation of SPFX in human serum (Hea-Young et al., 2006) while Argekar and Shah, (1999), Marona and Schapoval, (1999) and Nurun Nahar Rahman and Ahmad, (2007) developed methods for marketed products and stability testing. Another HPLC method was reported for simultaneous determination of sparfloxacin, gatifloxacin and moxifloxacin using levofloxacin as internal standard by Srinivas et al. (2008). Degradation products studies by HPLC have been carried out by Marona et al.**,** (1999).

Almost all previously reported methods were either for determination of sparfloxacin in dosage form or either in serum plasma but there is no efficient, reliable and precise method for determination of SPFX in bulk, dosage form and human serum together. Here, we report a simple, easy, quick and inexpensive isocratic RP-HPLC method with ultraviolet detection at 290 nm for the determination of SPFX in bulk, dosage form as well as in human serum using gemifloxacin (GFX) as internal standard (IS). Low limit of detection (LOD) and limit of quantification (LOQ) values also merit this method for the determination of sparfloxacin in clinical samples. Moreover, this method was further applied for determination of SPFX in three different marketed formulations.

Aim of the study

The aim of the present study was to establish an efficient, reliable, accurate, sensitive and reproducible method for the quantitative determination of SPFX in bulk, pharmaceutical formulations and human serum samples. As this would allow more efficient generation of quantifiable data and could be performed at more diffident cost.

EXPERIMENTALS

Materials and reagents

Standard bulk drug sample of sparfloxacin were supplied by Abott Pharmaceuticals Pakistan (pvt). Gemifloxacin used as internal standard (IS, Figure 2), was obtained from PharmEvo (Pvt) Ltd, Pakistan; three different formulations of sparfloxacin were used including sparaxin (Abott Pharmaceuticals Pakistan (Pvt)), quspar (100 mg) Schazoo Zaka (Pvt.) Ltd., sparkure (100 mg) Elko organization (Pvt) Ltd. HPLC grade acetonitrile and methanol were obtained from Merck Schuchardt OHG, Darmstadt, Germany.

Softwares

Standard regression curve analysis was performed by use of STATISTICA version 7.0 (USA), without forcing through zero. Linearity graphs were obtained by use of Micro-soft Excel 2007 software. Statistical package for social sciences (SPSS) software version 10.0 (Carry, NC, USA) was used for the calculation of means, standard deviations, homoscedasticity of the calibration plots, analysis of variance (ANOVA) and Student*'s t*-test.

Instrumentation

HPLC system equipped with Shimadzu LC-20 AT VP Pump, SPD-20AV VP Shimadzu UV visible detectors and second HPLC system consisted of an LC-10 AT VP Shimadzu pump, SPD-10AV VP Shimadzu UV visible detector, both connected by CBM-102 communication Bus Module Shimadzu to Intel Pentium 4 machine with Shimadzu CLASS-GC10 software (Version 5.03) and Rheodyne manual injector fitted with a 20 µl loop. Separation was achieved on a Hiber, RT, Purospher STAR C_{18} (25 cm × 4.6 mm, 5 µm) (Merck, Germany) and Discovery C_{18} (25 cm × 4.6 mm, 5 µm) (Supelco, USA). The chromatographic analysis was integrated using a Mobile phase which was sonicated by DGU-14 AM on-line degasser, and filtered through 0.45-micron membrane filter, calibrated Pyrex glassware was used for the solution and mobile phase preparation.

Preparation of solutions and quality control samples

Standard solutions of SPFX and GFX (100 ppm in100 ml) were prepared using mobile phase as solvent. Working solutions were prepared separately by making serial dilutions from the standard solution to obtain concentration between 2.5 to 100 µg mL^{-1} for SPFX and IS (GFX). For quality control (QC) samples, twenty tablets of each formulation were powdered finely and an amount equivalent to 10 mg of SPFX was weighed and then dissolved in the mobile phase. Solutions with high, medium and low concentrations that is; 8, 10 and 12 µg mL^{-1} were prepared, then filtered through a 0.45 µm Millipore filter in order to separate out the insoluble excipients by the same procedure as calibration standards but using different stock solutions. All these solutions and QC samples were stored at 20°C. Once prepared, analyzed daily for inter and intra-day variations of the method, 20 µl of these solutions were injected into LC system and chromatographs were observed.

Procedure for human serum sample

Plasma sample obtained from healthy volunteers were collected and stored at -20°C. Then, 1.0 ml of frozen plasma was mixed with

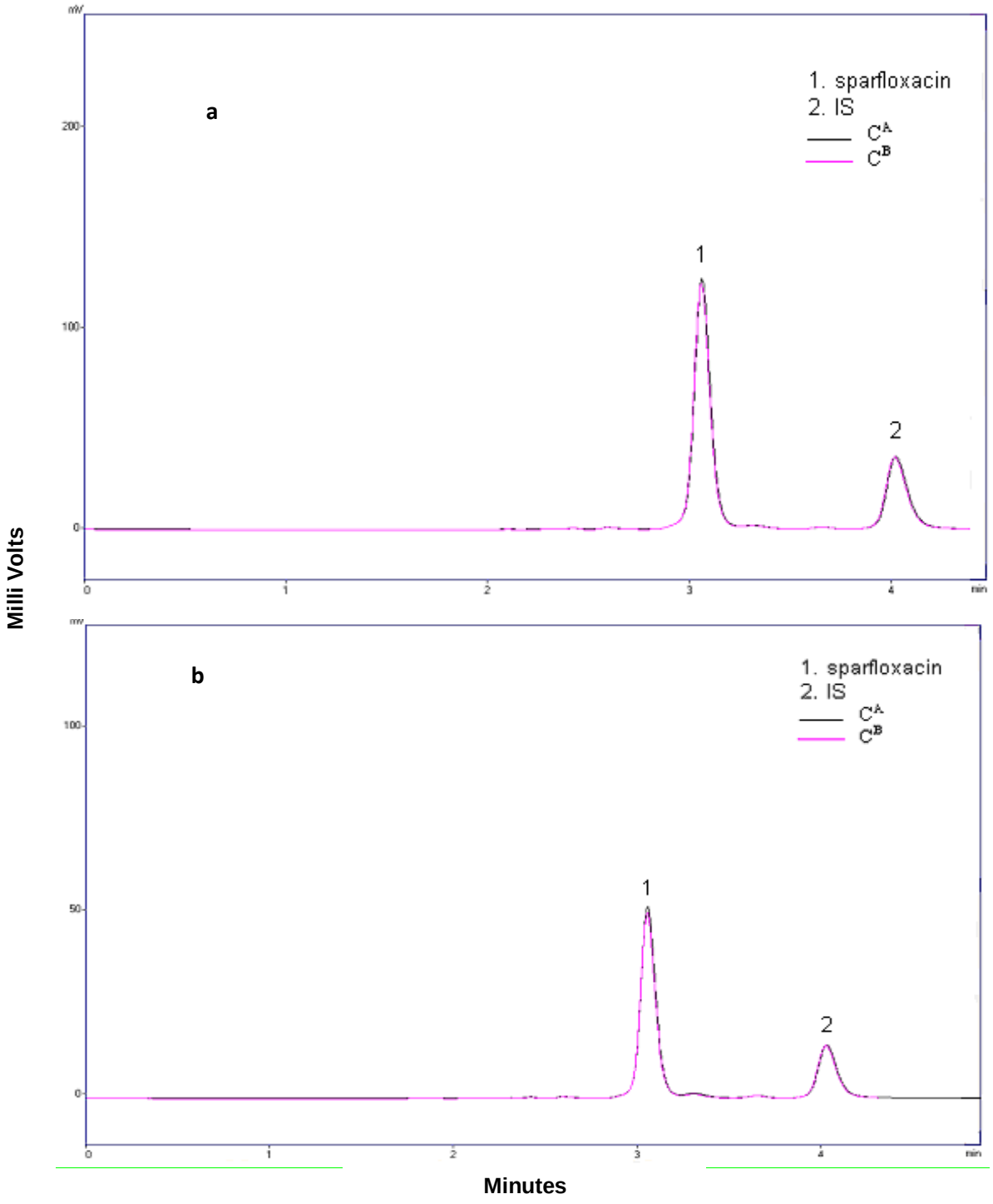

Figure 2. (a) Representative chromatogram of SPFX and IS at 290 nm using System LC 20; (b) representative chromatogram of SPFX and IS at 290 nm using System LC 10.

10 ml of acetonitrile. The mixture was vortexed for one minute and then centrifuged for 10 min at 10,000 rpm and the supernatant was prepared by filtration (0.45 µ pore size membrane filter). An aliquot serum sample was fortified with SPFX to get final concentrations of 2.5 to 100 µg mL^{-1}.

RESULTS AND DISCUSSION

Presented work has been designed to develop a simple, isocratic, precise, accurate and sensitive HPLC method

with UV detection for sparfloxacin determination in bulk, dosage form and in human serum samples. Moreover, the developed method has been applied for quantification of SPFX in its different marketed brands.

Method optimization and chromatographic conditions

For selection of optimal chromatographic conditions, different C_{18} stationary phases have been tried. Best separation, adequate resolution, short retention time and symmetric peak of SPX and IS were achieved by two difference columns which were C_{18} Hiber RT 250-4.6 Purospher STAR RP-18 (25 cm × 4.6 mm, 5 µm) (Merck, Germany) and Discovery C_{18} (25 cm × 4.6 mm, 5 µm) (Supelco, USA). Appropriate wavelength was investigated for determination of SPFX and IS by scanning solution of both drugs on UV-visible spectrophotometer. It was observed that the maximum absorbance of drug was obtained at 290 nm (Figure 3). Same solution was injected in HPLC at 230, 260 and 290 nm. At 290 nm, SPFX showed maximum absorbance. At 230 nm, both SPFX and GFX (IS) showed absorbance but peaks of GFX was not so prominent. While at 260 nm, GFX showed good absorbance but SPFX absorbance became low. Initially, methanol and water were tried in the ratio of 80:20 (v/v), as a result, SPFX and IS did not separate properly, so the above mobile phase was varied as 70:30 (v/v), both drugs separated but the peak was not symmetrical. For best response, acetonitrile was added to the mobile phase and final mobile phase was composed of methanol/water/ acetonitrile in the ratio of 60:30:10, v/v/v at which both drugs showed good resolution with typical peak nature and symmetry. To select the optimum mobile phase, pH range 2.5 to 4.0 were investigated, excellent performance was achieved at pH 2.75 adjusted with phosphoric acid. Total run time was 7 min; short analysis times are essential for routine analysis.

Effect of pH on mobile phase was also studied in the range of 2.5 to 4.0 adjusted with phosphoric acid. The pH of mobile phase had a little impact on resolution and the best separations were observed at 2.70. Retention time of SPFX was 3.6 ± 0.2 and 6.0 ± 0.2 min for IS, at a flow rate of 1.0 ml min^{-1}. The specificity of the method was established through the study of resolution factor of sparfloxacin peak. Peaks were identified using retention times, after injecting separately.

Method validation

The developed method was validated by various parameters which include system suitability, selectivity, specificity, linearity, accuracy test, precision, robustness, ruggedness, sensitivity, limit of detection and quantification, according to International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use, ICH (2005) and USP (2005).

System suitability

It is an essential component of method validation to make certain that the operational system is running appropriately throughout the analysis (Shabir, 2003; Ermer, 2001; USP, 2007). The system was equilibrated with the initial mobile phase composition, followed by 10 injections of the same standard. These 10 consecutive injections were used to evaluate the system suitability on each day of method validation (Table 1).

Linearity of the method

Linearity is generally reported as the variance of the slope of the regression line and was performed with known concentrations of SPFX, 2.5, 5, 10, 25, 50 and 100 µg mL^{-1}, respectively. Linear least squares regression procedure was used for obtaining calibration curves as given in Table 1. The correlation coefficient (r^2) value is ± 0.998. Homoscedasticity of the calibration plots, tested by Friedman's tests were found to be significantly linear over the tested ranges.

Accuracy of procedure

The accuracy of an analytical procedure measures the closeness of agreement between the values. Recovery tests were performed by adding known amounts of standard solutions to sample followed by analysis using proposed method. Three runs were performed for every concentration and result range was 98.11 to 102.83% indicating its high rank of accuracy (Table 2). The average recovery for each level was calculated as indicated by Association of Official Analytical Chemists International (Somia et al., 2012; AOAC International, 2002).

Intraday and inter-day precision

The precision of the proposed method was investigated with respect to repeatability. Intra-day and inter-day precision were determined by observed responses of freshly prepared solutions after replicate (n = 6) injection of sample solutions (Table 3). The precision of the method was analyzed as relative standard deviation (RSD%) throughout the linear range of concentrations (Shabir, 2003; Ermer, 2001; USP, 2007). All the results

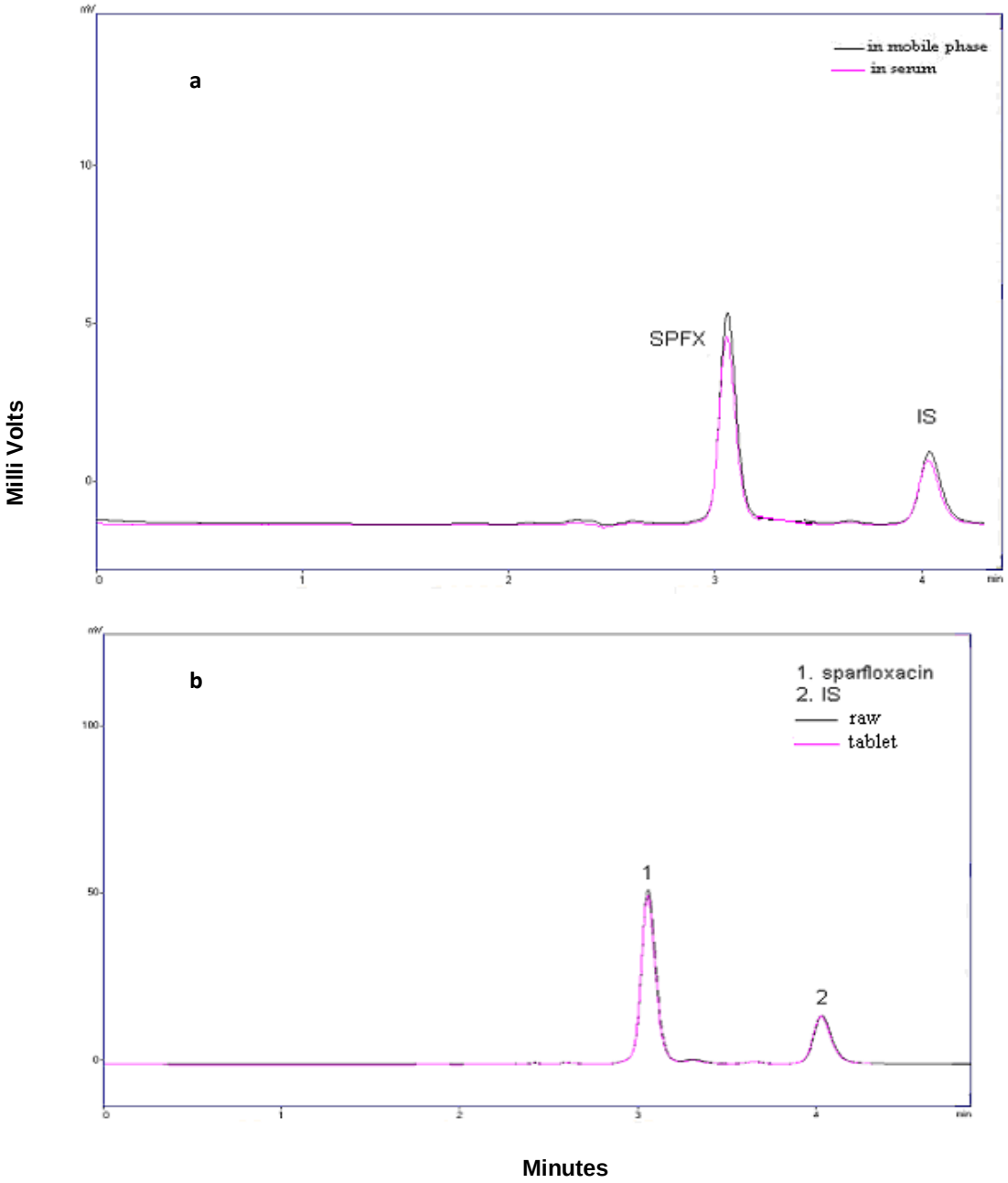

Figure 3. (a) Representative chromatogram of SPFX and IS at 290mm in both mobile phase and human serum; (d) representative chromatogram of SPFX and IS at 290 mm with and without excepients.

were correlated and found non-significant by student*'s t*-tests indicating no remarkable difference in intra and inter day precision.

Analysis of marketed products

Assay was done by taking three different brands of sparfloxacin. The peak of SPFX in marketed tablets was observed at 2.5 min and all the results were found to be in acceptable limits. The percent recovery was in good agreement with the label claims thereby suggesting that there is no interference from any excipients, which are normally present in tablets. The active content of drug in tablets was found to be 100.12% ± 0.994. Results were further verified by statistical evaluation, using one way analysis of variance (ANOVA) and the *F*-ratio at 95% confidence level as shown in Table 4 and found

Table 1. System suitability parameters and regression characters of the proposed method.

System	Column	t_R	K'	N	T	(R_s)	r^2	SEE	S.E	Y	Regression equation	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
LC 10	C^A	3.057	0.33	6564	1.11	3.27	0.9991	19985.75	11164.32	-46821.5	Y=16203X-46822	0.00648	0.01963
	C^B	3.054	0.46	6594	1.11	3.25	0.9991	14276.13	7974.842	-9623.6	Y=11110X-9623.6	0.00231	0.007
LC 20	C^A	3.051	0.46	6333	1.13	2.99	0.9987	44651.51	24942.95	-54558.5	Y=29925X-54559	0.00094	0.02856
	C^B	3.049	0.26	6374	1.13	3.16	0.9983.	52202.34	29160.95	-44623.5	Y=29715X-44624	0.00093	0.00283

C^A = Purospher STAR, C^B = Discovery, t_R = Retention time, K'= Capacity factors, N = Theoretical plates, T = Tailing factor, R_s = Resolution, SEE = Standard error of estimate, SE = standard error, Y = Intercept, LOD = Limit of detection, LOQ = Limit of quantitation, Conc. 2.5-100 µgmL-1.

Table 2. Accuracy of sparfloxacin.

Column System	Parameter	C^A				C^B			
		Assay (spiking method)		Assay in serum		Assay (spiking method)		Assay in serum	
	Conc. (µg mL^{-1})	Conc. Found	% Recovery	Conc. Found	%Recovery	Conc. Found	%Recovery	Conc. found	% Recovery
LC 10	8	8.148	101.85	8.152	101.9	8.129	101.617	8.124	101.55
	10	10.038	100.387	10.035	100.35	10.029	100.293	10.019	100.19
	12	12.33	102.75	12.35	102.961	12.205	101.711	12.195	101.625
LC 20	8	8.2	102.62	8.16	102	7.956	99.455	8.01	100.125
	10	10.25	102.57	10.19	101.9	10.283	102.833	10.279	102.79
	12	11.77	98.142	11.89	99.08	12.292	102.437	12.293	102.441

non-significant.

Limit of detection and quantitation

The LOD and LOQ of this method were determined from the coefficient of variation of a known concentration of SPFX. The LOD and LOQ for this assay were calculated from three and ten times the noise level of the response (Shabir, 2003; Ermer, 2001; USP, 2007) which are given in Table 1. The LOD and LOQ were 0.0009 to 0.0064 and 0.0028 to 0.0196 µg mL^{-1}, respectively (Table 2).

Specificity and selectivity

The selectivity and specificity of proposed method was evaluated during the entire study through possible interference due to excipients present in the pharmaceutical formulations. The method confirmed good resolutions (Table 1). It was found to be free of interference from the excipients used in pharmaceutical formulation and it indicated the specificity of the system. Specificity was also determined by screening four different samples of controlled human serum which were free from interfering endogenous plasma components.

Robustness

Robustness of the method was assessed by

Table 3. Precision of sparfloxacin.

Column	C^A			C^B		
Systems	Formulation (%RSD)		Serum (%RSD)	Formulation (%RSD)		Serum (%RSD)
	D_1	D_2	D_1	D_1	D_2	D_1
LC 10	0.495	0.575	0.494	0.712	0.799	0.717
	0.226	0.366	0.225	0.277	0.256	0.273
	0.201	0.565	0.201	0.254	0.215	0.251
	0.475	0.577	0.572	0.499	0.497	0.492
	0.366	0.367	0.368	0.156	0.153	0.155
	0.565	0.566	0.569	0.115	0.114	0.113
LC 20	0.383	0.396	0.384	0.400	0.431	0.403
	0.236	0.213	0.239	0.384	0.323	0.387
	0.108	0.109	0.106	0.371	0.377	0.372
	0.296	0.293	0.292	0.231	0.233	0.235
	0.013	0.012	0.013	0.023	0.021	0.024
	0.091	0.094	0.093	0.077	0.074	0.073
t-Test: paired two sample for precision						
Systems	Columns	t stat	P (T<t) two-tail	-	-	-
LC 10	C^A	-2.093	0.091	-	-	-
	C^B	-.197	0.852	-	-	-
LC 20	C^A	0.345	0.744	-	-	-
	C^B	0.363	0.732	-	-	-

Where, C^A = Purospher STAR, C^B = Discovery, D_1 = Intra-day and D_2 = Inter-day variations

deliberate variations made to the method parameters such as composition (± 5) of the mobile phase, pH (± 0.2), flow rate (± 0.2), and detection wavelength (± 30). Therefore, five repeated samples were injected under small variations of each parameter. The method proved to be fairly steady as there is no considerable drift in the factors as given in Table 5.

Ruggedness

The ruggedness of the method was established in two different labs. Lab 1 was Research Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Karachi while 2nd lab was lab 9, Department of Chemistry, Faculty of science, University of Karachi. Work was carried out on two different instruments; LC 20 and LC 10, using two different columns Purospher STAR C_{18} and Discovery C_{18} for the study on different days by different analysts. All the results were correlated by applying one-way ANOVA and the differences were found non significant. Concluding that the method was capable within acceptable limits in precision, but the peak area was affected with change of wavelength (Table 1).

Application of the proposed method

The proposed method is not only applicable for the routine quality control assay of sparfloxacin in bulk, pharmaceutical dosage form and serum but also for the clinical evaluation, pharmacokinetic, bio-equivalence and interaction studies where low volume of blood or plasma is needed due to the simplicity of the separation procedure, shorter run time (7.0 min) and the cheaper mobile phase. The proposed method also gains its significance, being applicable to the wider range of detection wavelengths.

Conclusion

A simple and reliable HPLC method has been developed successfully for the monitoring of SPFX, in bulk, pharmaceutical dosage formulation and human serum. This method has been established for the first time and was not reported earlier. The above developed method has

Table 4. Application of the proposed method for the determination of marketed products

Brands	Sparaxin™	Sparkure™	Quspar™	
sample	conc. Found (mg)/% recovery	conc. Found (mg)/% recovery	conc. found (mg)/% recovery	
1	99.77	99.65	99.68	
2	99.88	99.95	99.94	
3	99.86	99.82	99.85	
4	99.67	99.86	99.88	
5	99.56	99.36	99.37	
6	99.94	99.77	99.72	
7	100.26	100.18	99.96	
8	100.27	100.57	100.06	
9	100.12	100.07	100.17	
				total
Count	9	9	9	27
Sum	899.33	899.23	898.63	2697.19
Mean	99.925	99.914	99.847	99.895
S.D	0.2495	0.342	0.2355	0.827
Variance	0.0622	0.117	0.0554	0.2962
Result	***SS***	**df**	***MS***	**F**
Between Groups	0.105	3	0.0349	
Within Groups	1.878	32	0.0586	0.596
Total	1.983	35	-	

Where, *label claim is 100 mg.

Table 5. Robustness of the proposed method (n=6).

Parameters	Level	t_R	K'	T	(R_s)
pH of mobile phase					
2.5	-0.2	3.051	0.31	1.11	3.27
2.7	0	3.057	0.33	1.11	3.25
2.9	0.2	3.055	0.33	1.13	3.32
Mean± S.D (n=6)		3.053±0.0028	0.323±0.011	1.116±0.011	3.28±0.036
Flow rate (ml min^{-1})					
0.8	-0.2	3.057	0.26	1.11	3.37
1	0	3.059	0.33	1.13	3.25
1.2	0.2	3.053	0.36	1.13	3.32
Mean± S.D (n=6)		3.055±0.0028	0.316±0.051	1.123±0.011	3.313±0.060
Percentage of water in mobile phase (V/V/V)					
25	-5	3.051	0.27	1.13	3.24
30	0	3.054	0.33	1.11	3.27
35	5	3.057	0.37	1.11	3.32
Mean± SD (n=6)		3.054±0.0042	0.323±0.0503	1.116±0.011	3.27±0.040
Wavelength (nm)					
230	-60	2.49	4.7	1.42	2.39
260	-30	2.51	4.3	1.43	2.36
290	0	3.057	0.33	1.11	3.25
Mean± SD (n=6)		2.685±0.014	2.515±3.090	1.32±0.1819	2.66±0.505

t_R = Retention time, K'= Capacity factors, N = Theoretical plates, T = Tailing factor, R_s = Resolution.

been conducted on three different wavelengths, validated as per ICH guidelines and the results were then correlated. Results indicate that the 290 nm is the wavelength where the area under curve (AUC) of SPFX is maximum, in comparison to 230 and 260 nm, providing the same chromatographic conditions. Therefore, we can report that the best suited wavelength for the determination of SPFX is 290 nm provided the same chromatographic conditions were followed.

The limit of quantification, small sample volume and short chromatographic time of this method are particularly adapted for routine assay. The short analysis time (< 7 min) enables its application in routine and quality control analysis of finished products. The proposed method has been effectively applied to quantities SPFX in three different commercially available brands.

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Full Length Research Paper

Ototoxicity in tuberculosis treatment in South Africa: Exploring the current status

Katijah Khoza-Shangase

University of the Witwatersrand, Private Bag 3, Wits, South Africa.

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As a result of the known ototoxic effects of some of the medications used for tuberculosis (TB) treatment, healthcare workers involved in treating TB patients need to be aware of the ototoxic signs and when to refer to an audiologist for hearing monitoring. The main objective of the present paper was to determine the knowledge and management of ototoxicity related to TB treatment by South African healthcare workers. Telephonic interviews using a semi-structured interview schedule consisting of closed- and open-ended questions were conducted on 100 healthcare workers involved in the treatment of patients with TB. Findings were collated and analyzed via thematic analysis and quantitative descriptive statistics procedures. A large percentage of participants were not aware of what ototoxicity is and what the role of the audiologist was in the management of TB patients. All participating public health facilities do not work with an audiologist as a member of the team in the management of TB patients, and a stark lack of ototoxicity monitoring programs exists. Results highlight the need for education of health-care workers as well as patients regarding ototoxicity. Additionally, audiologists need to become more involved in establishing and implementing ototoxicity monitoring programs in facilities where ototoxic medications are prescribed.

Key words: Aminoglycosides, ototoxicity, tuberculosis, health care.

INTRODUCTION

Despite the fact that tuberculosis (TB) is a preventable and treatable disease, it is the oldest of the world's current pandemics (Dye et al., 2005). In South Africa, the TB epidemic is one of the greatest challenges facing post-apartheid South Africa, alongside the control of the concomitant human immunodeficiency virus (HIV) (Karim et al., 2009). Karim et al. (2009) assert that HIV continues to spread relentlessly, and that tuberculosis has been declared a national emergency, with the rising drug resistance and HIV co-infection, making South Africa one of the world's worst tuberculosis epidemics. The continued upsurge in the number of TB cases largely due to co-infection with HIV was earlier predicted by Clarke et al. (2006). Besides the contribution of human immuno-deficiency virus/acquired immune deficiency syndrome (HIV/AIDS), neglect of TB control also plays a role in the increased rate of TB infection, resulting in the emergence of drug resistant TB (Aziz et al., 2006), increased rate of recurrent TB (Korenromp et al., 2003), and extensively drug-resistant TB (XDR-TB) (Morbidity and Mortality Weekly Report (MMWR), 2006; Dye, 2006). Treatment therefore often consists of second-line drugs that are more costly and toxic than first-line anti-tuberculosis drugs (MMWR, 2006). Some of the drugs used in the treatment of TB fall under the umbrella term of antibiotics called 'aminoglycosides' (Smith and MacKenzie, 1997; Cohn, 1981). These antibiotics are most notorious for being ototoxic (Edmunds et al., 2006). As a result of the

E-mail: Katijah.Khoza@wits.ac.za. Tel: 011 717 4565. Fax: 086 553 6055.

known ototoxic effects of some of the medications used for TB treatment, healthcare workers involved in treating TB patients need to be well aware of the ototoxic signs as well as know when to refer to an audiologist for hearing monitoring.

Streptomycin was the original antibacterial agent to be used successfully to combat the scourge of TB (Schuknecht, 1974), and its ototoxic effects were also documented and highlighted that early on (Talaska and Schacht, 2005), when a substantial number of TB patients treated with streptomycin were found to develop irreversible cochlear and vestibular dysfunction (Edmunds et al., 2006). Despite its documented ototoxic effects, streptomycin continues to be in wide use in the present day (de Lima et al., 2006). Symptoms of streptomycin ototoxicity include: high-frequency hearing loss (usually not affecting lower frequencies which are utilized in conversational hearing), tinnitus; as well as vestibular symptoms (Khoza-Shangase et al., 2009).

Patients may present with unsteadiness, ataxia, nausea and/or vomiting (Wofford, 1981; Catlin, 1981; De Jager and van Altena, 2002). These symptoms can have a significant impact on the patient's quality of life and therefore, early diagnosis and management is important (de Lima et al., 2006). It is the belief of the current author that pre-treatment counseling around these symptoms could also contribute toward adherence and compliance with treatment plans. Hence, it is essential that health care workers involved in TB treatment are aware of the ototoxic effects so that they can be in a better position to educate patients who may not notice ototoxic hearing loss until a communication problem becomes apparent, signifying that hearing loss within the frequency range which is critical for understating speech has already occurred. Similarly, by the time a patient complains of dizziness due to the medication, permanent vestibular system damage may have probably already occurred (Cohn, 1981; Edmunds et al., 2006). Furthermore because late and slowly progressive hearing loss occurring several months and years later through synergic effects between drugs and other known toxic agents such as noise exposure is possible (Edmunds et al., 2006; Martini et al., 2003), patients need to be educated and pre-counseled to avoid excessive noise exposure during and post-exposure to ototoxic drugs, at least for some time.

When a deadly disease necessitates treatment with ototoxic drugs, preserving the quality of the patient's remaining life develops into an important objective. This is particularly serious where documented aminoglycosides which are infamous for their ototoxic effects have to be used; where high prevalence of infectious, life-threatening diseases such as TB makes their use understandable and often unavoidable. It can be argued that hearing loss is not a life-threatening condition; however, Khoza-Shangase et al. (2009) argue that it is a threat to essential quality of life indicators unless intervention occurs early. These authors emphasize the adverse effects of a hearing loss on cognitive-linguistic skills and psychosocial behaviour, as well as the vocational, social, and interpersonal consequences for the patient (Khoza-Shangase et al., 2009). It is thus important that the patient is educated about what to expect so that they are aware of possible side-effects and know what management options are available.

The aim of the current study was therefore to determine the knowledge and management of ototoxicity related to TB treatment by South African healthcare workers. The analysis was made in three relevant perspectives:

1. To determine the TB treatment protocol followed by health care workers.
2. To determine the health care workers' awareness of ototoxicity and its management
3. To determine the extent of involvement of an audiologist in the treatment of TB

MATERIALS AND METHODS

Subjects

A total of 100 healthcare workers were interviewed for this study (Table 1). Convenient sampling of health care workers from five healthcare facilities involved in TB treatment in Gauteng was done (Lavrakas, 1987). For the purpose of this study, all participants had to be familiar with the running of the TB clinic, including the medications used; and had to be fluent in English since the interviews were conducted in English. Semi-structured telephonic interviews, consisting of open- and closed-ended questions were used. This design allowed for a large geographic coverage (Salant and Dillman, 1994) and was less expensive than personal interviews (Lonsbury-Martin and Martin, 2001). Ethical clearance was obtained from the University of the Witwatersrand, Human Research Ethics Committee (medical) before the study could be conducted and the ethical considerations of consent, anonymity, confidentiality, and feedback provision were observed.

Interview schedule

The interview questions were developed in accordance with the aims of the study, and were divided into 5 subsections:

1. General occupation: aimed at determining the position the participant held at the health site as well as the amount of experience they had dealing with TB patients.
2. TB treatment: aimed to determine the participants' awareness of the treatment currently used to treat TB, multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), including knowledge of alternative drugs with less severe side effects.
3. Ototoxicity: focused on participants' awareness of ototoxicity, including the ototoxic effects of the TB medication.
4. Audiology and TB management: to ascertain the participants' awareness of audiology as a profession and services offered by an audiologist as well as to establish the perceived role of an audiologist in TB management programs.

Table 1. Description of participating healthcare professionals (N = 100).

Occupation	Participant's number
Clinical director	5
Clinical executive	5
Deputy director	3
Assistant director	4
Doctor	5
Pharmacist	11
Senior nurse	19
Professional nurse	26
Enrolled nurse	13
Community care worker	9

Table 2. Participants' responses to the medication used for treating TB (N = 100).

Parameter	TB	MDR-TB	XDR-TB
Rifafour	82	-	-
Ethambutol	16	9	-
Isoniazid	9	-	9
Rifampicin	14	9	-
Refinah	27	5	5
Pyrazinamide	9	9	-
Streptomycin	18	14	9
Pyrodoxin	21	-	-
Don't know	18	50	59

5. Protocols and regulations: aimed at determining what protocols were available in health settings concerning assessment and monitoring of renal function, blood-serum level, and hearing function during treatment as well as to determine recommendations provided to patients. According to Sharma and Mohan (Burns and Grove, 2001), patients receiving treatment for TB should be closely followed-up through reviewing clinical, radiological, laboratory and microbiological parameters to assess the patients' response to treatment.

Pilot study

Prior to the commencement of the study, a pilot study was conducted to assess the suitability and applicability of the research tool (Greeff, 2002). The pilot study proved the tool to be appropriate following minor changes to some of the questions in the interview schedule.

Data analysis

After the researcher transcribed the interviews, data was analyzed using a qualitative statistical approach through descriptive statistics and content analysis (Altman et al., 2005; Lancaster et al., 2004; Flick, 2002).

RESULTS AND DISCUSSION

The results of the study are reported following the format of the interview schedule.

General occupation

Participants' occupation included clinical directors, clinical executives, and deputy directors, assistant directors, doctors, pharmacists, senior nurses, professional nurses, enrolled nurses, and care workers. The range of length of experience working with the TB population was from 1.1 years to 10 years, with an average length of experience of 4 years.

TB treatment

Participants' responses to the question on the treatment protocols followed with TB patients were varied. As shown in Table 2, 82% indicated that Rifafour was used in the initial phase of treatment for a period of two months. Drugs that were reported to be used during the continuation phase were ethambutol (16%), isoniazid (9%), rifampicin (14%), refinah (27%), pyrazinamide (9%), streptomycin (18%), and pyrodoxin (21%). These responses are in accordance with literature on TB treatment. Rifafour is reported as the first line of treatment against TB (Clarke et al., 2006), while Refinah is used during the continuation phase. The rest of the 'single drugs', including streptomycin, are used to re-treat cases or when a patient develops resistance against one of the other drugs (Clarke et al., 2006). The remaining 18% of participants indicated that they were not sure of the treatment for TB, however, they were aware that drugs such as pyradoxin (21%), ethambutol (16%), and Refinah (27%) were used. This was of significant concern since lack of this knowledge could negatively impact on the healthcare workers' ability to properly counsel patients regarding treatment side effects. It further implied that they would in turn not be aware of the side-effects and as a result, would be unable to neither offer proper counseling nor refer patients for monitoring for symptoms of side effects such as ototoxicity.

Furthermore, as can be noted in Table 2, a higher percentage (50%) of the participants indicated that they did not know which drugs are currently being used to treat MDR-TB, and a similarly high number (59%) did not know what treatment was used for XDR-TB. This is of concern since health care workers' knowledge is crucial in the successful implementation of the South African government's plan to contain and eradicate TB as one of the infectious diseases that it has to manage. Upon further investigation, it was found that healthcare workers with minimal knowledge pertaining to treatment of MDR-

Table 3. Participants' awareness of ototoxicity (N = 100).

Questions	Participants' responses (%)	
	Yes	No
Do you know anything about medications causing a hearing loss (referred to as ototoxicity)?	26	74
Of the participants who responded 'yes' to the question above (n=26)	**Yes**	**No**
Are you aware of the ototoxic effects of some TB medications?	40	60
Do you know the symptoms a patient with ototoxicity would present with?	22	78
Do you ask patients about family history of hearing loss (ototoxicity)?	18	82
Do you ask patients about their ears and hearing status while on TB treatment?	0	100
Do you have an ototoxicity-monitoring program in place?	0	100
Is there documentation of the ototoxic effects of various medications available for professionals to read?	0	100
Do you routinely consider patients' renal function during treatment?	42	58
Do you routinely monitor patients' blood-serum levels during treatment?	26	74

TB and XDR-TB were predominantly those working in healthcare facilities where there was minimal contact with patients infected with MDR-TB and XDR-TB. Currently in Gauteng, health facilities refer MDR-TB and XDR-TB patients to a specialized facility, where a diagnosis is confirmed. The patient's treatment is then specifically instituted, and once rendered non-infectious; the patient is then referred back to the local health facility to continue treatment.

When asked whether the health care workers were aware of alternative medications that could be used in the treatment of TB if the patient had severe side effects, 87% of participants stated they were not aware of any alternative medications. Only 13% of the participants indicated that there were alternative drugs, however, these respondents were of the belief that these drugs are not as cost-effective and as efficient as drugs currently used. This response is in accordance with literature, which states, for an example that, although streptomycin has adverse ototoxic effects it is the most commonly used aminoglycoside in the fight against TB, especially in developing countries, because of its efficiency and cost-effectiveness (MMWR, 2006).

Ototoxicity

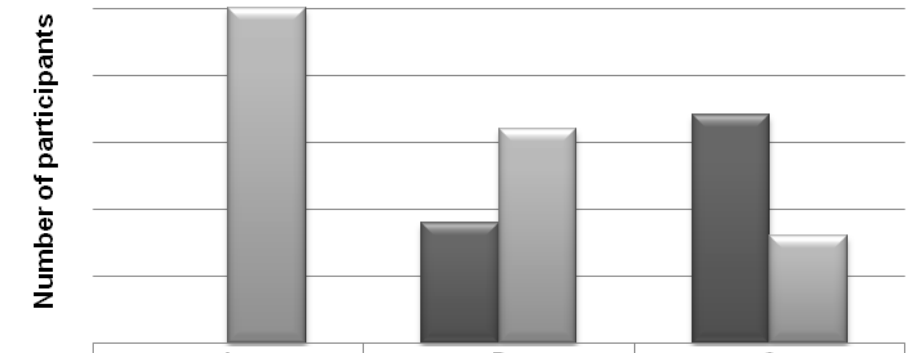
Table 3 reveals particularly concerning results about the healthcare workers' awareness of ototoxicity. These results highlight the amount of educational campaigns required from audiologists aiming at marketing the profession as well as in ensuring that patients receive the appropriate audiological services when required. The results in Table 3 indicate that a large number of health-care workers (74%) lack awareness in terms of ototoxicity and the symptoms a patient may present with as a result of exposure to ototoxic drugs. Out of those health-care workers who were aware of ototoxicity (n = 26), only 40% were aware of the ototoxic effects of the TB drugs, while only 22% were aware of the type of symptoms to look out for in the case of exposure to ototoxic drugs. From Table 3, it is also evident that there were no formal ototoxicity monitoring programs in place at health facilities where the current study was conducted nor were there available protocols for healthcare workers to refer to regarding ototoxicity.

These results reveal that healthcare workers, particularly those working in non-specialized TB treatment facilities, lack knowledge in terms of ototoxicity, symptoms of ototoxicity as well as management of patients on potentially ototoxic medications. Without the necessary knowledge of ototoxicity, ototoxic medication and the presenting symptoms, these healthcare workers are in no position to impart this knowledge to their patients or to refer to an audiologist or an otorhinolaryn-gologist when a patient presents with symptoms such as tinnitus, vertigo or hearing loss.

The fact that a large majority of the healthcare workers that were aware of ototoxicity (82 to 100%) reported that they did not enquire about family history of hearing impairment nor about patients' ear and hearing status is also of concern. If no formal audiological assessments exist at the TB clinics that patients can access; and minimal or no subjective inquiries are made regarding patients' hearing status, the disjuncture between what established theoretical knowledge has proven and what is being practiced is highlighted.

Audiology and TB management

Comprehensive management of TB patients that includes identification and treatment of side effects of drugs (in this case, ototoxicity) can only be done if the healthcare



	A	B	C
YES	0	36	68
NO	100	64	32

Figure 1. Summary of participants' responses to audiology and TB management sub-section (A = Do you have an audiologist as part of the TB management team? B = Do you refer TB patients to an audiologist? C= Do you know what the role of an audiologist is?).

workers know what audiologists are, what role the audiologists play in patient management, when to refer to audiologists, and work with audiologists as part of the TB management team. Figure 1 reveals that audiologists are not routine members of the TB management team. This again highlights the need for more active lobbying by audiologists to have posts created in such institutions so that ototoxicity monitoring programs can be established. Furthermore, Figure 1 indicates that even though 68% of the participants reported that they knew what the role of an audiologist is, only 36% actually refer patients to an audiologist. This again brings to the fore the need for audiologist to educate other healthcare workers about their many roles.

Protocols and regulations

This section aimed at determining protocols that were being followed for ototoxicity monitoring. As can be seen in Table 3, none of the participating facilities had protocols for ototoxicity monitoring that was prescribed when and how often the patients' hearing status can be evaluated. Furthermore, over half (58%) indicated that the patients' renal functioning was not considered and 74% indicated that the patients' blood-serum levels were not monitored during TB treatment. The main reason provided by a majority of respondents for lack of monitoring was that it was the attending doctor's responsibility to refer the patient. Of concern here is that a significant number of patients on TB treatment are not always seen by doctors, but rather by nurses, therefore shifting the responsibility to the doctors may negatively impact on the patients' quality of life.

Recommendations for TB patients

As can be seen in Figure 2, a significant amount (46%) of information provided to patients focused on adherence to the treatment regimen, with the other recommendations covering establishing and maintaining a healthy lifestyle (24%), reporting side effects such as skin rash, nausea and vomiting (21%), with only 9% focusing on ototoxicity and hearing conservation. Recommendations concerning hearing conservation were only made to patients complaining of hearing loss prior to discharge from the health facility.

Conclusion

The results of this study have highlighted the lack of awareness of ototoxicity amongst the healthcare workers from various health facilities involved in the treatment of TB. Healthcare workers are not aware of the symptoms of ototoxicity and as a result symptoms are attributed to side-effects that will wear-off and are therefore not investigated further. Healthcare workers also lack knowledge with regards to the role of the audiologist and as a result do not refer patients to an audiologist and/or employ the services of an audiologist during management of patients exposed to ototoxic drugs. The fact that audiologists do not form part of the treatment team involved in TB treatment influences the healthcare workers' knowledge as well as the referral rate of patients to audiologists for ototoxicity monitoring. Audiologists are responsible for imparting knowledge to healthcare workers as well as advocating for their role within the TB treatment team. They are responsible for ensuring that

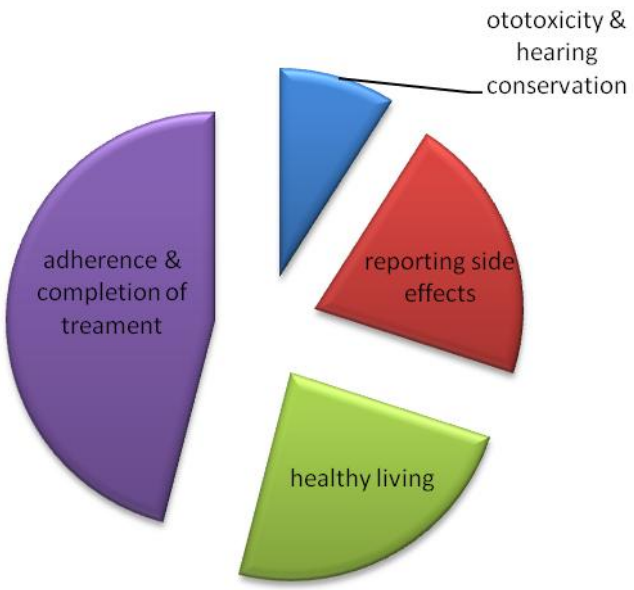


Figure 2. Summary of recommendations made to TB patients.

they get involved as one of the key members of the team that manages patients receiving ototoxic drugs, including implementing and managing ototoxicity-monitoring programs in healthcare facilities involved in treating diseases using ototoxic drugs. Without proper facilities and monitoring programs, patients are at a risk of the adverse psychological, social and occupational impacts that result from the presence of an unidentified and untreated hearing impairment.

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Full Length Research Paper

Assessment of toxicity in puffer fish (*Lagocephalus lunaris)* from South Indian coast

Niharika Mandal, Soumya Jal, K. Mohanapriya and S. S. Khora*

Medical Biotechnology Laboratory, School of Biosciences and Technology, VIT University, Vellore, Tamil Nadu, India.

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Puffer fish intoxication is one of the most common fish intoxications along the coasts of Asia. *Lagocephalus lunaris* is the most commonly available species in the South Indian coast and it is eaten by the locals. This study was undertaken to assess the risk of poisoning due to consumption of *L. lunaris* collected along the Southeast Indian coast. Swiss albino mice were intraperitoneally injected daily with liver and muscle extracts of *L. lunaris* (1 ml/100 g of body weight), for 10 days. Control mice received injections of NaCl (0.9%). No mortality was recorded. The treatment led to: (1) decrease in body weight and increase in organ (liver and kidney) weights; (2) oxidative stress evidenced by an increase in lipid peroxidation and a decrease in antioxidant enzymes activities in tissues (blood cells, liver, and kidney); (3) a decrease in alanine aminotransferase and alkaline phosphatase activities in serum; and (4) development of mydriasis. The study suggests that *L. lunaris* collected from Southeast Indian coast is toxic, particularly, the muscle and therefore, it is not fit for consumption.

Key words: Fish toxins, food safety, antioxidant activity, toxicology, risk assessment.

INTRODUCTION

Puffer fish intoxication is the best known of all types of fish intoxications and has been recognized from ancient times. It is probably the most common fish intoxication along the coasts of Asia. (Ahasan et al., 2004; Hwang et al., 2002; Wu et al., 2005). The puffer fish are also variously known as toadfish, blowfish, globefish, swellfish and balloon fish, belonging to the order Tetraodontiformes (Halstead, 1967). They contain a potent neurotoxin known as tetrodotoxin (TTX) which has the ability to selectively block the ion transport of the sodium channel (Baselt, 2008). Puffer fish from the South Indian coast have not been studied yet. *Lagocephalus lunaris* popularly known as green toadfish is the most commonly available species in south Indian coast. This species is reported to be toxic elsewhere (Berry and Hassan, 1973; Monaliza and Samsur, 2011; Shiomi et al., 1985), but is eaten by the locals. This explicitly demands toxicity study. To determine the toxicity, standard mouse bioassay (Kawabata, 1978) was followed. In this case, the mice exhibited some symptoms but no mortality was recorded. This prompted us to carry out biochemical assays. Toxicity assessment of puffer fish *Lagocephalus lagocephalus* from Tunisian coast showed that the fish extracts could produce oxidative damages in liver, kidney and erythrocyte tissues of the rats (Sauodi et al., 2008a, 2008b). In this work, the toxicity of *L. lunaris* is investigated in Swiss albino mice. Mice were daily injected for 10 days with muscle and liver tissue extracts of *L. lunaris*. To assess the toxicity, the clinical symptoms, body weight (BW), organs (kidney and liver) weight and mortality were recorded. The activities of liver biomarker enzymes, alanine aminotransferase (ALT), and

*Corresponding author. E-mail: sskhora@vit.ac.in. Tel: +91 416 2202472;09486274015; Fax: +91 416 2243092

alkaline phosphatase (ALP) in serum were also recorded as they are important parameter in toxicity studies (Saoudi et al., 2008). Since a few marine toxins were found to induce oxidative stress (Dittmann and Wiegand, 2006; Li et al., 2003; Scinska et al., 2006), lipid peroxidation level and antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities were also investigated in blood cells, liver and kidney of mice.

MATERIALS AND METHODS

Sample collection

Specimens of puffer fish *L. lunaris* (Bloch and Schneider, 1808) were collected from Chennai coast, Tamil Nadu in the Southeastern part of India, during the month of February, 2011 and transported to the laboratory in dry ice. Samples were frozen at -20°C until use. The specimens were identified by Professor S. S. Khora, Department of Medical Biotechnology, VIT University.

Fish extract preparation

Muscle and liver was carefully dissected from *L. lunaris*. 10 g of each organ/tissue was taken and minced properly. To each minced organ/tissue 2.5 volumes of 0.1% acetic acid was added and boiled in water bath for 10 min. Then these samples were cooled and centrifuged at 3000 rpm for 10 min and the supernatants were collected. This process was repeated thrice, to make up 5 volumes of the sample taken (Khora, 1991). The supernatants containing the toxins were finally stored at -30°C.

Animals

Adult male Swiss albino mice weighing about 25 to 35 g were obtained from VIT animal house (VIT University, Vellore, Tamil Nadu, India). They were housed at 22 ± 3°C with light/dark periods of 12 h and a minimum relative humidity of 40%. Mice were fed with a commercial balanced diet (V.R.K. Nutritional Solutions) and drinking water was offered *ad libitum*. This study was carried out in strict accordance with the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Guidelines for laboratory animal facility. The protocol was approved by the Institutional Animal Ethical Committee, VIT University (Permit Number: VIT/SBST/IAEC/IIIrd/01; Registration number: 1333/c/10/CPCSEA; Date of registration: 30.03.2010). All surgery was performed under anesthesia by intraperitoneal injection of chloral hydrate, and all efforts were made to minimize suffering.

Experimental design

After acclimatizing to the laboratory conditions for one week, 54 mice were divided into three groups. They were daily intraperitoneally (i.p.) injected with (1) 1 ml of a saline solution (0.9% NaCl)/100 g of BW for the controls group (C), (2) 1 ml of muscle extract/100 g BW for the muscle treated group (M), and (3) 1 ml of liver extract/100 g BW for the liver treated group (L). After 2, 5 and 10 days of treatment, 6 mice of each group were sacrificed under anaesthesia by i.p. injection of chloral hydrate. The blood was collected without anticoagulant by heart puncture, centrifuged (4000 rpm/15 min, 4°C) and serum and blood cells were obtained and kept at -30°C. The liver and kidneys were removed, weighed, rinsed with ice cold saline and kept at -30°C. The frozen liver, kidney and blood cells samples were homogenized (1/2, w/v) in an ice cold buffer (TBS: 50 mM Tris, 150 mM NaCl, pH 7.4) and centrifuged at 5000 rpm for 30 min at 4°C, supernatants obtained were frozen at -30°C (Saoudi et al., 2008b).

Biochemical assays

In serum, activity of ALT and ALP was determined using commercial kits (Span Diagnostics, India). The enzyme activity was expressed is International Units (IU)/ml.

In blood cells, kidney and liver, superoxide dismutase (SOD) activity was determined according to the method of Marklund and Marklund (1974) which involves pyrogallol auto-oxidation at pH 8.0. It is expressed in units/mg protein. One unit of enzyme activity is defined as the amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation and the absorbance was read at 420 nm. Catalase (CAT) activity was determined by the method of Sinha (1972). In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced was measured calorimetrically at 610 nm. The specific activity of catalase has been expressed as µmol of H_2O_2 consumed/min/mg protein. Glutathione peroxidase (GPx) activity was determined spectrophotometrically by using Ellmans reagent (DTNB) as a coloring reagent following the method described by Rotruck (1973) and the absorbance was read at 412 nm. The specific activity of GPx is expressed as µg of glutathione (GSH) utilized/min/mg protein.

Lipid peroxidation was determined by the procedure of Ohkawa et al. (1979) as thiobarbituric acid reactive substances (TBARS). It was expressed as nmol of malondialdehyde (MDA) formed/mg protein. The absorbance was read at 532 nm. The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Statistical analysis

Statistical analysis was performed using the Prism software package (GraphPad, InStat Version 3). Data were expressed as the mean and the standard deviation of the mean (SD). The analysis of variance (ANOVA) was used to compare the differences between the groups. Differences were considered significant at the 95% confidence level ($p < 0.05$).

RESULTS

Clinical manifestations

After the injection of muscle and liver tissue extract of *L. lunaris*, mice exhibited symptoms like hopping, scratching its body, stretching of hind limbs and lower back and concave curvature of spinal cord. The mice mostly remained motionless but no mortality was recorded. A significant decrease in the body weights of mice of M group was recorded. On the other hand a significant increase in the liver weights of mice of M and L groups were recorded especially after the 5th day of treatment

Table 1. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on body, liver and kidney weights of control and treated mice after 2, 5 and 10 days of treatment.

Parameter and treatment		Body weight (g)	Liver weight (g)	Kidney weight (g)
After 2 days	C	29.2 ± 2.68	1.22 ± 0.13	0.45 ± 0.07
	L	28 ± 1.58	1.47 ± 0.23	0.5 ± 0.04
	M	25.2 ± 1.79*	1.24 ± 0.9	0.4 ± 0.01$^{\#}$
After 5 days	C	28 ± 1.58	1.06 ± 0.12	0.38 ± 0.02
	L	25.8 ± 1.3	1.57 ± 0.06***	0.44 ± 0.05
	M	23 ± 2.82**	1.53 ± 0.2***	0.44 ± 0.01
After 10 days	C	28 ± 2.83	1.52 ± 0.18	0.42 ± 0.06
	L	25.25 ± 0.96	1.8 ± 0.09*	0.52 ± 0.06*
	M	21.33 ± 1.53**	1.87 ± 0.11*	0.57 ± 0.03*

Values are mean ± SD; n = 5; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{\#}$p ≤ 0.05: M group vs. L group.

Table 2. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities in serum of control and treated mice after 2, 5 and 10 days of treatment.

Parameter and treatment		ALT (IU/L)	ALP (IU/L)
After 2 days	C	71.69 ± 1.18	50.39 ± 2.37
	L	71.41 ± 2.37	28.4 ± 1.75***
	M	64.65 ± 1.68***$^{\#\#\#}$	38.21 ± 1.65***$^{\#\#\#}$
After 5 days	C	87 ± 1.5	48.17 ± 1.5
	L	37.02 ± 1.6***	39.89 ± 1.04***
	M	33.33 ± 2.35***$^{\#}$	30.05 ± 1.22***$^{\#\#\#}$
After 10 days	C	86.64 ± 2.21	52.69 ± 2.24
	L	35.19 ± 1.4***	42.6 ± 1.72***
	M	18.12 ± 0.76***$^{\#\#\#}$	24.6 ± 1.3***$^{\#\#\#}$

Values are mean ± SD; n = 5; ***p ≤ 0.001: significant from control. $^{\#\#\#}$p ≤ 0.001: M group vs. L group.

and also a significant increase in kidney weights of mice belonging to M and L groups were recorded after 10th day (Table 1).

ALT and ALP activities in serum

After the 2nd day of treatment, no change in ALT activity in serum of mice belonging to L group was recorded while a significant decrease in ALT activity was recorded in mice belonging to M group. There was a significant decrease in the ALT activities after the 5 and 10th days of treatment in both M as well as L groups, the decrease being more evident in M group. Similarly, a significant decrease in the ALP activities in serum of mice belonging to M and L groups were recorded after the 2nd, 5th and 10th days of treatment (Table 2).

SOD activities in blood cells, liver and kidney

After the 2nd day of treatment, there was no significant difference in the SOD activities in blood cells, liver and kidney of treated mice when compared with control mice. But after the 5 and 10th days of treatment, there was an evidential decrease in the SOD activities in both the treated groups, M as well as L, the decrease being more evident in M group (Figures 1, 2, and 3).

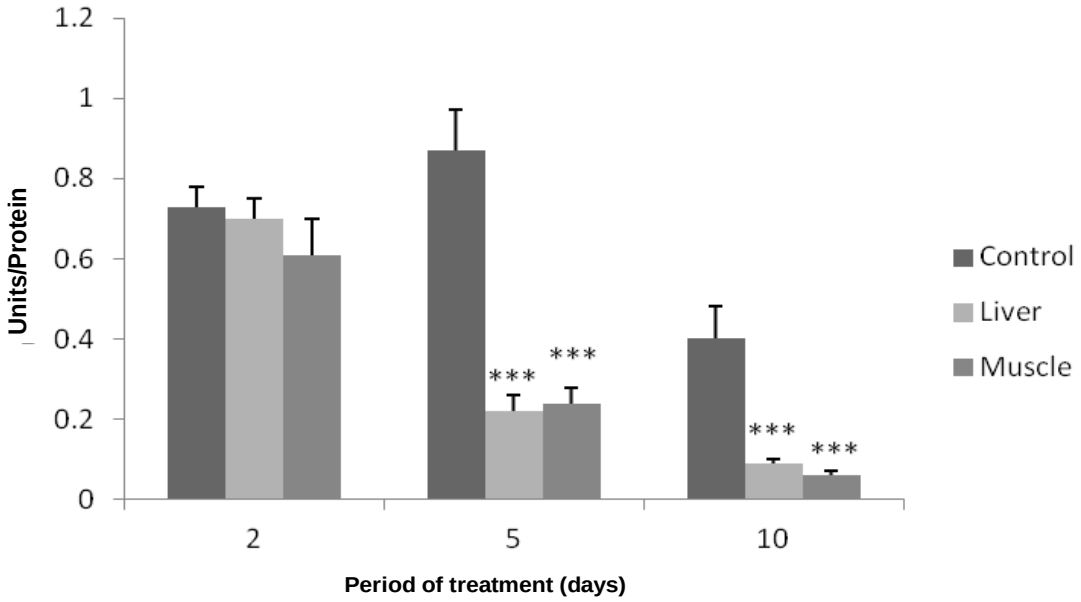

Figure 1. SOD activity in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on superoxide dismutase (SOD) activity in blood cells of control and treated mice after 2, 5 and 10 days of treatment. *p ≤ 0.05; ***p ≤ 0.001: significant from control.

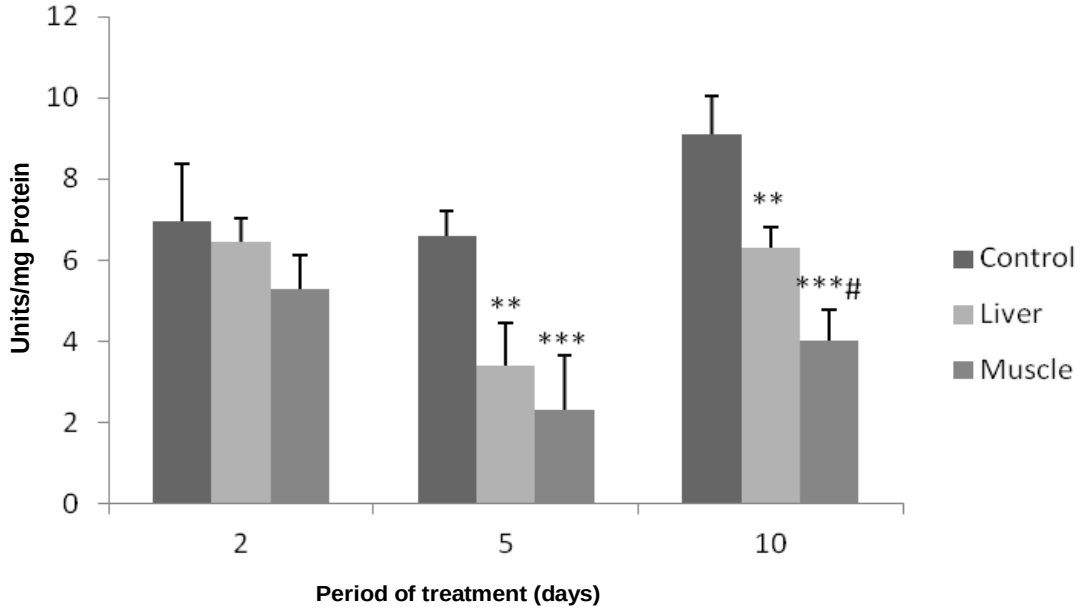

Figure 2. SOD activity in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on superoxide dismutase (SOD) activity in liver of control and treated mice after 2, 5 and 10 days of treatment.**p ≤ 0.01; ***p ≤ 0.001: significant from control. #p ≤ 0.05: M group vs. L group.

CAT activities in blood cells, liver and kidney

Similarly, there was no significant difference in the CAT activities after the 2nd day of treatment, but a significant decrease in CAT activity was recorded after the 5 and 10th days of treatment in mice belonging to M and L groups, the decrease being more evident in M group (Figures 4, 5, and 6).

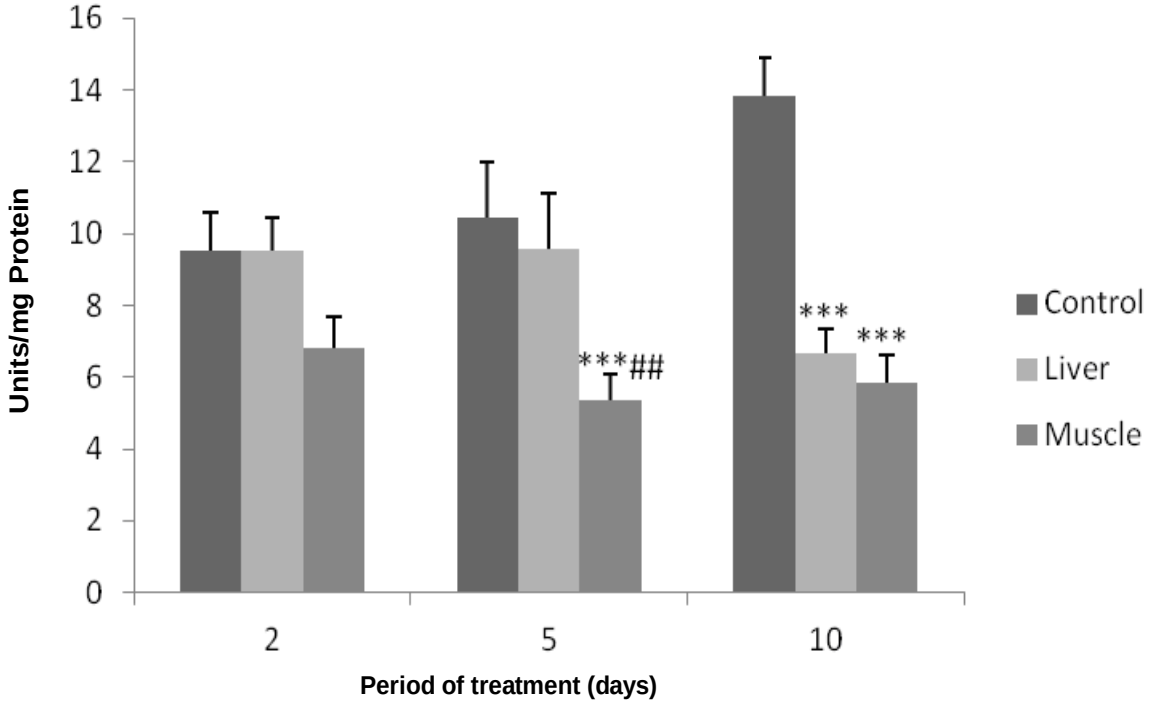

Figure 3. SOD activity in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on superoxide dismutase (SOD) activity in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. ##p ≤ 0.01: M group vs. L group.

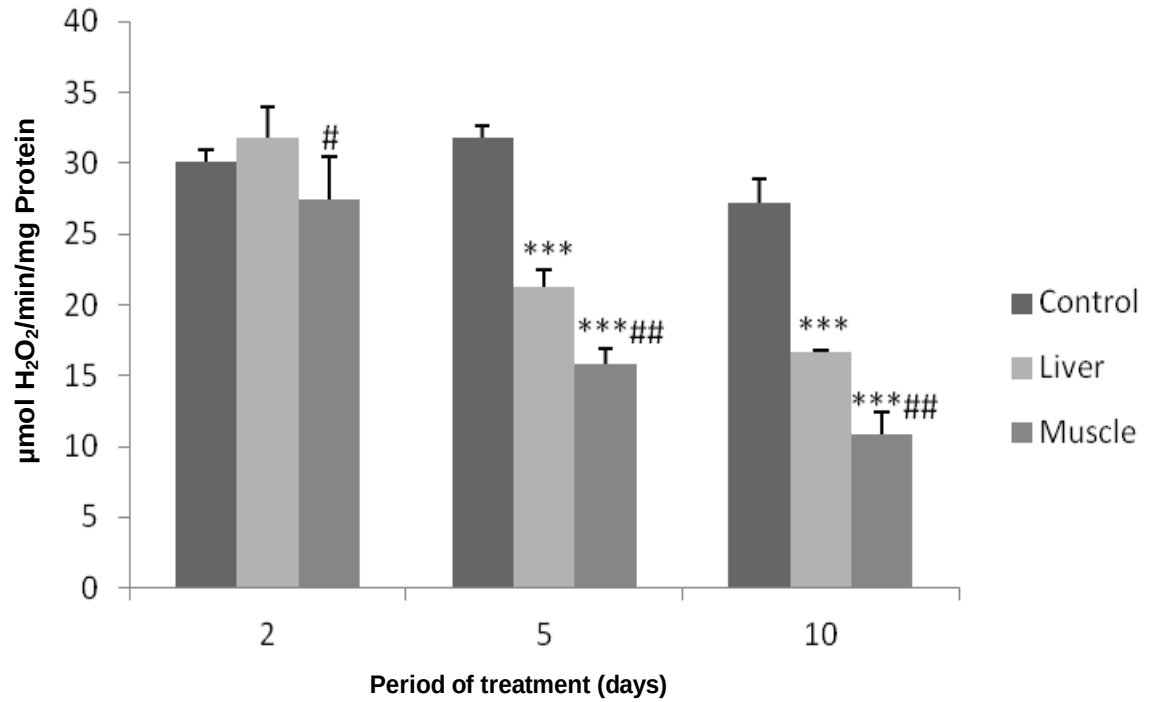

Figure 4. CAT activity in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on catalase (CAT) activity in blood cells of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control. #p ≤ 0.05; ##p ≤ 0.01: M group vs. L group.

µmol

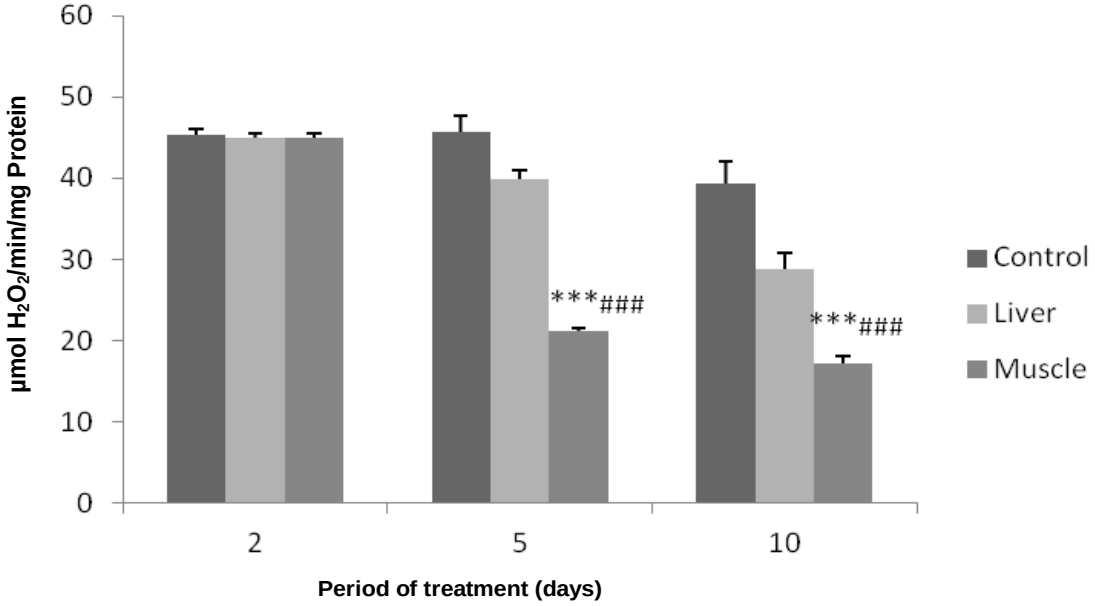

Figure 5. CAT activity in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on catalase (CAT) activity in liver of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control. $^{###}$p ≤ 0.001: M group vs. L group.

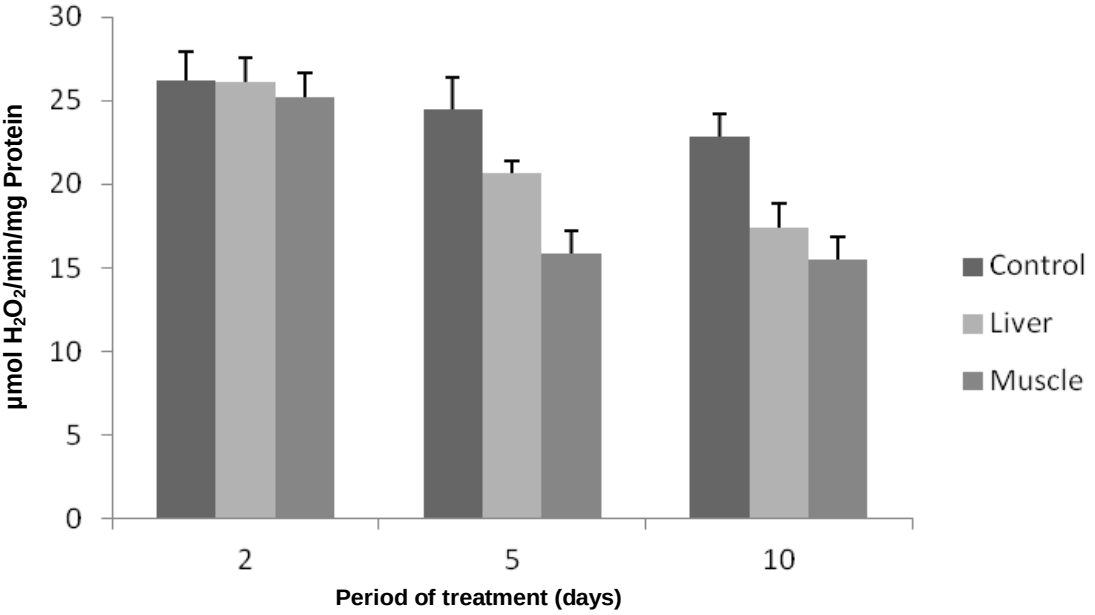

Figure 6. CAT activity in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on catalase (CAT) activity in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{##}$p ≤ 0.01: M group vs. L group.

GPx activities in blood cells, liver and kidney

Also, there was no significant difference recorded in the GPx activities after the 2nd day of treatment, but a significant decrease in GPx activity was recorded after the 5 and 10th days of treatment in mice belonging to M and L groups, again the decrease being more evident in M group (Figure 7, 8, and 9).

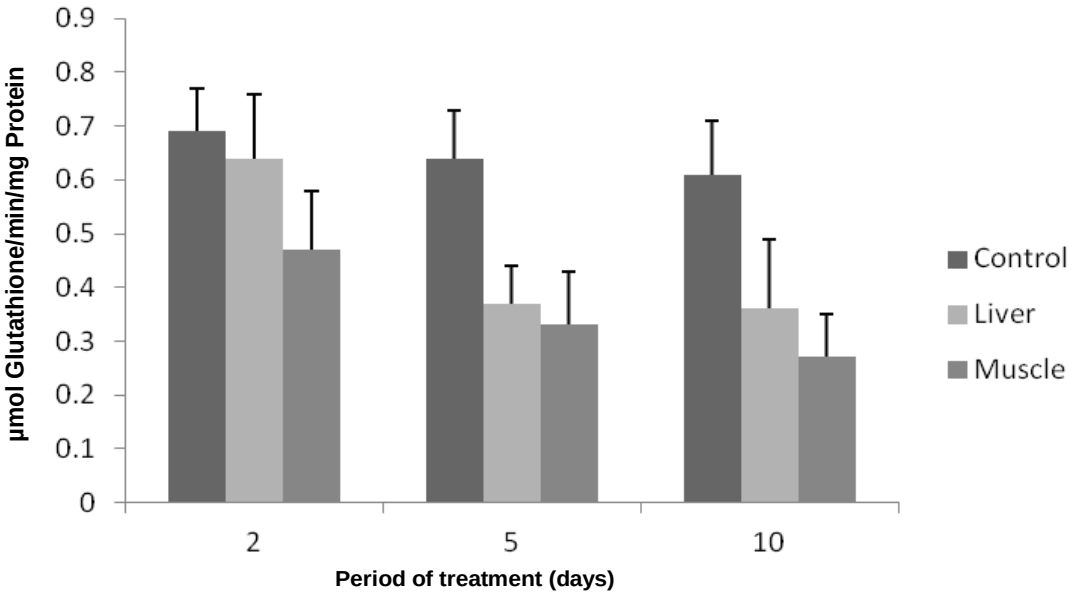

Figure 7. GPx activity in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on glutathione peroxidase (GPx) activity in blood cells of control and treated mice after 2, 5 and 10 days of treatment. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001: significant from control.

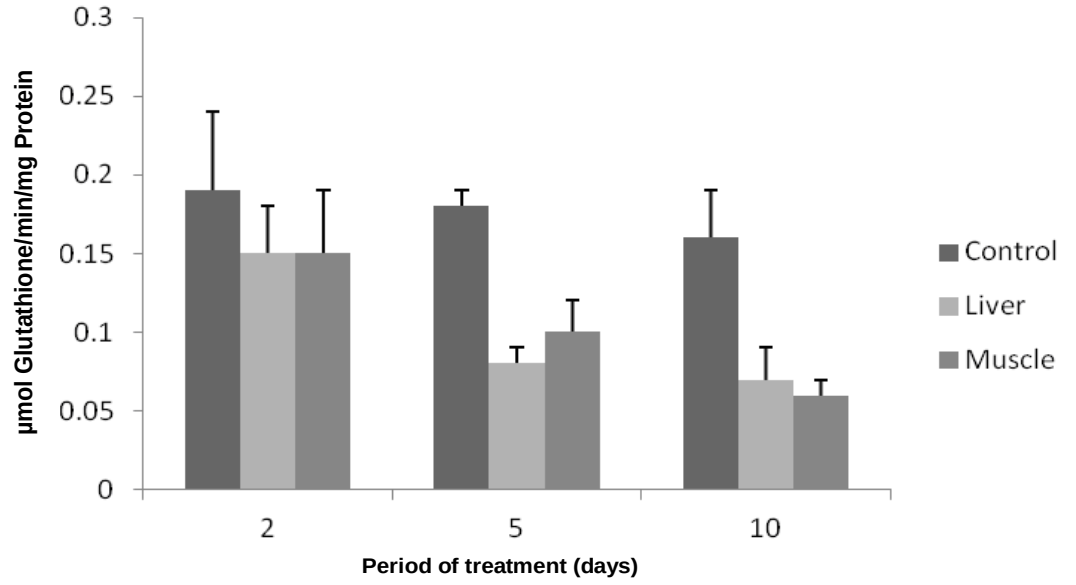

Figure 8. GPx activity in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on glutathione peroxidase (GPx) activity in liver of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control.

Lipid peroxidation levels in blood cells, liver and kidney

A significant increase in the TBARS levels were recorded in mice belonging to M as well as L groups and it was more evident in the blood cells than in liver and kidney of mice (Figures 10, 11, and 12).

DISCUSSION

This study showed that injection of *L. lunaris* liver and

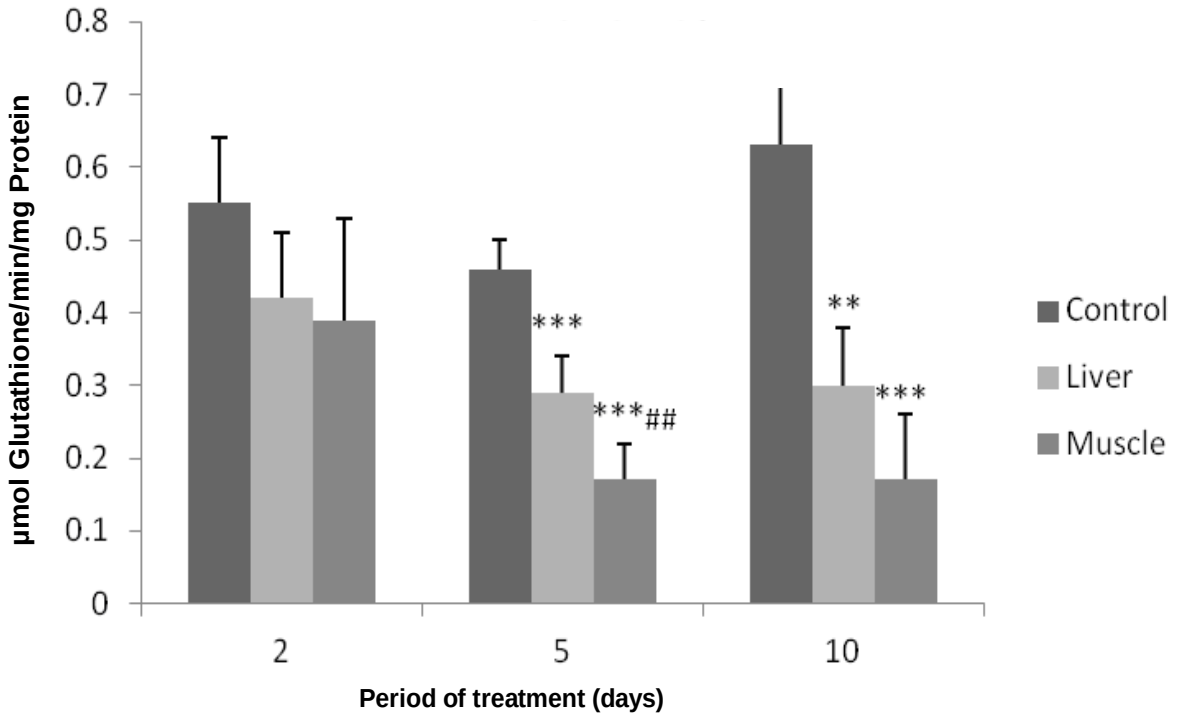

Figure 9. GPx activity in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on glutathione peroxidase (GPx) activity in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{##}$p ≤ 0.01: M group vs. L group.

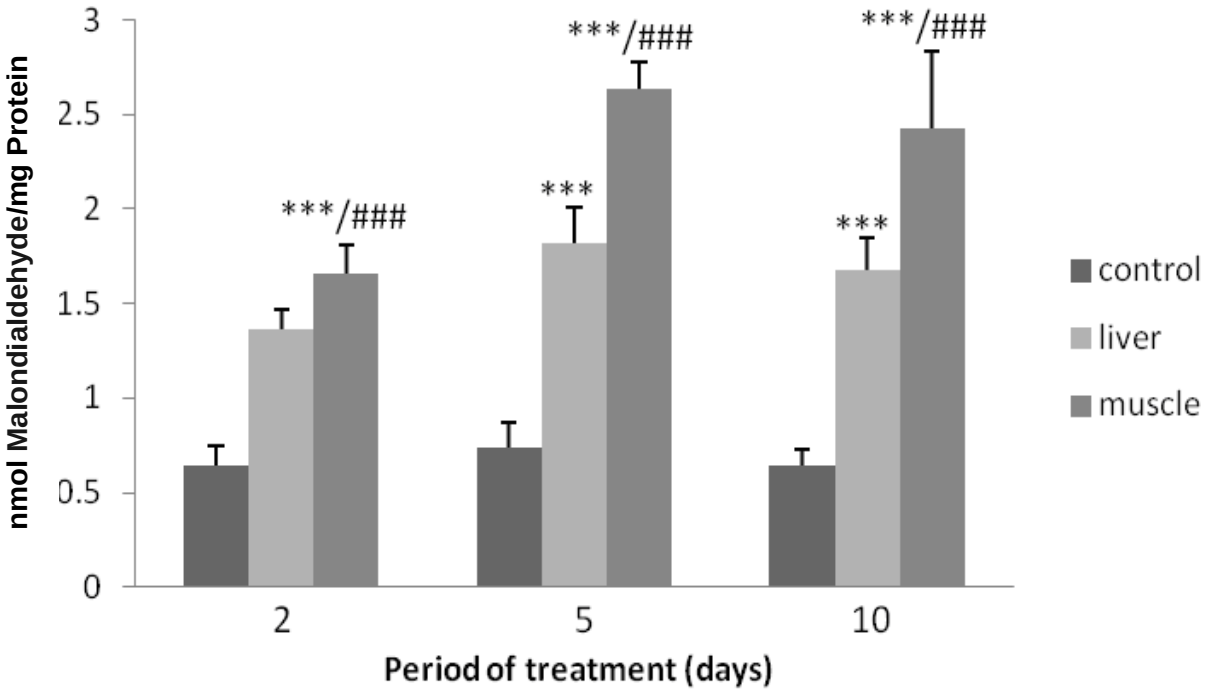

Figure 10. TBARS level in blood cells. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on lipid peroxidation (TBARS) level in blood cells of control and treated mice after 2, 5 and 10 days of treatment. ***p ≤ 0.001: significant from control. $^{###}$p ≤ 0.001: M group vs. L group.

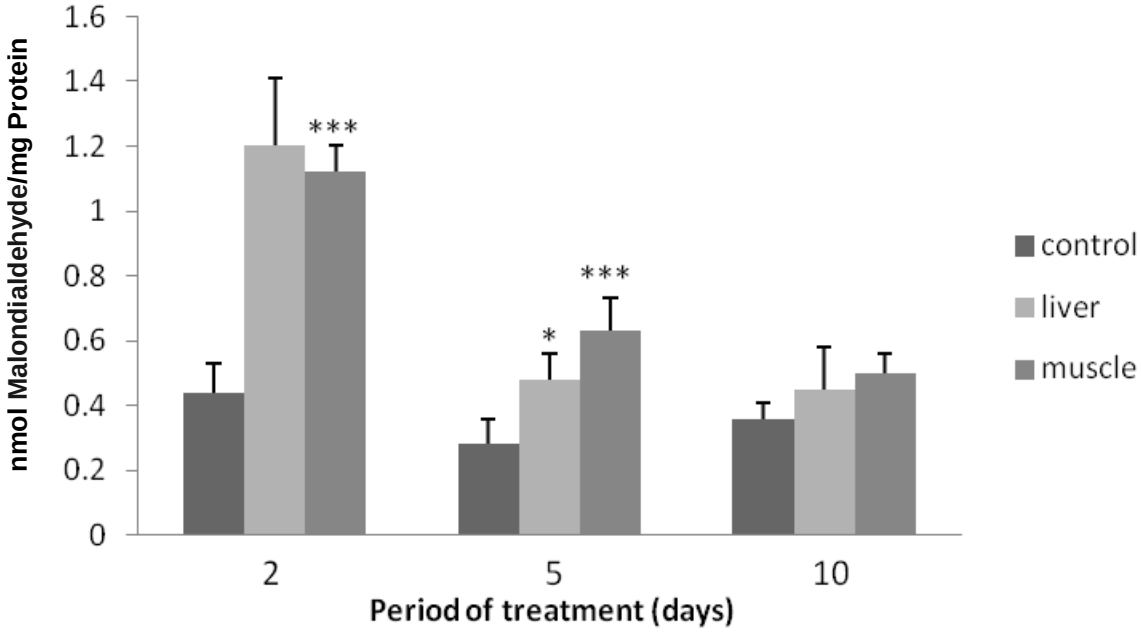

Figure 11. TBARS level in liver. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (L. lunaris) on lipid peroxidation (TBARS) level in liver of control and treated mice after 2, 5 and 10 days of treatment. *p ≤ 0.05;***p ≤ 0.001: significant from control.

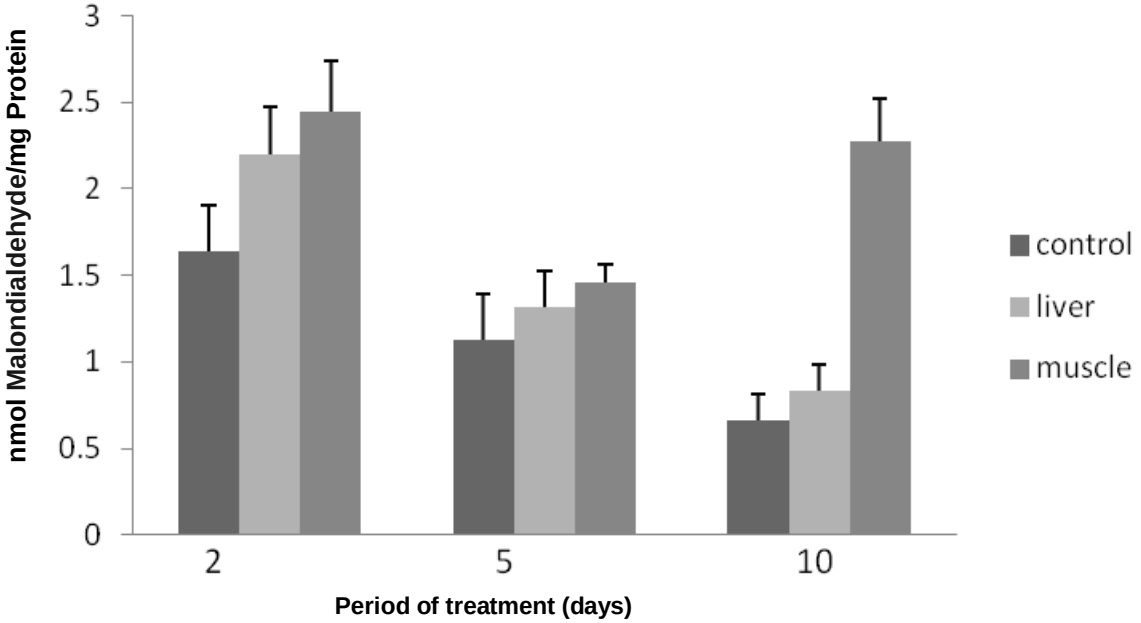

Figure 12. TBARS level in kidney. Effect of tissue extracts (1 ml/100 g, v/w) of liver and muscle of puffer fish (*L. lunaris*) on Lipid peroxidation (TBARS) level in kidney of control and treated mice after 2, 5 and 10 days of treatment. **p ≤ 0.01; ***p ≤ 0.001: significant from control. $^{###}$p ≤ 0.001: M group vs. L group.

muscle extracts into adult Swiss albino mice led to the decrease in body weight with significant increase in the organs (kidney and liver) weights which can be attributed to internal metabolic changes that may have occurred as same condition has been observed in other toxicity study (Soni et al., 2008).

The study showed a significant decrease in the ALP and ALT activities in serum of treated mice when compared with control. In liver enzyme, alkaline phosphatase is closely connected with lipid membrane in the canalicular zone, so that any interference with the bile flow, whether extra hepatic or intra hepatic leads to decrease in ALP activity (Vandenberghe, 1951). In this case, the muscle and tissue extracts of *L. lunaris* probably causes the cell membrane damage (lipid peroxidation) which leads to the imbalance between synthesis and degradation of enzyme protein, thus lowering the enzyme activity (Hardonk and Koudstaal, 1976). The findings of this study are in agreement with Saoudi et al. (2008b). A significant decrease in ALT activity was observed which could be due to the result of a down regulation of enzyme synthesis (Saoudi et al., 2008b). Interestingly, Solter et al. (1998, 2000) showed that ALT activity decreased with sub chronic exposure to hepatotoxin microcystin-LR. There was a significant decrease in the antioxidant enzyme (SOD, CAT, GPx) activities and therefore a high lipid peroxidation (TBARS) level which shows that the muscle and tissue extracts of *L. lunaris* resulted in oxidative stress. This study does not confirm the presence of a particular toxin since oxidative stress has been reported in various other toxicity studies (Ding et al., 1998; Li et al., 2003; Scinska et al., 2006).

From the 2nd day of treatment, a disorder in the eyes of most of the treated mice was observed. It started with a white spot at the centre of the pupil and as it progressed, the eye became reddish, the pupils were dilated, the membranes of the eyelids protruded and finally it was so much swollen that the mice could not open their eyes. The mice probably developed mydriasis due to the injection of toxin. Earlier mydriasis was reported in cats treated with ivory shell toxin (Hashimoto, 1976). Various toxins (botulinum toxin, prosurugatoxin, etc) have been known to induce mydriasis in rabbits and rats (Kosuge, 1985; Ishikawa, 2000). Perhaps, this is the first report of mydriasis due to puffer fish toxins in mice.

Generally, TTX is accumulated in the liver, gonads, intestine, muscle and skin of the puffer fish (Fuchi et al., 1991; Mahmud et al., 2000; Panichpisal et al., 2003). The liver and gonads are supposed to be more toxic when compared with the other parts. In another study, the skin was reported to be the most toxic part (Khora, 1991). Puffer fish accumulates TTX in their body through the food chain (Lee et al., 2003). Therefore, the toxicity of puffer fish also changes depending on the location they are collected from. It has also been reported that puffer fish raised in captivity are non toxic (Khora, 1994). Most of the earlier work on puffer fish showed the liver to be the most toxic part of puffer fish, and the muscle being the least toxic (Hashimoto, 1976; Matsui et al., 1981; Nagashima, 1999; Saoudi et al., 2008b). On the contrary, this study shows that the muscle can also be more toxic than liver.

Conclusively, this study reports for the first time, the development of mydriasis in mice due to puffer fish toxins. It also proves that the muscle of puffer fish can also be more toxic than the liver/gonad unlike earlier reports. Therefore, *L. lunaris* collected from Southeast Indian coast should not be consumed as it causes sub acute toxicity. Further studies are in progress to identify the implicated toxin.

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Full Length Research Paper

Evaluation of oxyclozanide and niclosamide combination as alternative antiparamphistomal therapy in buffaloes

Shaheen H.[1]*, Kadry M. Sadek[2] and Eman K. Bazh[3]

[1]Department of Pharmacology, Faculty of Veterinary Medicine, Damanhour University, Egypt.
[2]Department of Biochemistry, Faculty of Veterinary Medicine, Damanhour University, Egypt.
[3]Department of Pathology and Parasitology, Faculty of Veterinary Medicine, Damanhour University, Egypt.

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Paramphistomiasis causes enteritis and anemia in livestock and result in substantial production and economic losses. It is considered a neglected tropical disease, with lower effective trematodicidal compound for treatment. Keeping in view the importance of disease, the present study aims to evaluate the efficacy of oxyclozanide and niclosamide, as well as a combination of both drugs in treatment of *Paramphistomum* infection. Twenty buffalo males of native breed between eight months to 2 years of age, naturally infected with *Paramphistomes* were treated. Oxyclozanide and niclosamide were administered in 20 ml/100 kg and 125 mg/kg orally, respectively. The efficacies of the drugs were estimated on the bases of reduction in body weight, drug efficacy, paramphistomes egg count per gram feces, as well as, hematological and biochemical parameters. The obtained results revealed that Co administration of oxyclozanide and niclosamide resulted in amelioration of the most adverse effects associated with *Paramphistomum* infection and reflected significantly on decreased egg per gram count and oxidative stress, improved biochemical and hematological profiles.

Key words: oxyclozanide, niclosamide, antiparamphistomal, therapy, buffalo.

INTRODUCTION

Paramphistomiasis is largely a disease of young animals less than two years of age, because repeated infections of low intensity generally produce an almost complete immunity. Adult *Paramphistomes* are the main parasites in the rumen and reticulum of sheep, goats, cattle, and water buffaloes; the pathological effects of infection are almost entirely caused by the immature stages within the first part of the small intestine (Zahir et al., 2012). The immature worms penetrate the mucosa of the small intestine as deeply as the musculosa. This causes strangulation and the eventual necrosis of the piece of mucosa, leading to the development of erosions and petechiae. These lesions cause acute parasitic gastroenteritis with high morbidity and mortality rates, particularly in young animals (Rolfe and Boray, 1993). At the same time, hypoalbuminemia which is by losing seepage and other plasma protein into the gut, coupled with loss of appetite, seems to be the most important pathophysiological consequence of paramphistomiasis (Sissay, 2007). *Paramphistomum* infection provokes a lower feed conversion, a loss of weight, and/or a decrease in milk production, which results in economic loss (Rangel-Ruiz et al., 2003). *Paramphistomum cervi* is considered as one of the most important species of *Paramphistomes*, since they are cattle parasites with a cosmopolitan distribution (Hassan et al., 2005). Paramphistomiasis can be controlled by periodic treatment with a repertoire of drugs. Several drugs have been assessed and recommended

*Corresponding author. E-mail: dr_hazemshaheen3010@yahoo.com.

for the treatment of paramphistomiasis (Panyarachun et al., 2010). Although treatment for adult fluke has no direct benefit to the animal, it may reduce the source of infection for the snail intermediate host. This then reduces the size of the next generation of infective fluke larvae on pasture (Soulsby, 1982). The current research mainly focused on developing alternative drug formulations and evaluates the effect of both oxyclozanide and niclosamide on *Paramphistomes* applied on live animals, especially, the buffaloes.

MATERIALS AND METHODS

Animals

Twenty buffalo males of native breed between eight months to 2 years of age naturally infected with *Paramphistomes*, based on their fecal examination by sedimentation and floatation techniques (Kruse and Pritchard, 1982). Use of animals in this study was in accordance with Good Laboratory Practice standards and national welfare regulations.

Drugs and treatment

Oxyclozanide (Zanil® Fluke Drench 3.40 w/v%) was obtained from Schering-Plough Egypt. The recommended dose is 20 ml/100 kg orally, with two treatments given two days apart (Rolfe and Boray, 1987). Niclosamide (Niclosan® 500) was obtained from Misr Co. For Pharm. Ind. Cairo, Egypt. The recommended dose is 125 mg/kg orally (Einstein et al., 1994) and repeated after 14 days (Bishop, 2005).
Animals were divided into 4 groups, each of 5 animals as follows:

Group 1. Buffaloes were treated with oxyclozanide at oral dose of 20 ml/100 kg. Two doses were given two days apart.
Group 2. Buffaloes were orally treated with niclosamide at a dose of 125 mg/kg and repeated after each 14 days (five times) to overcome the prepatent period of *Paramphistomes*, 7-10weeks (Gerold and Hannah, 2007).
Group 3. Buffaloes were orally treated with oxyclozanide at a dose of 20 ml/100 kg. Two doses were administered two days apart and after the last dose of oxyclozanide, niclosamide at a dose of 125 mg/kg was orally administered and repeated after each 14 days (five times).
Group 4. Buffaloes were left as control infected non treated animals monitored weekly.

The animals of all groups were weighted every two weeks during the trial period.

Fecal sampling and parasitological examination

One week before and at the time of treatment (zero day) later weekly throughout the treatment trials, fecal samples were collected from each animal. The examinations of the fecal samples were done by direct smear subsequently floatation technique and fecal culture (Kruse and Pritchard, 1982) to ensure the status of infection with other parasites. Sedimentation technique and eggs count (eggs per gram of feces, EPG) were determined by the McMaster technique (Rieu et al., 2007) for a fecal count of *Paramphistomes* eggs. A complete description of each case was recorded. The efficacies of the drugs used were evaluated according to the following equation recorded by Khayatnouri et al. (2011).

$$\% \text{ of drug efficacy} = P\text{-}R/P \times 100$$

Where, R, Average number of parasite egg in a gram of fecal sample after treatment; P = average number of parasite egg in a gram of fecal sample before treatment.

Blood sampling

Every two weeks during the experimental period (ten weeks), two venous blood samples (six ml) were taken from each animal. The first blood sample was collected in test tubes containing heparin for hematological studies. While the second blood samples were allowed to coagulate at 4 °C and were then centrifuged at 3000 rpm for 15 min to separate the serum. The serum samples were frozen at −20°C.

Hematological analysis

The hematological parameters, red blood cells (RBCs) count, hemoglobin (Hb%), and packed cell volume (PCV%) were estimated by using an automatic cell counter (Exigo, Veterinary Hematology System, Boule Medical AB, Stockholm, Sweden.).

Biochemical analysis

Total protein, blood albumin, glutathione, malondialdehyde, serum glucose, blood sodium and blood potassium were measured in serum by commercially available kit methods. Globulins were estimated by electrophoretic analysis of serum protein.

Statistical analysis

The descriptive data are presented as the means ± SE. The statistical differences were calculated on the basis of two way test of ANOVA and $p < 0.05$ is considered as significant between the groups. The data were statistically analyzed by using one way ANOVA test for variance analysis (Student-Newman-Keuls) at $p < 0.05$, using the SPSS 13.0 Windows statistical package (2004).

RESULTS

All animals were clinically healthy throughout the experiment. None of the buffaloes in all groups suffered from identifiable reactions following the administration of oxyclozanide or niclosamide or the combination of both drugs. Oxyclozanide at a dose of 20 ml/100 kg, where two doses were orally given two days apart and after the last dose of oxyclozanide, niclosamide at a dose of 125 mg/kg was orally given and repeated every 14 days (five times) revealed significant effects on body weight, number of egg count/gram and drug efficacy (%) (Table 1, 2 and 3).

Body weight

Beginning with the 4th week of the experiment the drug combination improved significantly ($p < 0.05$) the body weight (kg) of animals (331.14 ± 9.32) with respect to (312.27 ± 6.30: 309.15 ± 8.33 and 319.16 ± 4.22) in

Table 1. Effect of orally administered oxyclozanide (20 ml/100 kg) or niclosamide (125 mg/kg) and both drugs on body weight (kg) of buffaloes naturally infected with *Paramphistomes* (n = 5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	288.22±4.60^{g}	297.55±6.70^{g}	309.15±8.33^{f}	319.25±6.28^{f}	327.12±5.16^{ef}	339.10±8.19^{e}
2nd group	289.14±7.18^{g}	300.26±5.12^{fg}	312.27±6.30^{f}	323.45±5.11^{ef}	337.34±5.56^{e}	357.64±9.57^{d}
3rd group	291.14±3.16^{g}	312.18±4.22^{f}	331.14±9.32^{e}	356.26±6.23^{d}	371.27±6.53^{c}	394.27±6.53^{a}
Control	290.50±5.32^{g}	302.62±7.21^{fg}	319.16±4.22^{f}	324.00±4.12^{ef}	336.14±6.33^{e}	356.14±9.43^{d}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different ($p < 0.05$).

Table 2. *Paramphistomes* egg count before and during treatment with oxyclozanide (20 ml/100 kg), niclosamide (125 mg/kg) or both drugs.

Weeks / Groups	One week before	0 day	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week	9th week	10th week
1st group	5.30±0.13^{ab}	5.30±0.13^{ab}	2.50±0.11^{e}	0.60±0.004^{g}	1.00±0.004^{f}	1.30±0.01^{f}	1.16±0.002^{f}	1.30±0.10^{f}	1.30±0.10^{f}	2.10±0.11^{e}	1.60±0.11^{f}	2.80±0.10^{e}
2nd group	5.50±0.13^{ab}	5.47±0.12^{ab}	5.50±0.13^{ab}	4.66±0.02^{c}	4.63±0.01^{c}	5.10±0.27^{b}	4.80±0.17^{c}	4.30±0.11^{c}	4.30±0.01^{c}	5.00±0.16^{b}	4.30±0.21^{c}	3.80±0.16^{d}
3rd group	5.22±0.07^{b}	5.62±0.11^{a}	2.50±0.001^{e}	1.36±0.001^{f}	0.33±0.001^{g}	0.33±0.001^{g}	0.33±0.001^{g}	0.00±0.00^{h}	0.00±0.00^{h}	0.00±0.00^{h}	0.00±0.00^{h}	0.00±0.00^{h}
Control	5.31±0.20^{ab}	5.52±0.10^{ab}	5.50±0.11^{ab}	5.67±0.22^{a}	5.31±0.20^{ab}	5.22±0.07^{b}	5.16±0.14^{b}	5.45±0.15^{ab}	5.54±0.22^{ab}	5.00±0.11^{b}	5.44±0.10^{ab}	5.83±0.14^{a}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

control animals, respectively. At the end of the experiment, 10th week, the significant differences increased (394.27 ± 6.53) vs. (357.64 ± 9.57; 339.10 ± 8.19 and 356.14 ± 9.43) in control animals, respectively.

The number of *Paramphistomes* eggs /gram

During the preparation of the experiment, some nematode eggs were recorded in some cases with our target fluke (*Paramphistomum* spp.) eggs. And so, these animals were excluded from the experimental trial. Infection of naturally infected buffaloes with *Paramphistomes* was significantly decreased after treatment until the 6th week of the experiment (0.00 ± 0.00) then remains stable until the end of the experiment. On the other side, in the control positive non treated group, the number of eggs per gram remain noticed until the end of the experiment (3.80 ± 0.16; 2.80 ± 0.10 and 5.83 ± 0.14) in control animals, respectively. The effect of oxyclozanide appeared in the 2nd week (0.60±0.004), but the recurrent appearance of the eggs showed an ascending manner from the 2nd week of treatment until the end of the trial (2.80±0.10). In niclosamide treated group, the results showed no obvious reduction in eggs per gram. While the group treated with the drug combination showed an effective decrease in *Paramphistomes* eggs from the first week (2.50±0.001), and shedding of eggs was completely stopped at the 6th week (00 EPG) until the end of experimental trial.

Drug efficacy (%)

Drug efficacy (%) significantly improved through the fall of the *Paramphistomes* eggs count for the 6th week (100) and stabilized until the conclusion of the experiment (100) vs. another drug tested groups (0 and 59.25, respectively).

Hematological findings

Oxyclozanide at a dose of 20 ml/100 kg, where

Table 3. Effect of orally administered of oxyclozanide (20 ml/100kg) or niclosamide (125 mg/kg) and both drugs on drug efficacy (%)of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week	9th week	10th week
1st group	62.9	77.77	77.77	70.3	70.3	66.66	66.66	59.25	66.66	59.25
2nd group	0	0	0	0	0	0	0	0	0	0
3rd group	80.76	96.15	96.15	96.15	96.15	100	100	100	100	100
Control	0	0	0	0	0	0	0	0	0	0

Table 4. Effect of orally administered of oxyclozanide (20 ml/100kg) or niclosamide (125 mg/kg) and both drugs on red blood cell count (x 10^6/ µl) of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	5.20 ± 0.15^{c}	5.40 ± 0.12^{bc}	5.42 ± 0.16^{bc}	5.44 ± 0.12^{bc}	5.44 ± 0.08^{bc}	5.40 ± 0.12^{bc}
2nd group	5.10 ± 0.13^{c}	5.50 ± 0.14^{bc}	5.50 ± 0.10^{bc}	5.70 ± 0.11^{b}	5.74 ± 0.10^{b}	5.78 ± 0.11^{b}
3rd group	5.10 ± 0.10^{c}	5.70 ± 0.13^{b}	5.70 ± 0.11^{b}	6.10 ± 0.12^{a}	6.08 ± 0.14^{a}	6.12 ± 0.10^{a}
Control	5.50 ± 0.22^{c}	5.56 ± 0.13^{bc}	5.50 ± 0.20^{bc}	5.60 ± 0.16^{b}	5.54 ± 0.11^{b}	5.60 ± 0.13^{b}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 5. Effect of orally administered of oxyclozanide (20 ml/100kg) or niclosamide (125 mg/kg) and both drugs on hemoglobin values (g/dl) of buffaloes naturally infected with *Paramphistomes* (n = 5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	8.40 ± 0.11^{cd}	8.45 ± 0.18^{cd}	8.34 ± 0.14^{d}	8.36 ± 0.14^{d}	8.30 ± 0.14^{d}	8.21 ± 0.13^{d}
2nd group	8.55 ± 0.13^{c}	8.47 ± 0.15^{c}	8.44 ± 0.14^{c}	8.49 ± 0.14^{c}	8.49 ± 0.12^{c}	8.48 ± 0.17^{c}
3rd group	8.46 ± 0.16^{c}	8.72 ± 0.10^{b}	8.9 ± 0.14^{b}	9.0 ± 0.12^{a}	9.0 ± 0.12^{a}	8.9 ± 0.11^{ab}
Control	8.21 ± 0.19^{c}	8.14 ± 0.12^{c}	8.13 ± 0.12^{c}	8.27 ± 0.14^{c}	8.26 ± 0.11^{c}	8.29 ± 0.13^{c}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 6. Effect of orally administered of oxyclozanide (20 ml/100kg) or niclosamide (125 mg/kg) and both drugs on packed cell volume values (%) of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	29.43 ± 0.25^{bc}	28.43 ± 0.88^{c}	28.76 ± 0.45^{c}	27.46 ± 0.66^{c}	28.75 ± 1.52^{c}	28.58 ± 0.42^{c}
2nd group	27.12 ± 0.55^{c}	27.27 ± 0.24^{c}	28.43 ± 0.74^{c}	28.08 ± 0.26^{c}	27.42 ± 1.43^{c}	29.92 ± 0.33^{c}
3rd group	28.36 ± 0.33^{c}	30.48 ± 0.33^{bc}	30.37 ± 0.36^{bc}	32.59 ± 0.74^{ab}	32.25 ± 1.73^{ab}	31.12 ± 0.46^{ab}
Control	26.44 ± 1.45^{c}	25.25 ± 2.14^{c}	26.42 ± 1.56^{c}	25.32 ± 1.75^{c}	25.62 ± 1.02^{c}	25.56 ± 1.43^{c}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

two doses were orally given two days apart and after the last dose of oxyclozanide, niclosamide at a dose of 125 mg/kg was orally given and repeated every 14 days (five times) revealed significant effects on red blood cell count, hemoglobin values and packed cell volume (%) (Table 4, 5 and 6). Beginning with the 6^{th} week of the experiment the drug combination improved significantly ($p < 0.05$) the red blood cell count (x10^6/µl) of animals (6.10 ± 0.12) and stabilized until the end of the experiment, 10^{th} week, 6.12 ± 0.10. Hemoglobin values (g/dl) improved significantly (p

< 0.05) by the 6th week of the experiment (9.0 ± 0.12) and continued until the end of the 10th week (8.9 ± 0.11) vs. treated groups. Packed cell volume values (%) improved significantly (p < 0.05) by the 6th week of the experiment (32.59 ± 0.74) and continued until the end of the 10th week (31.12 ± 0.46) vs. treated groups.

Biochemical findings

Values presented in Table 7 to14 indicate that the drug combination revealed significant effects on serum total protein, albumin, globulin, glutathione, malondialdehyde, glucose, sodium and potassium where, significant increasing (p<0.05) in serum total protein (g/dl) was recorded on the 6th week of the experiment (6.15± 0.29) and continued until the end of the 10th week (6.35± 0.11) with respect to different groups (Table 7).

Oxyclozanide and niclosamide combination caused significant increase (p<0.05) in blood albumin values (gm/dl) at the 6th week of the experiment (3.21 ± 0.16) and continued until the end of the 10th week (3.25 ± 0.12) with respect to different groups (Table 8). Significant increasing (p<0.05) in blood globulin values (gm/dl) were recorded on the 6th week of the experiment (2.94 ± 0.08) and continued until the end of the 10th week (3.10 ± 0.22) due to oxyclozanide and niclosamide combination with respect to different groups (Table 9). *Paramphistomes* infection caused significant decreasing (p<0.05) in serum glutathione values (µmol/g protein) from zero day, (13.76± 1.43) until the end of the experiment (14.73± 2.34). By treatment, these values were significantly increased (p<0.05) by the end of the experiment in other different groups (18.86± 1.72; 22.56± 3.12 and 20.74± 1.39, respectively) (Table 10).

Paramphistomes infection caused significant increase (p<0.05) in serum malondialdehyde levels (nmol /g protein) from zero day, (54.67± 1.52) until the end of the experiment (57.86± 2.54). By treatment, these values were significantly decreased (p<0.05) by the end of the experiment, especially in niclosamide and drug combination treated animals (34.86± 3.47 and 37.38± 2.24, respectively) (Table 11). Oxyclozanide, niclosamide and drug combination caused significant (p<0.05) improvement in serum glucose values (mg/dl) by the end of our trial (57.46± 2.73; 63.98± 3.26 and 69.47± 2.21, respectively) in comparison with the naturally infected non treated animals (52.98± 2.94) (Table 12). No statistical relevant differences in blood sodium values (mEq/L) remained within the same values between all groups (Table 13). No statistical relevant differences in blood potassium (mEq/L) remained within the same values between all groups (Table 14).

DISCUSSION

Paramphistomiasis is one of the most pathogenic diseases of domesticated animals, causing heavy losses to the livestock industry. It has been estimated that more than 500 million cattle worldwide are at risk due to parasitic infection (Juyal et al., 2003; Ilha et al., 2005). Clinical paramphistomiasis is usually diagnosed in cattle 4-18 months age as resistance developed after exposure to the parasite. This immunity protects the animal against the massive infections of immature fluke that causes such problems. Weaned cattle and lambs appear to be the most susceptible (Lloyd et al*.,* 2007).

It was confined to warmer tropical and subtropical areas of the world, and is associated with invasion of the duodenum and upper jejunum by large numbers of immature fluke (Rolfe and Boray, 1993; De Waal, 2010). Death due to immature *Paramphistomes* is very elevated and may reach to 80-90% in domesticated ruminants (Juyal et al*.,* 2003; Ilha et al*.,* 2005). *P. cervi* is considered to be one of the most important species of *Paramphistomes* since they are cattle parasites with a cosmopolitan distribution. The harm caused by the infection in bovine effects production, since these parameters provide a lower nutritious conversion, a loss of mass and a decrease in milk production, which causes economic losses (Ilha et al*.,* 2005).

Paramphistomiasis which is characterized by acute gastroenteritis occasionally occurred in cattle and buffaloes and rarely in sheep. Most infections of adult fluke are harmless although large numbers of fluke can cause a chronic ulcerative rumenitis with atrophy of rumenal papillae. Peak conical fluke numbers are usually seen in late summer or early winter following prolonged inundation of pasture (Smeal, 1995). Juvenile flukes attach to the intestinal mucosa. Catarrhal to necrotic and hemorrhagic duodenitis with less thickening may be seen in the early stages, progressing to be thickening (mucosal edema, submucosal hypertrophy), hemorrhages and ulceration. Anemia, hypoproteinemia (manifested as submandibular edema) and emaciation of the host ensue. After the juvenile fluke migrated to the rumen, the intestine repairs, leaving a thickened duodenum and jejunum as a result of diffuse mucosal and submucosal hypertrophy and fibrosis (Love and Hutchinson, 2003).

The hematological and biochemical findings revealed significant reduction in the total erythrocyte count, hemoglobin, packed cell volume, total protein, albumin, globulin and glucose. This could be attributed to the bloodsucking ability of parasites and hemorrhage that will lead to anemia as approximated to those reported by Gadre et al*.* (2008). Both sodium and potassium absorption occurred by distinct mechanisms in a major part of the intestine: in the jejunum, and potassium were mostly absorbed via co-transport, as a result of active uptake of sugars and amino acids; in the ileum, they were absorbed actively, against a significant electrochemical gradient. In the jejunum, sodium and potassium transports were greatly influenced by fluid movement and is stimulated by the presence of sugars; in the ileum, none of these

Table 7. Effect of orally administered of oxyclozanide (20 ml/100kg) or niclosamide (125 mg/kg) and both drugs on total protein (g/dl) of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	5.78±0.73^{bc}	5.78±0.15^{bc}	5.85±0.09^{bc}	5.88±0.26^{c}	5.91±0.34^{c}	5.96±0.18^{bc}
2nd group	5.88±0.42^{b}	5.91±0.24^{b}	5.94±0.24^{b}	5.95±0.18^{bc}	5.97±0.22^{bc}	5.99±0.24^{bc}
3rd group	5.80±0.23^{bc}	5.93±0.17^{b}	5.99±0.18^{b}	6.15±0.29^{a}	6.15±0.16^{a}	6.35±0.11^{a}
Control	5.45±0.73^{b}	5.48±0.03^{b}	5.62±0.16^{b}	5.80±0.22^{bc}	5.85±0.12^{bc}	5.48±0.13^{b}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 8. Effect of orally administered of oxyclozanide (20 ml/100kg) or niclosamide (125 mg/kg) and both drugs on blood albumin values (gm/dl) of buffaloes naturally infected with *Paramphistomes* (n = 5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	3.00±0.65^{c}	3.03±0.15^{c}	3.04±0.09^{c}	3.06±0.14^{c}	3.04±0.14^{c}	3.05±0.05^{c}
2nd group	3.04±0.42^{c}	3.04±0.24^{c}	3.05±0.24^{c}	3.07±0.12^{c}	3.06±0.24^{c}	3.10±0.24^{b}
3rd group	3.00±0.23^{c}	3.09±0.25^{b}	3.12±0.29^{b}	3.21±0.16^{a}	3.20±0.15^{a}	3.25±0.12^{a}
Control	3.06±0.22^{c}	3.03±0.03^{c}	3.03±0.10^{c}	3.06±0.15^{c}	3.04±0.19^{c}	3.05±0.13^{c}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 9. Effect of orally administered of oxyclozanide (20 ml/100 kg) or niclosamide (125 mg/kg) and both drugs on blood globulin values (gm/dl) of buffaloes naturally infected with *Paramphistomes* (n = 5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	2.78±0.12^{cd}	2. 75±0.14^{d}	2.81±0.11^{cd}	2.82±0.10^{c}	2.87±0.15^{c}	2.91±0.12^{b}
2nd group	2.84±0.14^{c}	2.87±0.18^{c}	2.87±0.19^{c}	2.88±0.12^{c}	2.91±0.08^{bc}	2.89±0.06^{bc}
3rd group	2.80±0.13^{cd}	2.84±0.17^{c}	2.87±0.14^{c}	2.94±0.08^{b}	2.95±0.11^{b}	3.10±0.22^{a}
Control	2.81±0.20^{c}	2.85±0.15^{c}	2.85±0.15^{c}	2.84±0.22^{c}	2.86±0.25^{c}	2.86±0.14^{c}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 10. Effect of orally administered of oxyclozanide (20 ml/100 kg) or niclosamide (125 mg/kg) and both drugs on glutathione (µmol/g protein) of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	12.43± 2.31c	16.56± 3.25b	18.89± 2.44b	18.55± 1.53b	19.67± 2.47ab	18.86± 1.72b
2nd group	14.65± 3.43c	19.12± 2.64ab	19.21± 3.86ab	20.24± 3.55a	23.43± 2.68a	22.56± 3.12a
3rd group	11.43± 1.32c	16.62± 2.74b	17.42± 2.86b	18.67± 1.65b	19.43± 2.28ab	20.74± 1.39a
Control	13.76± 1.43c	13.87± 1.22c	12.45± 2.54c	13.23± 1.46c	13.37± 1.63c	14.73± 2.34c

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

factors affect sodium and potassium movement. They were also actively absorbed in the colon (Church, 1993). These facts could explain the non-statistically relevant differences in both blood sodium and potassium values

Table 11. Effect of orally administered of oxyclozanide (20 ml/100 kg) or niclosamide (125 mg/kg) and both drugs on malondialdehyde level (nmol /g protein) of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	54.88±3.42^{b}	62.88±2.33^{a}	61.88±2.52^{a}	57.88±1.67^{ab}	52.88±2.46^{b}	52.88±2.35^{b}
2nd group	51.27±3.46^{b}	40.27±3.57^{c}	37.27±2.35^{d}	36.27±3.52^{d}	34.27±1.52^{d}	34.86±3.47^{d}
3rd group	54.38±2.34^{b}	49.38±3.46^{bc}	46.38± 2.46^{c}	45.38±1.58^{c}	42.38±3.63^{c}	37.38±2.24^{c}
Control	54.67±1.52^{b}	58.98±2.42^{a}	55.76±1.76^{b}	59.87±1.73^{a}	57.98±2.41^{ab}	57.86±2.54^{ab}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 12. Effect of orally administered of oxyclozanide (20 ml/100 kg) or niclosamide (125 mg/kg) and both drugs on serum glucose (mg/dl) of buffaloes naturally infected with *Paramphistomes* (n = 5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	53.13±2.43^{c}	52.34±2.55^{c}	49.62±2.23^{c}	56.58±2.47^{b}	58.97±2.33^{b}	57.46±2.73^{b}
2nd group	54.26±3.45^{c}	60.74±3.53^{ab}	61.63±3.72^{ab}	62.53±3.57^{ab}	64.16±3.73^{ab}	63.98±3.26^{ab}
3rd group	54.76±2.42^{c}	62.45±2.57^{ab}	63.30±2.32^{a}	62.52±2.64^{ab}	65.26±2.73^{a}	69.47±2.21^{a}
Control	54.57±3.52^{c}	54.08±3.43^{c}	54.67±3.35^{c}	55.39±3.46^{b}	57.94±3.53^{b}	52.98±2.94^{c}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 13. Effect of orally administered of oxyclozanide (20 ml/100kg) or niclosamide (125 mg/kg) and both drugs on blood sodium values (mEq/L) of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	128.25±2.45^{ab}	129.40±6.12^{ab}	133.76±4.13^{a}	135.46±4.56^{a}	136.47±4.55^{a}	137±4.22^{a}
2nd group	129.45±4.16^{ab}	134.56±2.45^{a}	136.22±5.13^{a}	135.13±5.10^{a}	138.12±7.35^{a}	136.16±6.43^{a}
3rd group	130.16±4.76^{ab}	133.24±7.42^{a}	135.44±3.12^{a}	135.76±3.45^{a}	134.25±6.12^{a}	135.22±3.36^{a}
Control	134.14±4.12^{a}	135±2.45^{a}	135.10±5.13^{a}	137.20±2.46^{a}	136.14±5.43^{a}	134.24±5.42^{a}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

Table 14. Effect of orally administered of oxyclozanide (20 ml/100 kg) or niclosamide (125 mg/kg) and both drugs on values of blood potassium (mEq/L) of buffaloes naturally infected with *Paramphistomes* (n=5).

Weeks / Groups	0 day	2nd week	4th week	6th week	8th week	10th week
1st group	4.04±0.7^{c}	4.20±0.14^{b}	4.18±0.14^{b}	4.43±0.21^{a}	4.52±0.15^{b}	4.44±0.14^{a}
2nd group	4.02±0.05^{c}	4.16±0.16^{b}	4.24±0.11^{b}	4.44±0.11^{a}	4.65±0.05^{b}	4.66±0.14^{a}
3rd group	4.00±0.16^{c}	4.22±0.14^{b}	4.28±0.17^{b}	4.38±0.07^{ab}	4.35±0.18^{ab}	4.37±0.04^{ab}
Control	4.05±0.12^{c}	4.10±0.11^{bc}	4.20±0.04^{b}	4.45±0.07^{a}	4.42±0.13^{a}	4.46±0.21^{a}

Values are expressed as Mean ± SE. The means which carry different letters in the same column were significantly different, ($p < 0.05$).

that remained within the same values between all groups in our trial.

The high intracellular content of glutathione (GSH) in the liver is congruous with the detoxification functions of

this organ. Regular dietary intake of precursor sulfur containing amino acids will maintain hepatic intracellular GSH levels in the 5–10 mM range. Alterations in liver GSH are either the cause or the effect of a number of pathologies (Townsend et al*.,* 2003). So, absorption impairment by intestinal diseases (paramphistomiasis) to sulfur containing amino acids (like: cyst(e)ine) could decrease liver production of glutathione that approximated in our findings.

Furthermore, intestinal affection could decrease the activity of key enzymes (gamma-glutamylcysteine synthetase and gamma-glutamyl transferase) involved in GSH synthesis accompanied by a decreased availability of cyst(e)ine for GSH synthesis contribute to mucosal GSH deficiency in IBD. As the impaired mucosal antioxidative capacity may further promote oxidative damage, promoted reactive oxygen species contribute to tissue injury through polyunsaturated fats can readily undergo peroxidation to yield lipid hydroperoxides that are potentially toxic to the intestine and other tissues when absorbed from the lumen (Sido et al*.,* 1998; Serdar et al*.,* 2008).

That could explain the decreased body weight gain in naturally infected non treated buffaloes beside the previously mentioned effect of the parasite on the absorption of nutrient from the intestine.

During the tissue invasion by immature stages of *Paramphistomum* spp., it is exposed to elevated amounts of exogenous reactive oxygen species (ROS), such as superoxide radical anions (O_2^-) and hydrogen peroxide (H_2O_2). Additionally, the parasite can stimulate the activation of the host's immune response, which results in production of the cytokines TNF-α and IFN- α, and these increase respiratory burst (and ROS) on phagocytes. These highly toxic molecules cause severe damage to biological macromolecules (such as lipids, proteins and DNA) leading to metabolic malfunctions. Lipid peroxidation reflects the interaction between ROS and polyunsaturated fatty acids, and induces oxidation of various breakdown products of the latter. Among these, malondialdehyde (MDA) is a reliable marker of oxidative damage (Sido et al*.,* 1998; Serdar et al*.,* 2008).

As a result, lipid peroxidation causes changes in membrane permeability and selectivity and ultimately leads to alterations in cell volume homeostasis and cellular metabolism. Moreover, hydroperoxides and aldehydes are directly toxic to cells and organelles (Namıduru et al., 2011). So, the obtained data suggested that reactive oxygen metabolites mediate injury is important in both primary and downstream secondary pathophysiological mechanisms underlying intestinal inflammation. At the same time, these parameters (glutathione and malondialdehyde) could be of importance to be used as a supplement the conventional microscopic method for reliable diagnostic method of intestinal parasitism especially in case of the parasite not revealed by examination of a single fecal sample. Periodic medication will not only help in the prevention of outbreaks of acute paramphistomiasis but also in preventing fecal contamination of environments by reducing egg output, thus interrupting the life cycle of the parasite. In view of this, our trial was begun with evaluation of the combination of oxyclozanide and niclosamide.

Oxyclozanide [2,3,5-trichloro-N-(3,5-dichloro-2-hydroxyphenyl)-6-hydroxybenzamide] was a salicylanilide anthelmintic that mainly acts by uncoupling oxidative phosphorylation in flukes (Jo et al*.,* 2011). Early investigations were carried on oxyclozanide in mice. They were infected by gavage with one or two metacercariae treated over different periods covering the whole of the immature phase of migration of *Fasciola hepatica* and then killed either 21 or 31 days after infection. Only the examined drug was generally ineffective over -1 to six days after infection, and displayed varying activity over the other periods. Flukes recovered from the treated animals were retarded in size and there were corresponding reductions in liver pathology. Oxyclozanide had little effect on 21-day-old flukes at 0.1% in the diet but had 100% efficacy at concentrations of 0.2 and 0.5%. The 0.5% level was 88% effective at 7 and 14-day-old infections (Probert et al*.,* 1981). Oxyclozanide was effective against adult liver flukes (*F. hepatica*), but only partially against later immature stages (Coles and Stafford, 2001). After oral administration, oxyclozanide was found mainly in liver, kidneys and gut. It was slowly metabolized and excreted through the bile and the feces (Jo et al*.,* 2011).

Both oxyclozanide and niclosamide belong to salicylanilides. The molecular mode of action of salicylanilides is not completely elucidated. They all are uncouplers of the oxidative phosphorylation in the cell mitochondria; inhibit the coupling between the electron transport and phosphorylation reactions and thus inhibit ATP synthesis, the cellular "fuel." This impairs the parasite's motility and probably other processes as well. Niclosamide acts on the tapeworms also through inhibition of glucose absorption (Terada, 1990 ; Mehlhorn, 2008).

The chemotherapeutic value against rumen flukes (*Paramphistomum* spp) could be limited and also evident in this study, the significant continuation of eggs shedding and partial low drug efficacy (%) all over the experimental period could be attributed to the survival of immature stages, and so they are capable of being mature as a second wave of infection. Another possible mechanism of survival suggested that the drug could cause incomplete atrophy to the generative tissues (gonads) and this depend upon the physiological status of the parasite (decreasing the energy caused by oxyclozanide) as mentioned before by Stammers (1975). Another data recorded by Rolfe and Boray (1987) mentioned that oxyclozanide at 18.7 mg/kg reduced parasite (*Calicophoron calicophorum*) numbers in the small intestine, abomasum and rumen-reticulum by 61 to 96.1%, 50.0 to 92.6% and 56.5 to 98.1%, respectively. When 2 doses were given 3 days apart, oxyclozanide was 99.9%, 100 and 100% effective, correspondingly, in the above organs, and produced

improvement in clinically affected calves. This disagreement with our mentioned data could be attributed to species of examining parasites.

Furthermore, the limited efficacy of oxyclozanide was approximated by the significant reduction in red blood cell count, hemoglobin, packed cell volume, total protein, blood albumin, blood globulin, glutathione, malondialdehyde, and serum glucose which began from the 6th week of the experiment to the end of the trial. Finally, these findings reflected on the body weight gain which significantly decreased when compared with the drug combination group of buffaloes naturally infected with *Paramphistomes*.

Niclosamide was poorly absorbed in the gut and was excreted through the feces almost completely as the unchanged parent compound (Saeb-Parsy et al*.,* 1999). Niclosamide was effective for immature tapeworms (for example, *Taenia*, *Moniezia* spp), and against several blood flukes (*Schistosoma* spp.). It was originally introduced as a molluscicide, that is, a snail killer, to control those snails that transmit schistosomiasis (Mehlhorn, 2001). The earlier study was conducted by Rolfe and Boray (1987). They controlled tests to assess the efficacy of anthelmintics against immature *Paramphistomes*, predominantly *Calicophoron calicophorum*, in 127 calves, which were exposed to contaminated pasture for seven weeks, treated and slaughtered. Niclosamide efficacy at 160 mg/kg given as single or two doses three days apart were 91.1 and 92.6% effective, respectively, against the parasites in the small intestine (the immature stages of *Paramphistomum* spp.).

Based on fecal egg count data, the continuation and nearly constant rate of egg production clearly demonstrated that niclosamide could effect on the immature stages in the intestine rather than the mature worms that maintain egg production. As niclosamide is poorly absorbed in the gut, so the drug was difficultly accumulated around the developed worms to be ingested by the parasite and could affect it like soluble form or the drug is more rapidly absorbed when worms are contacted in the earlier times of their lives (Kumchoo et al*.,* 2007). Rangel-Ruiz et al*.* (2003) mentioned that adult *Paramphistomes* attached to the villi in the rumens of definitive ruminant hosts and feed on nutrients from the rumens, omasums and abomasums, causing weight loss and a decrease in milk production although they can wander into the bile and pancreatic ducts, as do other trematodes.

Depending on these data, our trial clearly demonstrated that the limited efficacy of niclosamide effect on immature stages of *Paramphistomum spp*., was approximated by the relevant reduction in red blood cell count, hemoglobin, packed cell volume, total protein, blood albumin, blood globulin, glutathione, malondialdehyde, and serum glucose which began from the 6th week of the experiment to the end of the trial. Significant continuation of egg shedding and sharp low drug efficacy (%) all over the experimental period could be attributed to the survival of mature stages, and so they are capable of egg production and affecting animal health as well. Finally, these findings reflected in the body weight gained that significantly decreased when compared with the drug combination group of buffaloes naturally infected with *Paramphistomes*.

Oxyclozanide and niclosamide combination in the recommended dosage regimen is considered of choice for the treatment of *Paramphistomum* infection in buffaloes. In the present trial, the obtained data showed that, this combination significantly reduced number of egg count and improved drug efficacy (%), hematological and biochemical profiles. Besides, this combination can overcome the oxidative stress due to the infection by improving GSH concentration that gradually increased and lowering the level of malondialdehyde level in serum at the end of the trial. Finally, we could concluded that, treatment of infected animals with the tested drugs (oxyclozanide and niclosamide) resulted in amelioration of the most adverse effects associated with this infection, decreased egg count and oxidative stress, improved biochemical and hematological profiles.

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Full Length Research Paper

Acute modulation of rat plasma glucose byan aqueous garlic extract

Meherzia Mokni[1], Sonia Hamlaoui[1]*, Ferid Limam[2], Mohamed Amri[1] and Ezzedine Aouani[2]

[1]Laboratoire de Neurophysiologie Fonctionnelle et Pathologies, Département des Sciences Biologiques, Faculté des Sciences de Tunis. Campus Universitaire El Manar II-2092 Tunis, Tunisie.
[2]Laboratoire des Substances Bioactives, Centre de Biotechnologie, Technopole Borj-Cedria, BP-901, 2050 Hammam-Lif, Tunis, Tunisie.

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In this study, the putative antidiabetic effect of garlic was re-investigated. Aqueous crude garlic solution was prepared at high concentration (2 g/ml) and extracts were obtained by ethanol precipitation followed by chromatography on C18 Sep-Pak cartridge. Garlic or extracts were administered by single intraperitoneal injection to euglycaemic rats. Plasma glucose, insulin and nitric oxide (NO) were determined after 30 min, 1 and 2 h, respectively. Garlic induced hypoglycemia and hyperinsulinemia which is mimicked by an ethanol soluble and non polar extract. This active principle appeared different from S-allyl-cystein sulfoxide based on physico-chemical properties and mode of action. Data of thin layer chromatography experiments indicated the presence of at least four molecular species, indicating a more non polar nature, with Rf values higher than S-allyl-cystein sulfoxide. The mechanism of action seemed to involve nitric oxide as its glucose induced lowering activity is abolished by diphenyleneiodonium which is a selective constitutive nitric oxide synthase inhibitor.

Key words: Garlic, Plasma Glucose, Insulinemia, Nitric Oxide, Thin Layer Chromatography.

INTRODUCTION

Garlic (*Allium sativum* L.), an indigenous dietary component, belongs to the Liliaceae family and is widely used as a condiment. Besides, it is also used widely in home remedies and pharmacotherapy against debilitated pathologies because of its antioxidant (Lieben et al., 2012), anticardiovascular (Ginter and Simko, 2010), and antihyperglycemic (Kumar et al., 2013) activities. The antidiabetic effect of garlic is still controversial. Although some investigators (Swanston-Flatt et al., 1990; Baluchnejadmojarad et al., 2003) were unable to detect any glucose lowering activity in garlic preparations, some others described plasma glucose lowering activity and insulin secretagogue effect on a sulfur derived amino acid identified as S-allyl-cysteine-sulfoxide (SACS) (Bordia et al., 1977; Sheela and Augusti, 1992; Kook et al., 2009). Moreover this insulin secreting activity was only demonstrated *in vitro*, using isolated cells from normal rat pancreas (Augusti and Sheela, 1996).

Although the mode of garlic's action or its derivatives is still uncertain, nitric oxide (NO) was suggested as a putative mediator (Mokni et al., 2006; Lieben et al., 2012) especially in antihypertensive effects (Pedraza-Chaverri et al., 1998). NO is synthesized from L-arginine by NO synthase (NOS) which exist in three isoforms: neuronal, endothelial constitutive and inducible form (Kerwin et al., 1995). NO, derived from constitutive NOS, is reported to modulate vasomotor tone, inhibition of platelet or leukocyte aggregation and adhesion to the endothelium

*Corresponding author. E-mail: sonia_hamlaoui@yahoo.fr. Tel: 216 98 968 113. Fax: 216 71 885 480.

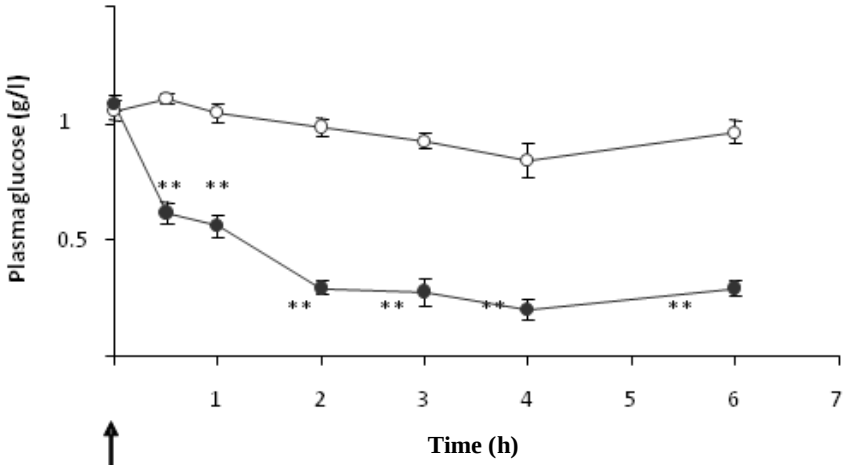

Figure 1. Effect of garlic on glycaemia (time course study). Rats were IP injected with aqueous crude garlic extract (80 mg / kg bw ●) or vehicle (○) and glycaemia determined at different times. Results are expressed by mean ± SEM (n = 10). **p < 0.01 vs control. The arrow indicates start of injection.

that suggests its anti-atherogenic properties (Moncada et al., 1991). In fact, a selective constitutive NOS (cNOS) inhibitor overcame the effect of aged garlic extract (AGE) (Morihara et al., 2006).

This research was aimed at studying the putative glucose lowering effect of aqueous extract of garlic on euglycaemic rats. In addition, attempts were made to identify the active component as well as its mechanism of action. We described a newly reported active principle, with a rapid onset of action and different from SACS based on physico-chemical properties and mode of action.

MATERIALS AND METHODS

Plant material and extraction

The raw garlic (*A. sativum* L.) cloves, purchased from local market, were peeled, weighted and blended with an electric mincer. The extraction was done using bi-distilled water at ambient temperature. The blended raw garlic was then dissolved in bi-distilled water at a concentration of 2 g/ml on the basis of the weight of the starting fresh material and centrifuged at 10,000 g for 15 min at 4°C (Beckman J20). Supernatant was sonicated with an ultraschall processor (UP 400S) and centrifuged again. Clear supernatant was then aliquoted and stored at -80°C until use. Aqueous solution (G) was subject to the extraction with ethanol as follows: briefly one volume of aqueous garlic was precipitated twice with seven volumes of ethanol and centrifuged at 10,000 g for 15 min at 4°C. Supernatant was dried using a rotavapor, dissolved in double distilled water and referred as ethanol-soluble extract (AS). After washing with ethanol/water (7v/1v) and drying, pellet was dissolved in double distilled water and referred as ethanol-insoluble extract (AP). AS was further subjected to chromatography on Sep-Pak C18 reverse phase cartridge. After extensive washing first with ethanol then with double distilled water loading of the cartridge with ethanol-soluble, extract gave two fractions: a polar fraction (Phile) eluted with double distilled water and a non polar fraction (Phobe) eluted with 10% ethanol.

Thin layer chromatography (TLC) analysis

30 µl corresponding to 1 mg dry product of Phobe extract was subject to TLC on silicagel plates 60 F_{254} (Merck, Germany) using butanol/acetic acid/water (12/3/5). Pure SACS (0.5 mg, Fluka, France) was run as control.

Animals and treatment

Male and female Wistar rats (Pasteur's institute, Tunis, Tunisia) weighting 180 to 220 g (6 to 7 weeks old) were maintained under standard laboratory conditions at 22 ± 2°C, on a light/dark cycle (12 h) supplied with standard pellet diet and tap water *ad libitum*. Procedures involving laboratory animals and their care were conducted in conformity with institutional guidelines of Tunis University and in accordance with the NIH guidelines. To determine the effects of aqueous extract of crude garlic on glycaemia, animals were divided in two groups: Group I was kept as control and received vehicle (H_2O) and Group II received aqueous crude garlic (G) (Figure 1). Each group contained 10 rats. To test the effect of the partially purified fraction of garlic, each group of rats received only one fraction. Rats were divided into 6 groups of 8 rats each (group I was kept as control, group II received aqueous crude garlic, group III received AP fraction, group IV received AS fraction, group V received Phobe fraction and group VI received Phile fraction) (Figure 2). Garlic or extracts were acutely administered by a single intraperitoneal injection (IP) at time = 0. Diphenyleneiodonium chloride (DPI, Fluka Aldrich, France) at 1 mg/kg body weight was dissolved in double-distilled water and IP injected 2 h prior to garlic or extract injection. Experimental duration never exceeded 3 h after which rats were anesthetized with urethane, sacrificed by decapitation and plasma used for glucose, insulin and NO determinations.

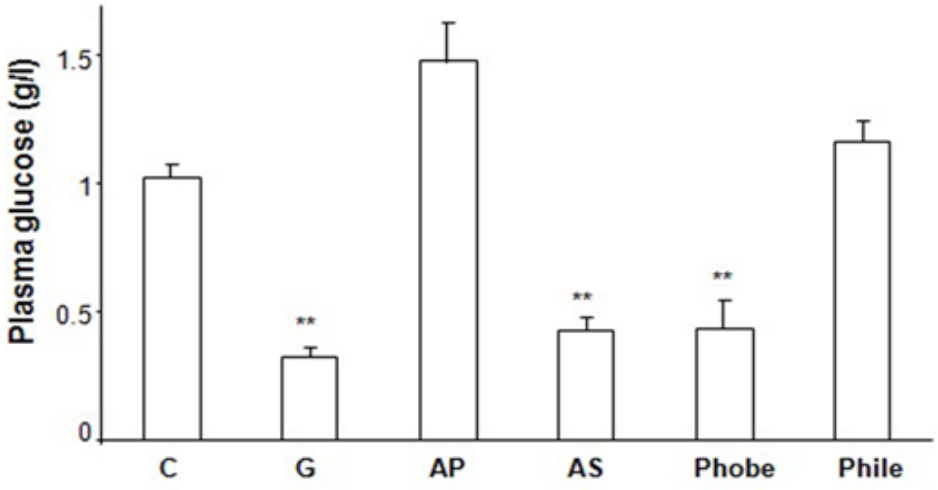

Figure 2. Effects of partially purified extracts from garlic on plasma glucose levels. C: control; G: aqueous crude garlic (80 mg / kg bw); AP: ethanol-insoluble extract; AS: ethanol-soluble extract; Phobe: non polar extract; Phile: polar extract. Extracts were IP administered to rats and glucose levels determined after 3 h of incubation. Results are expressed by mean ± SEM (n=8). **, $p < 0.01$ vs control.

Measurement of plasma glucose, insulin and NO levels

Glucose levels and plasma insulin were determined enzymatically using commercially available glucose oxidase (Sigma, France) and RIA kit (Immunotech, France), respectively. Plasma NO was measured by quantification of the NO metabolites nitrite and nitrate. These later were determined colorimetrically using a commercial kit (Roche diagnostics, France) according to Green et al. (1982).

Statistical analysis

Results are expressed by mean ± standard error of mean (SEM). Data were analyzed by unpaired Student's t-tests and expressed as means ± SEM, and $p < 0.05$ was considered significant.

RESULTS

Figure 1 shows the time related effects of aqueous extract of crude garlic on glycaemia. Data showed that garlic drastically induced hypoglycaemia from the first hour till several hours (6 h). The acute effects of crude garlic or partially purified extracts on plasma glucose levels were tested (Figure 2). All extracts were intraperitoneally injected (IP) at time 0 and glucose levels determined after 3 h. As expected, garlic exerted a glucose lowering effect, which is mimicked by the ethanol-soluble (AS) and the non polar extract (Phobe) but not by the ethanol-insoluble (AP) or the polar extract (Phile). Figure 3 showed that garlic as well as Phobe extract exerted their glucose lowering effect by increasing insulinemia (7-fold over control). Phobe extract was further subject to TLC on silicagel plates. Data from Figure 4 showed the presence of at least 4 spots in Phobe extract. However, none of them corresponded to SACS as assessed by Rf values.

The ability of Phobe extract to modulate plasma NO levels was also tested. Figure 5 showed the effect of Phobe extract either alone or in the presence of the specific constitutive NOS inhibitor DPI on plasma glucose (Figure 5A) and NO (Figure 5A) levels. Data clearly showed that Phobe extract lowered plasma glucose and simultaneously increased NO levels. It was clear that these effects are abolished by DPI.

DISCUSSION

The present work deals with a re-evaluation of the putative antidiabetic effect of garlic. We confirm that aqueous extracts exerts real glucose lowering effect *in vivo* (Sher et al., 2012), which is preceded by an increase in insulinemia (Sheela and Augusti, 1992). Some previous studies failed to show any antidiabetic effect probably because of the unappropriate use of streptozotocin-induced diabetic animals which no longer respond to any agonist (Baluchnejadmojarad et al., 2003). In this respect, it is generally recognized that an antidiabetic agent could exert a beneficial effect in the diabetic situation by enhancing insulin secretion and/or by mimicking insulin action (Gray and Flatt, 1999; Eidi et al., 2006). This lacking effect can also be the result of the use of too much low concentration of garlic, unable to elicit any detectable effect *in vivo*. In fact, neither garlic oil (100 mg/kg bw) nor DADS (40 or 80 mg/kg bw) significantly affected fasting blood glucose concentrations throughout the investigation period (Liu et al., 2006).

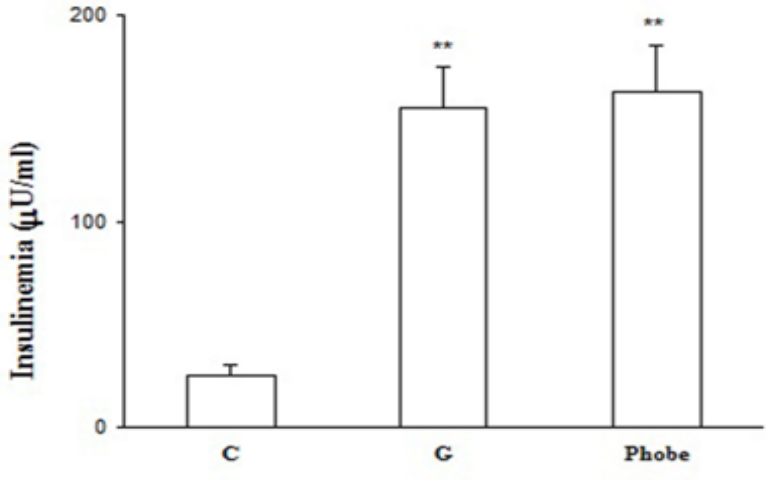

Figure 3. Effect of Phobe extract on insulinemia. Rats were IP injected with vehicle (10 % ethanol) or garlic (80 mg / kg bw) or Phobe extract (equivalent to garlic dose) and insulinemia determined by RIA after 1 h of incubation. Results are expressed by mean ± SEM (n = 8). **p < 0.01 vs control.

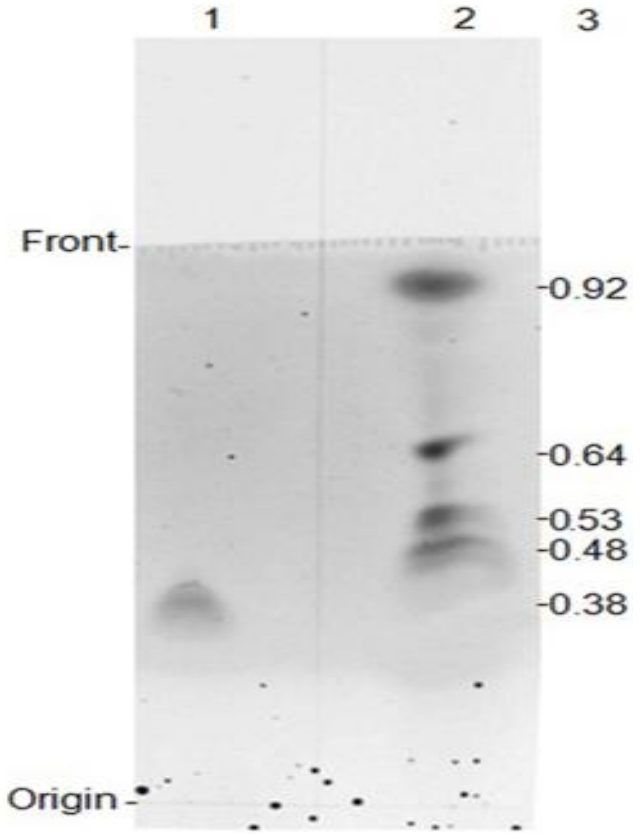

Figure 4. TLC analysis of Phobe extract. Phobe extract (line 2: 1 mg) or pure SACS (line 1: 0.5 mg) were subjected to reverse phase C18 silica gel plates using butanol/acetic acid/water (12/3/5). Line 3 indicate Rf values for pure SACS and 4 different spots of Phobe extract. Staining was performed with iodine.

In our hand garlic exerted dose related effects only at high concentrations. Indeed, on the basis of the weight of the starting material, our garlic preparation is approximately 1000 mg/kg/day which corresponds to 70 to 100 g crude garlic per day for a 70 kg adult, which is not safe (Alnaqeeb et al., 1996). These doses, which are much higher than previously reported in chronic (Ali and Thomson, 1995) or in acute experiments (Pantoja et al., 2000), outline the difficulty of comparing the two kinds of experiments in term of doses. In this respect, it is also well known that garlic activity depends closely on its mode of extraction or processing (Staba et al., 2001), doses (Banerjee et al., 2001) and ways of administration (Alnaqeeb et al., 1996; Sundaram and Milner, 1996). Our data rather support that garlic can no longer be used as a nutritional supplement (Ali and Thomson, 1995) but as a source of bioactive components and of potential new antidiabetic agents as yet to be isolated and identified (Saravanan and Ponmurugan, 2012).

Based on TLC experiments, SACS was identified as the major sulphur amino acid from aqueous extract of garlic implicated in insulin secretagogue effect (Augusti and Sheela, 1996). When submitted to TLC in the same conditions, Phobe extract exhibits at least 4 molecular species with Rf values higher than SACS, indicating a more hydrophobic nature (Rabinkov et al., 1998).

Phobe extract mode of action involved NO increase as found in kinetic as well as dose response experiments (data not shown). From pharmacological experiments on which we use selective constitutive NOS inhibitor as DPI, Phobe extract no longer induced glucose lowering and NO increasing activity. To our knowledge, our report is the first one that links garlic induced glucose lowering

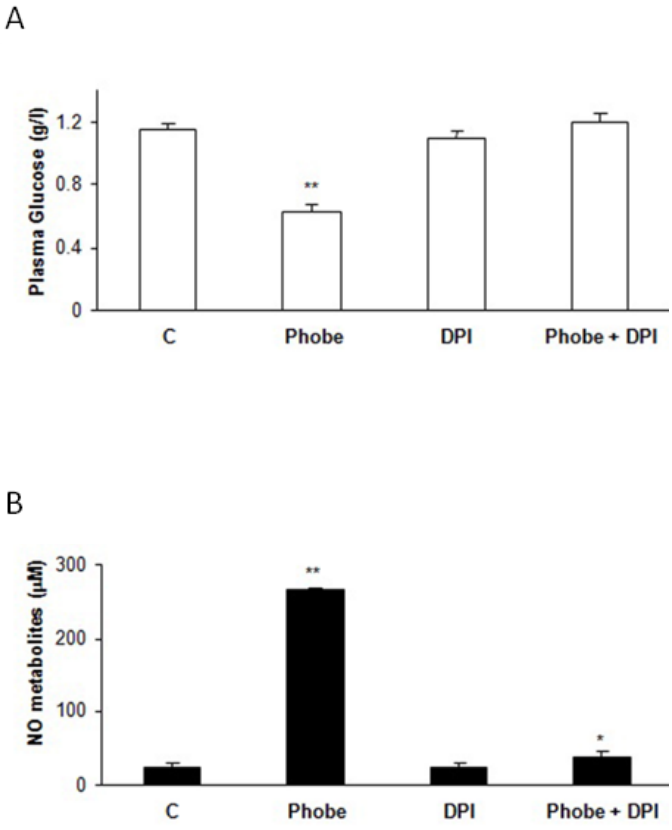

Figure 5. Effect of DPI on Phobe extract induced glucose (A) and NO (B) levels. Rats were pre-treated with DPI (1 mg / kg bw) during 2 h and IP injected with vehicle or Phobe extract. Plasma glucose levels were determined after 2 h (Figure 5A) and plasma NO after 30 min (Figure 5B). Results are expressed by mean ± SEM (n=8). *$p < 0.05$ vs control.

activity with NOS activation in euglycaemic rats. Our data also support that Phobe extract could not be alliin-derived products which have been previously shown to act by NO independent way (Morihara et al., 2002; Das et al., 1996). Further experiments using diabetic animals are underway to assess:

(i) The effectiveness of such new activity; (ii) the exact molecular nature of this active principle which might be a saponin (Matsuura, 2001); (iii) and the implication of constitutive NOS in glucose lowering and insulin secreting activity. Indeed NOS inhibition has been shown to reduce glucose uptake during exercise in individuals with type II diabetes more than in control subjects (Kingwell et al., 2002). In conclusion we described a new and not yet identified glucose lowering and insulin secreting activity from garlic exhibiting a rapid onset of action *in vivo*.

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Full Length Research Paper

Anxiolytic- and antidepressant-like effects of the ethanolic extract from *Citrus limon* plant widely used in Northeastern Brazil

Francisco Rodrigo de Asevedo Mendes de Oliveira[1], Gilberto Santos Cerqueira[2], Rizângela Lyne Mendes de Freitas[2], Joaquim Soares Costa Júnior[3], Chistiane Mendes Feitosa[4] and Rivelilson Mendes de Freitas[1]*

[1]Post-Graduation Program in Pharmaceutics Science, Federal University of Piaui, Teresina, Piaui, Brazil.
[2]Federal University of Ceará, Fortaleza, Ceará, Brazil.
[3]Department of Chemistry, Federal Institute of Piaui, Teresina, Piaui, Brazil.
[4]Department of Chemistry, Federal University of Piaui, Teresina, Piauí, Brazil.

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Anxiolytic and antidepressant effects and acute toxicity of ethanolic extract (EE) from *Citrus limon* were studied in mice. Anxiolytic activity was evaluated using open field and elevated plus-maze tests. The antidepressant effect of the extract was studied by forced swimming test in mice. Phytochemical screening of the ethanolic extracts indicated the presence of cumarin and triterpenoids/steriods. In the open field test, the oral route administration EE alone showed significant sedative and antidepressant activities in mice ($p < 0.05$). EE did not alter motor coordination. The EE, at three doses tested, showed antidepressant effect and produced decrease in immobility time. It is concluded that the EE of the aerial parts of *C. limon* have a sedative effect, which may be mediated by benzodiazepine-type receptors, and also an antidepressant effect where noradrenergic and serotoninergic mechanisms will probably play a role.

Key words: Antidepressant, anxiolytic, *Citrus limon*, ethanolic extract, sedative.

INTRODUCTION

Depression and anxiety are the most frequent mental disorders. More than 20% of the adult population suffers from these conditions (Buller and Legrand, 2001). The World Health Organization (WHO, 1999) predicts that depression will become the second leading cause of premature death or disability worldwide by the year 2020. The genus *Citrus* possesses about 70 species of subshrubs and shrubs that can be grown or naturally found in Germany, Spain, Mexico, Venezuela, Cuba, Jamaica, Ecuador and Northern and Northeastern Brazil (Lorenzi and Matos, 2002). The *Citrus* are also known to exhibit various biological activities such as antioxidant (Campêlo et al., 2011a, b; Misharina and Samusenko, 2008), antimicrobial (Choi et al., 2000), anti-inflammatory (Benavente-Garcia and Castillo, 2008), insecticide (Karr and Coats, 1988) and inhibition of acetylcholinesterase (AChE) enzyme (Conforti et al., 2007).

Citrus fruits are known to contain natural antioxidants in its oil, pulp, seed and bark. According to Pereira (Pereira, 1996), the methanol extracts of seeds of lemons has

*Corresponding author. E-mail: rivelilson@pq.cnpq.br. Tel: +55-86-3215-5870.

antioxidant activity. Studies show that ethyl acetate and methanol extract from *Citrus limon* leaves have anticholinesterasic activity as seen with the standard galanthamine, which is considered to be the most effective compound in the treatment of Alzheimer's disease (Feitosa et al., 2011). Citrus peel and seed represent natural sources of phenolic compounds, including phenolic acids and flavonoids (Bocco et al., 1998).

C. limon Burms (Rutaceae), popularly known as "limão", has as the major constituents essential oils limonene (52.77%), geranyl acetate (9.92%) and trans-limonene-oxide (7.13%) (Campêlo et al., 2011b). Other studies also showed that *C. limon* essential oil presents larvicidal activity against *Aedes aegypti* L. (Furtado et al., 2005). Studies showed the insecticidal properties of d-limonene major constituents of *C. limon* essential (Karr and Coats, 1988).

Previously, we showed in our laboratory that essential oil of *C. limon* leaves reduces the lipid peroxidation and nitrite content as well as increase the glutathione reduced (GSH) levels and enzymatic antioxidant activities (superoxide dismutase, catalase and glutathione peroxidase) in mice hippocampus. These findings strongly support the hypothesis that oxidative stress in hippocampus might occur during neurodegenerative diseases, proving that hippocampal damage induced by the oxidative process plays a crucial role in brain disorders, which implies that a neuroprotective effect could be achieved using *C. limon* essential oil as antioxidant treatment (Campêlo et al., 2011b). Other studies suggest a possible depressant action of *C. limon* essential oil on central nervous system (CNS) and anticonvulsant activity in seizures model induced by pentylenetetrazole (Campêlo et al., 2011a).

The purpose of the present work was to analyze the effects produced by the acute administration of the ethanolic extract from *C. limon* using the open field, elevated-plus-maze, rota rod, and forced swimming tests in order to evaluate the anxiolytic and antidepressant activities of this medicinal plant, attempting to clarify their mechanism of action.

MATERIALS AND METHODS

Plant

C. limon was identified and collected by Chistiane Mendes Feitosa in February, 2010, at the city of Picos, state of Piaui, Brazil. The voucher specimen (number 26.453) was deposited at the Graziella Barroso Herbarium of the Federal University of Piauí. Ethanolic extract of *C. limon* leaves was prepared in Laboratory of Chemistry of Natural Products at Federal University of Piauí, Teresina, Piauí.

Ethanolic extract preparation

The leaves collected from the *C. limon* were dried at 55°C under shade and powdered mechanically. 10 g of crushed leaves was extracted with water (70%, w/w), followed by ethanol (EtOH) (30%, w/w). The extract was concentrated in a vacuum evaporator. The concentrated extract was finally freeze-dried to get the yield of 10% of ethanolic extract. The dried extract was kept at 4°C in a refrigerator in the air tight bottles until use.

Drugs and reagents

The ethanolic extract (EE) was emulsified with 0.5% Cremophor (Sigma, USA) in distilled water. Diazepam (DZP) was purchased from the (União Química, Brazil) and used as standard. Reserpine sulphate (RESERP) was purchased from the Sigma Chem. Co. (St. Louis, MO, USA). Imipramine (IMI) and Paroxetine (PAROX) were purchased from the Novartis Biociências S.A. (São Paulo, Brazil) and Glaxo Smith Kline Brasil Ltda (Rio de Janeiro, Brazil), respectively. All other drugs were of analytical grade.

Animals and behavioral tests

Male Swiss mice (25 to 30 g) were used. All animals were maintained at a controlled temperature (25 ± 1°C) and a 12-h dark/light cycle. Animals had free access to water and food. All behavioral tests were conducted in quiet rooms at the same controlled conditions referred above. Different groups of mice were used for each behavioral task. For each experiment, mice were randomized into five groups (7 mice per group): one control group treated with the vehicle, three groups treated with EE at 50, 100 and 150 mg/kg, and a fifth drug reference group treated with imipramine (25 or 50 mg/kg; forced swimming test), paroxetine (10 or 20 mg/kg; forced swimming test), reserpine (0.25 mg/kg; forced swimming test) or DZP (0.75 or 2 mg/kg; open field, rota rod). Treatment with EE or vehicle was given orally (*p.o.*) via gastric gavage (10 ml/kg) for 30 days. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals, from the US Department of Health and Human Services, Washington DC, 1985. Experimental protocols and procedures were approved by the Ethics Committee on Animal Experiments at the Federal University of Piauí (CEEA/UFPI # 013/2011).

Experimental protocol and behavioral screening

After the treatment, each animal was submitted to a series of tests in the manner described below. Firstly, the animal was observed in a closed room at constant temperature (25 ± 1°C). Then, the animal was placed inside a plus maze and observed for 5 min. Immediately after the plus maze test, it was placed in the open field area for more 5 min. After that, the animal was removed to the rota rod where it was evaluated for 1 min. Finally, its temperature was taken with a digital thermometer. All the tests were performed between 08:00 a.m. and 10:00 p.m. The animals had free access to food and water during testing. The behavioral screening of the mice was performed according to the parameters described by Almeida et al. (1999). Animals were observed at 24 h after the EE *C. limon* administration (50, 100 and 150 mg/kg, *p.o.*). During 24 h we observed the occurrence of the following general signs of toxicity: piloerection, prostration, writhing, increased evacuation, grooming, discrete groups, dyspnoea, sedation, analgesia and palpebral ptosis.

Open field test

Mice were randomly allocated to the following groups: control (0.05% Tween 80, dissolved in 0.9% saline, *p.o.*), diazepam (2.0

mg/kg, *i.p.*) and ethanolic extract from *C. limon* (50, 100 and 150 mg/kg, *i.p.*), that received acute treatment acute with single dose before open field test. The open-field arena was made of acrylic (transparent walls and black floor, 30 × 30 × 15 cm), divided into nine squares of equal areas. The open field was used to evaluate the exploratory activity of the animal (Archer, 1973). The observed parameters were the number of squares crossed (with the four paws) and number of grooming and rearing, recorded for 5 min testing period.

Elevated plus maze test (EPM)

Mice were randomly allocated to the following groups: control (0.05% Tween 80, dissolved in 0.9% saline, *p.o.*), diazepam (0.75 mg/kg, *i.p.*) and ethanolic extract from *C. limon* (50, 100 and 150 mg/kg, *i.p.*), that received treatment acute with single dose before of open field test. This test has been widely validated to measure anxiety in rodents (Lister, 1987). The elevated plus maze (EPM; 30 × 6 × 6 cm, each arm) made of wood and consisting of two open and two closed arms across each other, respectively is placed 60 cm above the ground level. After treatment, the animal was placed at the center of the plus maze with its nose in the direction of one of the closed arms, and observed for 5 min, following the parameters: number of entries in the open and closed arms, and time of permanence in each of them. The time of permanence measures the time spent by the animal in the open and closed arms (Lister, 1987).

Forced swimming test

Mice were randomly allocated to the following groups: control (0.05% Tween 80, dissolved in 0.9% saline, *p.o.*), imipramine (25 and 50 mg/kg, *i.p.*), paroxetine (10 and 20 mg/kg, *i.p.*), reserpine (0.25 mg/kg, *i.p.*), and ethanolic extract from *C. limon* (50, 100 and 150 mg/kg, *i.p.*), that received treatment acute with single dose before behavioural test. For assessing antidepressant activities, we employed the method described by Porsolt et al. (1977a, b; 1978). The development of immobility when mice were placed inside an inescapable cylinder filled with water reflects the cessation of persistent escape-directed behavior. Briefly, mice were individually placed in a circular tank (46 cm tall × 20 cm in diameter) filled with tap water (25 ± 1°C) to a depth of 20 cm and left there for 5 min. During this period, the behavior of the animals was recorded by an observer. Mice were considered immobile when remained floating without struggling and making only slight movements necessary to maintain the head above the water.

Rota rod test

Mice were randomly allocated to the following groups: control (0.05% Tween 80, dissolved in 0.9% saline, *p.o.*), diazepam (0.75 mg/kg, *i.p.*) and ethanolic extract from *C. limon* (50, 100 and 150 mg/kg, *i.p.*), that received acute treatment with single dose before rota rod test. The mice were trained before the experiment to develop the ability to remain for 1 min on a 25 mm diameter rod rotating at 17 rpm. Two or three trials were usually enough for the animals to learn this task. For the rota rod test, for each animal, the number of falls (up to three falls) and the time of permanence on the bar for 1 min were recorded (Dunham and Miya, 1957).

Rectal temperature

Animals rectal temperatures were taken at the end of the tests with a digital thermometer.

Statistical analysis

Results were expressed as means ± SEM and analyzed with analysis of variance (ANOVA) and the *t*-Student-Neuman-Keuls as *post hoc* test ($p < 0.05$).

RESULTS

Phytochemical screening of the ethanolic extracts indicated the presence of cumarin and triterpenoids/steroids and the absence of alkaloids, flavonoids, tannins and saponins. The determination of the chemical constituents was carried out according to the previous methodology proposed (Matos, 2009; Barbosa, 2004). Ethanolic extract from *C. limon* at doses of 50, 100 and 150 mg/kg *p.o.* caused behavioral changes in animals during 30 days of treatment: decrease of spontaneous activity, palpebral ptosis, ataxia, analgesia, and sedation. Behavioral changes were more evident on the second day of treatments.

In the open field test (Table 1), EE (50 mg/kg, *p.o.*) decreased in 31% the number of rearings in comparison with the control. Similarly, EE 100 (100 mg/kg, *p.o.*) and EE 150 (150 mg/kg, *p.o.*) decreased the number of rearings (31 and 70%, respectively) and also led to grooming reduction. In EE 50 group there was decreased number of crossings by 33% when compared to control. In the same way, EE 100 and EE 150 decreased the number of crossings, as compared to control at the doses used in the present work (51 and 61%, respectively). Diazepam showed sedative effect at the dose used (2 mg/kg, *i.p.*).

Table 2 shows the effects of EE from *C. limon* in the plus maze test. Among the parameters used for evaluation of the anxiolytic activity, the control group presented the following data: number of entries in the open arms (NEOA); time of permanence in the open arms (TPOA). EE 50 mg/kg (*i.p.*) decreased NEOA ($p < 0.05$) and increased TPOA ($p < 0.05$). Differently, the highest dose of EE decreased NEOA, TPOA ($p < 0.01$). NEOA and TPOA were also increased by EE, 100 mg/kg *i.p.* when compared to control. Diazepam (0.75 mg/kg, *i.p.*) significantly increased all these parameters. An anxiolytic compound reduces the natural animals' aversion to the open arms and promotes the exploration thereof. On the other hand, the forced or voluntary passages of the animal into the open arms of the EPM are associated with hormonal and behavioral changes indicative of increased anxiety (Lister, 1987; Hogg, 1966).

The possible antidepressant effect of EE after oral administration was studied in the forced swimming test (Table 3). Under this condition, EE was used at higher doses (50, 100 and 150 mg/kg, *p.o.*), since at these three doses the antidepressant effect is not masked by the sedative and anxiolytic effects. The results showed that EE presents a significant antidepressant effect (50, 100

Table 1. Effects of EE from *C. limon* on the open field test in mice.

Groups	Number of squares crossed	Rearing	Grooming
Control	91.38±10.41	38.00±7.59	4.75±2.18
DZP 2	35.20±6.76[a]	18.20±5.26[a]	1.60±0.89[a]
EE 50	63.60±19.76[a,b]	25.40±2.40[a,b]	3.60±0.89[a,b]
EE 100	63.40±19.93[a,b]	18.60±5.77[a,c]	3.80±1.30[a,b]
EE 150	27.40±4.83[a,b,c,d]	14.80±2.38[a,b,c,d]	4.00±1.41[a,b,c,d]

Values are the mean ± S.E.M. for number of squares crossed, rearing and grooming of 7 mice (per group) used in the experiments. [a]p<0.01 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from control. [b]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from DZP. [c]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 50 group. [d]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 100 group.

Table 2. Effects of EE from *C. limon* on the plus maze test in mice.

Groups	NEOA	PEOA	TPOA	PTOA
Control	8.71±2.63	34.86±1.51	116.71±3.54	38.90±1.18
DZP 0.75	15.71±0.75	62.86±3.02	206.86±7.15	68.95±2.38
EE 50	6.60±1.52[a,b]	26.40±6.07[a,b]	152.00±25.64[a,b]	50.67±8.55[a,b]
EE 100	13.60±1.95[a,b,c]	54.40±7.80[a,b,c]	173.00±17.89[a,b,c]	57.67±5.96[a,b,c]
EE 150	4.40±1.34[a,b,c,d]	17.60±5.37[a,b,c,d]	83.60±4.16[a,b,c,d]	27.87±1.39[a,b,c,d]

Each values represents mean ± S.E.M. of NEOA, number of entries in the open arms; PEOA, percentage of entries in the open arms; TPOA, time of permanence in the open arms; PTOA, percentage of time in the open arms. Values are the mean ± S.E.M. for 7 mice (per group) used in the experiments. [a]p<0.01 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from control. [b]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from DZP. [c]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 50 group. [d]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 100 group.

and 150 mg/kg), as seen in decreasing of the time of immobility (28, 51 and 70%, respectively). The lowest dose (25 mg/kg) was devoid of any significant effect. The association of EE 150 mg/kg with imipramine (IMI) showed a decrease of 6 and 15% in the immobility time, as related to the groups treated with EE alone (150 mg/kg, $p < 0.01$) or IMI ($p < 0.05$) alone, respectively. In addition, the association of EE with paroxetine ($p < 0.01$) alter the effect observed with EE or paroxetine alone ($p < 0.05$), suggesting that the serotonergic system is involved in the antidepressant effect of EE. On the contrary, the EE activity was totally blocked by the previous administration of reserpine. These data suggest that the noradrenergic and serotoninergic system participates in the EE antidepressant action.

Table 4 shows the effects of EE from *C. limon* in the rota rod test, a method used for evaluating motor coordination and presence of any muscle relaxation effect. It revealed that there was no change in the number of falls after EE administration (50, 100 and 150 mg/kg, *p.o.*) when compared to controls (Table 4). Additionally, only EE at 150 mg/kg, *p.o.* decreased in 18% the time of permanence on the bar in relation to control. Diazepam (0.75 mg/kg, *i.p.*), like EE 50 and 100, was devoid of effect. The rectal temperature of the control group has been show in Table 5. The doses of 50, 100 and 150 mg/kg, *p.o.* decreased the rectal temperature, in a dose independent way.

DISCUSSION

In the present work, the central effects of the ethanolic extract (EE) of leaves from *C. limon* were evaluated. EE was firstly analyzed on the open-field test which gives a good indication of the animal's emotional state. The results showed that EE was able to significantly decrease not only the number of crossings, indicative of a possible sedative effect, but also grooming and rearing. The action mechanism of the sedative effect of this EE has not been elucidated yet. However, the reduction in the number of rearings and crossings in the open field test confirms the central activity of this EE, since it is known that rearing is a function of the excitability level of the central nervous

Table 3. Effects of EE from *C. limon* in mice in model of forced swimming.

Groups	Time of immobility (s)	Values of Immobility (%)
Vehicle	223.70±1.11	-
EE 50	161.80±10.91[a]	↓ 72
EE 100	109.60±19.92[a]	↓ 49
EE 150	67.60±4.01[a,b,c]	↓ 30
IMI 25	115.0±1.41[a]	↓ 51
IMI 50	75.00±1.67[a]	↓ 66
PAROX 10	82.67±2.66[a]	↓ 63
PAROX 20	133.0±8.02[a]	↓ 41
EE 100 + IMI 50	106.3±4.08	↑ 42
EE 150 + IMI 50	63.67±3.44[d,e]	↓ 15
EE 150 + PAROX 20	85.3±3.72[f]	↓ 64
RESERP 0.25	263.7±5.28[a]	↑ 18
RESERP 0.25 + EE 150	193.3±6.53[d]	↓ 27

Experiments performed as described in materials and methods. Extract ethanolic (EE) was administered orally. Values are the mean ± S.E.M. of time of immobility for 7 mice (per group) used in the experiments. [a]p<0.01 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from control. [b]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 50 group. [c]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 100 group. [d]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 150 group. [e]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from IMI 50 group. [f]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from PAROX 20 group. EE = ethanolic extract; IMI = imipramine; PAROX = paroxetine; RESERP = reserpine. Drugs were administered 10 min before EE and the test performed 30 min later.

Table 4. Effects of EE from *C. limon* in the rota rod test in mice.

Groups	Number of falls	Time of permanence (s)
Control	1.71±0.75	53.71±1.11
DZP 0.75	2.50±1.22	52.40±2.30
EE 50	1.80±0.34	55.80±3.11
EE 100	1.72±0.44	54.00±1.58
EE 150	1.69±0.84	44.00±1.58[a,b,c,d]

Values are the mean ± SEM of number of falls and time of permanence for 7 mice (per group) used in the experiments. [a]p<0.01 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from control. [b]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from DZP. [c]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 50 group. [d]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from EE 100 group.

system. These effects are indicative of a dopaminergic-type action (Mora et al., 2005; Almeida et al., 2012). This test is classical model for screening CNS actions, since it provides information about psychomotor performance, anxiety and locomotor activity. It is well known that benzodiazepines act as anxiolytics (at low doses), anticonvulsants, and also produce sedation and a myorelaxant effect at higher doses (Melo et al., 2006). Anxiety, a symptom accompanying various CNS disorders and a disorder by itself, is characterized in humans by a tense and exhaustive physical alertness (Jackson and Turkington, 2005). Other species display a variety of defensive reactions in response to predators, some understood as correlated states of anxiety (Rodgers et al., 1995).

In order to study the possible anxyolitic effect of EE, the elevated-plus-maze test was used. The results showed that EE was able to significantly decrease the time of permanence as well as the number of entrances in the open arms, indicating a negative response. Our results point out that the sedative effects of EE possibly involve the $GABA_A$ receptor complex. A sedative action was already shown by essential oil of leaves from *C. limon* (Campêlo et al., 2011a) and *Lavandula angustifolia* P.

Table 5. Rectal temperature of mice after administration of EE from *Citrus limon*.

Groups	Rectal temperature (°C)
Control	38.00±0.81
Diazepam 0.75	37.98±0.83
EE 50	34.80±0.11[a,b]
EE100	34.70±0.35[a,b]
EE150	34.50±0.58[a,b]

Values are the mean ± S.E.M. of rectal temperature for 7 mice (per group) used in the experiments. [a]p<0.01 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from control. [b]p<0.001 (ANOVA followed by Student-Neuman-Keuls *t*-test), significantly different from DZP.

Miller (Lis-Balchin and Hart, 1999), as assessed by the elevated-plus-maze test in rodents. Active constituents of *C. limon* are primarily monoterpene compounds chemically similar to EE. These monoterpenes exhibit sedative activity that is thought to be due to GABAergic mechanisms. Furthermore, an essential oil from *C. limon* was shown to exert a dose-dependent increase in antioxidant parameters in mice hippocampus (Campêlo et al., 2011b).

The forced swimming test is a behavior test which, in rodents, gives an indication of the clinical efficacy of various types of antidepressant drugs. Nowadays, antidepressants are known to act by several distinct mechanisms at the receptor level, probably also stimulating similar pathways at the sub-cellular level (Yildiz et al., 2002). EE was also able to decrease the immobility time of mice (50, 100 and 150 mg/kg). At higher doses (100 and 150 mg/kg), however, the antidepressant effect was not masked by sedative and hypnotic actions of the drug (data not shown). The EE effect was increased by imipramine, a tricyclic antidepressant (TCA) which blocks the reuptake of both serotonin and norepinephrine. In addition, alterations were seen after EE association with paroxetine, a known selective serotonin reuptake inhibitor. Additionally, EE effects were totally blocked by the reserpine pretreatment, a known inhibitor of the vesicular catecholamine transporter (that facilitates vesicular storage). A similar process occurs at storage sites for 5-HT, which can finally result in a depletion of biogenic amines. Furthermore, this finding proposes that the antidepressant effect of EE is probably related, at least in part, to the increase in CNS noradrenergic and serotoninergic activities.

In order to assess whether the EE produces loss of motor coordination of animals, a test was performed on the rota-rod apparatus. Our results suggest that the highest dose of EE produces loss of motor coordination of mice. This result also suggests that increasing the dose leads to an EE amnesic effect, an important characteristic of the benzodiazepine drugs. Thus, lack of coordination and muscle relaxation in the test of the Rota-rod is characteristic of a drug that reduces the CNS activity such as sedatives and hypnotics (Almeida et al., 2012; Silva et al., 2011). Rectal temperature decreased at all doses tested. This result can probably be associated with possible action on the GABAergic system.

Conclusions

Our results gave support to the idea that EE interacts with the $GABA_A$ receptor, probably at the receptor subtypes that mediate BDZ effects, to produce sedative and hypnotic activities, and also acts to increase the noradrenergic and serotoninergic activities that is the main factor responsible for its antidepressant activity. Additional studies, however, are needed to fully clarify the mechanism of sedative and antidepressant effects of ethanolic extract of leaves from *C. limon*.

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Full Length Research Paper

Analgesic and neuromodulatory effects of sea anemone *Stichodactyla mertensii* (Brandt, 1835) methanolic extract from southeast coast of India

**Sadhasivam Sudharsan[1], Palaniappan Seedevi[1], Umapathy Kanagarajan[2], Rishikesh S. Dalvi[2,3], Subodh Guptha[2], Nalini Poojary[2], Vairamani Shanmugam[1], Alagiri Srinivasan[4] and Annaian Shanmugam[1]*

[1]Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, Tamil Nadu, India.
[2]Central Institute of Fisheries Education, Off Yari Road, Versova, Mumbai-400061, Maharashtra, India.
[3]Maharshi Dayanand College, Dr. S.S. Rao Road, Mangaldas Verma Chowk, Parel, Mumbai-400012, Maharashtra, India.
[4]Department of Biophysics, All India Institute of Medical Sciences, New Delhi-110 029, India.

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The biological activity of crude methanolic extract (CME) of sea anemone *Stichodactyla mertensii* was screened. The CME was fractionated using diethylaminoethyl (DEAE – cellulose) and screened for hemolytic activity, mice bioassay, analegsic activity and neuromodulatory activity. The presence of protein was estimated to be 0.292 mg/ml in crude, followed by 0.153, 0.140 and 0.092 mg/ml in Fractions 1, 2 and 3, respectively. The crude extract and 3 fractions showed the hemolytic activity of 109.58, 52.28, 57.14, 43.47 HT/mg on chicken blood, while in human blood it was recorded as 27.39 and 26.14 HT/mg in crude and F1 fraction and 26.14, 28.57 HT/mg in F1, F2 fractions of 'AB' and 'O' blood groups. Whereas the F2 and F3 fractions in 'AB' group and F3 fraction alone in 'O' group did not show any activity. The lethal dose 50% (LD_{50}) in mice was found to be 108.24 mg/kg in the crude extract. The crude extract and the active fractions reported moderate analgesic activity at 93.75 mg/kg concentration. The impact of sea anemone crude extract was found to increase neuromodulatory activity in Na^+ K^+ ATP-ase at 86 to 91%, Mg^{++} ATP-ase at 266 to 438% and AChE at 67 to 24% activities, though the study has provided some justification of sea anemone extract which showed moderate hemolytic activity and prompt analgesic activity, as well as acting variably on the presynaptic sodium pump and promoting the cholinesterase activity.

Key words: *Stichodactyla mertensii*, hemolytic titration assay, analgesic drug, mice bioassay.

INTRODUCTION

Sea anemones (Actinaria, Cnidaria) are armed with venom-secreting nematocytes, which help in capturing of prey and protection from predators. Venom from most sea anemones is harmless to humans or may induce mild dermatitis, but few species of sea anemones possess highly toxic venoms which may cause some serious

*Corresponding author. E-mail: shanpappu48@gmail.com.

effects to humans. *Phyllodis cussemoni* is one of the most dangerous (Kwietniewski, 1897). The biological role of toxins delivered by nematocysts include the capture and killing of prey, digestion, repelling of predators and interspecies spatial competition (Macek, 1992). Most of the coelenterate venomous animals, sea anemones, contain a variety of interesting biologically active compounds and some potent toxins (Vincent et al., 1980). Previously, different types of protein toxins have been isolated from several species of sea anemones which are neurotoxins of 46 to 49 amino acid residues cross-linked by three intra molecular disulfide bonds having specific affinity for sodium channels associated with synaptic nerve terminals and hemolysins, basic and cysteine-less polypeptides (Schweitz et al., 1981; Thomson et al., 1987). In addition to previous studies, cardio stimulatory activity has also been reported for some toxins from sea anemones (Norton et al., 1990; Simpson et al., 1990).

Further studies dealing with the depiction and the mechanism of action of venoms and their constituents produced by sea anemones are imperative to explicate the function of central nervous system and thus become noteworthy tools for the study of synaptic transmission (Kozlov et al., 2009; Peterson, 1977). Thus in the present study, the crude and fractionated extracts comprises an initial effort to evaluate the bioactivity of secondary metabolites from sea anemone *Stichodactyla mertensii* collected from Devipattinam coast of India.

MATERIALS AND METHODS

Extraction of methanolic extract

S. mertensii was collected from the depth of 6 m along the Devipattinam (Lat. 09° 28' N; Long.78° 53' E) Southeast coast of India. The sea anemones were first thoroughly washed with seawater, followed by tap water, and later with distilled water to remove the epiphytes and other materials attached on their body surface. Tentacles were separated from the anemones and ground in methanol using tissue homogenizer (Remi RQ-127, India), following the method of Kozlov et al. (2009).

Partial purification of the crude extracts (column chromatography)

A properly cleaned glass column (55 × 2.75 cm) was mounted vertically in a stand and filled with DEAE cellulose, 15 cm from the bottom of the column. The column was pre - equilibrated with phosphate buffer before loading the sample. The crude extract was dissolved in Phosphate Buffer Saline (PBS pH 7.4) at a concentration of 5 mg/ml and was eluted with a linear gradient of 0.1 M to 1 M Nacl in phosphate buffer saline.

Estimation of protein

Protein contents of the crude extract and the fractions eluted from the DEAE column were estimated following the method of Peterson (1977) using Bismuth sulfite agar (BSA) as the standard.

Hemolytic activity

The hemolytic activity was estimated following the method of Pani Prasad and Venkateshvaran (1977). The hemolytic assays were performed in 96-well 'V' bottom microtiter plates. The crude extract and the fractions were prepared (5 mg/ml) in 100 µl of normal saline, and 100 µl of 1% erythrocyte suspension was added to all wells. The positive and negative controls were maintained using distilled water and normal saline, respectively. The plates were gently shaken and allowed to stand for 3 h at room temperature (26°C). Occurrence of uniform red-coloured suspension in the wells was considered as positive hemolysis whereas formation of a button at the bottom of the wells was considered as absence of hemolysis. The hemolytic titer was defined as the reciprocal of the highest dilution giving positive hemolysis (HT/ml). Specific activity was expressed as hemolytic units per mg of soluble protein (HT/mg).

Mice bioassay

An acute toxicity test of the extract and fractions was carried out as per the Association of Official Analytical Chemists (AOAC, 1990). Clinically healthy male albino mice (weigh 20 ± 2 g) were maintained at the animal holding facility of CIFE, Mumbai and were housed under standard conditions of temperature (25 ± 2°C), relative humidity (60%), 12/12 light/dark cycle, and fed with standard pellet diet and tap water following the guidelines of the Institute's Ethical Committee. Animals (Regulation number FGB/CPCSEA/2001-02) were fasted prior to dosing and the test substances were administered in a single dose intraperitonially (i.p.). The dried crude methanolic extract and fractions (F1 to F3) was dissolved in PBS (5 mg/ml) and was injected intraperitonially to mice in dosages ranging from 0.25 ml (1.25 mg), 0.50 ml (2.5 mg), 0.75 ml (3.75 mg) and 1.0 ml (5.0 mg). All the experiments were performed in triplicates. Control mice were injected with a corresponding volume of PBS. Behavioral changes during the experimental period and the time required for death of each animal were recorded. The LD_{50} was estimated following the method of Lorke (1983).

Analgesic activity

Analgesic activity was assessed by tail flick method following the method of Gray et al. (1970) using Tail Flick Analgesia Meter (Harvard, USA) with a variable 150 W, 25 V lamp as the heat source. Male albino mice (20 ± 2 g) were housed under standard conditions as described above. Prior to the experiment, the animals were restrained in a plastic tube, for 10 min twice a day for three days, for adaptation. The crude methanolic extract and fractions (F1, F2 and F3) were dissolved in PBS (1.25 mg/ml) and 0.25 ml of this solution was injected intraperitonially to mice, and tested at 30, 60 and 90 min after injection. Mice without administration of any extract were used as controls while those injected with a corresponding dose of paracetamol served as reference standards. All the experiments were performed in triplicates. The application site of the heat on the tail was maintained about 5 cm from the tip of the tail of each animal. The tail flick response was noted and cut-off time of 15 s was maintained. Analgesic activity was expressed as a ratio between the difference in reaction time of mice treated with the samples and control.

Neuromodulatory activity

In vitro effect of the sea anemone crude methanolic extract on the ATPase activity of mice brain was given below.

P_2 fraction preparation

Clinically healthy male albino mice (weight 20 ± 2 g) were housed as mentioned above. P_2 fraction (mitochondrial nerve endings) from the brain of mice was prepared following the method of Green et al. (1957). Brain was homogenized in chilled sucrose solution (0.32 M) and centrifuged (Remi -refrigerated centrifuge, India) at 2,500 rpm for 15 min at 4°C to remove cell debris, nuclei and plasma membrane fragments. The supernatant was collected in separate tubes and centrifuged at 15,000 rpm for 20 min at 4°C. Later, the supernatant was discarded and the pellet was dissolved in sucrose solution and centrifuged at 15,000 rpm for 20 minutes at 4°C. This step was repeated twice to wash the pellet and resuspended in same sucrose solution, and kept in glass vials at -20°C until use as the enzyme source. Protein was estimated following the method of (Peterson, 1977) using BSA as the standard.

ATPase assay for inorganic phosphate was performed according to Lowry and Lopez (1946). For total ATPase reaction mixture, 0.8 ml of imidazole buffer (0.135 mM) with 100 mM NaCl, 20 mM KCl and 5 mM MgCl, were added in each test tube and 0.1 ml of enzyme (a quantity that depends on the protein mg/h of enzyme source) was added and stirred. To this mixture, 0.1 ml of crude extract at each of four concentrations (250µg, 500µg, 750µg, and 1000µg) was added immediately by using micropipettes. For the Mg^{2+}-ATPase reaction mixture, 0.07 ml (1 mM) was added as inhibitor for Na+-K+-ATPase in addition to the above mixture. Triple distilled water (0.1 ml) was added to the total ATPase reaction mixture and 0.03 ml of triple distilled water was added to Mg^{2+}-ATPase mixture to bring the reaction mixture to a total volume of 1 ml. The reaction was started by adding 50 µl of Adenosine-5'-triphosphate (ATP) substrate (4.5 mM) in each tube. All the tubes were gently shaken and incubated at 37°C for 30 min in a water bath. By adding 0.5 ml of 10% TCA, the reaction was stopped and the contents of all tubes were centrifuged and the supernatants were collected. To this supernatant, 0.3 ml of 0.1 N sodium acetate solution followed by 0.4 ml of ammonium molybdate (1%) and H_2SO_4 (0.05N) solution were added to each tube. The color development was measured at 800 nm in a spectrophotometer after 15 min. The control experiment was run simultaneously with 100 µl of triple distilled water instead of toxins.

$$ATP = \frac{\text{Absorbance of the sample} \times \text{Amount of phosphate present in the standard} \times 60}{\text{Absorbance of the standard} \times \text{Amount of protein in the sample} \times \text{incubation time}}$$

***In vitro* effect of the sea anemone crude methanolic extract on the AchE activity of mice brain**

The mice brain AchE enzyme activity was assessed according to Ellman et al. (1961). The brain isolated from the male albino mice weighing 20 ± 2 g was homogenized with 0.25 M (8.55 g in 100 ml) ice cold sucrose solution and 2% (w/v) tissue homogenate was prepared in the same sucrose solution and stored in the freezer as the enzyme source. Three millilitres of phosphate buffer (pH 8.0) was placed in each tube, to which 0.1 ml of enzyme source (2% w/v homogenate) was added and stirred. Then 100 µl of 0.01 M DTNB (0.5-5- dithiobis-2-nitrobenzoic acid) was added and the initial color was measured spectrophotometrically at 412 nm. The test solutions of crude extract (100 µl) at each of four concentrations (250, 500, 750 and 1000 µg) were added. Control experiment was run simultaneously with 100 µl of triple distilled water instead of crude extract. To start the reaction, 20 µl of acetyl thiocholine iodide (ATCl) (0.075 M) was added to each tube as substrate and then the reaction was allowed to incubate for 15 min at room temperature. The colour development was measured as the final spectrophotometric reading at 412 nm.

$$AChE = \frac{\text{Absorbance of the sample} \times \text{Amount of Ach present in the standard} \times 60}{\text{Absorbance of the standard} \times \text{Amount of protein in the sample} \times \text{incubation time in minutes}}$$

Statistical analysis

The values were expressed as the mean value ± standard error, and the data were subjected to statistical analysis (ONE-WAY ANNOVA).

Histopathology

For the histopathology examination, all the organs (brain, heart, liver, kidney and lungs) were dissected out from one mice in each group of experimental animal. The organs was fixed in 10% formalin and after proper dehydration, the tissue were embedded in paraffin wax. The sections of 6 µ thickness were cut using a rotary microtome and stained with hematoxylin and eosin (Pantin, 1962). The sections were observed under Motic trinocular stereozoom digital microscope (DMWB1 series) and photographs were taken.

RESULTS

About 2.3 g of crude methanolic extract was obtained from a starting material of 265 g of whole body tentacle. Protein content in the crude methanolic extract was estimated to be 0.292 mg/ml. The crude extract was purified with DEAE cellulose and fractions of 5 ml (F1 to F10) were collected. Among them, fractions with higher protein content were considered for further screening. Hence, the partially purified fractions F1 (0.153 mg/ml), F2 (0.140 mg/ml) and F3 (0.092 mg/ml) were dialyzed

against distilled water, freeze dried and used for further study.

Hemolytic assay

The crude methanolic extract, F1, F2 and F3 fractions showed a specific hemolytic value of 109.58, 52.28, 57.14 and 43.47 HT/mg, respectively, on chicken erythrocytes (Table 1). Whereas crude and F1 fraction reported 27.39 and 26.14 HT/mg (in 'AB' blood group) and crude, F1 and F2 fractions recorded 27.39, 26.14 and 28.57 HT/mg (in 'O' blood group) hemolytic activity in human blood. In the case of 'AB' blood group the F2 and F3 fractions and in 'O' blood group only the F3 fraction did not exhibit hemolytic activity (Table 2). The crude extract showed higher hemolytic titer on chicken than on the human erythrocytes.

Mice bioassay

The mice bioassay was done by crude and fractionated samples from *S. mertensii* by various concentrations 0.25 ml (1.25 mg), 0.50 ml (2.5 mg), 0.75 ml (3.75 mg) and 1.0 ml (5.0 mg) respectively and their response is give in Table 3.

Analgesic activity in tail flick method

The analgesic activity of crude and fractions showed moderate analgesic ratio when compared to standard (Table 4). The analgesic ratio of the crude extract was 0.49, whereas of the F1, F2 and F3 fractions was 0.17, 0.14 and 0.10 respectively.

Neuromodulatory activity

Effect of Na+-K+ ATP-ase, Mg++ ATP-ase and AChE activity were evaluated by using different concentrations (250, 500, 750 and 1000 µg/ml) of crude extract to observe the neuromodulatory activity (Figure 1). Na+-K+ ATP-ase activity was inhibited by methanolic (86 to 91%) extract, but when the concentration was increased from 250 to 1000 µg/ml it showed the positive modulation. Whereas its crude extract showed 266% of Mg^{++} ATP-ase activity at 250 µg/ml and 438% at 1000 µg/ml concentration (Figure 2). The crude extract exhibited the positive modulation in AChE activity up to 67% at 250 µg/ml and 24% at 1000 µg/ml concentrations (Figure 3).

Histopathology

Observed histopathological changes in the test mice suggest that all the main organs: brain, heart, liver, kidney and Lungs were affected by sea anemone toxins (crude extract and fractionated) Figure 4 to 8.

DISCUSSION

Natural products play a vital source of novel therapeutic agents for various conditions, including infectious diseases (Selim et al., 2013). Hence in the present study, the crude methanolic extract (CME) of sea anemone *S. mertensii* was investigated for their pharmacological potential to develop it as a therapeutic agent. The crude extract of 2.3 g from 265 g of sea anemone (tentacle tissue) was obtained. Earlier, Ravindran et al. (2010) reported that 500 g fresh weight of *Heteractis magnifica* yielded 9.73 and 7.84 g in *Stichodactyla haddoni* followed by 5.37 g in *P. sinensis* of crude extracts. The total protein was estimated to be 0.219 mg/ml in *S. mertensii* crude extract which is lower than that of *H. magnifica* (981 µg/ml), *S. haddoni* (820 µg/ml) and *Paracondactylis sinensis* (605 µg/ml) (Ravindran et al., 2010) in the low level of protein content may be depending on the habitat (in terms of abiotic parameters) from where it has been collected. Karthikayalu et al. (2010) reported the hemolytic property in sea anemone *H. magnificasea* crude extract at concentrations as low as 120 µg/ml, whereas in the present study the *S. mertensii* showed moderate hemolytic activity in both chicken and human erythrocytes at 5 mg/ml concentration.

Vakorina et al. (2005) previously reported that actinoporin compound isolated from sea anemone *Radianthus macrodactylus* showed the hemolytic activity, which significantly correlates with our present study and indicates the presence of considerable hemolytic unknown toxic compound in the *S. mertensii* tentacle extract. The lethal dose (LD_{50}) in mice was found to be 108.24 mg/kg in the crude extract and changes in their behaviour was observed based on the dosage (Table 3). Ravindran et al. (2010) observed toxicity in mice for three species of sea anemone. *H. magnifica, S. haddoni* and *P. sinensis* at different concentrations (48, 30 and 15 µg/ml) tested wherein death rate occurred in 68.3, 135, 86.33 seconds, respectively. The aqueous ethanol extract from root bark of the plant *Ximenia americana* L. (Olacaceae) showed LD_{50} of 345 mg/kg of body weight of mice besides inhibiting the oedema, pain, cell migration and increasing vascular permeability (Olabissi et al., 2011).

Similarly, CO_2 extract from *Ilex paraguariensis* showed acute toxicity in mice at 500 mg/kg. Further significant changes were also observed in the histology of liver and kidney cells when compared to the normal cells (Pasquali et al., 2011).

Sea anemones are known to contain mainly polypeptides and proteins, which account for most of their

Table 1. Hemolytic activity in Chicken blood against *Stichodactyla mertensii*

S/No	Type of extract	Protein (mg)	Source of blood		
			Chicken blood		
			Total hemolysis (up to dilutions)	Hemolytic titer	Specific hemolytic activity (HT/mg)
1	Methanol (crude)	0.292±0.001	5	32	109.58±0.67
2	F1	0.153±0.001	3	8	52.28±0.96
3	F2	0.140±0.002	3	8	57.14±0.75
4	F3	0.092±0.001	2	4	43.47±0.87

Table 2. Hemolytic activity in Human blood against *Stichodactyla mertensii.*

S/No	Type of extract	Protein (mg)	Source of blood					
			Human blood "AB" and "O" group					
			Total hemolysis (up to dilutions)		Hemolytic titer		Specific hemolytic activity (HT/mg)	
			AB	O	AB	O	AB	O
1	Methanol (crude)	0.292±0.001	4	4	8	8	27.39±0.87	27.39±0.63
2	F1	0.153±0.001	2	2	4	4	26.14±0.42	26.14±0.43
3	F2	0.140±0.002	ND	2	ND	4	-	28.5±0.59
4	F3	0.092±0.001	ND	ND	ND	ND	-	-

*Non detectable.

Table 3. Mice bioassay for crude and their fractions.

S/No	Extract/ fractions	Injected volume (ml)	Death time (min)	Symptoms	Mortality
1	Control	1.00 (saline)	NA	NA	NA
2	Crude	0.25	NA	Escape reaction, defecation	Non lethal
	Crude	0.50	NA	Palpitation, escape reaction, defecation	Non lethal
	Crude	0.75	2.68±0.025	Palpitation, escape reaction, defecation, over active, dragging of hind limb	Lethal
	Crude	1.00	2.45±0.015	Palpitation, escape reaction, defecation, sniffing and scratching, dragging of hind limb	Lethal
3	F1	0.25	NA	Escape reaction, defecation	Non lethal
	F1	0.50	NA	Escape reaction, defecation	Non lethal
	F1	0.75	1.33±0.020	Palpitation, escape reaction, sniffing and scratching, dragging of hind limb	Lethal
	F1	1.00	1.20±0.025	Palpitation, escape reaction, micturition, dragging of hind limb, excess breathing	Lethal
4	F2	0.25	NA	Escape reaction, defecation	Non lethal
	F2	0.50	NA	Palpitation, escape reaction	Non lethal
	F2	0.75	NA	Palpitation, escape reaction,	Non lethal
	F2	1.00	1.23±0.025	Palpitation, escape reaction, micturition	Lethal
5	F3	0.25	NA	Escape reaction, defecation	Non lethal
	F3	0.50	NA	Over active, escape reaction	Non lethal
	F3	0.75	NA	Over active, escape reaction	Non lethal
	F3	1.00	NA	Over active, palpitation, escape reaction	Non lethal

*Not applicable.

Table 4. Analegsic activity of *Stichodactyla mertensii*

S/No	Weight of mice in (g)	Type of Extract	Amount of sample injected 93.75 mg/kg	Time of tail flick (Sec)	Analegsic ratio
1	20±2	Crude	0.25	29.3±1.81	0.49±0.1
2	20±2	F1	0.25	20.1±0.68	0.17±0.1
3	21±2	F2	0.25	18.2±1.69	0.14±0.1
4	20±2	F3	0.25	43.6±1.12	0.10±0.1
5	21±2	Standard	0.25	15.2±0.73	1±0.57
6	20±2	Control	-	15.2±0.14	-

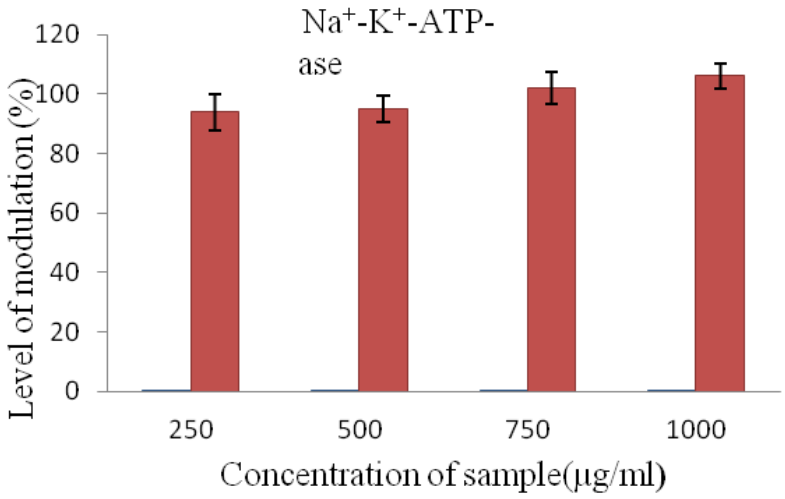

Figure 1. *In vitro* Evaluation of the effect of the Seaanemone crude methanolic extract on mice Brain ATP – ase enzyme.

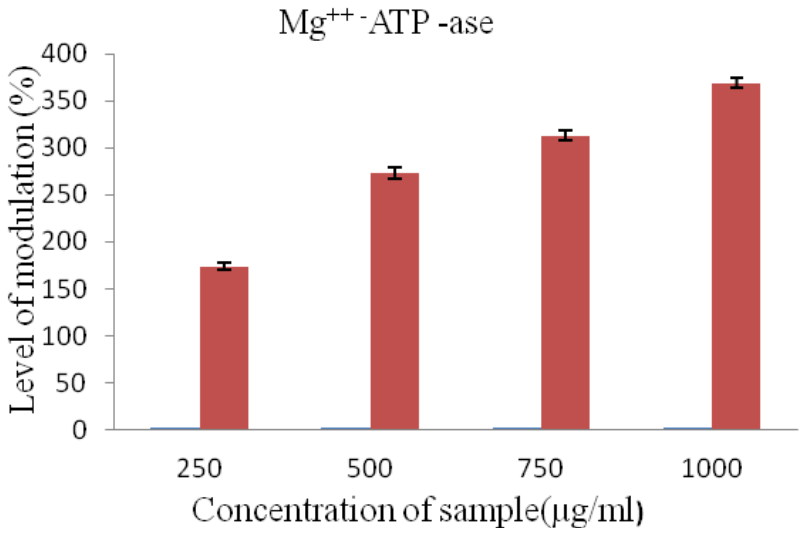

Figure 2. *In vitro* Evaluation of the effect of the sea anemone crude methanolic extract on mice brain Mg^{++} ATP-ase.

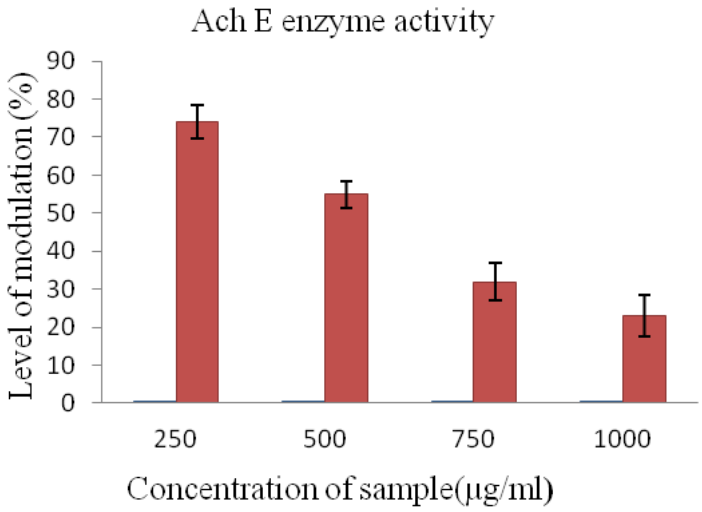

Figure 3. *In vitro* evaluation of the effect of the sea anemone crude methanolic extract on mice Brain Ach E enzyme.

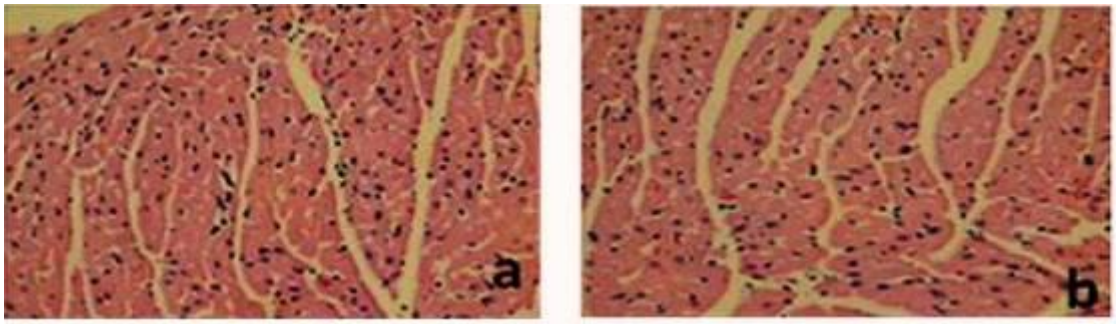

Figure 4. Histopathology of brain from mice. (a) Control; b) sample treated normal.

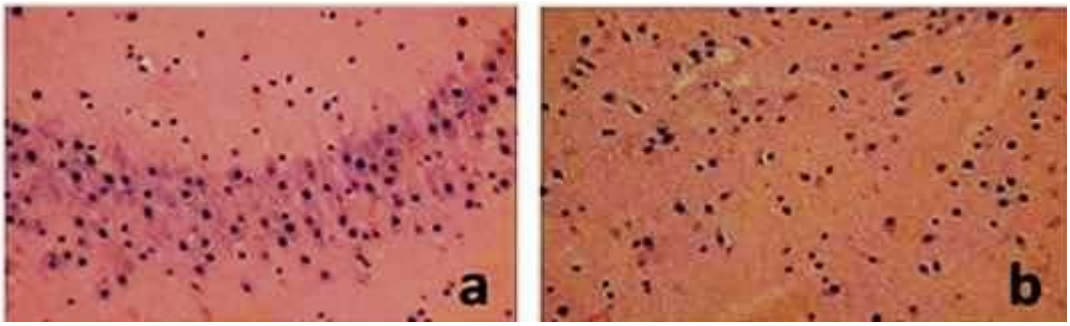

Figure 5. Histopathology of heart from mice. (a) Control; b) sample treated normal.

pharmacological properties (Hessinger et al., 1973; Khoo et al., 1993). The peptides (APHC2 and APHC3) from *Heteractis crispa* sea anemone showed analgesic activity in mice at the dosage of 0.1 mg/kg (Kozlov et al., 2009). Likewise, the aqueous extract of jelly fish *Crambionella stuhalmanni* and *Chrysaora Quinquecirrha* exhibited the significant analgesic activity at 5 mg/ml (Suganthi et al., 2011) which greatly corresponds with our present results.

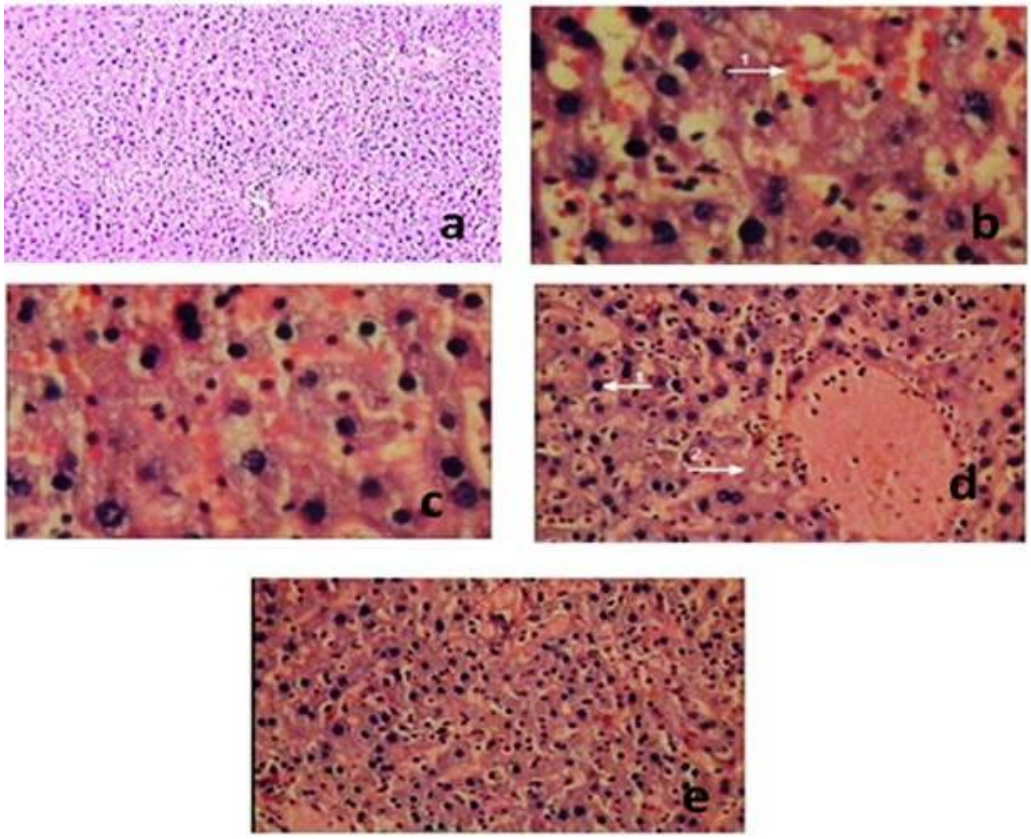

Figure 6. Histopathology of liver from mice. (a) Control b and c) Hepatocyte degeneration and vacuolation and infilteration of mononuclear cells d and e) Pyknotic nuclei and centrilobular necrosis disorganization of hepatic laminae.

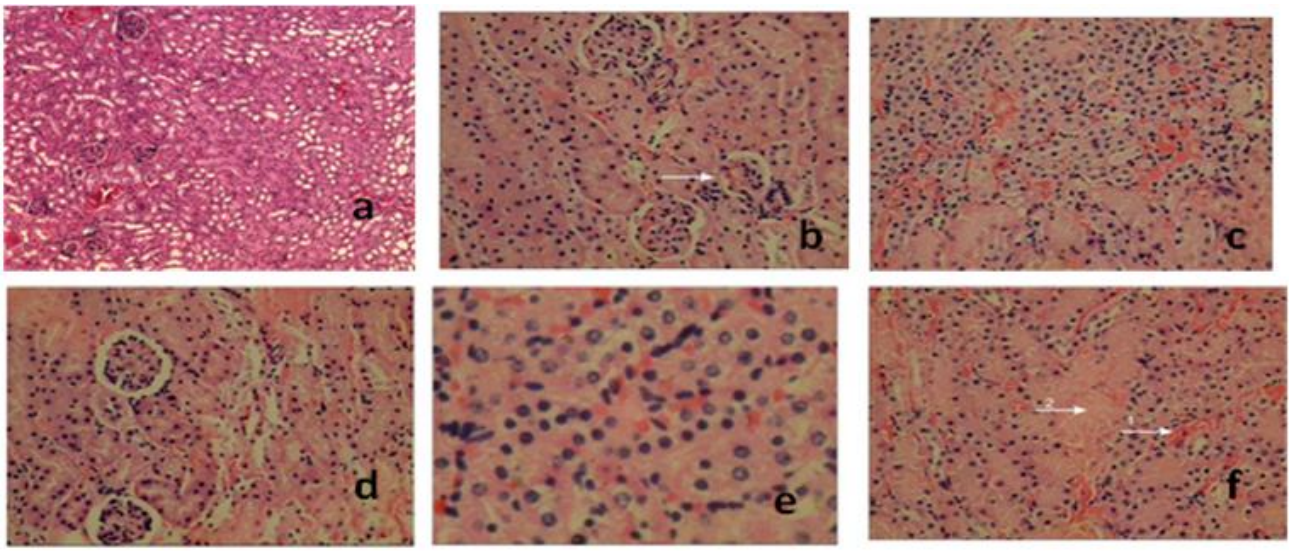

Figure 7. Histopathology of kidney from mice. (a) Control; b and e) Hemorrhage and tubular degeneration; c) interstitial congestion and tubular necrosis; d) tubular necrosis; f) disruption of the renal corpuscles in some areas and infiltration of inflammatory cells was also observed.

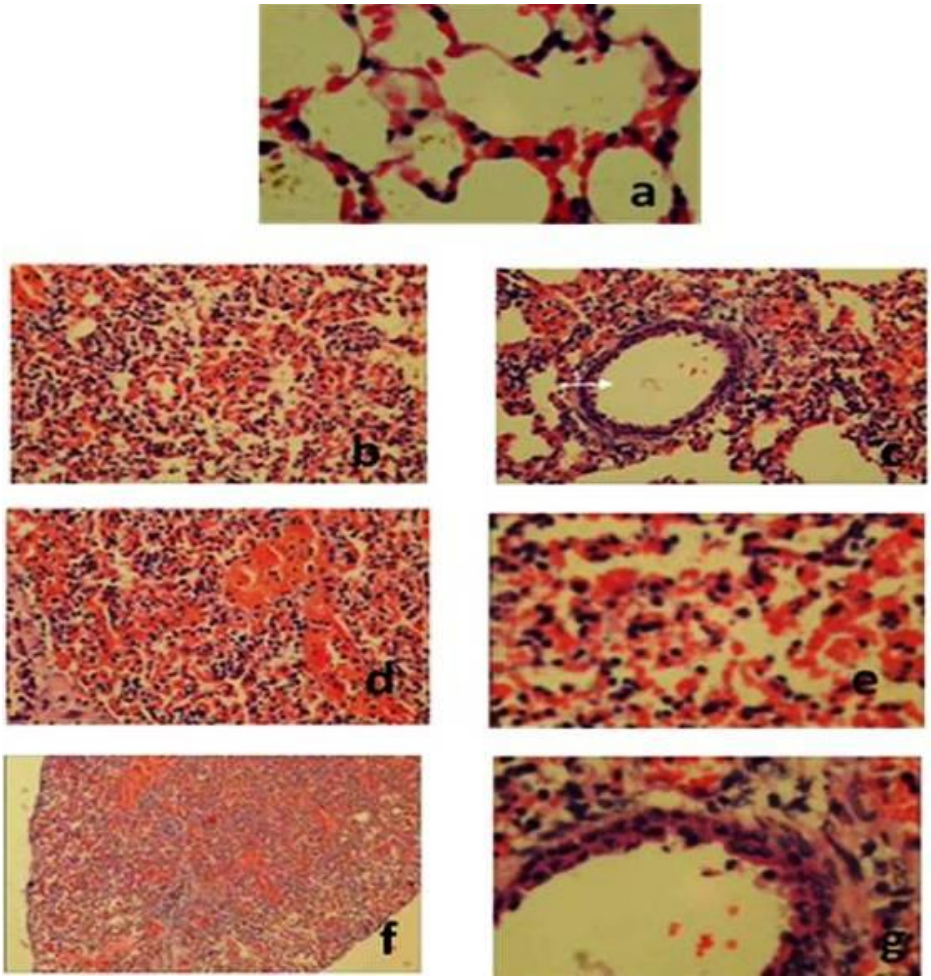

Figure 8. Histopathology of lungs from mice. (a) Control; b) disruption of alveoli or air spaces are seen; c) excessive congestion near the bronchiole; d) excessive congestion in lungs and massive infiltration of macrophages; e, f and g) RBC's and macrophage and excessive congestion in lungs.

Further, the crude nematocyst extract of seaanemone *Paracondactylis indicus, Paracondactylis sinensis, Heteractis magnifica* and *Stichodactyla haddoni* showed analgesic activity at 2 mg/ml concentration (Bragadeeswaran et al., 2011).

The ATPase enzyme system is widely accepted as deriving a part of its energy from ATP hydrolysis for active transport of Na^+-K^+-ATPase. The Na^+-K^+-ATPase (sodium pump) is a pre synaptical membrane protein of higher organisms which hydrolytes cytoplasmic ATP, interacts with neighbouring membrane proteins and organized cytosolic cascades of signalling proteins to send messages to intercellular organelles (Xie et al., 2002). In the present study, the analgesic activity was carried out in *S. mertensii* tentacle methanolic extract. The crude extract and fractionated possessed the analgesic activity at the dosage level of 1.25 mg/ml concentration, and their values are expressed in analgesic ratio. It is interesting to note that the CME protein is capable of stimulating the sodium pump based on the dosage level. In the present study the CME of *S. mertensii* tentacle inhibited the level of Na^+ K^+ATP.ase (86 to 91%). However, the CME showed dose dependant effect on the activity of the sodium pump, the enzyme which is responsible for ATP activity is higher at low dosage of CME (250 µg) and it gives diminished activity at higher concentration (1000 µg). Mg^{++} ATP-ase activity of methanolic extract of sea anemone was elevated in lower concentration (250 µg) and the activity was inhibited at higher concentration (1000 µg), which is comparable to previous reports in *Gyrostoma helianthus* neurotoxins which delayed the activation of K^+ channel

and showed reversible acetylcholine esterase inhibition in both brain and blood of mice (Kamal et al., 2006).

Wankhede (1996) observed similar neuroinhibitory activity by bile extracts of freshwater crabs on the Na^{+}-K^{+} ATP-ase enzyme system in mammalian models. Besides them crude toxin from *Protonibea diacanthus, Otolithoides biauritus* and *Muraenesox talabonoides* also elevated the Mg^{++} ATP ase activity (Malarvannan, 2002). The ATP-ase enzyme system is widely accepted as a structure that employs part of the free energy from ATP hydrolysis for active transport of Na^{+}-K^{+}. The methanolic extract of sea anemone inhibited AchE enzyme activity in lower concentration and elevated in higher concentration. In the *in vitro* neuromodulatory activity screening, CME from *S. mertensii* increased the AchE activity on dose dependent manner. It is characterized as neuromuscular transmission which leads to the behavioural changes on mice palpitation, escape reaction, defecation, over active, dragging of hind limb and micturition. Likewise, puffer fish *Arothron hispidus* exhibited positive modulation *in* Na^{+}-K^{+}ATP ase, Mg^{++} ATP ase and Ach E enzyme activity (Bragadeeswaran et al., 2010).

Conclusion

The present study demonstrated that the marine sea anemone extract have potent pharmacological properties and without any series of toxic effects at low dosage level. These finding strengthen the health care industry and indigenous medicine, and it can be used as remedies for analgesic and neurological disorder.

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Full Length Research Paper

Development and comparative evaluation of extended release indomethacin capsules

Buket Aksu[1], Aysu Yurdasiper[2]*, Mehmet Ali Ege[2], Neslihan Üstündağ Okur[2] and H. Yesim Karasulu[2]

[1]Santafarma Pharmaceuticals, Okmeydani, Boruçiçeği 20 Sisli, Istanbul, Turkey.
[2]Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, 35100, Izmir, Turkey.

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The aim of this study was to develop a new extended release capsules of indomethacin. The formulation has been prepared to enhance its dissolution which could provide better oral absorption of indomethacin (IND). Therefore, the effects of the component nature, their proportion in the release rate and the dissolution mechanism were investigated. Extended release capsules of IND were prepared by physical mixing using plasdone (PVP K-90) and compritol-HD5 ATO (Comp) at various drug-polymer ratios. Flow properties of the physical mixtures were evaluated by calculation of the Carr's index, angle of repose and Hausner ratio. According to the United States Pharmacopeial (USP) drug release criteria of IND extend release capsules, the release results of formulations F2 and F3 were found to be similar to the USP ($P < 0.05$). Certain mathematical models were used for evaluation of release profiles and the results supported by multiple regression analysis. It was observed that the best-fit model to determine the mechanism of the formulation which has shown the highest release was Higuchi square-root of time model ($r^2 = 0.969$). According to the dissolution results, dissolution efficiency, relative dissolution rate and mean dissolution time were also evaluated. The results of the study indicated that new extended release hard gelatin capsules can be a promising alternative for the other oral formulations of IND.

Key words: Indomethacin, drug release, kinetic evaluation, hard gelatine capsule, stability, multiple regression analysis.

INTRODUCTION

Generally known as an analgesic and antipyretic drug, Indomethacin (IND) refers to the compound 1-(4-chloro-benzoyl)-5-methoxy-2-methyl-IH-indole-3-acetic acid. It should be used attentively unlike any simple analgesic due to its potential adverse effects (Goodman and Gilman, 1980). Its areas of use could be listed as follows: Treating gout, rheumatoid arthritis, relieving pain, inhibiting cyclo-oxygenase with a diminishing effect on prostaglandia synthesis and also on body temperature of febric patients (Taha, 2009; Taha et al., 2009). Gastric output of prostaglandins and intestinal maintenance of mucoid secretion on gastrointestinal canal facing is inhibited by IND. Thus, as a result of its use, peptic ulcers are observed to occur, which is a common occasion with all other nonselective cyclo-oxygenase inhibitor drugs (Eis et al., 1998; Taha, 2009). Therefore it is thought that new delivery systems can be devised to overcome the side effects by controlling the drug release (Friend, 2005; Karasulu et al., 2003). Additionally, for poor soluble highly permeable (Class II) drugs, such as IND, the rate of oral absorption is often controlled by the dissolution rate in the gastrointestinal tract (Nokhodchi et al., 2005). Moreover, solubility and dissolution behavior of a drug is one of the key determinants of its oral bioavability. Indomethacin

*Corresponding author. E-mail: aysuyurdasiper@hotmail.com

may show low and erratic oral bioavailability due to poor dissolution of the drug in the fluids of the gastrointestinal tract (Alsaidan et al., 1998; Elchidana and Deshpande, 1999).

In recent years, the number of poorly soluble drug candidates has increased tremendously. The formulation of poorly soluble drugs for oral delivery presents a challenge to the formulation scientists (Dehghan and Jafar, 2006). Furthermore, Indomethacin is a drug possessing a safety risk during usage. Depending on this data, United States Pharmacopeial (USP) has some restrictions on extended release indomethacin preparations relating the *in vitro* drug release versus time. Apart from this, IND follows linear pharmacokinetics. These features allow the great development of a modified-release dosage form in which large variations in plasma concentration are reduced. The use of the sustained-release formulation is a more convenient way of prescribing indomethacin and is especially suited for patients who tend to be non compliant.

Solid dosage forms are important for oral administration because they have a high-metering accuracy, their application is easy and their stability is pretty good. Drug-polymer solid dispersion can improve the dissolution rate of drugs and lead to higher bioavailability (Lin and Huang, 2010). Thus, a capsule formulation often is in the industry in the first dosage form for early clinical studies. Moreover, capsules improve drug stability because the content is tightly enclosed by the capsule shell and thus protected from oxygen, humidity and light (Edgar et al., 2001).

The present study was designed to prepare a new extended release capsules of IND and to investigate the influence of plasdone (PVP K-90) as a hydrophilic polymer, compritol-HD5 ATO (Comp) as a lipophilic polymer on the *in vitro* dissolution of IND from hard gelatin capsules and to explore the mechanism of drug release through mathematical modeling of dissolution data for all formulations. Dissolution efficiency (DE), relative dissolution rate (RDR) and mean dissolution time (MDT) parameters were used to also evaluate the dissolution profiles of extended release IND capsules. In addition, multiple regression analyses have been performed to develop and evaluate a novel formulation of IND for oral delivery. Apart from this, the stability of all formulations in terms of drug content was analyzed during 3 months in order to have a suitable extended release IND formulation.

MATERIALS AND METHODS

Chemicals

Indomethacin was a gift from Deva Holding Inc. (Turkey). Plasdone (PVP K-90) was supplied by ISP, Tech. Inc. (USA), compritol-HD5 ATO was obtained from Gattefosse (France). Aerosil and avicel pH-101 were given kindly from Santafarma Pharmaceuticals (Turkey). All other chemicals and solvents were of analytical grade.

Preparation of extended release capsules

Physical mixture

A series of extended release capsules of IND were prepared in fixed concentration of IND (75 mg) and varying concentrations of plasdone (PVP K-90) and compritol-HD5 ATO (Comp). Each mixture were added 10% avicel pH 101 and 5% aerosil. Then all materails meant for mixing were taken into a cubic mixer at 10 min. The resultant physical mixtures were passed through 35-mesh sieve. The prepared mixtures were sealed and stored in desiccator until used for further studies. All samples which were used in dissolution studies, were analysed for drug content. Before the dissolution studies, these powders were hand filled into zero-size hard gelatin capsules using capsule filling apparatus. Hard gelatin capsule formulations are shown in Table 1.

Drug content estimation

An accurately weighed quantity of physical mixtures was transferred to a 100 ml volumetric flask containing 10 ml of ethanol and dissolved. The volume was made up to 100 ml with phosphate buffer pH 6.2. The solution was filtered and the absorbance was measured after suitable dilutions by using UV-Spectrophotometer at 320 nm (Lakshmi Narasaiah et al., 2011).

Determination of particle size distribution

Particle size analysis was carried out to determine mean particle size of the formulations by Master Sizer 3000 Aero S (Malvern Instruments Ltd. UK). Tests were performed in triplicate.

Determination of flow properties of the physical mixtures

Carr's index

A pre-weighed quantity of dry powder was placed in a graduated 10 ml cylinder. The apparent volume occupied by the powder was then noted before and after the application of 1250 taps to the cylinder using a tap density tester (Varian, Inc. USA). Carr's index formulas are calculated according to Equation (1) (Khan et al., 2012; Staniforth et al., 1996).

$$Carr's\ index = \frac{Tapped\ density - bulk\ density}{Tapped\ density} * 100 \quad (1)$$

Angle of repose

The angle of repose can be defined as the constant three dimensional angle measured relatively to the horizontal base, assumed by a cone-like pile of material formed when the powder is passed through a funnel-like container (Khan et al., 2012; Rios, 2006; Abdullah and Geldart, 1999; Carr, 1965). Angle of repose of the powder material was calculated by using the formula Equation (2):

$$\theta = \tan \frac{h}{r} \quad (2)$$

Where h = height of the pile, r = radius.

Hausner ratio

The basic procedure is to measure the unsettled apparent

Table 1. Extended release capsules compositions of indomethacin.

Ingredients (mg)	F1	F2	F3	F4	F5
IND	75	75	75	75	75
PVP	-	-	-	5	37.5
Comp	-	5	10	25	10

*Each formulation contained 10% avicel pH 101 and 5% aerosil.

volume,V_0 and the final tap volume, V_f of the powder tapping the material until no further volume changes occur. The Hausner ratio was calculated according to Equation (3) (Khan et al., 2012).

$$Hausner\ ratio = \frac{v_0}{v_f} \quad (3)$$

Differential scanning calorimetry

Formulations F1-F5 were weighed and hermetically sealed in flat bootomed aluminum pan with crimped on lid. The pans were positioned on sample pan holder of a Perkin-Elmer DSC 8000. The samples were heated in an atmosphere of nitrogen at a flow rate of 20 ml/min over a temperature range of 0 to 300°C with a constant heating rate of 10°C/min.

Drug release studies

The dissolution rates of the extended release capsules of indomethacin were measured by using USP XXIII apparatus I (rotating basket). The dissolution medium was 900 ml phosphate buffer with a pH 6.2 kept at 37 ± 1°C according to the USP drug release Test 2 criteria (Table 3). Samples of 5 ml were withdrawn at specified time intervals and analyzed spectrophotometerically at 320 nm using Shimadzu 160-A spectrometer, the samples withdrawn were replaced by fresh buffer solution. Each dissolution study was carried out twelve times and mean values were calculated.

Determination of mean dissolution time and dissolution efficiency

Dissolution efficiency (DE) was calculated from the area under the dissolution curve at time t and expressed as a percentage of the area of the rectangle described by 100% dissolution in the same time (Equation 4) (Khan, 1975). Mean dissolution time (MDT) was employed for comparison of dissolution profiles (Polli et al., 1997), calculated according to Equation (5).

$$D.E. = \frac{\int_0^t y * dt}{y_{100} * t} * 100\% \quad (4)$$

Where y is the drug percent dissolved at time t.

$$MDT = \frac{\sum_{j=1}^{n} \hat{t}_j \Delta M_j}{\sum_{j=1}^{n} \Delta M_j} \quad (5)$$

Where *j* is the sample number, n is the number of dissolution sample times, $\hat{t}_j$ is the time at midpoint between t_j and t_{j-1} (easily calculated with the expression $(t_j + t_{j-1})/2$) and ΔM_i is the additional amount of drug dissolved between t_i and t_{i-1}. Relative dissolution rate (RDR) is the ratio between amount of drug dissolved from optimized formulation and that dissolved from the conventional formulation (Gurrapu et al., 2012).

The analysis of release profiles

Kinetic evaluation

Kinetic evaluation of certain release from capsules were applied using a computer based kinetic programme. Three mathematical models (Zero order model, First order model, Higuchi square root of time model) were chosen to describe the release patterns of the indomethacin extended release capsules (Ege et al., 2001). The large value of the coefficient of determination (r^2) indicated a superiority of the dissolution profile fitting to mathematical models.

Determination of release mechanism

Determination of indomethacin release from capsules were estimated (Ozyazici et al., 2006; Ritger and Peppas, 1987) by the Korsemeyer- Peppas Equation (6):

$$Mt/M\infty = kt^n \quad (6)$$

Mt/M∞; the fraction of drug released, t; released time, k; release rate constant.

Dissolution stability studies of extend release IND capsules

For dissolution stability evaluation, extended release capsules of IND were investigated over 3 months under different temperature and relative humidity (RH) conditions at 25 ± 2°C, 65% RH and 40 ± 2°C, 75% RH. Samples were withdrawn at various time points and analyzed for dissolution using the methods described above.

Statistical analysis

Statistical analyses were conducted by one-way analysis of variance (ANOVA) using target significance levels of 0.05 ($P < 0.05$). Multiple regression analysis was undertaken using a computer program SPSS 10.0.

RESULTS AND DISCUSSION

Determination of flow properties of the physical mixtures

The drug content in physical mixtures were found to be in the range of 97.3 to 99.4%. The effect of formulation conditions on the flow properties of IND capsules are shown in Table 2 (Staniforth, 1996). The particle size affects flow rates and angle of repose. F4 presented higher particle size average than F2 and F3. Based on the obtained results, it can be suggested that the amount of the Comp in the formulations could be affected by the angle of repose. The flowability of IND powder was poorer than the flowability of pysical mixtures. Physical mixtures containing polyvinylpyrrolidone (PVP)

Table 2. Flow properties of extend release Indomethacin capsules.

Parameter	F1	F2	F3	F4	F5
Angle of repose	75.37±6.74	62.97±2.79	49.34±2.71	35.82±3.19	66.70±7.45
Carr's index	89.76±0.44	89.76±0.62	91.60±0.38	86.48±0.45	84.71±1.75
Hausner ratio	9.78±0.42	9.77±0.61	11.92±0.55	7.40±0.24	6.59±0.76
Particle size	3.61±0.01	5.71±0.13	7.34±0.50	9.00±0.51	5.62±0.41

Table 3. The percentages of indomethacin dissolved in a phosphate buffer of pH 6.2 and USP XXIII criteria of indomethacin extend release capsules according to time.

Time (h)	Formulations					USP XXIII criteria
	F1	F2	F3	F4	F5	Amount dissolved
1	27.48±0.01	23.71±0.08	19.68±0.07	17.87±0.09	49.96±0.05	Between 12-32%
2	36.67±0.04	45.74±0.05	37.96±0.06	55.19±0.10	58.90±0.08	Between 27-52%
4	49.20±0.07	63.96±0.06	53.09±0.02	80.36±0.04	66.79±0.03	Between 50-80%
12	74.51±0.03	99.22±0.04	82.35±0.05	90.41±0.03	82.20±0.07	Not less than 80%

possessed slightly higher flow rate than formulations including only Comp. This may be responsible for the high bulk density obtained for IND including PVP. Also, the angle of repose is mostly affected by the presence of PVP. The values of Hauser ratio and Carr's index obtained for the formulations were found to be in conformity with their flow rates. Both the Carr's index and Hauser ratio indicated poor flow property in all formulations (Ozyazici et al., 1996). To sum up, as the particle size decreased, the cohesion of the particles increased and the inter-particulate forces between the powders became stronger. Therefore the high percentage of smaller particles displayed an influence on IND's lack of flowability.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) studies have been conducted to understand the role of the physical interaction among PVP, Comp and IND. To explain more about the current studies, it is crucial to examine the DSC thermogram of IND, PVP, Comp, avicel, aerosil and all formulations which are exhibited in Figure 1. The DSC curve shows IND has an endothermic peak at 164.9°C corresponding to its melting and indicating its crystalline nature. Additionally, Comp showed a single endothermal peak at 64.8°C and PVP showed at 184.7°C. Melting of IND can be observed in physical mixtures of drug: PVP and Comp. The DSC thermogram of the physical mixtures showed a slight change in melting peak of the IND, suggesting the alteration in crystallinity of IND. Also, a peak intensity corresponding to the drug has decreased in all thermograms. It can be seen more conspicuous in those with higher proportions of PVP and Comp. In the study, the heat of fusion pertaining to IND has been higher (478.8 J/g) than to all other formulations. Should the results be mentioned, a decrease has been monitored in the crystallinity of IND in the presence of a higher amount of PVP and Comp (Wu et al., 2009).

Drug release studies

In vitro drug release was determined using the USP basket method. Release profiles of the extended release capsules containing IND were showed in Figure 2. The percentages of indomethacin dissolved in phosphate buffer of pH 6.2 according to USP XXIII criteria were given in Table 3.

When the dissolution results have been compared with USP XXIII criteria, it was observed that the release results of F2 and F3 capsule formulations were similar to the USP criteria (Table 3). The dissolution results indicate that the release of F5 formulation including large amount of PVP is faster than pure IND. This may be due to the presence of PVP, which appears to facilitate the dissolution of IND as it is more soluble and could hydrate in an easier manner andalso possibly due to the increase in its wettability (Oliverira et al., 2013). An increase in the concentration of PVP may prevent drug aggregation or raise drug wettability resulting in a higher solubility (Oliveria et al., 2013). For this reason, it was observed that F5 formulation has shown faster release profiles in early stage of dissolution compared to F2, F3 and F4 formulations. Moreover, dissolution results showed that the formulations including Comp takes longer compared to formulations with PVP. Hydrophobic interaction between IND and Comp appears to possess a key function for a slower diffusion process in the capsule formulations (Roberts et al., 2012). Additionally, multiple regression analysis was applied to the results obtained

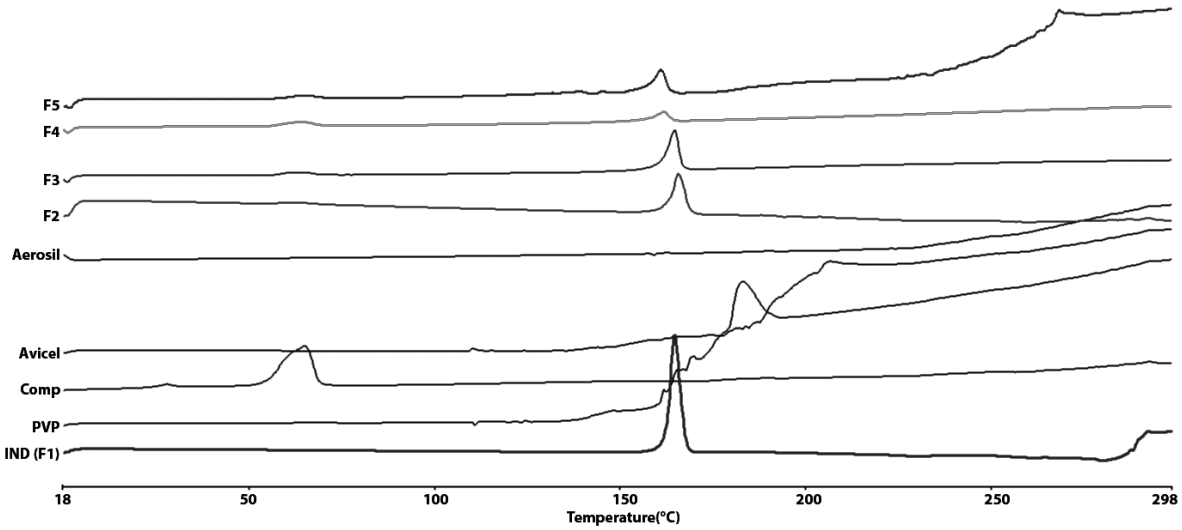

Figure 1. DSC thermograms of IND (F1), PVP, avicel, Comp, aerosil and F2 to F5.

from *in vitro* drug release studies. The analysis results have shown that concentration of PVP and Comp had an influence on drug release of IND capsule formulations, however Comp concentration had much less influence on the drug release (Figure 3).

The analysis of release profiles

Release profiles have been evaluated with the help of various mathematical models. According to the analysis of all mathematical models, it is clearly seen that Higuchi square-root of time model show obviously a better fit for IND extented release capsules (Orelli and Leuenberger, 2004) (Table 4). The Higuchi square root equation describes that the rate of drug release from systems is related to the rate of drug diffusion (Ertan et al., 2000; Iravani et al., 2011). This data confirms that the extended release of IND capsule formulations have been generated in a Higuchian diffusion fashion, which is statistically proven by the release curves in comparison with their correlation coefficients. To mention the formulations shortly, when F2 and F3 formulations are released in dissolution medium pH 6.2 phosphate buffer, the slope value arrives at its highest point. Formulations F4 and F5 as physical mixture of IND: PVP provided faster release than individual IND (F1) and physical mixture of IND: Comp (F2 and F3) in dissolution media.

Dissolution mechanism of the formulations has been shown to be significantly diffusion-controlled during which the high amount of PVP and Comp is the main factor to control the dissolution rate. Furthermore, dissolution release data were studied using Korsmeyer-Peppas release model. The release exponent (n) values from the power law Peppas equation have provided an insight to understand the release mechanism from the dosage form (Pritchard et al., 2010). Formulation F5 exhibited anomalous (non-Fickian transport) diffusion mechanism with "n" value 0.916 (Table 4). This suggests that some level of swelling must be operating within the system, causing deviation from the Fickian release because of higher amount of PVP. In the first 2 h, F4 and F5 formulations containing PVP showed higher release rate than the other formulations leading to a boost at the wettability and an acceleration of solvent penetration in the capsules to dissolve the drug thereby more rapidly, and they get diffused out (Mohana Raghava Srivalli et al., 2013). The "n" values of F2, F3 and F4 formulations have been found to be more than 1. This result indicates that the drug release from the polymer matrix formulations suggests super Case II transport, that is the mechanism of drug release has been administered by both diffusion and polymer relaxation (Apu et al., 2009).

As a foot-note to the above, the improvement in dissolution characteristics of a drug is described in terms of dissolution efficiency (DE) and RDR. In line with the outcome of the study, the RDR of 1 h have been acquired more slowly from F2, F3 and F4 formulations owing to the poor wettability of compounds compared to F5 formulation prepared with a high concentration level of PVP (Table 5). After 1 h, the wettability starts to become visible and within 2 to 6 h, RDRs of all formulations appear to be higher by comparison with F1 formulation. When RDR is greater than 1, this leads us to a dissolution enhancement. It is observed that all formulations were more than 1 values (Table 5) (Gurrapu et al., 2012). When formulations including Comp increase wettability, dissolution efficiency of formulations increased in the meantime. It is noticeable as given in Table 5 that the value of DE% 2 h was augmented for F2 and F3 formulations. Consequently, the percent dissolution efficiencies are noted considerably higher for F2, F3, F4

Table 4. Mathematical models of extended release capsules of indomethacin obtained after fitting the drug release data.

Formulations	Zero order		First order		Higuchi		Peppas	
	Slope	r^2	Slope	r^2	Slope	r^2	n	r^2
F1	5.183	0.886	-0.104	0.973	22.169	0.976	0.658	0.980
F2	7.788	0.867	-0.351	0.951	33.545	0.969	1.427	0.966
F3	6.464	0.867	-0.146	0.976	27.842	0.969	1.208	0.956
F4	6.791	0.693	-0.209	0.866	30.624	0.849	1.137	0.974
F5	6.258	0.724	-0.153	0.891	24.261	0.858	0.916	0.932

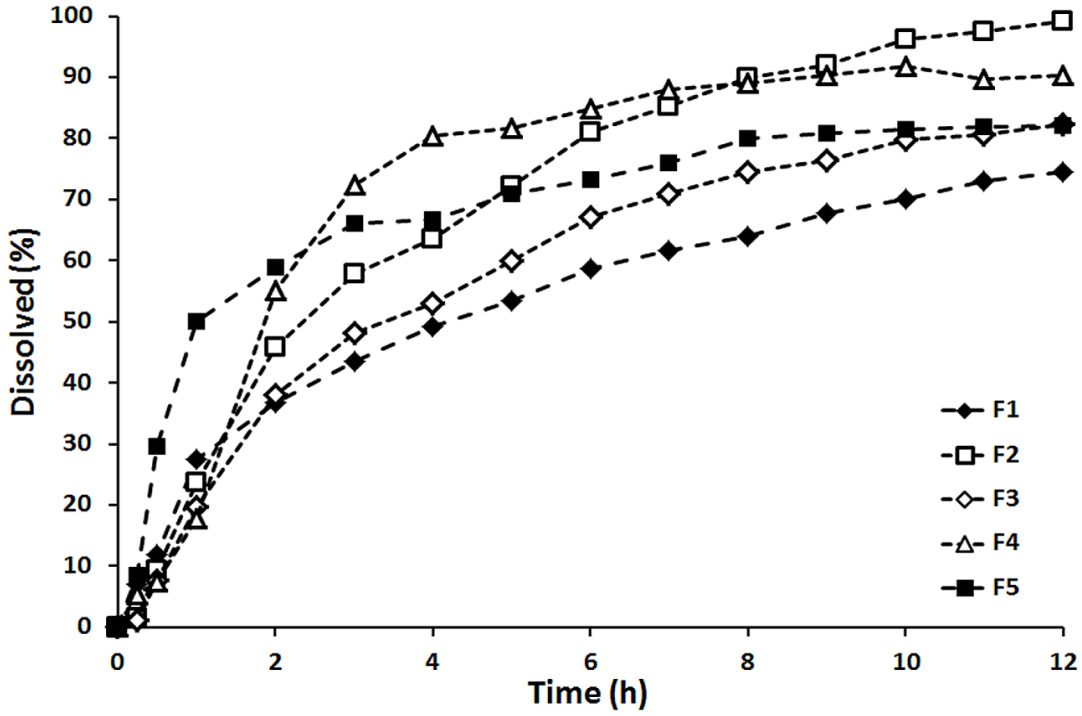

Figure 2. Comparison of release profiles using PVP and Comp by physical mixtures with pure drug (F1) (n=12). Error bars smaller than the symbols are not shown.

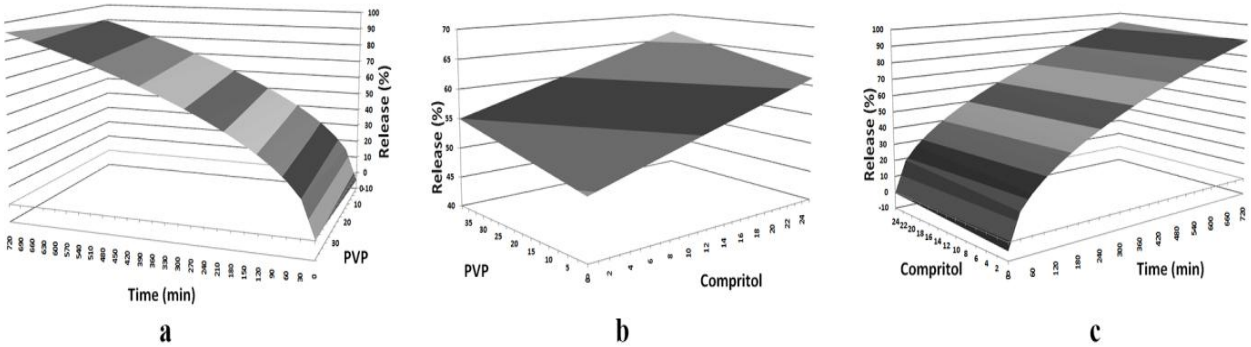

Figure 3. Response surface plot of the effect of PVP concentration and time on drug release percentage (a).and PVP concentration and Comp concentration on drug release percentage (b). and Comp concentration and time on drug release percentage (c).

Table 5. Dissolution parameters of extended release capsules of indomethacin.

Time (h)		Formulations				
		F1	F2	F3	F4	F5
1	MDT	31.45	35.20	35.20	31.23	29.10
	DE (%)	13.08	9.80	8.14	8.57	25.74
	RDR	1.00	0.86	0.72	0.65	1.82
2	MDT	46.12	61.57	61.59	70.98	38.34
	DE (%)	22.58	22.29	18.48	22.55	40.08
	RDR	1.00	1.25	1.04	1.50	1.61
4	MDT	79.39	92.46	92.46	101.71	52.12
	DE (%)	32.92	39.32	32.64	46.31	52.28
	RDR	1.00	1.30	1.08	1.63	1.36
6	MDT	115.21	136.26	136.26	112.81	72.91
	DE (%)	39.85	50.34	41.78	58.27	58.34
	RDR	1.00	1.38	1.15	1.45	1.25
8	MDT	140.14	164.69	163.76	126.44	102.55
	DE (%)	45.25	59.05	49.06	65.57	62.83
	RDR	1.00	1.41	1.17	1.39	1.25

and F5 formulations compared to F1 formulation after 6 h ($P < 0.01$) (Table 5).

At this point, it is necessary to state that MDT value is generally used to characterize the drug release rate from a dosage form and it indicates the drug release retarding efficiency of a polymer (Mohana Raghava Srivalli et al., 2013). MDT reflects the period of time for the drug to dissolve and is the first statistical data for the cumulative dissolution process that provides an accurate drug release rate (Elchidana and Deshpande, 1999). A higher MDT value indicates a greater drug retarding ability. In terms of the study, the MDT values of all formulations at 1 h were attested to be similar, which suggested a similar dissolution rate compared to F1 (IND powder). Furthermore, provided that dissolution release profiles of F4 and F5 formulations have come to a steady state after 8 h. Therefore, RDR, DE and MDT values incident to none of the formulation were taken under evaluation (Figure 1). Overall increase in the dissolution performance of the optimized formulations described in terms of dissolution parameters (MDT, DE, RDR) in comparison with F1 formulation could be due to increased wetting properties, solubility and enhanced surface area of drug particles (Kakran et al., 2012; Seedher and Kaur, 2003).

Stability of indomethacin capsules

The stability of new extended release Indomethacin capsules was investigated in terms of drug content in a time period of 3 months and the values were calculated to be at 25 ± 2°C, 65% RH and 40 ± 2°C, 75% RH. Dissolution data of stability studies on IND capsule formulations are presented at Table 6. No significant alteration was observed for the drug content values of the formulations ($P > 0.05$). The stability study has indicated that all formulations were stable at 25 ± 2°C, 65 ± 5% RH and 40 ± 2°C, 75 ± 5% RH. Herewith, the results showed that extended release IND capsule formulations could be employed as effective replacements for the conventional marketing products of IND which are currently used in the pharmaceutical area.

Conclusion

Taken together, in this study, the effect of component nature and the proportion at the release rate and the mechanism were investigated by virtue of IND extended release capsules. It was found that release results of formulations F2 and F3 were detected to be proper to the USP Drug Release Criteria. By this means, safety of the drug was improved by the usage of this new capsules. It would be an ideal formulation for 12 h release profile, they were considered to be suitable for prescribing the formulations for twice a day administration. It also indicates that these capsule formulations can be an effective dosage form for modified release formulations.

Table 6. Dissolution stability studies of extend release IND capsules.

Formulations		Time (h)			
		1	2	4	12
F2	Initial	23.71±0.08	45.74±0.05	63.96±0.06	99.22±0.04
	25°C±2	22.83±2.73	40.50±3.59	53.33±5.28	72.67±5.41
	40°C±2	26.50±3.10	52.50±2.75	63.33±4.27	81.83±1.46
F3	Initial	19.68±0.07	37.96±0.06	53.09±0.02	82.35±0.05
	25°C±2	20.33±1.97	41.67±2.87	62.50±2.81	83.83±2.27
	40°C±2	31.17±3.13	51.00±2.52	63.50±2.14	78.83±2.11

In conclusion, these findings suggest that the formulations F2 and F3 could be promising candidates for oral sustained drug delivery systems, especially for poorly soluble drugs such as IND.

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Full Length Research Paper

An audit of drug consumption and wastage during subarachnoid block

Amucheazi Adaobi Obianuju[1]*, Ajuzieogu Obinna V[1], Ezike HA[1] and Onuora CE[2]

[1]Department of Anaesthesia College of Medicine, University of Nigeria.
[2]Department of Anaesthesia, University of Nigeria Teaching Hospital.

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Our aim was to estimate the amount of anaesthetic drug wastage during spinal anaesthesia in the operation room of a tertiary hospital and suggest ways of reducing such wastage. The study is a retrospective survey conducted using the anaesthesia records. Drug wastage was considered as the amount of drug left unutilized in the vials after completion of a procedure. An estimation of the cost of wasted drug was made. There was wastage of heavy bupivacaine and lignocaine. Maximal wastage was associated with lignocaine. An equivalent of (89.9%) of lignocaine was wasted against 35.6% of prescribed bupivacaine. The cost of the wasted drugs for the duration was 121800 and 127820 Naira respectively for the period. Drug wastage and financial loss occurred during spinal anaesthesia. Gender contributed to the loss. Reduction in the amount of drugs in the vials would be prudent.

Key words: Bupivacaine, lignocaine, drug wastage, subarachnoid block.

INTRODUCTION

Current trends favour the use of regional blocks in anaesthesia where possible. Many reasons have been cited for this and include patient safety, early mobilisation and reductions in cost. The reduction in cost has important implications for patients especially in developing countries where out of pocket payment for medical services may be much more frequent than health insurance (Onwujekwe et al., 2013). This necessitates that every effort be made to reduce wasting of resources. In anaesthesia, drug waste is surprisingly common (Weinger, 2001, Nava-Ocampo, 2004). They also add to the cost of anaesthesia (Gillerman and Browning, 2000). Therefore waste reduction strategies are relevant and attempts should be made to discover subtle ways drug waste occur and deal with such promptly. Doing this will benefit the patients that pay on an out-of-pocket basis as well as centres that procure drugs for their patients with health insurance.

For this study, waste was defined as the drugs left in ampoules or vials. 0.5% hyperbaric bupivacaine comes in 4 ml ampoules in our centre while 2% lidocaine with or without adrenaline comes in 50 ml vials. The volume wasted was obtained by subtracting that administered to the patient from this known volume.

Aims/objectives

1. To assess and estimate the amount of local anaesthetic drug wastage during subarachnoid block.
2. To analyse financial implications to patients, determine reasons for such occurrence and suggest appropriate ways to minimize or prevent waste.

METHODOLOGY

This retrospective study was conducted in a tertiary care hospital that caters to a variety of surgical patients. Surgeries conducted

*Corresponding author. E-mail: adamucheazi@yahoo.com

under spinal anaesthesia for all adults 18 years and above were included in the study while those performed under local infiltration/ peripheral nerve blocks and combined spinal/epidural blocks were excluded from January to December 2012. It was not possible to obtain data from the anaesthesia charts on propofol, atropine and ephedrine dispensing and administration so these were excluded. Drugs are purchased by individual patients from the hospital pharmacy on an out of pocket basis. Therefore each patient procures all drugs and consumables required. Heavy bupivacaine (0.5% hyperbaric bupivacaine) is supplied to our hospital in 4 ml vials while 2% lidocaine with or without adrenaline comes in 50 ml vials. Spinal anaesthesia is usually instituted with 0.5% hyperbaric bupivacaine (23/25G needles are used) after local infiltration with about 5 ml plain lignocaine. For this study, drug wastage refers to drugs left in ampoules after the procedure. By surveying the anesthesia records, the study aimed to measure drug wastage during anesthesia care in the operating rooms of our teaching hospital. An estimation of the cost of wasted drug was made using the retail price of the drug in the hospital pharmacy at the time of study.

RESULTS

Four hundred and sixty seven (467) patients were documented to have had different surgical procedures performed under subarachnoid block in the hospital over the period January to December 2012. The obstetrics and gynaecology specialty had the most patients (Figure 1). Among the patients under study, 395(84.6%) were females who were administered a modal dose of 2.5 ml of 0.5% bupivacaine. The dose given to the males was higher 3 ml (Table 1). The left over drugs amongst females was 1.5 ml of heavy bupivacaine which amounts to of 592.5 ml. This represents an equivalent of 148 ampoules which would be 31.7% of prescribed ampoules per year being wasted. The retail price of each ampoule of heavy bupivacaine is seven hundred and seventy naira (₦ 770). This would have cost 148 ampoules multiplied by ₦ 770; a total of ₦ 113960. This means that for every seven hundred and seventy naira spent purchasing an ampoule of bupivacaine, there is wastage of ₦ 289 for each female patient.

The total left over among the seventy-two males was 72 ml. This is equivalent to 18(3.9%) ampoules per year which would cost ₦ 13860. This amounts to 193 naira wastage for each male patient.

Regarding plain lignocaine total of 23,350 ml were obtained by patients. 2335 ml were used and 21,015 (89.9%) were left unused which is about 420 vials of plain lignocaine. Each vial of lignocaine costs ₦290 giving a total of ₦121800. This amounts to wastage of ₦ 26 per patient.

DISCUSSION

In Nigeria, health care expenditure is usually financed on an out of pocket basis because health insurance is yet to reach optimal coverage (Onwujekwe et al., 2013). Thus, drug costs take on even more significance for the patient concerned. Current evidence suggests that wastage may comprise a large part of anesthesia costs (Gillerman and Browning, 2000). Usually, drugs including those for anaesthesia are packaged in specific quantities and cater for a wide range of patients. It may not be possible to package patient-specific volumes. The decision to use a specific volume then lies on the anaesthetists when attending to a patient.

This study examined retrospectively the consumption of local anaesthetic agents in the hospital. Intravenous and inhalational agents were excluded. This was because the study was reviewing the activities of the previous year, it would not have been possible to have the records of the drug supplied the amounts used and those discarded. One of the limitations of this study is that broken ampoules were not documented and so would have been missed thereby underestimating the wastage.

Other studies have examined drug wastage in anaesthesia. Hannah Dee (2012) conducted a prospective study regarding drug wastage and was concerned with wasted drugs in syringes, ampoules, or IV bags and also considered the wastage of unused materials such as arterial lines, hot lines, laryngeal masks, oropharyngeal and nasopharyngeal airways (OPAs and NPAs), and endotracheal (ET) tubes. The study revealed that lidocaine was among the most wasted drugs by volume (from syringes and vials) in addition to propofol, neostigmine, succinylcholine, ephedrine, and phenylphrine. They also noted a high amount of waste resulting from discarded and partially used IV bags. This study differed from ours. Ours focused on local anaesthetics while this included a wide range of drugs and consumables. However, like ours, they also noted wastage of lidocaine and bupivacaine.

Chaudhary et al. (2012) also conducted a prospective observational study that included surgeries performed under both general and regional anaesthesia. They showed that lignocaine (93% waste of lignocaine) was among the drugs that accounted for the maximum wastage and drugs amounting to Rs. 16,044.01 were wasted. This wastage of lignocaine and money agrees with our study. Our study showed that there was wastage of ₦ 289 for each female patient and ₦ 193 wastage for each male patient for each ampoule of 0.5% bupivacaine prescribed.

Our study also differed from that of Weinger (2001) who considered the drugs remaining at the end of a standard workday. However both studies are in agreement that drug wastage and inadvertent wastage of resources occurred.

Gillerman and Browning (2000) used computerised anaesthesia records to track drug prescription and administration. They recorded drug waste data for six large cost/volume drugs (propofol, Rocuronium etc) for a fiscal year and surveyed providers' knowledge of departmental drug waste. Although lignocaine and

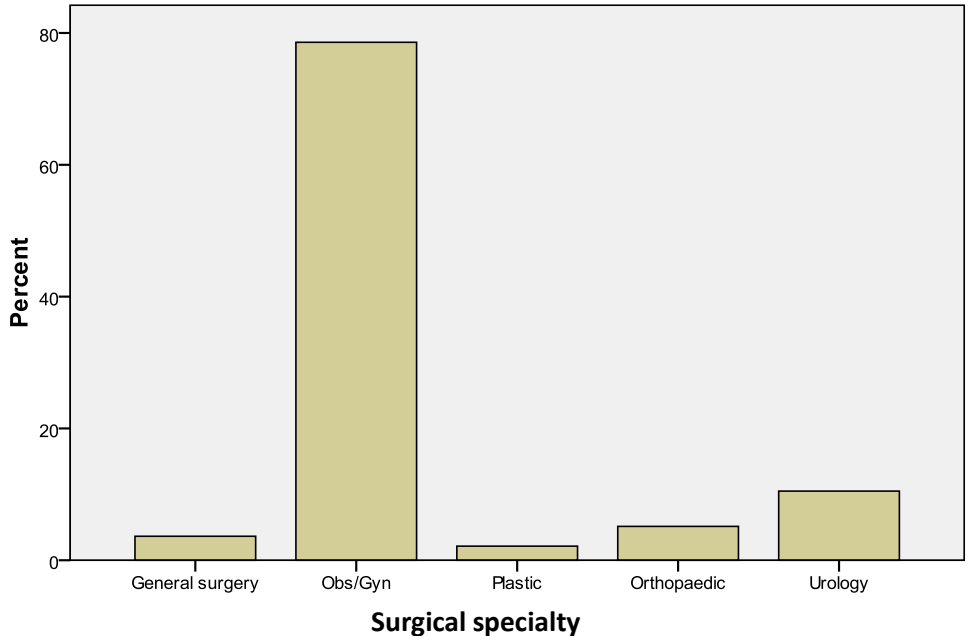

Figure 1. Showing surgical specialty.

Table 1. Table showing amount of administered bupivacaine.

Parameter		Amount of bupivacaine given			
		Frequency	Percent	Valid percent	Cumulative percent
Valid	2.5 ml	395	84.6	84.6	84.6
	3 ml	72	15.4	15.4	100.0
	Total	467	100.0	100.0	

bupivacaine were excluded, their results showed wastage of drugs.

Mankez (2012) collected his data by analyzing waste bins at the end of each procedure. This is potentially dangerous but he used protective gloves and showed that lignocaine and bupivacaine were among the wasted drugs. This agrees with our study.

It was noticed that larger volumes of heavy bupivacaine was used in males as opposed in females in whom smaller volumes were used. This calls for more studies on the reasons for such practice. From this study, there are a lot of leftover drugs because of the large volumes provided in the ampoules. Plain lignocaine was provided in 50 ml ampoules, but anaesthetists in the hospital rarely used more than 5 ml from the vial. The amount leftover per vial was 45 ml. This suggests that wastage of lignocaine would be significantly reduced if the drug was provided in smaller volumes. 5 ml or at the most 10 ml vials or if provided in pre-filled syringes. Bellefleur et al. (2009) compared the consumption and cost of ephedrine with respect to two packages-ampoules and prefilled syringes. Their result showed that use of the pre-filled syringes significantly reduced the wastage of ephedrine allowing cost minimization. By extension, using pre-filled lignocaine syringes rather than ampoules, may achieve the same results. Furthermore, regarding 0.5% (heavy) bupivacaine, a total of 664.5 ml were wasted which amount to 166 ampoules. This means that the 4 ml preparation of the 0.5% bupivacaine was never administered to a patient as a single dose. On the other hand, multispike vials can be obtained and stored at right temp and for max duration recommended while maintaining sterility.

Our findings agree with the findings of Goyal and Bhargava (2012), who studied the records of 2566 subarachnoid blocks performed over one year and found that ≤ 3 ml bupivacaine was used in 99.65% of the cases. They have questioned the need to manufacture 4 ml ampoule and have suggested that more than 3 ml of bupivacaine which is most widely used by local anaesthesia providers is not required in any ampoule/vial. There is a need to find out how other centres are working as regards minimizing drug wastage. Gillerman and Browning (2000) have suggested the use of “clean boxes” in the anesthesia workplace to reduce drug wastage. They use these clean boxes to prevent the wastage of unused drug syringes between cases. By keeping drug syringes in a sterile environment until they

are used, it may be possible to prevent the wastage of unused syringes. However, they added that such policy would require practitioners to keep careful track of the syringes in order to follow the protocols regarding the maximum amount of time drugs may be stored in syringes.

For more accurate determination of drug consumption and wastage, either a computerised drug dispensing system or using anaesthesia information management system (AIMS) need to be established in every centre. Gillerman and Browning (2000) have shown that they help to show how much of a prescribed drug is actually administered to patients.

Regular audits may also help provided the results are disseminated to the healthcare providers. Lectures and posters regarding drug cost and consumption may also be useful. Hawkes et al., (1994) have suggested raising cost awareness within hospitals. This however needs to be maintained otherwise there would be a fall in cost-reducing behaviors (Weinger, 2001). Lin and Miller (1998) studied the impact of price-labeling of muscle relaxants on cost-consciousness of anesthesiologists. In this retrospective study, they noted an increase in expenditures for less costly muscle relaxants and a decrease in expenditure for the expensive ones. Feed-back through audits or studies such as this may help in changing practice and making appropriate adjustments.

Conclusion

Drug wastage occurs during spinal anaesthesia due to the volume of drugs in the ampoules or vials. A reduction in the volume of drugs in ampoules may be beneficial. Further studies may be indicated to measure the extent of wastage of intravenous drugs and other consumables in operating suites.

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