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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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Case Report

Neurofibromatosis 1 with invasive spinal cord compression (case report)

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Accepted 17 May, 2013

Neurofibromatosis, being found as one incident in 2500-3000 births, is a genetic disease that involves either skin system, nervous system or both systems simultaneously and that leads to important cosmetic and functional disorders. Although, cervical cord compression is not frequently found in nervous system compression, nevertheless, it takes a very important place since it may lead to severe neurological deficits and it may be treated by combination of surgical intervention with physical medicine and rehabilitation in early stage. There are not many treatment options in the advanced cases. In our study, we aimed to discuss treatment approaches by evaluating a patient with complex case of neurofibromatosis in whom whole spinal cord is full of neurofibromas accompanied by cervical cord compression.

Key words: Neurofibromatosis1, spinal cord compression, physical medicine and rehabilitation.

INTRODUCTION

Neurofibromatosis type 1(NF1) is a genetically transmitted autosomal dominant disease with incidence of one in 2500-3000 births (Huson et al., 1989; Kluwe et al., 2003). It is a kind of neurocutaneous disease type and a defect of NF1 gene located on chromosome 17q11.2 is responsible for this disease (Leroy et al., 2001).

NF1 is diagnosed by the presence of two or more diagnostic criteria. Diagnostic criteria include six or more café-au-lait spots (greater than 5 mm in diameter before puberty and greater than 15 mm in diameter after puberty), two or more neurofibromas or a plexiform neurofibroma, axillary or inguinal freckles, optical glioma, two or more Lisch nodules, various bone lesions (sphenoid dysplasia, pseudoarthrosis, scoliosis) and first-degree relative diagnosed with neurofibromatosis (Apaydın et al., 1997; Washington et al., 2010). The cervical cord compression due to cervical root neurofibroma is an important clinical consideration in patients with NF type I. However, this case has been rarely reported (Leonard et al., 2007). In this study, we aimed to present a complex case of neurofibroma that filled bilateral cervical neural foramina at all levels of cervical position and caused cervical cord compression.

CASE

A 46-year-aged male patient diagnosed with neurofibromatosis type1 appealed to our clinic due to complaints of progressive fatigue in his right arm and leg, neck and right arm pain and frequent fallings. His medical history revealed that he was diagnosed of NF1 depending via tests performed for fibromas observed on his skin at 18 years old. The complaints of pain and fatigue started suddenly 7 years ago in the patient. This fatigue and pain have increased by time and affected his professional and social life. Feeling fatigue on his right leg started without pain in the recent 1 year.

Walking disturbances and fallings became frequently due to this fatigue. The family history of the patient included diagnosis of NF1 in his 1st and 2nd degree relatives. His 12-year-old son was diagnosed with optic

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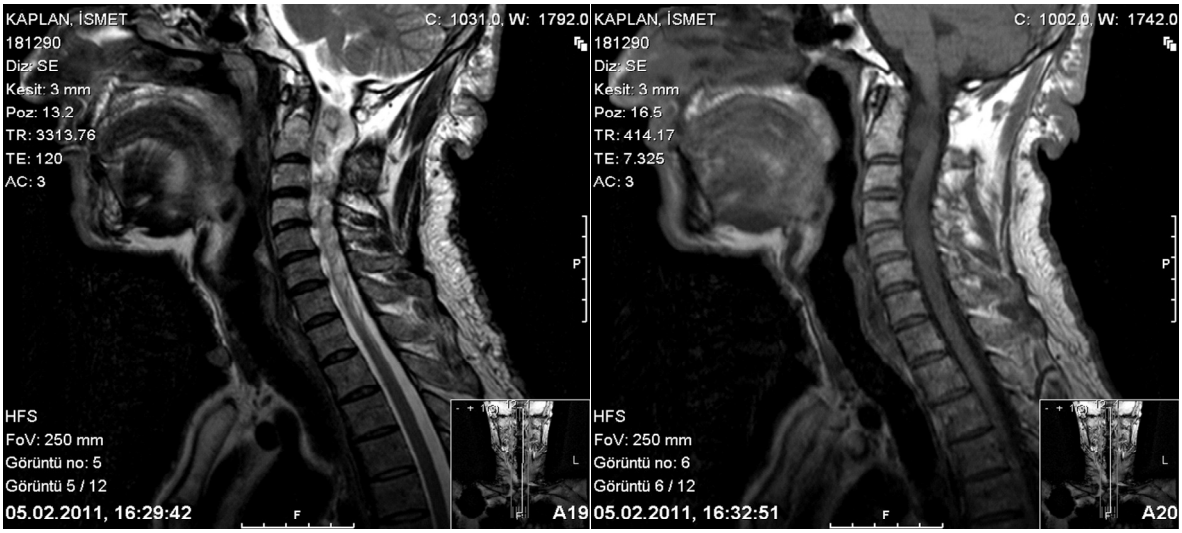

Figure 1. Spinal magnetic resonance imaging (MRI) of the case.

glioma. The patient had no additional disease. He had no use of medication and alcohol. He had smoking history of 80 pockets/year.

The physical examination of the patient revealed invasive neurofibromas in his body. The range of motion (ROM) in the cervical region which had multidirectionally active limited motion, lateral flexions and rotations were painful. The ROM of his right shoulder showed active and passive limited motion and was multidirectionally painful, his left shoulder was open and painless. The ROM of right elbow and right wrist were passive open, active limited and painful, ROM of left elbow and left wrist were open painless. Muscle strengths of upper extremities were 2/5 on right while it was normal on the left. The sensation was bilaterally normal. The deep tendon reflexes (DTR) of the upper extremities were brisk (4+). Hoffmann reflex was bilateral positive. The ROM of lumbar region was multidirectionally open and painless. The ROM of right hip was limited and painless while left hip was open painless. The knee was bilaterally open and painless. The ROM of right ankle was active limited and passive open painless. Left ankle was normal. The straight leg raising test and Trendelenburg test were negative. The muscle strength hip flexion of right lower extremity was 3/5, while knee extension was 5/5, the ankle dorsiflexion, toe dorsiflexion and foot plantar flexion were 2/5. The muscle strength was normal on the left side. The sensation of lower extremity was normal. His DTRs were bilateral corresponding (4+), Babinski was bilateral positive. The patient had clonus. The patient did not have urinary and fecal incontinence.

The laboratory evaluation revealed normal values for whole blood count, sedimentation, C-reactive protein, alkaline phophatase, parathormone, calcium, hepatic and renal function tests and whole urinary test. The performed magnetic resonance imaging (MRI) for spinal cord revealed neurofibromas that filled bilateral neural foramina at all levels of cervical region and increased T2 signaling compatible with myelomalacia causing a thinning of spinal cord at the level of cervicomedullar region and C3-C4 (Figure 1). The massive formations accompanied by neural foraminal widenings at all dorsal levels were found compatible with neurofibroma by performed MRI. The massive formations with subcentimetric dimensions in lumbar and sacral foramina and massive formations of sacral plexus compatible with extraforaminal nerve root process were found compatible with neurofibroma via Lomber MRI.

A remarkable thickening of cutaneous-subcutaneous tissue and contour lobulation were monitored in cranial MRI. The findings were found compatible with neurofibroma. No compression was encountered in the parenchyma. The patient was consulted with Neurosurgery Clinic. No surgical intervention was considered because the patient had high muscle strength loss, compression level may lead to several complications during and after operation and that recurrence probability is high.

The patient's neck, shoulder and ankle ROM were performed 2 times a day, and each set of exercises in 20 replicates. Stretching exercises were performed 2 times a day in the form of stress and rest for 10 s. And strengthening exercises, 10 repetitions were given 2 times a day. Transcutaneous electrical stimulation (TENS) applied to the shoulder and neck and neuromuscular electrical stimulation (Compex) was applied to the ankle for 30 min a day. Plastic high-intensity walking mold (PAFO) was given to prevent frequent fallings. As medication, pregabalin was initiated two times a day totally 50 mg while baclofen was initiated daily 10 mg and increased up to 30 mg.

The patient underwent 8 sessions of physical medicine

and rehabilitation programme because the patient wanted to go out of hospital. The patient reported decreased falling frequency and reduced fatigue in his right leg. However, there was no significant change in his muscle strength. The patient was discharged due to his request. The patient applied 20 sessions of radiotherapy to his cervical region 2 months later (In the Department of Radiation Oncology, Kocaeli University, Uganda). After treatment, the patient had increased muscle fatigue on his left arm and left leg and impaired walking problems during particularly when descending and climbing stairs.

DISCUSSION

NF1 has been first described by Freidrich von Recklinghausen in 1882. Neurofibromatosis (NF1, NF2, Schwannomatosis) in the United States affects more than 100,000 people. It also affects more than 2 million people worldwide (Huson et al., 2010). The majority of the cases (71%) such as our patient- had a family history. NF1 gene is found on 17th chromosome and a tumour-suppressor gene which codes the protein neurofibromine (Leroy et al., 2001; Gutmann, 2001).

Neurofibroma is one of the major characteristic features. Single or multiple cutaneous neurofibromas were observed in above 95% cases. The plexiform neurofibroma (PN) is found in 30% cases (Saltik et al., 2005). The symptoms of PN depend on localization site of the lesions and compression of neighbour structures. Intestinal/airway obstruction, findings of brain and cranial systems such as hydrocephalia, mental retardation, dementia, epilepsy, osseous changes such as lordosis, kyphoscoliosis, pseudoarthrosis and spina bifida, and endocrine disorders such as acromegaly, cretinism, hypoparathyroidism, myxedema and pheochromacytoma may be observed due to plexiform neurofibromas (Huson et al., 2010; Bayramgürler et al., 2003 ; Ragge 1993). (It has been reported that majority of the adult patients with cervical plexiform neurofibroma are asymptomatic cases however cervical plexiform neurofibroma may also lead to massive formations, pain and neurological deficits in the neck (Ward et al., 1994). Our case had a plexiform neurofibroma which involved whole spinal cord accompanied by multiple skin neurofibroma and caused spinal cord compression at the cervical level.

İn patients with NF1, the cervical cord compression occur due to compression of neurofibromas to the cervical nerve root in the patients diagnosed NF1. However, they have not been frequently reported (Leonard et al., 2007; Créange et al., 1999). Physical therapy is useful in mild cases. The duration of physical therapy programme which is effective may be changed for patients. Severe cases may require surgery which is the pressure of the spinal cord (Taleb et al., 2011; Craig and Govender, 1992; Tonogai et al., 2008)

Leonard et al. (2007) have reviewed approximately 1500 patients with NF1 followed-up by two major centers between the years 1996 to 2006 in their retrospective study. The cervical cord compression was detected in 13 patients aged between 9 and 61 years. The cervical cord compression was at the levels of C2 and C3 in majority of the patients. The 7 patients had progressive quadric-paresis while totally 3 patients had paraparesis, found at lower extremity in 2 patients and at upper extremity in 1 patient, also incontinence was detected in 1 patient while 3 patients without kyphotic deformity had complaints of cervical pain. The 11 of 13 patients were had undergone a single and multiple-level cervical laminectomy and a subtotal resectomy for neurofibromas. A secondary operation was required in 2 patients by the advancing time (Leonard et al., 2007).

Créange et al. (1999) have reviewed approximately 158 patients with NF1 and they found spinal cord compression or cauda equina syndrome in five patients. Despite the surgery, two patients with cervical cord compression developed quadriplegia and one of them died. Quadriplegia and neurological sphincter dysfunction were developed in two patients because of cauda equina syndrome. İntraspinal neurofibromatosis did not show any progression in 1 patient for 7-year follow-up (Créange et al., 1999). Sarıca et al. (2008) have present-ed a 32-year-old case diagnosed NF1 with quadriparesis who had neurofibromas along whole spinal cord and cord compression at the level of C4-C5 and who had undergone a partial resection at the level of C3 and a total resection at the levels of C4,C5, C6. Postoperatively, clinical course of the patient significantly improved by application of physical rehabilitation and myorelaxant medication (Sarica et al., 2008).

In the present study, the patient had hemiparesis differently from the cases reported above. An operation was not decided in this patient considering the level of cervical cord compression, advanced clinical condition and postoperative recurrence probability. Duong et al. (2011) have made a retrospective study between 1980-2006 and examined 1895 patients with NF1. Death rate due to NF1 was investigated and found that 56 patients died due to complications of NF1. Spinal cord compressions was found in 3% as part of the complications (Duong et al., 2011). Helmers and Irwin (2009) have reported a 17-year-old case with NF1 with complaints of cervical pain and headache who had 4/5 extremity muscle strength and was treated with 20 sessions of cervical stabilization, posture exercises, stretching, interferential current and US during 13 weeks. The muscle strength of the patient increased up to 4+/5 and his complaints significantly decreased (Helmers and Irwin 2009). Although, the case in the present study could not continue physical medicine for more than 8 sessions because of social reasons, nevertheless, he expressed that frequency of fallings and fatigue of lower extremity decreased at the end of this process. We conclude that non-significant change in muscle strength resulted from high loss of muscle strength, inability to eliminate the compression causing this clinical condition and necessity

of earlier onset and longer duration of physical medicine.

Conclusion

Conclusively, surgical intervention should be decided on the patients with NF1 accompanied with spinal cord compression considering compression level, invasion grade and clinical condition of the patient if a neurological deficit is present. The best outcomes are obtained when the patient has minimal preoperative neurological deficit. Also preoperative and postoperative treatment modalities are the important instruments in reversing muscle strength loss, reducing pain and improvement of the normal daily activities of the patient depending on the clinical condition.

ABBREVIATIONS

NF1, Neurofibromatosis type 1; **ROM,** range of motion; **DTR,** deep tendon reflexes; **MRI,** magnetic resonance imaging; **TENS,** transcutaneous electrical stimulation; **PN,** plexiform neurofibroma.

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Full Length Research Paper

Evaluation of the antioxidant properties of *Vitis vinifera* juice extract in rifampicin dispersions

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The purpose of this study was to evaluate the antioxidant properties of *Vitis vinifera* juice extract (grape) in rifampicin suspensions and to compare with that of ascorbic acid. Grape juice was extracted and used as antioxidant in both pure sample and commercial reconstituted samples of rifampicin. The rate of drug degradation was determined by spectroscopic method in a time dependent manner. Also, the antioxidant property of grape juice was compared with that of ascorbic acid. Phytochemical screening of the constituents in grape juice extract was carried out according to standard methods. The results revealed that rifampicin dispersions containing ascorbic acid (batches A1 to A5) showed significantly higher stability and had higher amount of drug remaining over time than the control (batch A6) containing no antioxidant ($p < 0.05$). However, the results revealed that grape fruit juice extract had higher antioxidant properties than the ascorbic acid ($p < 0.05$) in rifampicin dispersions. The results also indicated that increase in amount of both antioxidants significantly reduced the rate of drug degradation through oxidation, thereby causing a corresponding increase in the amount of rifampicin remaining over time. Grape juice extract contain alkaloids, carbohydrates, saponins, reducing sugars, steroids, tannins, proteins, flavonoids, resins, oils, terpenoids and acid compounds. Glycosides were however not found in the juice extract. Therefore, grape fruit juice extract could be used as a natural antioxidant in rifampicin suspensions.

Key words: *Vitis vinifera,* grape juice, antioxidant, rifampicin, suspensions.

INTRODUCTION

Natural antioxidants have gained considerable interest in recent years for their role in preventing the auto oxidation (El- Hawary et al., 2012). *Vitis vinifera* L. (grapes) (Vitaceae) is considered as a natural antioxidant source and contains many chemical constituents such as phenolic acids, flavonoids, anthocyanins, proanthocyanidins, sugars, sterols, amino acids, and minerals (Sokar, 1991; El- Hawary et al., 2012). Grapes are also used as demulcent, laxative, refrigerant, stomachic and diuretic. Moreover, it is useful in bilious dyspepsia, haemorrhage, dysuria, in chronic bronchitis, heart diseases and gout. Grape juice is given to children to prevent constipation (El- Hawary et al., 2012; Bunea et al., 2012). Dried grapes or raisins are useful in thirst attendant on fevers, cough, catarrh, jaundice, and in sub-acute cases of enlarged liver and spleen. The pharmacokinetics of grapefruit revealed that fruit have a potent inhibitory effect on the intestinal cytochrome P450 system. For this reason, grapefruit has a high potential for interaction with drugs (Ulbricht and Seamon, 2010). The grape berries are important since they are consumed as fruits, wine, juice or raisins and are largely cultivated for the wine

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industry. Antioxidant activities of grapes are due to the presence of antioxidant components such as flavonoids, phenolic acids, anthocyanins and carotenoids (Guedes et al., 2001; Lutz et al., 2011; El-Hawary et al., 2012; Bunea et al., 2012).

Antioxidants are molecules capable of slowing or preventing the oxidation of other molecules (Hamid et al., 2010). Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells (Hamid et al., 2010). Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Mendes-Pinto et al., 2004; Sies, 1997; Mulero et al., 2010; Hamid et al., 2010; Vinkovic et al., 2011). Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. Free radical damage may lead to cancer. Examples of antioxidants include beta-carotene, lycopene, vitamins A, C, E and other substances (Sies, 1997; Hamid et al., 2010).

Rifampicin is the drug of choice in treatment of tuberculosis. Also, it is effective in treatment of various bacterial infections. Rifampicin is bactericidal agent against wide range of microorganism (Lubna et al., 2006; Dhanapal et al., 2012). It is slightly soluble in water, thus is suitable for suspension dosage form (Lubna et al., 2006). Rifampicin undergoes oxidation when exposed to light, air or moisture and hence requires incorporation of suitable antioxidant and should also be stored under nitrogen in an airtight container, protected from light, at a temperature not exceeding 25°C (BP, 2009). The aim of the work is to evaluate the antioxidant properties of grape fruit juice in rifampicin dispersions and to compare the antioxidant properties of grape juice with that of ascorbic acid in rifampicin suspensions.

MATERIALS AND METHODS

Rifampicin, hydrochloric acid (Sigma-Aldrich, Germany), ascorbic acid (BDH, Poole, England), distilled water from (UNN Water Resources Management Laboratories Ltd., UNN, Enugu State, Nigeria), and *V. vinifera* (grape) juice was processed in our laboratory. All the chemicals used were of analytical grade and was used as supplied without further purification.

Processing of grape juice

V. vinifera (grape) fruits were purchased from Ogige market Nsukka, in Enugu state, Nigeria in the month of February, 2008. The fruit was identified by a plant taxonomist, Mr. P.O. Ugwuozor of the herbarium section of Botany Department, University of Nigeria, Nsukka. The fruits were washed and peeled to remove the bark. The juice was squeezed out by hand, and filtered using a non adsorbent filter paper (Whatman no. 1). The extract was later preserved with 30 % alcohol in a bottle and stored at room temperature (25°C) until used.

Phytochemical analysis

Phytochemical tests were carried out on the grape juice extract for the presence of alkaloids, saponin, steroids, terpenoids, glycosides, acidic compounds, carbohydrates, reducing sugars, tannins, proteins, fats and oil. The tests were carried out using standard procedures of analysis (Harborne, 1993; Sofowora, 1993; Trease and Evans, 2002).

Test for stability of rifampicin using ascorbic acid as the antioxidant

A 5 mg quantity of rifampicin was dispersed in 100 ml of distilled water, different concentrations of ascorbic acid was incorporated into the dispersions as presented in Table 1. The rifampicin dispersions were stored at room temperature (25°C) until used. The concentration of rifampicin remaining in the dispersion was determined in time dependent manner 1, 4, 8 and 12 days using a spectrophotometer (Jenway 6305, UK). The absorbance readings were recorded and the concentration of rifampicin in sample at any given time was calculated with reference to Beer's plot previously obtained for rifampicin at concentration range of 0.2 to 1 mg/% in 0.1 N HCl at a predetermined wavelength of 470 nm. The test was repeated using grape fruit extract as the antioxidant at concentrations listed in Table 1. Also, commercial reconstitutable samples of rifampicin was reconstituted with distilled water, ascorbic acid (batches C1 to C5) and grape fruit (batches D1 to D5), respectively was added to the dispersions and the amount of rifampicin remaining in each sample was also determined spectrophotometrically in time dependent manner with reference to Beer's plot.

Data and statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) and differences in means were assessed using a two tailed student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Stability of rifampicin dispersions

The results of the percentage amount of rifampicin remaining in the dispersion are shown in Figures 1 to 4. The results show that batch A1 containing 10 mg of ascorbic acid had 52.5 and 18.8% rifampicin remaining at 1 and 12 days, respectively while batch A5 containing 30 mg of ascorbic acid had 52.5 and 32.9% of rifampicin. However, batch A6 containing no antioxidant had 32.5 and 12.9% at 1 and 12 days, respectively as shown in Figure 1. Also, (Figure 2) batch B1 containing 2.8 ml of grape fruit had 68.8 and 3.3% rifampicin remaining at 1 and 12 days, respectively. Batch B4 containing 7 ml of grape juice had 77.9 and 54.6% rifampicin remaining at 1 and 12 days, respectively while 95.8 and 69.2% rifampicin were remaining at 1 and 12 days, respectively

Table 1. Contents of rifampicin preparations.

Batch	Rifampicin (mg)	Ascorbic acid (mg)	Grape juice (ml)	Distilled water q.s. (ml)
A1	5.00	10.00	-	100
A2	5.00	15.00	-	100
A3	5.00	20.00	-	100
A4	5.00	25.00	-	100
A5	5.00	30.00	-	100
A6	5.00	0.00	-	100
B1	5.00	-	2.80	100
B2	5.00	-	4.20	100
B3	5.00	-	5.60	100
B4	5.00	-	7.00	100
B5	5.00	-	8.40	100
B6	5.00	-	0.00	100

Key: A1, A2, A3, A4 and A5 contain ascorbic acid 10, 15, 20, 25, 30 mg, respectively as the antioxidant, while A6 has no antioxidant. B1, B2, B3, B4 and B5 contain grape fruit juice 2.8, 4.2, 5.6, 7.0 and 8.4 ml, respectively as the antioxidant, while B6 has no antioxidant.

Table 2. Results of phytochemical constituents of *Vitis vinifera* juice.

Constituent	Remark
Alkaloids	+++
Saponins	+++
Reducing sugar	++
Tannins	+++
Carbohydrates	+
Fat and oil	+
Flavonoids	+++
Resins	+++
Steroids and terpenoids	+++
Proteins	+
Glycosides	-
Acid compounds	+++

+++ = Present in high quantity, ++ = present in moderate quantity, + present in low quantity, - absent.

for batch B5 formulated with 8.4 ml of grape juice. However, the negative control containing no grape juice (batch B6) exhibited 32.5 and 12.9% rifampicin remaining at 1 and 12 days, respectively.

Also batches C1 to C5 (Figure 3) formulated with commercial rifampicin powder for reconstitution and containing 10 to 30 mg of ascorbic acid showed significant reduction in the amount of rifampicin remaining with time just like the rifampicin pure samples as above, however batches D1 to D6 (Figure 4) also formulated with commercial rifampicin powder for reconstitution, and containing grape fruit juice had 100% amount of drug remaining at 12 days for batches C4 and C5 as shown in Figure 4.

Phytochemical constituents of grape juice

The results of phytochemical constituents of *V. vinifera* (grape) juice are shown in Table 2. The results revealed the presence of alkaloids, saponins, tannins, flavonoids, resins, acidic compounds, steroids and terpenoids in very high quantity. Reducing sugars were found in moderate quantity, while carbohydrates, proteins, fat and oil were found in low quantity. Glycosides were however not found.

DISCUSSIONS

Stability of rifampicin dispersion

The results of antioxidant effect of ascorbic acid in rifampicin dispersions studied over time show that ascorbic acid exhibited good antioxidant properties. However, rifampicin dispersions containing ascorbic acid (batches A1 to A5) showed higher stability ($p < 0.05$) and amount of drug remaining over time than the control (batch A6) containing no antioxidant. Also, the results of the antioxidant properties of grape fruit juice revealed that it had significantly higher antioxidant properties than the ascorbic acid ($p < 0.05$) in rifampicin dispersions as shown in Figures 2 and 4. The results also indicated that increase in amount of both antioxidants significantly reduced the rate of drug degradation through oxidation, thereby causing a corresponding increase in the amount of rifampicin remaining over time.

The antioxidant activities of grapes are due to the presence of antioxidant components such as flavonoids, phenolic acids, anthocyanins and carotenoids (El-Hawary et al., 2012; Bunea et al., 2012). These constituents

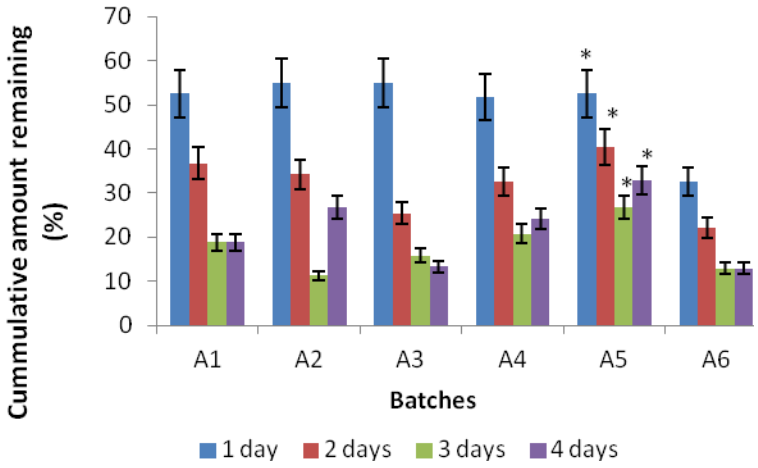

Figure 1. Plot of amount of pure sample of rifampicin remaining with time; A1, A2, A3, A4 and A5 contain ascorbic acid 10, 15, 20, 25, 30 mg respectively as the antioxidant, while A6 has no antioxidant; *statistically significant at $p < 0.05$ compared to control.

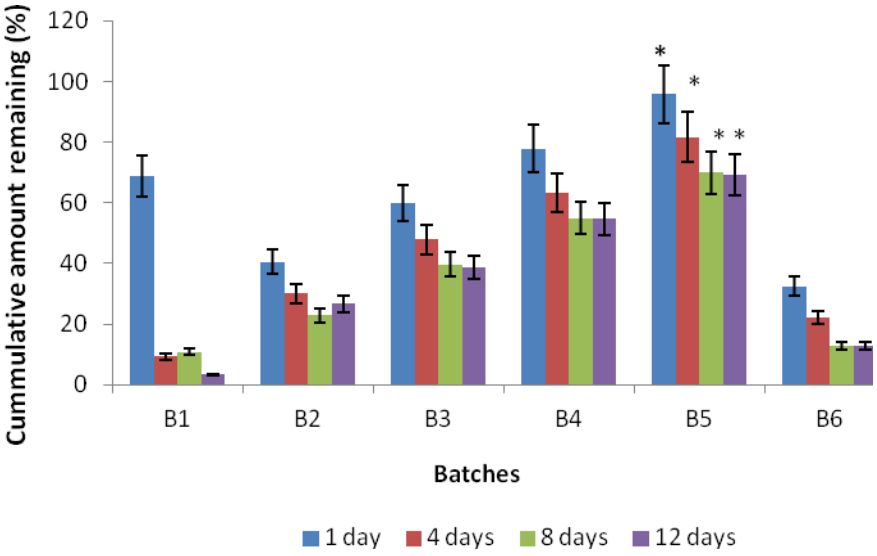

Figure 2. Plot of amount of pure sample of rifampicin remaining with time; B1, B2, B3, B4 and B5 contain grape fruit juice 2.8, 4.2, 5.6, 7.0 and 8.4 ml, respectively as the antioxidant, while B6 (control) has no antioxidant; *statistically significant at $p < 0.05$ compared to control.

terminate chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves (Sies, 1997; Hamid et al., 2010). Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. This natural antioxidant have several advantages over synthetic antioxidants, grape fruit juice is an edible fruit juice therefore, it is physiologically inert hence, may have better biocompatibility over synthetic antioxidants, it is relatively cheap and readily available compared to most antioxidants used in drug formulation. These have led to sudden interest in grape juice as antioxidant in drug formulation.

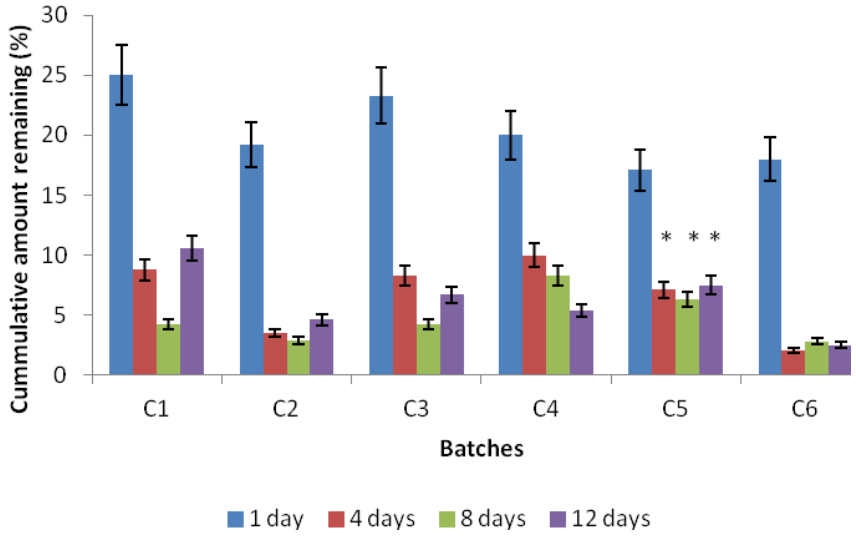

Figure 3. Plot of amount of rifampicin commercial sample (reconstitution) remaining with time; C1, C2, C3, C4 and C5 contain ascorbic acid 10, 15, 20, 25, 30 mg, respectively as the antioxidant, while C6 has no antioxidant (control); *statistically significant at $p < 0.05$ compared to control.

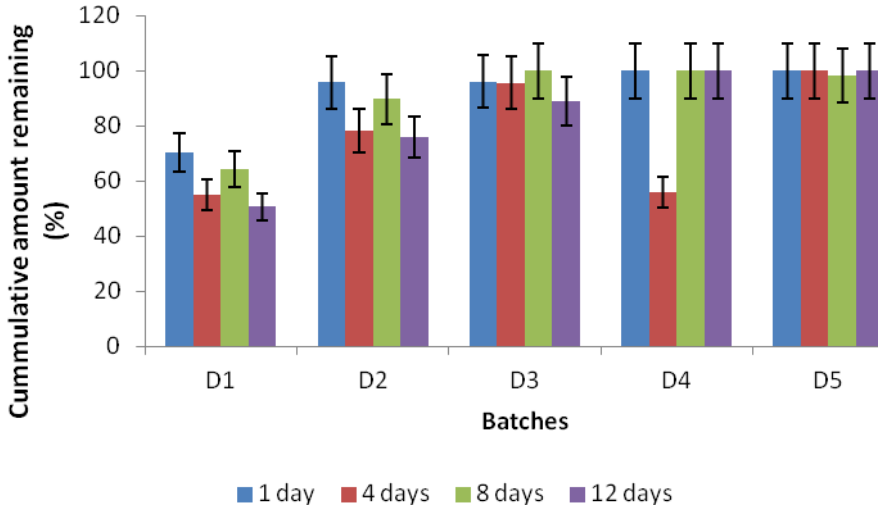

Figure 4. Plot of amount of rifampicin commercial sample (reconstitution) remaining with time; D1, D2, D3, D4 and D5 contain grape fruit juice 2.8, 4.2, 5.6, 7.0 and 8.4 ml, respectively as the antioxidant.

Phytochemical constituents of grape juice

The results of the phytochemical screening of the grape juice indicate the presence of very important phyto-chemicals at different concentrations. The juice extract contain alkaloids, carbohydrates, saponins, reducing sugars, steroids, tannins, proteins, flavonoids, resins, oils, terpenoids, acid compounds, but glycosides were however, not found in the juice extract. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plants produce these chemicals substances to protect themselves, and they are also believed to protect humans against certain diseases (Edeoga et al., 2005).

Conclusion

Grape fruit juice extract was successfully used as an antioxidant in rifampicin dispersions. The results revealed

that grape juice had significantly higher antioxidant properties than ascorbic acid in rifampicin dispersions. Natural antioxidants such as grape juice extract may have advantages over the synthetic and semi synthetic ones due to the fact that they are edible fruits, relatively inexpensive, better biocompatibility among others. More research into this field of study is however required in order to scale up all its aspect.

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Full Length Research Paper

Evaluation of mechanism of hepatotoxicity of leflunomide using albino wistar rats

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Leflunomide (LEF) is used for the treatment of rheumatoid arthritis via inhibition of dihydroorotate dehydrogenase and tyrosine kinase enzymes. It is metabolised by cytochrome 2C9 enzyme to active form A771726 (melononitrilamide) which is responsible for both its pharmacological and toxicological responses. Food and Drug Administration (FDA) gave black box warning in 2010 due to acute liver failure in humans. While the exact mechanism of its toxicity remains unknown, it has been postulated that the formation of toxic reactive metabolites and elevation of serum transaminase level may be responsible for its toxicity. The purpose of this study is to investigate whether oxidative stress has any role in inducing hepatotoxicity of this drug. LEF was administered orally in 10 mg/kg body weight to albino wistar rats. Then, liver and blood were collected at 4, 8, 12 and 16 h intervals. Liver glutathione (GSH), oxidized lipid (malonaldehyde MDA), superoxide dismutase (SOD), catalase (CAT) and plasma alanine transaminase (ALT), aspartate transaminase (AST) levels were measured to determine the level of toxicity. GSH, CAT and SOD levels were found to be decreased with respect to control at all time points, whereas MDA level was significantly increased which signified liver cell injury after drug administration. The plasma ALT and AST levels were also increased at the same time, denoting liver tissue damage. Our result collectively indicated that oxidative stress might be responsible for LEF inducing hepatotoxicity.

Key words: Leflunomide, A771726 metabolite, hepatotoxicity, oxidative stress.

INTRODUCTION

Leflunomide (LEF, Figure 1) is used for the treatment of rheumatoid arthritis since 1998 and this action is mediated through inhibition of dihydroorotate dehydrogenase and tyrosine kinase enzymes (Alcorn et al., 2009; Fox 1998). On the other hand, this action is triggered through inhibition of T- and B-lymphocytes proliferation *in vitro* (Cutolo et al., 2009). Researcher proposed that LEF induces tumor necrosis factors-α and interleukin-1β factors during its pharmacological response (Déage et al., 1998). Cytochrome 2C9 is the key enzyme which metabolized LEF to its active form A771726 (melononitrilamide, major), 4-trifluoroaniline and other minor metabolites (Rozman, 2002). Food and Drug Administration (FDA) categorised this drug through black box warning in 2010 due to its acute liver failure in humans (FDA Drug Safety Communication, 2010). The

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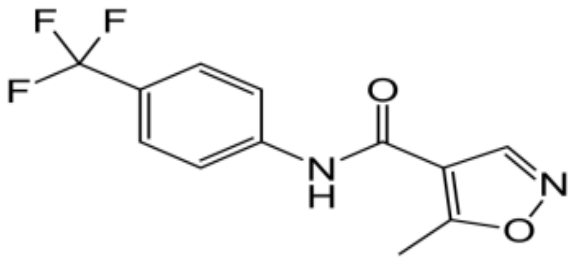

Figure 1 Chemical **s**tructure of LEF

exact nature of its acute liver failure inducing effect has not been completely established but several mechanisms have been proposed. One reason may be the formation of toxic reactive intermediates due to increasing activity of cytochrome 2C9 enzymes during metabolism (Yuyuan and Xuqing, 2010). Researcher also proposed that elevation of 10% serum transaminase level during LEF metabolism may be another reason of liver cell damage (Yuyuan and Xuqing, 2010; Gupta et al., 2011). At the same time, Shi et al. (2011) suggested that cytochorme P450 enzymes might be responsible for cytotoxicity in primary culture rat hepatocytes. Therefore, the question arose whether LEF produced hepatotoxicity via any other mechanisms in humans without formation of toxic reactive metabolites. To get the answer, we performed experiment where LEF was given orally to albino wistar rats in a single dose. Various biochemical parameters are measured at varying time intervals to evaluate the mechanism of LEF induced hepatotoxicity.

Glutathione (GSH), oxidised lipid (malonaldehyde, MDA), tissue catalase (CAT), superoxide dismutase (SOD) in liver and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were measured to evaluate the mechanism of toxicity. It is demonstrated for the first time that LEF produced liver toxicity at normal dose in albino rats.

MATERIALS AND METHODS

LEF was kindly donated by IPCA Laboratories Ltd. Rampur, Dehradun. Disodium ethylenediaminetetra acetic acid (EDTA), sodium citrate, disodium hydrogen phosphate, trichloroacetic acid, potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from SD Fine Chemicals, Mumbai, India. Glacial meta phosphoric acid, 5,5-dithiotris-2-nitro benzoic acid, tris buffer, sodium carbonate, sodium chloride, sodium potassium tartarate were purchased from Loba Chemicals, New Delhi, India. All other chemicals were obtained from Himedia, Mumbai, India. All chemicals and solvents were of analytical grades and double distilled water was used throughout the experiment.

Animals and dose schedule

Albino Wistar rats of both sexes (weight 90 to 140 g) were purchased from Animal House, CARPS, Shobhit University, Meerut, India (Registration No. 1279/AC/09/CPCSEA, approval date 05.03.2012) and were kept in polypropylene cages under standard conditions of temperature (25 ± 1°C) with 12 h light and dark conditions, diet and water *ad libitum* for seven days. Animals were randomized and divided into the following two groups, control and test (six in each group). LEF was suspended in 1% sodium carboxy methyl cellulose given to test animals according to 10 mg/kg body weight. Blood samples were collected after 4, 8, 12 and 16 h of drug treatment from Retro orbital plexus and then 6 rats from each group were sacrified by the cervical dislocation at the above given time points. Livers were removed and kept in -20°C for further biochemical assays. Blood samples were taken and centrifuged at 2,500 g for 30 min. Plasma was separated, kept at -20°C and subjected to biochemical estimations.

Biochemical estimations

Plasma was subjected to AST, ALT analysis (Reitman and Frankel, 1957). Liver sample was further analysed for total GSH (Sedak and Kubdsay, 1968), CAT (Ellman, 1959), SOD (Claiborne, 1985) and MDA (Ohkawa et al., 1979) contents in tissues.

Plasma ALT

ALT was estimated according to method described by Reitman and Frankel (1957), with slight modifications and performed by using ALT assay kit obtained from Excel Diagonistic Pvt. Ltd., Hyderabad, India. Working solution was prepared by dissolving 4 ml of reagent 1 (mixture of L-alanine and α-ketoglutarate) and 1 ml of reagent 2 (mixture of nicotinamide adenine di neucleotide phosphate, NADP and lactate dehydrogenase, LDH) and kept at 2 to 8°C for future use. The whole assay was performed in 96-well plate and each plate contained 0.5 ml of working solution and 0.05 ml of plasma. This was incubated for 1 minute at 37°C and change in optical density (ΔA_{340}/min) was measured per minute for the next 3 min. Data was calculated by the following equations:

$$\Delta A_{340}/\text{min} = [A_{340}\ (\text{Time 2}) - A_{340}\ (\text{Time 1})] / [\text{Time 2 (min)} - \text{Time 1 (min)}]$$

$$\text{ALT Activity (unit/ml)} = \Delta A_{340}/\text{min} \times 1746 \times 10^{-3}$$

Plasma AST

AST was estimated according to method described by Reitman and Frankel (1957), with slight modifications and performed by using AST assay kit obtained from Excel Diagonistic Pvt. Ltd., Hyderabad, India. Working solution was prepared by dissolving 4 ml of reagent 1 (mixture of L-aspartate and α-ketoglutarate) and 1 ml of reagent 2 (mixture of NADP and malonate dehydrogenase, MDH) and kept at 2 to 8°C for future use. The whole assay was performed in 96-well plate and each plate contained 0.5 ml of working solution and 0.05 ml of plasma. This was incubated for 1 min at 37°C and change in optical density (ΔA_{340}/min) was measured per minute for the next 3 min. Data was calculated by the following equations:

$$\Delta A_{340}/\text{min} = [A_{340}\ (\text{Time 2}) - A_{340}\ (\text{Time 1})] / [\text{Time 2 (min)} - \text{Time 1 (min)}]$$

$$\text{ALT Activity (unit/ml)} = \Delta A_{340}/\text{min} \times 1746 \times 10^{-3}$$

Tissue GSH

Tissue GSH content was estimated by the method of Sedak and Kubdsay (1968), with slight modifications. 0.2 ml of 10% tissue homogenate suspension medium was taken in a tube and 1.8 ml of distilled water was added to it. 3.0 ml of precipitating solution (1.67 g of glacial meta phosphoric acid, 0.2 g disodium ethylenediaminetetra acetic acid and 30 g of sodium chloride in 100 ml distilled water) was added to the above mixture. The mixture was then allowed to stand for approximately 5 min and then filtered. 2.0 ml of the filtrate was added to 8.0 ml of the phosphate solution (0.3 M). 1 ml 0.4% 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added to it, vortex-mixed and centrifuged at 13,000 rpm for 1 min. A blank solution was prepared in the above mentioned procedure where tissue sample was absent. The Optical density (OD) was measured at 412 nm. The data was calculated by the following procedure:

GSH (µM/mg of protein) = 310.4 × E_i × OD/mg of protein

Where O.D. at 412 nm and E_i is correction factor (0.542).

Tissue MDA

Tissue MDA assay was performed by the following procedure of Ohkawa et al. (1979), with slight modification. 1 ml of 10% tissue homogenate, 0.5 ml of 30 % trichloroacetic acid and 0.5 ml of 0.8% thiobarbituric acid were taken in a test tube and covered with aluminum foil and kept in a shaking water bath for 30 min at 80°C. After 30 min, tubes were taken out and kept in ice-cold water for 15 min. They were then centrifuged at 3,000 rpm for 15 min. The absorbance of supernatants was read at 540 nm at room temperature against appropriate blank. Blank consisted of 1.0 ml distilled water, 0.5 ml of 0.8% TBA solution, and 0.5 ml of 30% TCA solution. In a separate experiment, total protein content of the sample was estimated by bicinchotannic acid (BCA) assay kit using bovine serum albumin (BSA) as standard. The amount of MDA present in a sample was calculated according to the equation:

nM of MDA/mg of protein = (V × OD at 540 nM) / (0.56 × protein concentration)

Tissue CAT

Livers were homogenized in 50 mM stock solution of potassium phosphate buffer with a weight to volume ratio of 1:10. The homogenates were centrifuged at 10,000 rpm for 20 min. 50 µl of supernatant was added to a cuvette containing 2.95 ml of 19 mM/Liter solution of hydrogen peroxide (H_2O_2) prepared in potassium phosphate buffer. Disappearance of H_2O_2 was monitored at 1 min interval for 3 min at 240 nm. Catalase activity was calculated as:

nM of H_20_2/min/mg of protein = (ΔA/min × volume of assay) / (19 × volume of sample × mg of protein)

Tissue SOD

The supernatant was assayed for superoxide dismutase activity by following the inhibition of pyrogallol auto oxidation. 100 µl of cytosolic supernatant was added to tris HCl buffer, pH 8.5. The final volume of 3 ml was adjusted with the same buffer. At last, 25 µl of pyrogallol was added and changes in absorbance at 420 nm were recorded at one minute interval for 3 min. The increase in the absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD. One unit of SOD is described as the amount of enzyme required, causing 50% inhibition of pyrogallol auto oxidation per 3 ml of assay mixture and is given by the formula:

Unit of SOD/mg or protein = [100 × {(A - B) / (A × 50)}]/mg of protein

Where A = change in absorbance per minute in control and B = change in absorbance per minute in test sample.

Statistical analysis

Statistical analysis was carried out using Graph Pad Prism 5.0 (Graph Pad Software, San Diago, CA). All results were expressed as Mean ± standard deviation (SD). The data was analysed by one-way analysis of variance (ANOVA) followed by Bonferrori multiple comparison test, and statistically significant data was accepted when $p < 0.05$.

RESULTS

Tissue GSH

Tissue GSH level in the control group was found to be 21.70 ± 0.89 µM/mg of protein (Figure 2, Table 1). There was significant decrease in the GSH level in the LEF-treated rats beyond 4 h, which were lowest at 12 h post-treatment (16.43 ± 3.16 µM/mg of protein). Statistically significant differences were observed between control and 8, 12, 16 h treated groups (one-way ANOVA, $p < 0.05$).

Tissue MDA

Lipid peroxidation assay is another indicator for oxidative stress. Total MDA was measured in both control and LEF-treated rats at varying time points. According to the data presented in Figure 3, (Table 1), it could be observed that MDA concentration was ~ 0.08 nM/mg of protein in control group whereas it increased to ~0.21 nM/mg of protein at 16 h post LEF treatment. Significant increase in MDA concentration was observed at and above 4 h treatments. Statistically significant differences were observed between control and 8, 12, 16 h treated rats (one-way ANOVA, $p < 0.05$).

Tissue CAT

CAT enzyme is most abundant in the liver which is responsible for the catalytic decomposition of hydrogen peroxide (H_2O_2) to oxygen and water. The H_2O_2 level was measured in control and LEF-treated rats at varying time

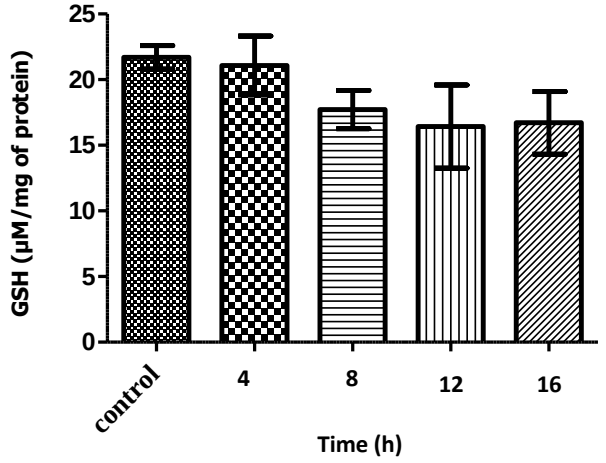

Figure 2. Effect of LEF on GSH level in liver with respect to control at varying time points in albino wistar rats (n = 6). GSH level were significantly reduced at and above 8 h treated rats with respect to control. Statistically significant differences were observed between control and 8, 12, 16 h treated groups (one-way ANOVA, $p < 0.05$).

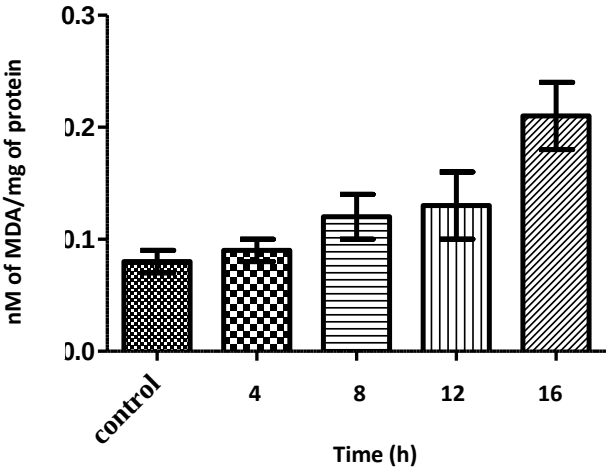

Figure 3. Effect of LEF on lipid oxidation (MDA) in liver with respect to control at varying time points in albino wistar rats (n = 6). MDA level was found to be increased at and above 8 h treated rats with respect to control. Statistically significant differences were observed between control and 8, 12, 16 h treated rats (one-way ANOVA, $p < 0.05$).

points. As could be observed from the data given in Figure 4, Table 1, there was increase in the concentration of the H_2O_2 from ~19 nM/min/mg of protein in control to ~126 nM/min/mg of protein at 16 h after LEF treatment. Although there was no change in H_2O_2 concentration at 4 h time point, its concentration was doubled in 8, 12 h, and four times higher in 16 h with respect to control. Increase in concentration of H_2O_2 depicted that there were less number of CAT enzyme available in the tissue to decompose the H_2O_2. Statistically significant differences were observed between control and 4, 8, 12, 16 h LEF treated rats (one-way ANOVA, $p < 0.05$).

Tissue SOD

SOD is a free radical scavenging enzyme which neutralizes superoxide free radical in normal physiological situations. From the data shown in Figure 5, Table 2, it is evident that there was significant reduction of SOD level with respect to control at and above 4 h. SOD level was 50% lower than control group at 16 h post-treated rats. Statistically significant differences were observed between control and 4, 8, 12, 16 h LEF treated rats (one way ANOVA, $p < 0.05$).

Plasma AST

AST is an important enzyme in human physiological system which helps in transamination of amino acids. Its level is increased during hepatic pathologic conditions. It was observed that there was slight increase of plasma AST level at 12 h albino rats after LEF administration (Figure 6, Table 2). Statistically significant difference was observed between control and 12 h treated rats (one-way ANOVA, $p < 0.05$).

Plasma ALT

ALT is also a transaminase enzyme like AST and its level is also increased during liver damage. As could be observed from the data given in Figure 7, Table 2, plasma ALT level was increased dramatically after 4 h of LEF treatment. The concentration of plasma ALT was almost double during 12 and 16 h after drug treatment (~60 unit/ml) in comparison to control (~25 unit/ml). Statistically significant difference was observed between control and 8, 12, 16 h treated rats (one-way ANOVA, $p < 0.05$).

DISCUSSION

More than 1,100 drugs are thought to be hepatotoxic in nature world-wide (Biour et al., 1998). Drug-induced liver injury is the most frequent cause for withdrawal of marketed drugs, despite rigorous preclinical and clinical testing (Bissell et al., 2001). The major causes of hepatotoxicity related withdrawal of drugs are elevation of liver enzymes levels and depletion of GSH during drug induced oxidative damage. One possible reason of hepatotoxicity is the formation of reactive intermediates during drug metabolism which ultimately binds with nucleophile GSH. These reactive intermediates are highly

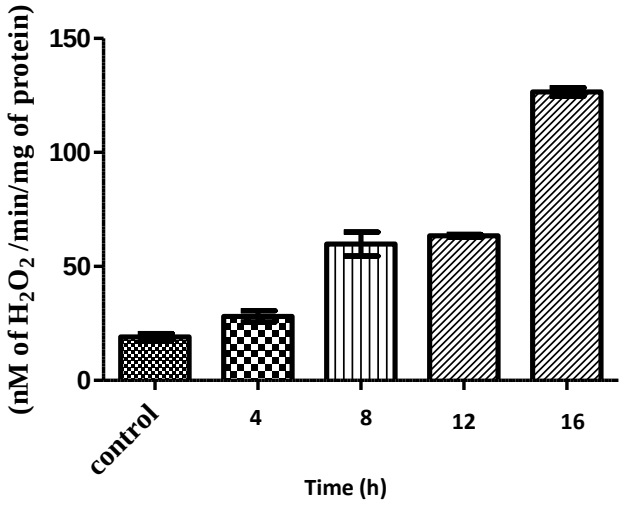

Figure 4. Tissue CAT was measured after LEF treatment at different time intervals with respect to control (n=6). Increase in concentration of H_2O_2 depicted that there were less amount of CAT available in the tissue to decompose the H_2O_2. Statistically significant differences were observed between control and 4, 8 12, 16 h LEF treated rats (one way-ANOVA, $p<0.05$).

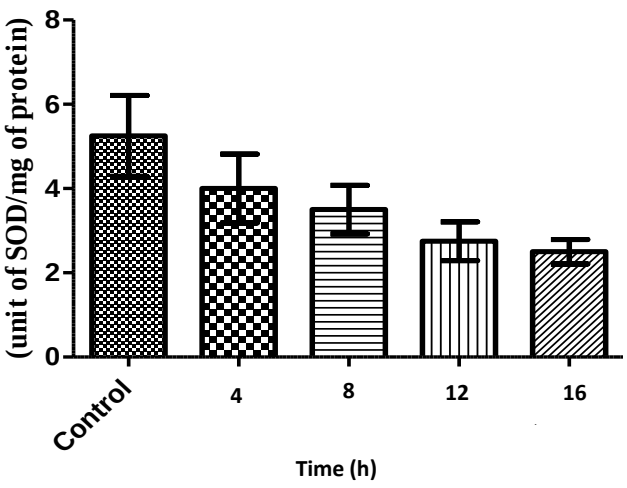

Figure 5. Tissue SOD was also measured at different time intervals with respect to control (n = 6). SOD level was decreased at all time points with respect to control. Statistically significant differences were observed between control and 4, 8 12, 16 h LEF treated rats (one way-ANOVA, $p < 0.05$).

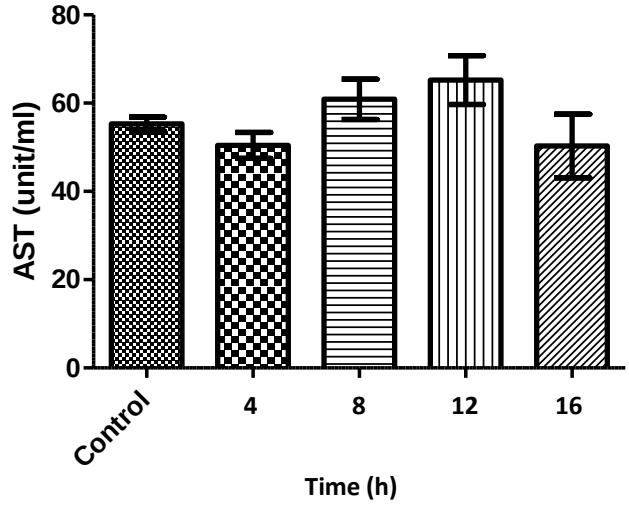

Figure 6. Plasma AST was measured at different time intervals with respect to control (n=6). No changes were observed on AST level after drug treatment. Statistically significant difference was observed between control and 12 h treated rats (one way-ANOVA, $p < 0.05$).

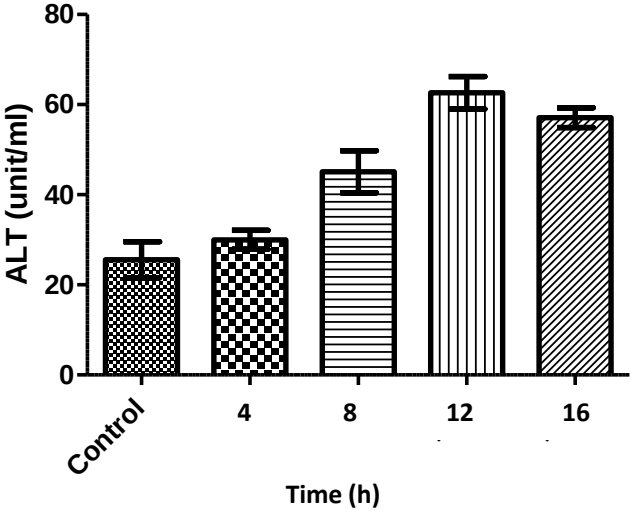

Figure 7. Plasma ALT was measured at different time intervals with respect to control (n=6). ALT levels were increased at and above 8 h with respect to control. Statistically significant difference was observed between control and 8, 12, 16 h treated rats (one way-ANOVA, $p < 0.05$).

toxic in nature which detoxify in presence of GSH. As a result, there is a reduction of GSH in liver which leads to liver injury. For example, five reactive intermediates have been identified for troglitazone in orally administered Sprague-Dawley rats which bound with GSH, and may lead to liver toxicity (Kassahun et al., 2001). This is the major cause for withdrawal of troglitazone from market in 2001 (Jaeschke, 2007).

On the other hand, few drugs also cause hepatotoxicity due to other mechanisms like changes in liver mitochondrial functions, alteration of expression of liver genes and oxidative stress (Russmann et al., 2009; Boverhof et al., 2005; Troudi et al., 2010). Oxidative stress induced hepatotoxicity is the major cause of drug withdrawal nowadays (Jaeschke et al., 2002). It is postulated that orally administered drugs may generate free radicals which cause reduction of GSH, CAT, SOD and elevation

Table 1. Effect of LEF on GSH, MDA, CAT, SOD and in liver at varying time points (n = 6).

Time (h)	GSH (µM/mg of protein)	MDA (nM of MDA/mg of protein)	Tissue CAT (nM of H_2O_2/min/mg of protein)	Tissue SOD (unit of SOD/mg of protein)
control	21.70±0.89	0.08±0.01	19.04±1.47	5.25±0.95
4	21.08±2.24	0.09±0.01	28.06±2.55	4.02±0.81
8	17.72±1.45	0.12±0.02	59.84±5.25	3.50±0.57
12	16.43±3.16	0.13±0.03	63.44±0.63	2.75±0.45
16	16.71±2.39	0.21±0.03	126.51±1.88	2.50±0.29

Table 2. Effect of LEF on ALT and AST levels in plasma at varying time points (n = 6).

Time (h)	AST (unit/ml)	ALT (unit/ml)
control	55.27±1.58	25.55±3.99
4	50.43±2.96	29.99±2.11
8	60.88±4.58	45.11±4.67
12	65.22±5.55	62.64±3.58
16	50.30±7.20	57.11±2.22

of MDA levels in liver. Elevation of plasma ALT and AST occurs during drug toxicity. Acetaminophen causes hepatotoxicity via oxidative stress mechanism in long term therapy (Reid et al., 2005).

Few recent surveys suggested that LEF has tendency to produce hepatotoxicity in human (Gupta et al., 2011). The final question arose how LEF produced hepatotoxicity in humans without formation of toxic reactive intermediates. To get the answer, we performed experiment where LEF was given orally to albino wistar rats in a single dose (10 mg/kg). One recent medical bulletin suggested that leflunomide is effective for organ transplantation at 10 mg/kg dose in rats (Drug Information Portal, Druglib.com). This information allowed us to perform the experiment at same dose to measure the toxicological responses. It is converted to major A771726 intermediate during first pass metabolism which is pharmacologically active than its parent drug (Rozman, 2002). A771726 is 99.3% plasma protein bound (albumin), has half-life ($t_{1/2}$) of 8 h and its concentration in blood is decreased after 12 h in rats (Silva et al., 1996). This data was very important to select time for collecting the liver and plasma from rats. Finally, we decided four time points (4, 8, 12 and 16 h) for collection after drug administration to get maximal effect. Various biochemical parameters were measured at varying time after drug treatment to evaluate the mechanism of LEF induced hepatotoxicity.

To evaluate, we performed GSH assay which explained that there was decrease in liver GSH level after drug treatment. Depletion of GSH by LEF has not been explained as the cause of the oxidative stress induced hepatotoxicity in rats. To get the satisfactory answer related to mechanism of toxicity, we measured MDA, CAT and SOD levels in liver.

Oxidation of lipids is an important parameter for measurement of oxidative stress in living system. Lipid is oxidized to MDA in presence of free radicals. From the result, it was observed that the MDA level was increased with time, which is an important indication of oxidative stress based hepatotoxicity of LEF. Antioxidant enzymes such as CAT are easily inactivated by peroxides or reactive oxygen species. It is most abundant in the liver, which is responsible for the catalytic decomposition of H_2O_2 to oxygen and water (Baudrimont et al., 1997; Reiter et al., 2000). The H_2O_2 level was measured and the values of control and LEF-treated groups were compared at varying time points. Increase in concentration of H_2O_2 in test rats depicted that there was less amount of CAT available in the tissue to scavenge the H_2O_2.

SOD is a free radical scavenging enzyme which neutralizes superoxide free radical in normal physiological situations (Karaman et al., 2006). SOD level was also decreased after LEF treatment. All these experiments suggested that LEF produced toxicity in liver via oxidative stress. To support this hypothesis, we further measured ALT and AST level in plasma. Both enzyme levels were also increased during pathophy- siological conditions and drug toxicity. Similar observation was found in our study where both these enzyme levels were increased after LEF treatment. It was also observed that both ALT and AST levels in plasma were higher at 12 h treated rats (Figures 6 and 7). These enzymes normally present within liver cells. If the liver is injured or damaged, the liver cells spill these enzymes into the blood, raising the enzyme levels in the blood. Increase in these enzyme levels in plasma might be due to LEF induced liver injury.

Conclusion

All biochemical assays suggested that LEF induced liver damage might be due to oxidative damage. LEF is converted to A771726 (major metabolite of LEF) via fast pass metabolism after oral administration in both rats and human. A771726 has higher plasma protein binding

capacity in rats after oral administration, and $t_{1/2}$ is 8 h (Baudrimont et al., 1997). From our findings, it is evident that LEF induced oxidative damage were higher at 12 and 16 h. It might be possible that LEF was converted to A771726 during oral administration which was responsible for liver damage at and above 12 h. The final conclusion of the study is that oxidative stress may occur during LEF hepatotoxicity. Prospective studies involving liver histopatological lesion is needed to confirm the toxic effect of LEF. There is no literature available for LEF induced oxidative stress related hepatotoxicity. This is the first report of LEF induced hepatotoxicity in a single dose after oral administration to albino wistar rats. Further studies can be performed with multiple dosing to evaluate the LEF induced hepatotoxicity as future scope of the work.

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Full Length Research Paper

Antimicrobial activities of some herbal anti-infectives manufactured and marketed in South-East Nigeria

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The aim of this study was to evaluate the anti-microbial activities of some liquid herbal anti-infectives manufactured and marketed in South-Eastern Nigeria and determine the extent of their microbial contamination. Twenty samples were randomly collected from herbal shops in the five states that make up the South-East Nigeria. Antibacterial activities of the herbal preparations were evaluated using agar-well-diffusion method. The samples that showed significant antibacterial activity against the test organisms were further subjected to cell killing rate test. Maximum inhibitory dilutions of the active compounds were obtained and their *in vitro* anti-infective activity against multidrug resistance *Staphylococcus aureus* (MRSA) and Extended Spectrum β-Lactamase (ESBL) organisms were evaluated also. Eight (40%) of the product showed some antibacterial activity and none have antifungal activity against the test fungi. Kill kinetic experiment showed that some products have some activity against the test bacteria. One of the products showed antibacterial activity against MRSA. Comparison of the antibacterial activity of the products and conventional antibiotics showed that there was no significant difference among the microorganism to the antibiotics (F = 0.498, P = 0.686) and herbal anti-infectives (F = 0.477, P = 0.700). Only 40% of the 20 products have some antibacterial activity but none have anti-fungal activity. All were heavily contaminated with microorganisms. Current good manufacturing practice may not have been applied in their manufacture.

Key words: Herbal anti-infectives, antibacterial activity, South-East Nigeria, liquid preparations.

INTRODUCTION

Health for all is a dream and a goal which humanity at large shares and strives for but is clear that modern pharmaceuticals are and will remain out of reach for a large proportion of the human population for the foreseeable future (Mosihuzzaman and Choudary, 2008). This gap has created the need for the use of alternative and traditional medicines, largely herbal in nature, to solve human health need. Alternative medicines, such as herbal medicines, are gaining popularity because of typically low side-effect profiles (Wilt et al., 2000), low cost (Vanderhoof, 2001), and a high level of acceptance by patients. Some managed care organizations now offer these therapies as an expanded benefit (Langyan and Ahuja, 2005). In Africa, traditional medicine has always been a part of the culture even though this form of medicine is not as well organized as, for example, in India and China (Ogunshe et al., 2006). Herbal medicine has become a popular form of healthcare at least in African and Asian countries being intertwined with modern medicine (Eisenberg et al., 1998; Esimone et al.,

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2002). The use of herbal medicine has always been part of human culture, as some plants possess important therapeutic properties (Barkatullah et al., 2013; Selim et al., 2013). The ideas that certain plants had healing potentials and contain antimicrobial principles were well accepted long before mankind discovered the existence of microbes (Rios and Recio, 2005). Traditional herbalists in Nigeria use various herbal preparations to treat various types of ailments, including diarrhea, urinary tract infections, typhoid fever and skin diseases (Sofowora, 1993).

In developing countries, "traditional medical practice is often viewed as an integral part of their culture" (Kunle *et al.,* 2012; Evans, 1996), traditional medical practice is often viewed as an integral part of their culture. Although it is generally believed that most herbal preparations are safe for consumption, some herbs contain biologically active substances that can be toxic or at least have undesirable side effects (Evans, 1996). There is no effective machinery to regulate manufacturing practices and quality standards (Kunle et al., 2012). Given the variable nature of products of plant origin, ensuring consistent quality of their products is vital for the survival and success of the industry (Bauer, 1998). In Nigeria, there appears to be an overwhelming increase in the public awareness and usage of herbal medical products in the treatments and/or prevention of diseases (Okunlola et al., 2007). With this increased usage, the safety, efficacy and quality of these medicines have been an important concern for health authorities and health professionals (Oluyege and Adelabu, 2010). Many of these products have bogus claims on their labels and these claims may have also led to the increase usage of the products. Advertising in various forms by the herbal practitioners is unparalleled in Nigeria. People now attend hospitals as often as they go to herbalists (Okunade, 2001).

This study was therefore meant to examine the antibacterial properties of some liquid herbal anti-infectives produced and marketed in South-East Nigeria and to estimate their level of contamination.

MATERIALS AND METHODS

The materials used include: nutrient agar media (Lab M, UK), MacConkey agar, (Fluka, UK), Xylose Lysine Deoxycholate Agar, Urease broth, Citrate agar, Saubouraud Dextrose agar (BIOTECH Laboratories LTD, UK), Mueller Hinton agar media (Oxoid, UK) and Peptone water. All the media were prepared aseptically following the manufacturers' instructions.

Study area

The study sampled herbal anti-infectives from South-Eastern Nigeria, West Africa. South-Eastern Nigeria comprises five states which include: Abia, Anambra, Ebonyi, Enugu and Imo states.

Samples collection

A total of twenty (20) different liquid herbal anti-infectives were purchased randomly from identified herbal shops and retail outlets across the South-Eastern states of Nigeria. The samples as shown in Table 1 were kept at room temperature (28±2°C) and used within two weeks of collection.

Test organisms

The microbial cultures were untyped clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Candida albicans* obtained from the Medical Microbiology Laboratory of Nnamdi Azikiwe University Teaching Hospital, Nnewi. They were properly identified and preserved on agar slants at 37°C as stock.

Identification and characterization of the bacteria isolates

Wire-loopful quantities of the products were stricken onto MacConkey agar, Blood agar, and Sabouraud agar. The plates, incubated aerobically at 37°C for 24 to 48 h, were examined for growth and biochemical tests carried out according to the methods described by Monica (2002) for proper identification of the organisms isolated.

Agar well diffusion method

About 0.1 ml of 0.5 Mcfarland standards of clinical isolates of *S. aureus, E. coli, P. aeruginosa, S. typhi* and *C. albicans* was taken and aseptically transferred into labeled sterile Petri dishes. Then 20 ml of molten sterile nutrient agar was poured into the seeded Petri dishes and swirled to distribute the medium homogenously. After solidification, holes of dept 3.5 mm were made aseptically with a 6 mm sterile cork borer and sealed. The stock and 2 fold dilution of the liquid herbal products (volume = 0.1 ml) were introduced into separate wells and allowed to diffuse into the medium. The whole set up was then incubated aerobically for 18 to 24 h at 37°C. One well containing sterile water served as control and another containing amoxicillin/clavulanic acid (AMC, 20/10 µg) served as a positive control in each plate. The antimicrobial activity of the various agents was determined by measurement of the inhibition zone diameter using a meter rule and compared with the control well (containing water).

Determination of ant-bactericidal activity of the herbal anti-infectives using kill kinetic method

Nutrient broth of volume (5 ml) was dispensed into six culture tubes provided for each indicator bacteria and was sterilized. These were labeled 0 to 5 h where 0 h serves as control. One milliliter of four different herbal anti-infectives (product 2, 3, 7, 11) that showed significant antibacterial activity by the agar diffusion method were added to the culture tubes containing the indicator bacteria. The suspension of the indicator bacteria and herbal medicine were thoroughly mixed and held at room temperature (28±2°C). Anti-bactericidal activity was determined by plating 0.1 ml of the suspension at hourly interval for up to 5 h. The plates were incubated and the colony forming units were counted

Determination of maximum inhibitory dilution (MID)

The MID of the aqueous herbal preparations which showed by the micro-broth dilution method. Serial dilutions of herbal anti-infectives in dilutions of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 of the broth and herbal products, respectively were prepared in sterile test tubes. Standard

Table 1. Some brands of herbal anti-infectives marketed in South-East Nigeria.

Product code	NAFDAC Reg. NO.	Contents	Therapeutic claim
1	04-8618L	*Carica papaya*, *Magnifera indica*, Newbouldlia leaves, *Azadricha indica*, *Jaminum officionili*, *Aloe barbedensis*, ginseng, treated water 60cl.	Antibacterial, antimalarial, antirheumatic, infertility, antiviral
2	1295	38 African roots, herbs, fruits, barks plus ginseng, aloe vera and garlic.	Antibacterial, antirheumatic, antifungal and antiviral
3	-	60% Herbs, 25% flower, 10% leaves, and 5% roots.	Antibacterial, Antirheumatic, antifungal, earlier menopause, painful and irregular menstruation
4	A7-0280L	Aloe vera plus 31 roots and herbs, fruits and barks	Antibacterial and antifungal
5	-	Water, herbs, root and fruits	Internal heat, pile, antibacterial, antimalarials, anti-parasitic and reduces blood sugar
6	NUOMHP NOHerbal:0 9840	-	Antibacterial, treatment of all kinds of eye infections
7	No.A7-0736L	*Nauclea diiderchi* 10%, *Hippocrates pallens* 20%, *Alluim sativum* 12.5%, *Cochios permum pianchoni* 5.5%, *Uvaria chame* 5%, *Punica granatum* 47%	Antibacterial, antimalarials
8	No	Aloe vera 40%, Olong tea 20%, flower and roots 40%, saracin.	Antibacterial
9	No	Aloe vera	Antibacterial anti-malarial, HBP, cough antirheumatism, etc.
10	No.A7-0220L	Aloe vera, flowers, and fruits seed barks.	Antibacterial, hypertension, antiviral, fibroid, stroke.
11	No	Honey (natural), lime juice, *Zingiber officinale*, and herbal seeds and roots.	Antibacterial and asthmatic cough.
12	No	-	Anti-bacterial antiviral, diabetes, reduces cholesterol.
13	No	25 different types of roots, herbs, seeds and flowers.	Anti-bacterial, anti-malaria, antirheumatic. Antifungal
14	No	Herbs, water, root and fruits.	Antibacterial, Internal heat, pile, Antiviral, Antirheumatic, Antifungal, Antiparatic.
15	No.Al-0240L	*Mangifera indica, Carica papaya* leaves*, Psidium guajava, Masularia acuminate* root, breadfruit bark, citrus lemon leaves, *Zingiber officinale* root*, Cymbopogon* spp.	Antibacterial, Treatment and prevention of toothache.
16	No	Awapa bark, white lotus, Golden seal, mahogany, Ukor root, Aloe barbaders, Mistletoe, Osisika Aguru, Uda roots, Uvuru ilu, lemon grass.	Antibacterial, antirheumatic and arthritis, venereal diseases.
17	No.A7-1482L	Aloe vera, Cadeperi salt, lime	Antibacterial, treatment and prevention of toothache.
18	No.A7-0912L	Lymbopogon citrates, *Carica papaya* leaves, *Magnifera indica*, bark, Treculia Africana, Citrus, Limonia, *Psiduim guajava, Zingibar officinale* root, *Alluim sativum.*	Antibacterial, antirheumatism, reduces sugar and cholesterol.
19	No.41563	Natural roots and barks.	Antibacterial, antiviral, purifies blood, detoxifies toxins, builds immune system, stops dizziness, weakness.
20	No	Nuclealatifolia, *Allium sativum*, Aloe vera bitter, Chick weed, *Preclina nitida, Hibiscus sabdrrifa*, Aqua, ethanol.	Antibacterial, antiparasitic, ulcer, constipation, fibroid, internal heat heart burn and diabetes

inoculums of the test microorganisms equivalent to 0.5 McFarland were prepared and 0.1 ml of the standard inoculum introduced into test tubes containing the dilutions of the herbal products. The tubes were incubated aerobically at 37°C for 24 h. After 24 h a loopful of the different concentration were taken from the tubes and streaked on agar plates, which were also incubated at 37°C for 24 h after which the presence or absence of growth was observed and matched with the particular concentration. The highest dilution (lowest Concentration) of the agent that produced no visible bacterial growth when compared with the control plate was regarded as the MID.

Determination of the antimicrobial activity of the products against MRSA and ESBL- expressing *E. coli* and *Klebsiella* species

About 0.1 ml of the standardized suspension (equivalent to 0.5 McFarland) of MRSA, ESBL expressing *Escherichia coli* and *Klebsiella* were obtained from the stock available in pharmaceutical and biotechnology lab, Faculty of Pharmacy, Nnamdi Azikiwe University, Agulu campus and aseptically transferred into labeled sterile Petri dishes. Then 20 ml of molten sterile nutrient agar was poured into the seeded Petri dishes and swirled to distribute the medium homogenously. After solidification, holes were made aseptically with a 6 mm sterile cork borer and 1 ml of the test solution of different concentrations introduced into the wells. The agents were allowed to diffuse into the medium and then incubated aerobically for 18 to 24 h at 37°C. The plates were examined for zones of inhibition, which indicate the degree of susceptibility of the test organisms. The antimicrobial activity of the various agents was measured with a meter rule and compared with the control well (containing water).

Determination of the level of microbial contamination of the products

Exactly 1 ml of each herbal anti-infectives was aseptically transferred into a corresponding sterile test tube containing 9 ml of sterile distilled water and ten-fold serial dilution was carried out. Using the Pour plate technique, 1 ml of dilutions 10^{-1}, 10^{-2} 10^{-3}, 10^{-4}, and 10^{-5} were transferred to clean sterilized Petri dishes and mixed with 20 ml of sterile molten nutrient agar and Sabouraud Dextrose agar which was cooled to 45°C for bacteria and fungi, respectively. These were done in triplicates and the plates were allowed to set and then incubated at 37°C for bacterial counts from 18 to 24 h, and 20 to 27°C for fungal count for 72 to 168 h. All counts were expressed in CFU/ml.

Statistical analyses

Analysis of variance (ANOVA) was performed to determine statistical significant differences in inhibition zone diameter (IZD) amongst the herbal products and the conventional antibiotics. The level of significance was set at 0.05.

RESULTS

Table 1 shows the different liquid herbal anti-infectives that were evaluated in this work. All the products (100%) had antibacterial claims, 6 (30%) claimed to have antifungal activity while 13 (65%) claimed to be effective for the management of non-infectious diseases/ conditions. None of the products investigated demonstrated any antifungal activity and only 40% of the products showed some antibacterial activity.

The antimicrobial activity of the herbal anti-infectives was evaluated against selected pathogenic bacteria and fungi as presented in Table 2 using agar well diffusion method. The table revealed that none of the herbal samples has antifungal effect, 3 out of the 20 inhibited the growth of *S. aureus,* with product 2 stock having the highest zone of inhibition (26 mm) and product 11 stock having the least (16 mm). Three of the herbal product inhibited the growth of *Salmonella typhi* with product 3 and 17 having a higher zone of inhibition of 12 mm than product 2 which has 10 mm IZD. The growth of *P. aeruginosa* was inhibited by 5 products. Product 2 had the highest IZD of 22 mm while products 5 and 9 have the least which is 10 mm. Growth of *E. coli* was inhibited by two products; product 7 (IZD of 25 mm) and product 11 (IZD of 14 mm).

The bactericidal activity of the products (2, 3, 7 and 11) that showed significant antibacterial against at least two of the target bacteria was characterized using killing rate kinetics method (Table 3). The MID of the products that showed significant antibacterial activity against the test organisms is given in Table 4 and the activity of the herbal products against MRSA and ESBL expressing *E. coli* and *Klebsiella* is shown in Table 5. Table 6 shows the comparison of IZD of the herbal anti-infectives and conventional antibiotics. Table 7 shows the level of microbial contaminations of the products.

DISCUSSION

The herbal medicinal products selected for this study were all liquid dosage forms. Products 2, 7, 10, 12, and 17 were purchased from Aba in Abia state, products 1, 5, 6, 9, and 16 were from Anambra state, products 3, 4, 13, 18, 19, and 20 were from Ebonyi and Enugu States while Products 8, 14, and 15 were from Owerri in Imo State. All the samples were within their shelf-life at the time of investigation. Ten (50%) of the products had NAFDAC registration number while the other 10 (50%) were not registered by the agency. This is contrary to the laws governing the manufacture, advertisement, sale and distribution of herbal medicinal products and indeed all foods and drugs in Nigeria which forbids such when the products are not properly registered by NAFDAC (HMRP, 2004). The European Agency for the Evaluations of Medicinal Products (EAEMP) and WHO have stated that the quality of the herbal drug should be given as a range corresponding to a defined quantity of constituents with known therapeutic activity and if constituents responsible for therapeutic activity are unknown, the quantity of the whole herbal drug preparation should be given (EMEA, 2002; WHO, 1996a). They also stipulated that the dosage

Table 2. Products' antimicrobial activity on the test organisms.

Product	IZD (mm)									
	E. coli		*P. aeruginosa*		*S. aureus*		*S. typhi*		*C. albicans*	
	Stock	Dilution	Stock	Dilution	Stock	Dilution	Stock	Dilution	Stock	Dilution
Product 1	-	-	18	18	-	-	-	-	-	-
Product 2	-	-	22	20	26	22	10	10	-	-
Product 3	-	-	16	16	-	-	12	12	-	-
Product 4	-	-	-	-	-	-	-	-	-	-
Product 5	-	-	10	10	-	-	-	-	-	-
Product 6	-	-	-	-	-	-	-	-	-	-
Product 7	25	18	-	-	18	18	-	-	-	-
Product 8	-	-	-	-	-	-	-	-	-	-
Product 9	-	-	10	10	-	-	-	-	-	-
Product 10	-	-	-	-	-	-	-	-	-	-
Product 11	14	12	-	-	16	12	-	-	-	-
Product 12	-	-	-	-	-	-	-	-	-	-
Product 13	-	-	-	-	-	-	-	-	-	-
Product 14	-	-	-	-	-	-	-	-	-	-
Product 15	-	-	-	-	-	-	-	-	-	-
Product 16	-	-	-	-	-	-	-	-	-	-
Product 17	-	-	-	-	-	-	12	12	-	-
Product 18	-	-	-	-	-	-	-	-	-	-
Product 19	-	-	-	-	-	-	-	-	-	-
Product 20	-	-	-	-	-	-	-	-	-	-

S = Stock Concentration, D = Diluted Concentration (2-fold), - = No inhibition.

Table 3. Anti-bacterial activity of the products with significant activity using kill kinetic method.

Time (h)	Bacterial load (CFU/ml)									
	S. typhi			*S. aureus*			*E. coli*		*P. aeruginosa*	
	Prod. 2	Prod. 3	Prod. 7	Prod. 2	Prod. 7	Prod.11	Prod. 7	Prod.11	Prod. 2	Prod. 3
0	221	221	248	49	41	41	44	19	77	112
1	209	202	236	37	19	29	24	22	56	95
2	204	185	204	26	38	26	3	15	48	81
3	180	179	146	20	56	20	0	12	44	66
4	170	172	87	17	10	17	0	0	29	51
5	162	162	51	0	0	10	0	0	19	39

Prod.: Product.

Table 4. MID of the products with significant antibacterial activity.

Test organism	MID of the products (% v/v)			
	Product 2	Product 3	Product 7	Product 11
S. typhi	66.6	25	66.6	-
P. aeruginosa	66.6	66.6	-	-
S. aureus	150	-	66.6	150
E. coli	-	-	66.6	150

form, therapeutic indications and expiry dates should be stated. However, 3 (15%) of the products did not have their content stated even though therapeutic claims were indicated either on the container or in the leaflet insert.

Table 5. Antibiotic activity of the products against MRSA and ESBL-Expressing Organisms.

Product	MRSA 1		MRSA 2		ESBL (*E. coli*)		ESBL (*Klebsiella*)	
	Stock	Dilution	Stock	Dilution	Stock	Dilution	Stock	Dilution
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
7	30	27	13	13	-	-	-	-
11	-	-	-	-	-	-	-	-

MRSA: Methicillin Resistant *Staphylococcus aureus*; ESBL: Extended Spectrum Beta-Lactamase; Dilution: Two-fold dilution; -: No inhibition.

Table 6. Inhibition Zone Diameter (mm) of the products and conventional antibiotics.

Test organism	P 2	P 3	P 5	P 7	P 11	OFX	CIP	TE	GN	CRO
S. typhi	10	26	-	24	-	15	32	14	26	-
P. aeruginosa	22	16	10	-	-	-	17	-	20	16
S. aureus	26	-	-	18	16	25	20	8	-	13
E. coli	-	-	-	18	14	-	15	-	16	-

P: Product; OFX: ofloxacin; CIP: ciprofloxacin; CAZ: ceftazidime; TE: tetracycline; AMP: ampicillin; SXT: sulfamethoxazole-trimethoprim; GN: gentamicin; CTX: cefotaxime; CRO: ceftriaxone; AMC: amoxycillin-clavulanic acid.

Table 7. Level of microbial contamination of the products.

Product code	Bacterial count (CFU/ml)				Fungal count (CFU/ml)			
	Plate1	Plate 2	Plate 3	$\dot{X}$±SEM	Plate 1	Plate 2	Plate 3	$\dot{X}$±SEM
1	28	35	23	2.9×10^{3} ±3.48	5	7	3	5×10^{4}±1.15
2	27	38	30	3.2×10^{2}±3.28	4	3	5	4×10^{4}±0.58
3	36	48	50	4.5×10^{4}±4.37	9	4	2	5×10^{5}±2.08
4	35	40	42	3.9×10^{3}±2.08	7	12	4	8×10^{3}±2.33
5	90	73	67	7.7×10^{2}±6.89	0	0	0	- -
6	28	47	32	3.6×10^{3}±5.78	16	2	1	6×10^{2}±4.84
7	7	7	7	7×10^{1}±0.00	46	20	26	3.1×10^{3} ±7.86
8	4	51	10	2.2×10^{4}±14.77	30	32	28	3.0×10^{3}±1.15
9	25	92	15	4.4×10^{3}±24.17	35	18	17	2.3×10^{2}±5.84
10	76	67	60	6.8×10^{4}±4.63	25	25	20	2.3×10^{4}±1.67
11	250	250	200	2.33×10^{6}±16.67	30	25	5	2.0×10^{3}±7.64
12	116	120	100	1.12×10^{5}±6.11	94	90	94	9.4×10^{5}±1.33
13	48	35	40	4.1×10^{5}±3.79	30	38	40	3.6×10^{3}±3.06
14	93	62	70	7.5×10^{3}±9.29	30	36	32	3.3×10^{4}±1.76
15	41	45	40	4.2×10^{3}±1.53	35	30	15	2.7×10^{5}±6.01
16	64	55	68	6.2×10^{3}±3.84	40	41	39	4.0×10^{5}±0.58
17	48	50	45	4.8×10^{3}±1.45	50	49	51	5.0×10^{5}±0.58
18	109	100	98	1.02×10^{6}±3.38	10	15	11	1.2×10^{5}±1.53
19	15	20	30	2.2×10^{2}±4.41	55	50	56	5.4×10^{4}±1.86
20	46	40	38	4.1×10^{4}±2.40	12	12	12	1.2×10^{2}±0.00

All the products (100%) had antibacterial claims, 6 (30%) claimed to have antifungal activity while 13 (65%) claimed to be effective for the management of non-infectious diseases/conditions. Medicinal products designed for the purpose of chemotherapeutic and pharmacological benefits should be effective against the target medical condition.

The evaluation of the antimicrobial activity of the products against test organisms (*S. aureus, E. coli, S. typhi, P. aeruginosa* and *C. albicans*) (Table 2) showed

that only 8 (40%) of the herbal products (products 1, 2, 3, 5, 7, 9, 11, and 17) have some antibacterial activity. Four out of the eight products showed significant activity. None of the products showed antifungal activity against the test fungi organism even though six (30%) of the products (2, 3, 4, 10, 13, and 14) claimed to have antifungal activity. The poor antimicrobial activity as shown by the herbal products against the test organisms could be attributed to the fact that the products were contaminated as shown in Table 7. The presence of microbial contaminants in non-sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the product and has the potential to adversely affect patients taking the medicines (Nakajima et al., 2005; Okunlola et al., 2007). Apart from possible microbial degradation of the active constituents contained in the herbal preparations, the presence of these contaminating microorganisms could constitute a source of infection and serious health risk to the consumers of the herbal preparations who were probably already overwhelmed by the serious medical conditions for which the herbal drugs were initially indicated (Mangram et al., 1999; Bowler et al., 2001). Spoilage of medicines involve initial or pioneer invading biodegrading microorganisms, which prepare the way for later invaders that biodegrade complex nutrient, thus altering the surrounding pH and increasing moisture content (Omwuliri and Wonang, 2005). On the other hand, the anti-microbial claims of the products may not be true.

Further evaluation on the bactericidal action of the herbal products with significant antibacterial activity was done using kill kinetic method. The herbal preparations showed bactericidal effect on the indicator bacteria as bacterial load decreased from the first hour to the fifth hour (Table 3). Product 7 seems to have erratic effect on *S. typhi*. The organism was not very susceptible to the products generally. *E. coli* was found to be susceptible to antibacterial actions of herbal preparations 7 and 11, *S. aureus* was found to be susceptible to the antibacterial actions of products 2 and 7 but less to product 11. Susceptibility of *P. aeruginosa* to the products 2 and 3 was also observed as the products were able to reduce the bacteria load significantly although more time was observably needed to clear the organisms. The MID of the herbal products that showed significant antibacterial activity was also evaluated (Table 4), and the result showed that at higher dilutions, the products have little or no antibacterial activity. Evaluation of the actions of the products against MRSA and ESBL expressing *E. coli* and *Klebsiella* spp. (Table 5) revealed that only one of the products (Product 7) showed activity against MRSA and none had activity against ESBL-expressing organisms. This shows that in cases of infections caused by these organisms, the products are powerless.

In the present study, it was observed that herbal products were grossly contaminated by fungal and bacterial agents, with the exception of one that was not contaminated with fungi (Product 5) (Table 7). Contamination by microorganisms is influenced by the environment, improper handling and storage of medicinal plants (Idu et al., 2010; Oleghe et al., 2011). Herbal medicinal plants usually contain bacteria and molds from soil and atmosphere. Microorganisms are everywhere. This can be supported by the results of the present research as the herbal products from all the five states were all contaminated. One of the major shortcomings of herbal preparations in developing countries is the unhygienic condition under which they are produced *(*Frazier and Westhoff*,* 2003).

From the ANOVA, the comparison of the herbal products with conventional antibiotic shows no significant difference. This suggests that both the herbal products and the antibiotics have the potential to produce anti-bacterial effect (Table 6), but aseptic techniques may not have been applied during the manufacture of the products. Some studies on the sterility of the manufacturing environment of these companies are hereby suggested.

Conclusion

Only 40% of the 20 products have some antibacterial activity, but none have anti-fungal activity. All were heavily contaminated with microorganisms. Current good manufacturing practice may not have been applied in their manufacture.

ABBREVIATIONS

EAEMP, The European Agency for the Evaluations of Medicinal Products; **EMEA,** European Medicines Evaluation Agency; **NAFDAC,** National Agency for Food Drug Administration and Control; **HMRP,** Herbal Medicines and Related Products; **MRSA,** methicillin resistant *Staphylococcus aureus*; **ESBL,** extended spectrum beta-Lactamase; **HMPWP,** Herbal Medicinal Products Working Party.

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Full Length Research Paper

Effects of Saikosaponin D on apoptosis genes expression profile of the colon cancer cells HT-29

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The present study aims to investigate the change of apoptosis genes expression of HT29 cells induced by Saikosaponin D (SSD), which is the major component of Xiao Chai Hu Tang, the classic Chinese medicine prescriptions. In this study, gene expression profiles of HT29 cells with or without the SSD treatment were obtained by means of using RT ProfilerTMPCR Array Human Apoptosis and quantitative real-time polymerase chain reaction (PCR) and analyzed to detect differential gene expression. According to results of 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, a final concentration of 10 mg /L of SSD for 24 h was used in the SSD group and the growth inhibition rate of HT-29 cells was about 5%. In SSD treated cells, expression levels of 14 kinds of apoptosis genes changed significantly. Compared with the control group, some apoptosis genes such as DIVA, CASP10, and TRAILR were expressed at much higher levels, while some anti-apoptosis gene such as HIAPI, NAIP, BCL2 were also expressed at much higher levels. SSD induces the apoptosis of HT29 through the apoptosis pathway of TRAIL, TRAIL-R and caspase10 and/or caspase8. And these data could provide targets for the treatment of colon cancer.

Key words: Saikosaponin D, colon carcinoma, apoptosis, gene expression.

INTRODUCTION

Xiao Chai Hu Tang first recorded in the Chinese Ancient medicine book "Febrile Diseases"is widely used in clinical treatment of inflammation, cancer prevention and control, and is a classic Chinese medicine prescription. Saikosaponin is the major component of Xiao Chai Hu Tang. There have been many studies on anti-tumor effect of the saikosaponin and its extracts by local and foreign scholars, confirming that saikosaponin D (SSD) has the strongest anti-inflammatory effect in the four kinds of of saikosaponin; same as some other saponins drugs with anti-tumor effects. However, the antitumor mechanism of SSD is still unclear (Wang et al., 2006; Zu 2000). It was confirmed that in leukemia, liver cancer, lung cancer and other tumor cells, SSD could induce apoptosis through different mechanisms, such as increasing FASL, FAS, and BAX protein expression, reducing B-cell lymphoma 2 (BCL2), BCL-XL expression, activating poly (ADP) polymerase (PARP) shear, or cracking inhibitors of apoptosis (IAP) family members XIAP and cIAP-2 among others (Hsua et al., 2004a; 2008;Yu et al.,2008). It was reported that as one of the four kinds of of saikosaponin, saikosaponin A (SSA) could induce apoptosis in colon cancer cells through activation of CASP2 and CASP8 (Kim et al., 2010), so whether SSD is able to induce apoptosis in colon cancer cells and the mechanism of SSD induced apoptosis is still unclear.

In this study, by means of using the apoptosis gene polymerase chain reaction (PCR) microarray and quantitative real-time PCR technology, we screened the SSD-induced differentially expressed apoptosis genes in the colon cancer cells. Here, we found and reported that there were 15 kinds of apoptosis genes whose expression were significantly changed, and these apoptosis genes might play a major role in SSD-induced

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Table 1. Inhibition effects of saikosaponin D on the growth of colon cancer HT-29 cell.

Group	Inhibition rate (%)		
	24 h	48 h	72 h
0 (mg/L)	0.11±0.02	0.09±0.012	0.14±0.021
5 (mg/L)	1.9±0.14	17.8±0.24	19.1±0.21
10 (mg/L)	5.0±0.17	31.2±0.12	33.4±0.28
20 (mg/L)	9.4±0.18	42.3±0.31	44.5±0.18

apoptosis of the colon cancer cells. These results laid the foundation for understanding the mechanisms of saikosaponin D-induced apoptosis of colon cancer cells, and could provide targets for the treatment of colon cancer.

MATERIALS AND METHODS

Materials

Saikosaponin D was purchased from Chinese medicine and biological products Institution (Beijing, China); Colon cancer cell line HT-29 cells were purchased from Shanghai Institute of Cell Biochemistry Life Sciences (Shanghai, China). Cell culture, RNA purification and reverse transcription reagents were mainly purchased from Invitrogen company and TakaRa company; RT ProfilerTM human apoptotic gene PCR microarray (PAHS-012A) were purchased from SABiosciences company.

Methods

Cell growth inhibition assay

Cells were seeded into 96-well plates (5×10^3 cells/well) for 12 h, followed by treatment with various concentrations of saikosaponin D. Cell viability was determined using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. In brief, 15 µl (5 mg/ml) MTT working solution was added to per well and then incubated at 37°C for 4 h. Then the supernatant was removed and 150 µl of dimethylsulphoxide (DMSO) was added to each well to dissolve the crystals. The absorbance of each well at 570 nm was measured under an ELx 808 Universal Microplate Reader (BIO-TEK, INC). Data were expressed as the percentage of cellular viability relative to the control culture (assuming 100%) in the present study.

Cells drug treatment and RNA purification identification

The same number of HT-29 cells were inoculated in the cell culture dish with an equivalent diameter of 10 cm. After 24 h, cells were treated with 0 mg / L SSD (control group) or 10 mg / L (experimental group), and for another 24 h, the cells were collected. Total RNA of the cells sample was extracted with Trizol reagent (Gibco, USA) as described by the manufacturer. RNA was purified using RNeasy ® MinElute ™ purification kit (Qiagen); quality, concentration and purity of RNA were measured using the agarose gel electrophoresis method and the UV absorption. RT-PCR was performed using SuperScript. III Reverse Transcriptase (invitrogen). PCR was performed for 30 cycles in 25 µl of reaction mixture.

Apoptosis gene PCR microarray assay

10 µl cDNA and SuperArray PCR master mix (Cat. No. PA-112) premixed solution was added to each well of PCR microarray. Real-time PCR procedure was set as follows: Denaturation at 95°C for 10 min, amplification of 40 cycles at 95°C for 15 s and 60°C for 1 min. After collecting the fluorescence, the melting curve was analysed.

Data were analyzed by ΔΔCt method, and the fluorescence threshold was set to 0.15. ΔCt was the mean number of the threshold cycle number of each gene subtracting the threshold cycle of housekeeping gene B2M, HPRT1, GAPDH, ACTB.Gene ΔΔCt was the ΔCt of the SSD group minus the control group ΔCt. The differences of each gene expression were calculated with 2-ΔΔCt calculation of DHA and control groups (Livak and Schmittgen, 2001). PCR microarray experiments technical services were provided by the Kangcheng Sheng Bio-Engineering Co., Ltd. (Shanghai, China).

RESULTS AND DISCUSSION

Identification of RNA samples from Saikosaponin D-treated Colon cancer cells

According to results of MTT assay (Table 1), a final concentration of 10 mg / L of SSD for 24 h was used in the SSD group, and the growth inhibition rate of HT-29 cells was about 5%, consistent with the results reported by Hsua et al. (2004b) in non-small lung cancer cells. The UV absorption ratio Abs260/Abs280 reflecting the purity of RNA samples from SSD and control groups were 2.00 and 2.02, respectively. As shown in Figure 1, two clear 28S, 18S ribosomal RNA bands and one RNA band with a little spread and low molecular weight were observed in both the SSD and control groups. And above the 28S ribosomal RNA band, the diffusion of high molecular weight substances or belt usually resulting from DNA contamination was not observed.

The analyses of SSD-induced apoptosis genes differential expression profile in HT-29 cells

As shown in Figure 2a, the column height of three-dimensional histogram meant multiples of differentially expressed genes, 2 ^-ΔCt ratio of gene mRNA expression from the SSD group to that from the control group. The measured genes distribution is shown in Figure 2b.

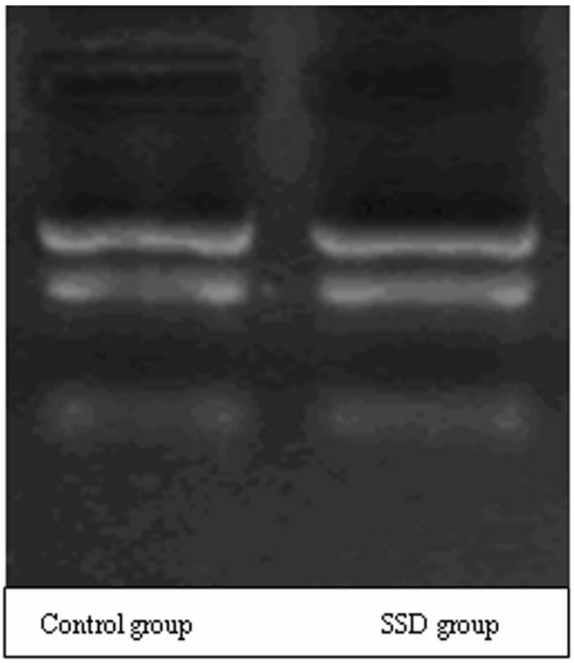
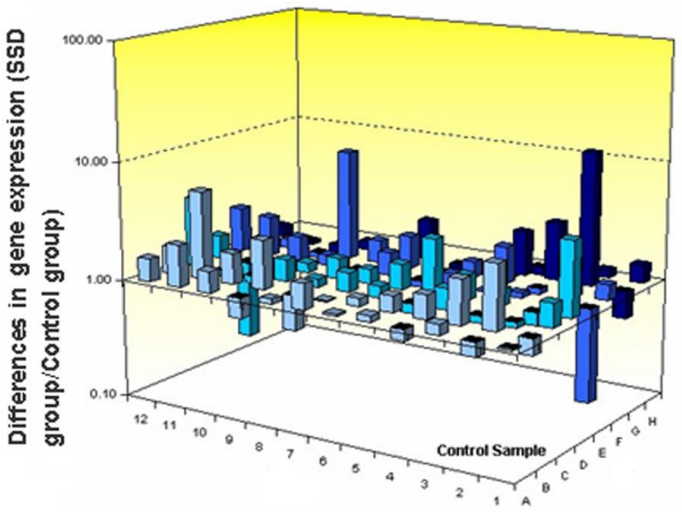


Figure 1. Purity identification of RNA samples by denatured agarose gel electrophoresis (left: control group; right: SSD group).

A



B

ABL1 A01	AKT1 A02	APAF1 A03	BAD A04	BAG1 A05	BAG3 A06	BAG4 A07	BAK1 A08	BAX A09	BCL10 A10	BCL2 A11	BCL2A1 A12
BCL2L1 B01	BCL2L10 B02	BCL2L11 B03	BCL2L2 B04	BCLAF1 B05	BFAR B06	BID B07	BIK B08	BIRC1 B09	BIRC2 B10	BIRC3 B11	BIRC4 B12
BIRC6 C01	BIRC8 C02	BNIP1 C03	BNIP2 C04	BNIP3 C05	BNIP3L C06	BRAF C07	CARD4 C08	CARD6 C09	CARD8 C10	CASP1 C11	CASP10 C12
CASP14 D01	CASP2 D02	CASP3 D03	CASP4 D04	CASP5 D05	CASP6 D06	CASP7 D07	CASP8 D08	CASP9 D09	CD40 D10	CD40LG D11	CFLAR D12
CIDEA	CIDEB	CRADD	DAPK1	DFFA	FADD	FAS	FASLG	GADD45A	HRK	IGF1R	LTA

Figure 2. The SSD-induced apoptosis genes differential expression profiles in colon cancer cell line HT-29 cells. A, SSD-induced apoptosis genes differential expression; b, PCR microarray gene distribution.

Table 2. Part of significantly up-regulated apoptosis-related genes induced by SSD in HT-29 cells.

Symbol	Well	2^-ΔCt		Fold change	Fold up- or down-regulation	Open gene table in web browser
		SSD group	Control group	SSD /control group	SSD /control group	Description
BCL2	A11	8.90E-04	3.99E-04	2.23	2.23	B-cell CLL/lymphoma 2
BCL2L10/DIVA	B02	4.35E-05	1.25E-05	3.48	3.48	BCL2-like 10 (apoptosis facilitator)
BCL2L11/BAM	B03	1.82E-02	7.82E-03	2.33	2.33	BCL2-like 11 (apoptosis facilitator)
NAIP	B09	4.13E-03	1.57E-03	2.63	2.63	NLR family, apoptosis inhibitory protein
HIAP1/BIRC3	B11	1.12E-03	2.02E-04	5.54	5.54	Baculoviral IAP repeat-containing 3
CASP10	C12	5.37E-04	1.37E-04	3.92	3.92	Caspase 10, apoptosis-related cysteine peptidase
IGF1R	E11	1.14E-02	5.25E-03	2.17	2.17	Insulin-like growth factor 1 receptor
LTA/TNFB	E12	1.01E-04	4.19E-05	2.41	2.41	Lymphotoxin alpha (TNF superfamily, member 1)
MCL1	F02	9.35E-02	4.12E-02	2.27	2.27	Myeloid cell leukemia sequence 1 (BCL2-related)
PYCARD	F04	2.71E-05	1.31E-05	2.07	2.07	PYD and CARD domain containing
TNFRSF9/CD137	G02	3.73E-05	2.87E-06	12.98	12.98	Tumor necrosis factor receptor superfamily, member 9
TNFSF10/TRAIL	G03	5.09E-05	1.62E-05	3.14	3.14	Tumor necrosis factor (ligand) superfamily, member 10
CD70	G04	8.33E-04	3.56E-04	2.34	2.34	CD70 molecule
TP53BP2	G07	6.07E-02	2.72E-02	2.23	2.23	Tumor protein p53 binding protein, 2
CIDEA	E01	1.30E-05	3.72E-05	0.35	-2.90	Cell death-inducing DFFA-like effector a

These results suggest that SSD could induce 14 kinds of significantly up-regulated apoptosis genes in colon cancer cell line HT-29, and the differences multiples of these genes were higher than 2 (Table 2). Interestingly, the gene CIDEA expression level was significantly reduced, but the difference multiple was less than 0.5, maybe because this gene expression level in both the SSD group and control group was low, the reduced significance in this experiment was not statistically significant.

SSD induced significantly up-regulated expression of 14 kinds of apoptosis genes in HT-29 cells, including pro-apoptotic genes TRAIL, CASP10, TNFB, BAM and DIVA, and anti-apoptotic genes BCL2, HIAP1, NAIP and so on. Meanwhile, there were also some genes, which were up-regulated but increased in less than 2-fold expression, including CASP8 (1.48), CASP9 (1.23), CASP3 (1.39) and TRAIL receptor gene TNFRSF10A (1.88), TNFRSF10B (1.55). These results suggest that SSD triggered apoptosis in HT-29 cells by the TRAIL death receptor pathway. In the TRAIL-induced apoptosis, CASP10 may replace CASP8 or synergy with CASP8 from the classic death receptor pathway (Kischkel et al., 2001). CD137, which has a significant increase in SSD-treated cells, might play an anti-tumor role by regulating the immune responses of tumor cells (Ju et al., 2003).

TNFB has been found to induce the apoptosis by the mitochondrial pathway, although the molecular mechanisms were still unclear. In HT-29

cells, SSD induced increased expression of TNFB possibly resulted in the cells apoptotic by the mitochondrial pathway. And moreover, the pro-apoptotic BCL2 family members BAM and DIVA were significantly up-regulated in SSD-treated cells and this suggested that the mitochondrial pathway played an important role in SSD-treated cells apoptosis. In addition, up-regulated TNFB heterotrimer binds with receptors, and may in turn recruit TRAF2, TRAF3 and cIAP1, and the BIR1 domain of cIAP1 were removed in this process, so that CASP9, CASP7, and CASP3 activity were activated (Kuai et al., 2003). In short, these data suggest SSD induced the apoptosis of HT29 through the apoptosis pathway of TRAIL, TRAIL-R and caspase10 and/or caspase8, and could provide targets for the treatment of colon cancer.

ACKNOWLEDGEMENTS

This work was supported by the grant from Science and Technology Program of Liaoning Province Science and Technology Agency (NO. 2009225008-9).

ABBREVIATIONS

SSD, Saikosaponin D; **PARP,** poly(ADP)polymerase; BCL2, B-cell lymphoma 2; **IAP,** inhibitors of apoptosis; **SSA,** saikosaponin A; **PCR,** polymerase chain reaction; **MTT,** 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; **DMSO,** dimethylsulphoxide.

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Full Length Research Paper

Electrophysiological changes in response to L-arginine infusion in isolated mammalian heart

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Arrhythmia is one of the major detrimental risk factors for cardiac arrest and death especially those associated with prolonged Q-T interval. Several antiarrythmic and cardiac agents prolong the Q-T interval as class I-a and class III anti-arrythmic agents. The cGMP is an important second messenger formed by the NO induced-guanylyl cyclase in response to L-arginine infusion. The aim of the present work is to investigate the relation between L-arginine infusion and different electrocardiograph (ECG) intervals. Isolated hearts from 6 male rabbits were perfused using Langendorff's apparatus in which the perfusion fluid was ringer-Locke solution, applied at constant flow rate and was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Each heart served as its own control before infusion of adrenaline and then L-arginine at concentration of 3 mmol/L. With the help of Power Lab data acquisition and analysis system and Chart 7 program (ADInstruments Australia), the force of contraction, heart rate, and ECG were recorded for 5 min. NO generation and cGMP generation produces negative chronotropic effect with significant decrease in the heart rate from (125.2 ± 8.320) to (93.67 ± 7.04) /min. and significant prolongation of the Q–T interval 34% from (199.5 ± 22.35) to (268.4 ± 9.948) m.sec. and the Q-Tc by 24% from (291.0 ±35.98) to (361.2 ± 13.23) m.sec. L-arginine infusion with NO generation in isolated mammalian produces negative inotropic effects as well as prolongs Q-T and Q-Tc intervals.

Key words: L-arginine, Q-T interval, arrhythmias, isolated heart.

INTRODUCTION

Arrhythmias is one of the major cardiovascular causes of mortality caused by abnormality in the generation or propagation of the cardiac electricity. Some of these arrhythmias are paroxysmal with life threats, others have tremendous effects ending with death as torsades de pointes (TdP) which is a polymorphic ventricular tachy-cardia characterized by a distinctive pattern of undulating QRS complexes that twist around the isoelectric line. TdP is usually self-terminating or can subsequently degene-rate into ventricular fibrillation, syncope, and sudden death (Blancett et al., 2005). The electro cardio graph (ECG) intervals includes R-R, P-R and QT intervals. QT interval (also termed electrical systole), the period between the beginning of the QRS complex and the end of the T wave of the electrocardiogram, reflects the ventricular action potential duration (APD) and represents the required period for ventricular depolarization and repolarization. This duration is determined by the balance of inward and outward currents occurring during depolarization and repolarization phases of ventricular action potential (AP). TdP has been associated with QT interval prolongation of the electrocardiogram; therefore,

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the QT interval has come to be recognized as a surrogate marker for the risk of TdP (Van et al., 2004).

Nitric oxide (NO), essential for the proper functioning of the cardiovascular system, is derived from L-arginine by NO synthase (NOS) in endothelial cells as shown in Figure 1. NO through cGMP generation produces negative inotropic and chrontropic effects on isolated mammalian heart (Sakr et al., 2010). NO donors or the precursor for NO synthesis, L-arginine, can ameliorate reperfusion-induced arrhythmias and reduce ischemic/ reperfusion injury in rabbits. Several previous studies investigated the effects of l-arginine on the Q-T interval and Q-Tc in the presence of other variables such as exercise (Bednarz et al., 2000) and hypercholesterolemia (Kumar et al., 2009). So the aim of the present work is to clarify the ECG intervals changes in response to L-arginine infusion on isolated mammalian heart in the absence of other variables.

MATERIALS AND METHODS

Animals

Six adult white adult newzealand male rabbits weighing between 2 and 3 kg were used for the experiments with the approval of Ethical Committee of the Medical School, King Khalid University, Abha, Saudi Arabia. The animals were obtained from the animal house of the College of Medicine of King Khalid University where they were fed with standard rabbit pellets and allowed free access to water. They were housed at a controlled ambient temperature of 25 ± 2°C and 50 ± 10% relative humidity, with 12-h light/12-h dark cycles. All studies were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH, 1996).

Experimental procedure

This experiment was carried out in accordance with the Langendorff (1985) procedure. Each rabbit was injected with 1000 IU of heparin intravenously through the marginal ear vein. Five minutes later, a blow on the neck of the rabbit made them unconscious. The chest was opened and the heart was dissected out with about 1 cm of aorta attached, and washed quickly as possible with oxygenated Ringer-Locke solution (NaCl; 45.0 g, $NaHCO_3$; 1.0 g, D-glucose; 5.0 g, KCl; 2.1 g, $CaCl_2.2H_2O$; 1.6 g, in 5 L of distilled water). The isolated heart was gently squeezed several times to remove as much residual blood as possible. The heart was then transferred to the perfusion apparatus (Radnoti isolated heart system, AD instrument, Australia) and tied to a stainless steel canula through the aorta. The perfusion fluid was worm Ringer-Locke solution which was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide and was applied at a constant perfusion pressure of 70 mm Hg (Langendorff, 1985). Temperature was continuously monitored by a thermo-probe inserted into the perfusion fluid tank and maintained between 36.5 and 37.5°C. The hearts were allowed to stabilize for 30 min before any drug interventions. 1 ml of Ringer-Locke solution containing 3 mmol/L of L-arginine was injected over 30 s with the aid of 1 ml syringe through the perfusion line above the aortic line, and the changes in the cardiac parameters were recorded (Figures 2 and 3). Parameters measured are heart rate (beats/min) and ECG for rhythm monitoring. During the experiments each heart served as its own control before infusion of each solution.

Statistical analysis

Results were expressed as the mean value ±SD. Statistical differences between groups were assessed using the Graph pad5 software by t- test. Values of $P<0.05$ were considered significantly different (95% confidence interval).

RESULTS AND DISCUSSION

Table 1 shows that the infusion of 3 mmol/L L-arginine reduces the heart rate significantly by 25% from (125.2 ± 8.320) to (93.67 ± 7.04) /min. Table 1 shows that the infusion of L-arginine increases the R-R interval significantly by 35% from (496.1 ± 25.83) to (670.2 ± 18.79) m.sec and decreases the P-R interval significantly by 40% from (52.00 ± 6.106) to (32.09 ± 2.401) m.sec. Also, NO generation in response to L-arginine infusion significantly prolongs the Q-T interval by 34% from (199.5 ± 22.35) to (268.4 ± 9.948) m.sec. and the Q-Tc by 24% from (291.0 ±35.98) to (361.2 ± 13.23) m.sec.

Nitric oxide (NO) synthetized by essentially all cardiac cell types exerts a key role in regulating cardiac function (Kelly et al., 1996). NO is a highly diffusible gas that spreads greatly from its site of synthesis and a free radical highly reactive with other species, notably oxygen, superoxide and iron-containing haeme groups which act as NO scavengers (Massion et al., 2003). For this reason, the half-life of NO is limited to seconds and its effects are localized close to where it is synthetized. NO generated within the cardiomyocytes can exert intracrine effects or modify the functional properties of adjacent cardiomyocytes (Schulz et al., 2005). NO generated from non-cardiomyocyte sources (coronary, endocardial, and endothelial cells, autonomic nerves and ganglia, and blood-formed elements) can exert direct effects on cardiomyocytes and indirect effects by modulating coronary blood flow and/or autonomic transmission (Ziolo et al., 2004; Seddon et al., 2007). The heart produces NO on a beat-to-beat basis in response to changes in coronary flow and myocardial loading. In rabbit hearts, NO levels reach peak values during diastole and lowest during systole. NO concentrations were 15% lower in rat hearts (Pinsky et al., 2007).

The ventricular AP Figure (5) can be divided into 5 phases. When a wave of depolarization reaches ventricular myocytes, a rapid opening of voltage-gated sodium channels (INa), allows for the influx of Na^+ into the ventricular myocytes; this produces phase 0 of ventricular AP, and produces depolarization, which is represented by the QRS complex on the surface ECG. Immediately after maximal depolarization of Phase 0, INa is in the inactivated stage, and repolarization begins with activation of the transient outward potassium current (Yan and Antzelevitch, 1996). This process causes a brief rapid repolarization and yields a notch on the ventricular action potential known as Phase 1. This phase is followed by a slower phase of repolarization called Phase 2 (the plateau). Phase 2 of the AP is generated mainly by

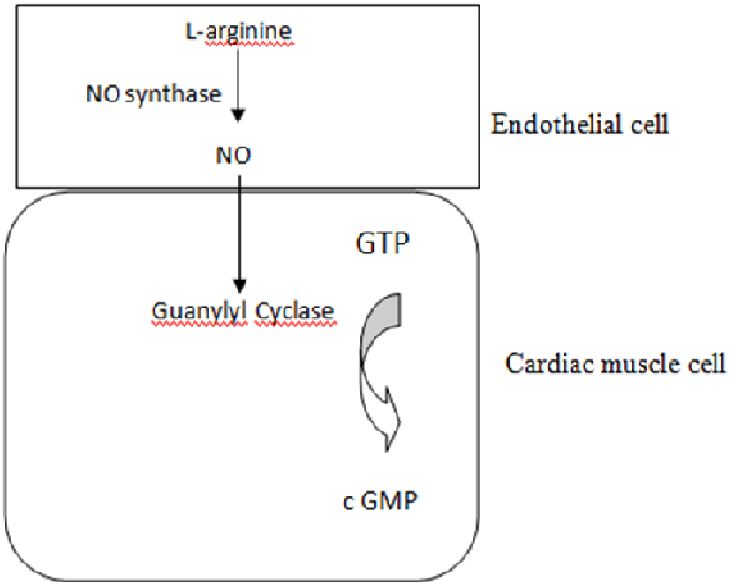

Figure 1. Mechanism of NO generation in the endothelial cells and its activation on gyanylyl cyclase, in the presence of endothelial nitric oxide synthase (eNOS), L-arg arginine is converted into NO. NO diffuses to the ventricular muscle fiber forming cGMP from GTP by the action of guanylyl cyclase.

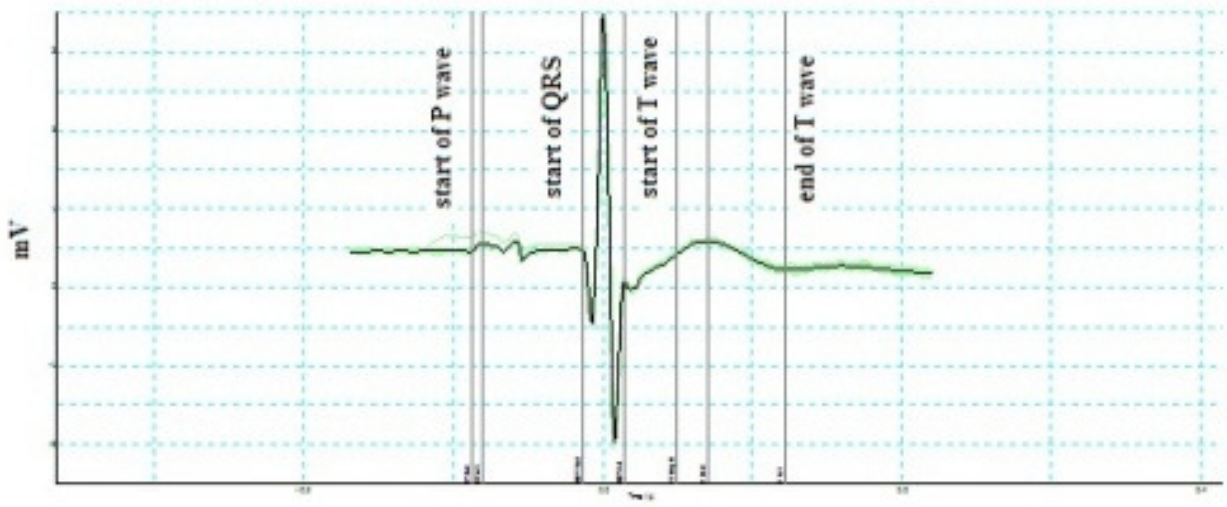

Figure 2. Baseline recording of ECG from the rabbit's heart.

the inward L-type calcium current (ICaL) and outward K^+ currents. The delayed rectifier potassium currents also begin to activate at this phase. The activation is slow and the currents have a reduced conductance at positive transmembrane potentials causing the prolonged AP (Sanguinetti and Tristani-Firouzi, 2006).

Our results showed that L-arginine infusion produced a significant negative chronotropic effect with decreasing the heart rate by about 25% and significant prolongation of the R-R interval. These data were previously concluded by other studies that proved that effect. NO generated under the influence of NO synthase stimulated the guanylyl cyclase yielding the highly important second messenger cGMP. cGMP decreased the rhythmicity by the activation of the acetyl choline dependent K channels in the sino-atrial node facilitating excess K efflux with hyperpolarization generation. Our data was in accordance with Kiziltepe et al. (2004) who discovered

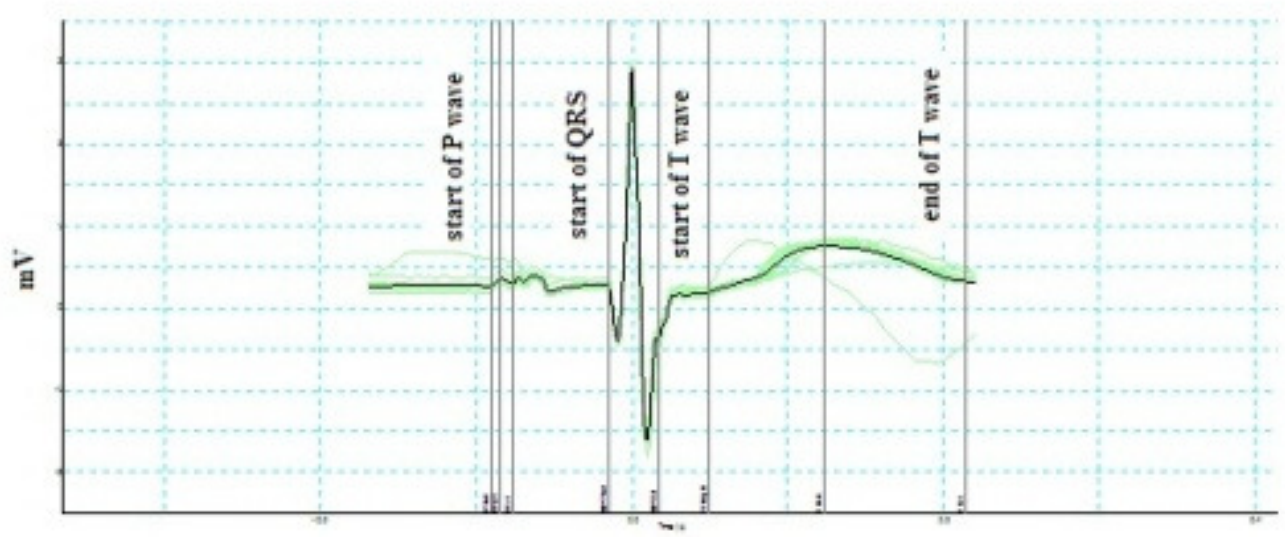

Figure 3. Recording of ECG from the rabbit's heart in response to L-arginine.

Table 1. The effect of L-arginine infusion 3 mmol/L on isolated mammalian heart on heart rate, R-R interval, P-R interval, Q-T interval and Q-Tc intervals.

Parameter	Baseline	L-arginine infusion	Percent of change
Heart rate /min	125.2 ± 8.320	93.67 ± 7.04	- 25 %
R-R interval (m.sec)	496.1 ± 25.83	670.2 ± 18.79	+ 35 %
P-R interval (m.sec)	52.00 ± 6.106	32.09 ± 2.401	+ 40 %
Q-T interval (m.sec)	199.5 ± 22.35	268.4 ± 9.948	+ 34 %
Q-Tc interval (m.sec)	291.0 ±35.98	361.2 ± 13.23	+24 %

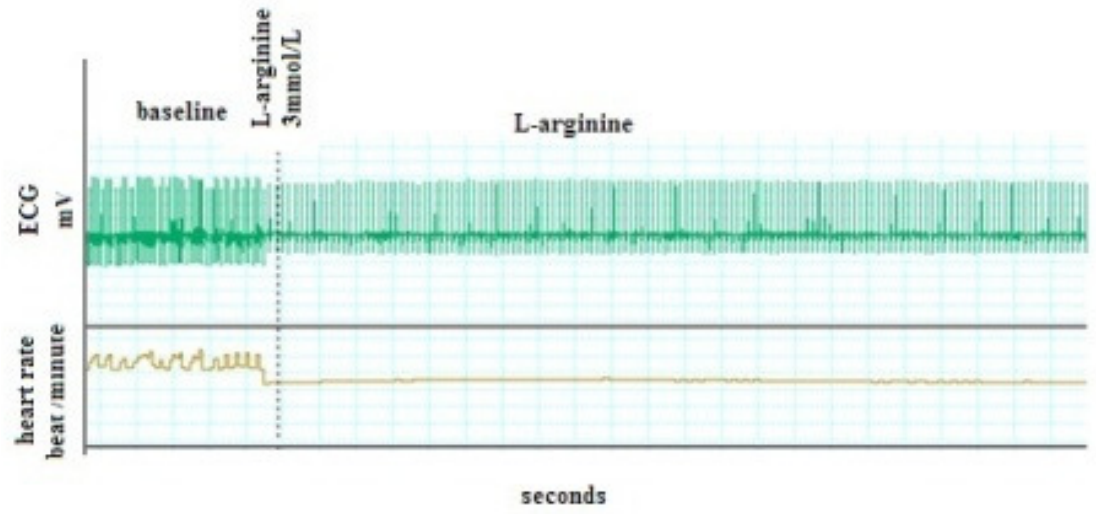

Figure 4. Effect of L-arginine (3 mmol/L) on isolated mammalian heart at horizontal scaling, showing the ECG recording, heart rate. (on scaling 500:1).

that L-arginine may be a natural anti-arrhythmic agent upon consideration of its effect in restarting normal sinus rhythm at the completion of heart surgery. The P-R interval introduces as idea about the conduction of the electrical impulse in the atrial wall as well as the atrio-ventricular node. Naturally the atrioventricular nodal

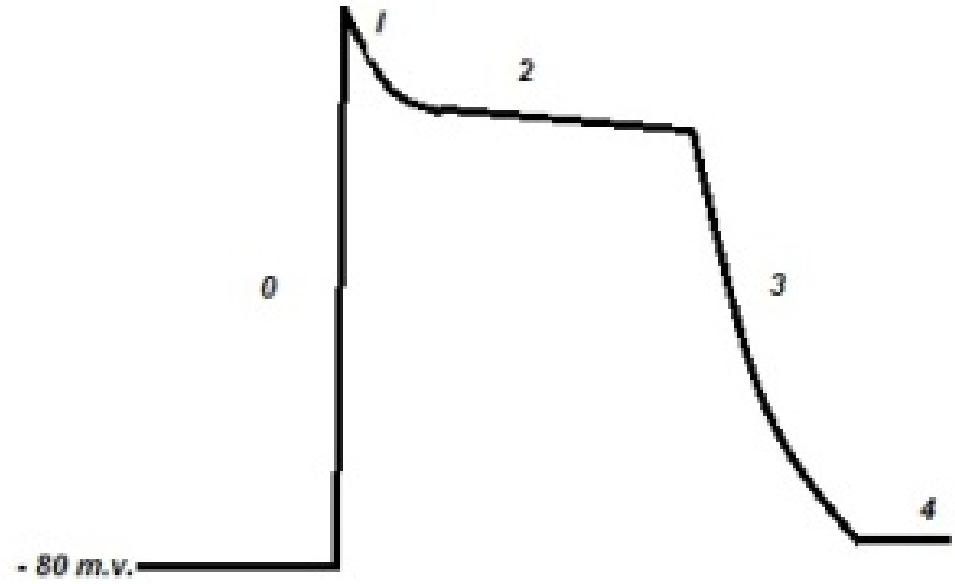

Figure 5. Action potential of ventricular muscle fiber phase 0: rapid depolarization, phase 1 slow partial repolarization, phase 2: plateau, phase 3: rapid repolarization and phase 5: complete repolarization.

(AVN) is characterized by the slowest velocity of conduction in the myocardium which offers sufficient time for atrial contraction before ventricular contraction and protects the ventricles from high atrial rhythm. In disagreement with our results, conduction through the AVN was previously studied by Khori et al. (2011) who concluded that NO generation in response to L-arginine had stimulatory effect on AV nodal properties through decreasing the refractory period. The mechanism of impulse conduction facilitation could be attributed due to the activation of protein kinase G in response to cGMP. Previous research suggested that the NO-cGMP-PKG pathway contributes to phosphorylation of K(ATP) channels in rabbit ventricular myocytes producing depolarization of the myocytes in the AVN and enhanced conduction (Tamargo et al., 2010)

Our results showed that NO generation produces prolongation of the Q-T and Q-Tc intervals significantly. The QT interval is measured from the beginning of the QRS complex to the end of the T wave, therefore it represents the duration of depolarization and repolariza-tion of ventricular muscle fibers which is roughly parallel to the ventricular absolute and relative refractory period. The QT interval consists of 2 components: the QRS complex represents ventricular depolarization and the JT interval, a measure of the duration of ventricular repolarization.

Since QT duration changes inversely with heart rate; the slower the heart rate the longer the QT interval. Hence, a QT correction formula is needed to substitute for each measured QT interval. The corrected QT (QTc) value corresponds to one that would have been measure-ed had each ECG tracing been recorded at the same heart rate (Bednar et al., 2010). The three most common correction methods are Bazett's equation [QTcB = QT/RR0.5; (Bazett, 1920)], Fridericia's equation [QTcF = QT/RR0.33; (Frodericia, 1920)], and Van de water's equation [QTcV=QT-0.087(RR-1000)]; (Van de water et al., 1989). The QT interval prolongation may arise from either a decrease in repolarizing cardiac membrane currents or an increase in depolarizing cardiac currents late in the cardiac cycle. Most commonly, QT interval prolongation is produced by delayed repolariza-tion due to reductions in either the rapidly or the slowly activating delayed rectifier cardiac potassium currents. Less commonly, QT interval prolongation results from prolonged depolarization due to a small persistent inward leak in cardiac sodium current or from a sustained sodium current. QT interval prolongation can be characte-rized as acquired (drug-induced QT prolongation) or congenital known as long QT syndrome (LQTs), a rare genetic disorder associated with life-threatening arrhythmias. Prolongation of ventricular repolarization and consequently lengthening of QT and/or QTc interval results in an increase in the absolute refractory period. This is the mechanism by which some antiarrhythmic drugs prevent or terminate ventricular tachyarrhythmias; however, prolongation of ventricular repolarization may be also implicated with arrhythmias especially TdP. Therefore, QTc prolongation is widely viewed as a surrogate marker of the arrhythmogenic potential of a drug. The precise relationship between the extent of QTc prolongation and the risk for TdP is unknown. Recently published data in humans showed that TdP rarely occurs

unless the QTc exceeds 500 ms (Bednar et al., 2001).

The mechanism of Q-T prolongation in response to L-arginine seems to be unclear. Several previous works by Horimto et al. (2000) and Stavrou et al. (2001) established that NO generation increases the ventricular muscle action potential duration and the absolute refractory period independent to the ATP sensitive K^+ channels, meaning that Q-T prolongation in response to L-arginine is not related to the change in the ventricular repolarization.

Previous work investigated the significant correlation between the activation recovery intervals and the action potential duration (Haws and Lux, 1990; Millar et al., 1985). Wang (2003) investigated the activation-recovery intervals from epicardial ECGs leads and recorded that intravenous administration of N^G-nitro-L-arginine, a NO synthase inhibitor, increased left ventricular systolic pressure from 101±7 to 118±10 mmHg (P=0.02), and left ventricular end diastolic pressure from 6.3±1.5 to 8.8±1.8 mmHg (P<0.01) without changing the heart rate (96±4 beats/min versus 94±3 beats/min, P=0.06). Wang (2003) concluded that NO synthase inhibition with N^G-nitro-L-arginine did not change the configuration of epicardial ECGs or influence the activation-recovery intervals. These data indicate that basal NO inhibition has no significant effect on ventricular repolarization.

Evidence also suggests that NO is involved in certain drug induced reduction of action potential duration. In guinea pig ventricular papillary muscles, inhibition of NO synthase with NG-monomethyl-L-arginine (L-NAME) atenuates lipopolysaccharide-induced shortening in action potential duration (Chen et al., 2000). In normoxic rabbit Purkinje fibres, NO donors, S-nitrosoglutathione and spermine NONOate shorten the action potential duration to the level seen in hypoxic preparations (Baker, 2001). The shortening of action potential duration can be abolished by an NO remover such as carboxy-PTIO (Baker, 2001). Another NO donor, sodium nitroprusside, decreases the duration of repolarization and increases the pacemaker activity of the isolated guinea pig sinus node. However, sodium nitroprusside has no significant effect on the action potential duration of ventricular papillary muscles (Joa et al., 2000).

Prolongation of the ventricular action potential and consequently the Q-T interval could be attributed to Na^+ and Ca^{2+} permeability. NO inhibits Na influx in isolated mouse and guinea pig ventricular myocytes without changing channel kinetics (Ahmmed et al., 2001). This inhibition is due to a decrease in open probability (Po) without changes in single-channel conductance and involves the activation of both protein kinase G (PKG) and protein kinase A (PKA). However, in rat ventricular myocytes, NO donors induce a late Na+ current (INaL) because Na^+ channels fail to inactivate completely or close and then reopen at depolarized potentials, that is, during the plateau phase of the AP(Ahern et al., 2000). Cardiac depolarization opens L-type Ca^{2+} channels (LTCC) generating an ICa that is responsible for the AP plateau and triggers a larger release of Ca^{2+} through the opening of RyRC. The ICa is also responsible for phase 0 depolarization and the slow diastolic depolarization in sinoatrial (SAN) and AVN cells.

NO produces contradictory effects on ICa, increasing,(Wang, 2000) decreasing, (Abi-Gerges et al., 2002) or producing a biphasic effect (Campbell et al., 1996). In human atrial myocytes, the NO donor SIN-1 stimulates ICa, an effect that decreases at concentrations of 1 mM (Stavrou et al, 2001). The increase in ICa is produced via cGMP-inhibited PDE3, which increases intracellular cAMP levels (Kirstein et al., 1995); however, it has also been attributed to a cAMP-independent activation of PKG (Wang, 2000).

In accordance to our data, the prolongation of the Q-T and Q-Tc may be related to the cGMP effect on the L-type Ca^{++} channels which was confirmed by Tohse et al. (1995) who studied the effect of the cGMP generated in response to the atrial natriuretic paptide on the rbbit ventricular muscle action potential and reviewed that cGMP inhibits the Ca^{++} current through blockade of the L-type Ca^{++} channels. These data was also confirmed by another study performed on guinea pig myocytes and evidenced that cGMP regulated the Ca^{++} current (Levi et al., 1989).

Conclusion

From these data we can conclude that inspite of its cardioprotective effects; NO generation in response to L-arginine infusion prolongs the Q-T interval and consequently the corrected Q-T. Further studies are needed to investigate the effects of NO generating drugs as Na nitrprosdie and hydralazine on the Q-T interval and their impact in patients with cardiac arrhythmia.

Conflict of interest

The authors have no conflict of interest to declare.

ABBREVIATIONS

PAAET, Public Authority for Applied Education and Training; **TdP**, Torsades de pointes; **ECG**, electro cardio graph; **APD**, action potential duration; **AP**, action potential; **NO**, nitric oxide; **NOS**, nitric oxide synthase; **Po**, open probability; **PKG**, protein kinase G; **PKA**, protein kinase A; **LTCC**, L-type Ca2+ channels; **SAN**, sinoatrial; AVN, atrioventricular nodal; ICa, inward calcium current; **CGMP,** Cyclic guanosine monophosphate, **RyRC**, bryanodine receptor calcium release channel.

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Full Length Research Paper

Protective effects of oxysophoridine on alcoholic hepatic injury in mice

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The aim of study is to detect the effects and relevant mechanisms of oxysophoridine on alcoholic hepatic injury in mice. Sixty male Institute of Cancer Research (ICR) mice were randomly divided into a normal control group, an alcoholic liver injury model group, a positive control (tiopronin) group and an oxysophoridine (250, 125, 62.5 mg/kg) group. Consecutive interventions were conducted on each group for 10 days; specimens were obtained according to requirements of the testing indicators 16 h after the last drug administration. The protective effects were evaluated by biochemical parameters including serum aspartate transaminase (AST), alanine transferase (ALT), reduced glutathione (GPx), liver malondialdehyde (MDA) and superoxide dismutase (SOD). The pathological changes of the liver in microstructure and ultrastructure were observed. A decreased level of serum ALT, AST activity and liver MDA content with increased liver SOD, GPx activity ($P<0.05$) were observed ($P<0.05$) in the oxysophoridine group compared with the alcoholic liver injury model group, in which elevated serum ALT and AST activity were recorded, along with a marked increase of liver MDA ($P<0.05$), decrease of liver SOD and GPx ($P<0.05$). The following changes of the liver were observed in the model group: Blurred contour of the hepatic lobule with punctated or focal necrosis in partial liver cells, multiple intracellular microvesicular steatosis, with lipid droplets formed in the cytoplasm, marked swelling of hepatocytes and disarrangement of hepatic cords, swelling of mitochondria, with disappeared or broken cristae, enlarged endoplasmic reticulum and condensed chromatin. Compared with the model group, a decrease in pathological changes of various degrees was observed in the oxysophoridine group at various doses. The result indicates that oxysophoridine prevents alcoholic liver damage in mice and the protective effect may be associated with anti-oxidative stress.

Key words: Alcohol, oxysophoridine, oxidative stress, antioxidants.

INTRODUCTION

Alcohol-related liver disease (ALD) is a major public health hazard in developed as well as in developing world

(Abdul et al., 2010). ALD is a multifactorial disease that progresses through a set of distinct stages. ALD involves hepatocellular injury induced by consumption of ethanol. ALD is considered to be progressive and associated with duration and quantity of alcohol consumed (Lucey et al., 2009). Upwards to 90% of individuals consuming alcohol on a daily basis develop fatty liver (steatosis) which can resolve upon cessation of alcohol consumption (Smathers et al., 2011). Despite extensive research, alcohol abuse remains one of the most common causes of acute and chronic liver diseases in the world. Despite our best efforts, alcohol remains one of the most common causes of both acute and chronic liver disease in the United States (Sofair et al., 2010). In Western countries, up to 50% of cases of end-stage liver disease have alcohol as a major etiologic factor (Orholm et al., 1985). Excessive alcohol consumption is the third leading preventable cause of death in the United States. Alcohol-related deaths, excluding accidents/homicides, accounted for 22,073 deaths in the United States in 2006 with 13,000 of those specifically attributed to ALD (Heron et al., 2009). Alcohol represents a major financial burden on the overall economy as well, with an estimated cost of US$185 billion annually lost productivity, motor vehicle accidents among others (Kim et al., 2002). In China, ALD incidence increased year by year. Due to increased frequency of drinking and change of diet construction, such as the increase of fat content, the incidence of alcoholic liver disease has increased in China, becoming another important risk factor for morbility and mortality in addition to viral hepatitis (Jing et al., 2008). Alcoholic liver disease is found to be a common disease in Zhejiang Province, indicating an urgent need for the Public education on alcohol abuse and the treatment on related health problems (Youming et al., 2003). Drugs including bifendate, tiopronin and bicyclol have been reported to have protective effect against ALD (Hirayama et al., 1983; Peng et al., 2003; Zhao et al., 2008); however, there is no satisfactory therapy for alcoholic liver disease at present. Except for the combination of abstinence from alcohol and supportive care (Bouneva et al., 2003), however, definite treatment strategies for ALD remain undefined. Developing agents possessing hepatoprotective effects from natural products and traditional Chinese formula has become the focus of research in recent years (Leeetal, 2007; Tsenget al., 2007; Chandan et al., 2008). Moreover, ALD imposes a significant economic burden from the lost wages family, high health care costs people, and lost productivity human. Chinese herbal medicine has the advantage in terms of multi-targeting efficacy, lower toxicity, as well as lower cost. In recent years, more and more researchers have begun to focus on herbs and their constituents in liver injury prevention and treatment.

Natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases.(Samy et al., 2013). The use of medicinal plants forms the basis of the traditional healing system in many developing countries (Steenkamp et al., 2013). The world health organization (WHO) highly appreciated the conventional medical practices for treatment and precautionary measure of diarrheal diseases (Barakat et al., 2013).

The plants of the genus *Sophora* (subfamily Papilionaceae in the family Leguminosae) range from the temperate to the tropic areas in the world. Some of the *Sophora* plants are important sources of Chinese drugs, such as ku-shen (roots of *Sophora flavescens* Ait.), Shan-dou-gen (roots of *Sophora tonkinensis* Gagnep), and ku-dou-zi (roots of *Sophora alopecuroides* L.). Phytochemical investigations show that there exist more than twenty chemical compounds, belonging to alkaloids, flavonoids, volatile oil, organic acid, amino acid, protein, saccharide among others (Hong et al., 2011). Alkaloids from *S. alopecuroides* L. show strong medicinal functions when used for treatment of many diseases such as depressant, analgesic, hypothermic, antipyretic, cardiotonic activities, virus, liver fibrosis, tumors and improves immunity (Xuegong et al., 2007; Hong et al., 2011).

Oxysophoridine (OSR) is a major alkaloid extracted from *S. alopecuroides* L. Previous studies have determined its pharmacological activities in terms of anti-inflammation, immune regulation, growth suppression of hepatocellular carcinoma (Jianqiang et al., 2001; Yao et al., 2012). Basic research has confirmed the hepatic protection effect of its structural analogue oxymatrine (Jue and Guojun, 2000); however, there has been no published account to demonstrate that OSR is beneficial in the treatment of ALD. This experiment was designed to observe the protective effect of OSR on alcoholic liver injury in mice, and to study its oxidation mechanism.

MATERIALS AND METHODS

Drugs

Oxysophoridine was provided by the Ningxia Institute of Materia Medica. It is a white powder with molecular weight (Mw) of 264, melting point (mp) of l62°C, purity of 99% with batch number, 991230. Tiopronin was obtained from the Henan Xinyi pharmaceutical Co., Ltd with batch number, 100909. Alanine transferase (ALT), aspartate transaminase (AST), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) kits were purchased from Jiancheng Bioengineering Institute (Nanjing). All other reagents used were of highest purity and were commercially available; the 50% ethanol solution was diluted from anhydrous ethanol, with a dose of 5 g/kg body weight.

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#These authors equally to this study, therefore they are both first authors.

Animals

Sixty male, healthy adult Institute of Cancer Research (ICR) mice weighing 18-22 g were provided by the Laboratory Animals Center of Ningxia Medical University (Yinchuan, China). The mice were housed in an animal care facility at room temperature of 25±1°C with a 12 h light/dark cycle, and were given free access to standard pellet diet and tap water. The mice were left for three days to acclimatize before the treatment. This study was approved by Institutional Animal Ethics Committee, at Ningxia Medical University. The study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (1985).

Treatment

The mice were randomly divided in to six groups with ten in each to evaluate the protective effect of OSR pretreatment on ethanol induced acute liver toxicity. The normal control (NC) group received only distilled water. The alcohol control (AC) group received only ethanol (5 mg/kg body weight). Group OSR I received ethanol and oxysophoridine (250 mg/kg body weight). Group OSR II received ethanol and oxysophoridine (125 mg/kg body weight). Group OSR III received ethanol and oxysophoridine (62.5mg/kg body weight). The positive control (PC) group received ethanol and tiopronin.

Experimental design

The experiment was carried out for 10 days. All mice underwent administration by gavage. At 7:00-8:00, saline was given to the NC and AC groups, oxysophoridine was given to all the experimental groups (OSR I – OSR III), the PC group mice were fed with tiopronin (30 mg/kg body weight). At 19:00-20:00, the 50% ethanol was given at 5 g/kg body weight to all groups except the NC group.

Preparation of serum and tissue homogenate

Preparation of serum and tissue homogenate were determined by the method of Leelavinothan and Arumugam (2008). At the end of the experimental period, the animals were sacrificed by cervical dislocation under mild anesthesia. Blood was collected and centrifuged for serum separation. The tissues were dissected out, weighed and washed using ice cold saline solution. A part of tissues were minced and homogenized (10% w/v) in 0.9% physiological saline buffer and centrifuged at 10000 r/min at 4°C. The resulting supernatant was used for various biochemical assays.

Light microscopic examination

Liver tissues were cut into ~3 mm-thick slices and fixed with 10% neutral formalin. The tissue slices were embedded in paraplast. Tissue sections of 5 µm were stained by hematoxylin and eosin (H and E) and observed with a Nikon Eclipse E400 light microscope.

Electron microscopic examination

To observe ethanol-induced ultrastructural changes by conventional electron microscopy, livers were fixed *in situ* by vascular perfusion with Karnovsky's fixative (2% para-formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4) and post-fixed in 1% osmium tetraoxide. Ultra-thin sections were stained by uranyl acetate and lead citrate and observed with a Philips transmission electron microscope.

Assay for serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

The activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed spectrophotometrically according to the standard procedures, using commercially available diagnostic kits (Jiancheng).

AST and ALT activities were determined by the method of Reitman and Frankel (1957). Each substrate, 0.5 ml (2 mMα-ketoglutarate and 200 mM L-alanine or L-aspartate) was incubated for 5 min at 37°C in a water bath. 0.1 ml serum was then added and the volume was adjusted to 1 ml with 0.1 M, pH 7.4 phosphate buffer. The reaction mixture was incubated for exactly 30 min at 37°C for ALT and AST, respectively. Then to the reaction mixture, 0.5 ml of 1 mM 2,4-dinitro-phenylhydrazine (DNPH) was added,after another 30 min at 37°C; the color was developed by addition of 5 ml of 0.4 N NaOH and the product read at 505 nm.

Hepatic malondialdehyde (MDA) determination

MDA was assayed by determining the rate of production of thiobarbituric acid-reactive components of Ohkawa et al. (1979). One gram of the liver was homogenized in 1.15% KCl buffer on ice. An aliquot of 0.2 mL was mixed with solution containing 20% acetic acid, 0.8% thiobarbituric acid and 8.1% sodium dodecyl sulfate, heated in water bath at 95°C for 60 min. The solution was centrifuged for 10 min at 4000 rpm, and the absorbance of the supernatant fraction was determined at a wavelength of 532 nm. The content of MDA was expressed in terms of nmol/mg protein.

Hepatic superoxide dismutase (SOD) Activity.

SOD was determined using the method of Winterbourn (1975), in which the light-triggered release of superoxide radicals from riboflavin leads to the formation of a blue complex through reaction with nitroblue tetrazolium. 1 g of the liver was homogenized in 0.1 M phosphate buffer pH 7.4 on ice and cleared by centrifugation at 3000 rpm at 4°C for 15 min. The supernatant fraction was incubated in solution containing 0.067 M phosphate buffer pH 7.8, 0.1 M ethlenediaminetetracetic acid (EDTA), 1.5 mM nitroblue tetrazolium (NBT) and 0.12 mM riboflavin for 10 min in an illuminated chamber with an 18 W fluorescent lamp. Absorbance was recorded at 560 nm, and SOD activity was expressed as units/mg protein.

Hepatic glutathione peroxidase (GPx) activity

GPx activity was measured by the method described by Ellman (1959). Briefly, reaction mixture containing 0.2 ml of 0.4 M phosphate buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of tissue homogenate (homogenate on 0.4 M phosphate buffer, pH 7.0), and 0.2 ml glutathione, 0.1 of 0.2 mM H_2O_2 was used. The content was incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% trichloroacetic acid (TCA) and centrifuged. Absorbance was recorded at 412 nm, and GPX activity was expressed as units/mg protein.

Statistical analysis

The data for various biochemical parameters were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using the statistical statistics software package (SPSS for Windows, V. 13.0). *P* values less than 0.05 were considered as statistically significant.

Table 1. Effect of OSR on hepatic markers (serum ALT and AST) in liver injury mice.

Group	ALT (IU/L)	AST (IU/L)
Control（NC）	12.51±5.02	22.97±2.89
50%Ethanol(AC)	88.31±7.88*	96.56±7.27*
Tiopronin(PC)	27.07±8.29*▲	42.06±4.20*▲
OSR250mg/kg	17.38±4.79▲	37.53±5.62*▲
OSR125 mg/kg	53.87±7.21*▲	42.98±3.20*▲
OSR 62.5 mg/kg	38.07±5.46*▲	45.36±4.94*▲

Table 2. Changes of activities of antioxidant enzymes in injury mice.

Group	MDA (nmol/mgprot)	SOD (U/ mgprot)	GPx (U/ mgprot)
Control(NC)	14.00±3.85	70.94±9.58	30.28±1.37
50%Ethanal(AC)	64.07±2.07*	47.97±3.67*	12.06±1.21*
Tiopronin(PC)	17.72±4.57▲	45.92±2.11*	22.71±2.87*▲
OSR250mg/kg	17.98±6.62▲	74.28±5.45▲	26.17±2.12*▲
OSR125mg/kg	25.46±3.50*▲	58.82±2.12*	21.63±2.03*▲
OSR62.5mg/kg	21.26±4.05*▲	31.45±3.77*	21.22±3.31*▲

Data were presented as mean (S.E.M). Number=10 per group. *$p < 0.05$ vs control group. ▲$p < 0.05$ vs AC group.

RESULTS

Effects of OSR on the hepatic enzymes

Results of the effect of OSR on ALT and AST are presented in Table 1. Protective effect of OSR on serum ALT and AST level were observed. Significance change in these parameters were found in the ethanol treated group as compared to control(p<0.05). The PC group was found significantly (p<0.05) effective in the normalization of these markers when compared to ethanol treated group. Pretreatment with OSR was found significantly (p<0.05) effective in the normalization of these markers when compared to ethanol treated group. The OSR groups show difference as compared to control. The activities of antioxidant enzymes SOD and GPx in liver are given in Table 2. A significant (P < 0.05) decrease in the activities of enzymatic antioxidants were observed in alcohol treated mice when compared with control mice. Administration of OSR to alcohol treated mice significantly (P < 0.05) increased the activities of enzymatic antioxidants when compared to alcohol treated mice.

MDA formation was measured to demonstrate the oxidative damage on LPO of ethanol induced liver injury in mice. A significant (p<0.05) increase of the MDA formation was found in the ethanol treated group when compared with control. We have observed that pretreatment with OSR at OSR I – OSR III leads to the significant (p<0.05) prevention of membrane damage when compared to ethanol treated group (Table 2).

Changes under light microscope

In the control group, light microscopy revealed clear hepatic lobules, orderly arranged hepatic plates, radial arrangement of hepatic cords, normal hepatic sinusoids, rich and pink cell plasma and clear structure of the nucleus, and a centered central vein (Figure 1A). However, hepatic changes were observed in the alcohol model group, including a blurred contour of the hepatic lobule, with punctate or focal necrosis in partial liver cells, as well as alcoholic foamy degeneration. The histological features of true microvesicular steatosis included hepatocyte enlargement, flocculent alteration of the cytoplasm as well as deep stained nucleus (Figure 1B). In the OSR groups, the structure of the lobules remained clear, hepatic cords were arranged radially and roughly in order, the hepatic sinusoids were relatively normal, and the cellular swelling was obviously alleviated with a few lipid droplets observed (Figure 1C).

Changes observed under the electron microscope

The hepatocytes of the control group were normally polygonal with oval-shaped nuclei and one or two nucleoli; the cytoplasm was filled with organelles, particularly rough endoplasmic reticulum, smooth endoplasmic reticulum, golgi apparatus, ribosomes, mitochondria and glycogen particles (Figure 2A). In contrast, the heaptocytes of the ethanol model group showed marked pathological alterations, which were represented by large

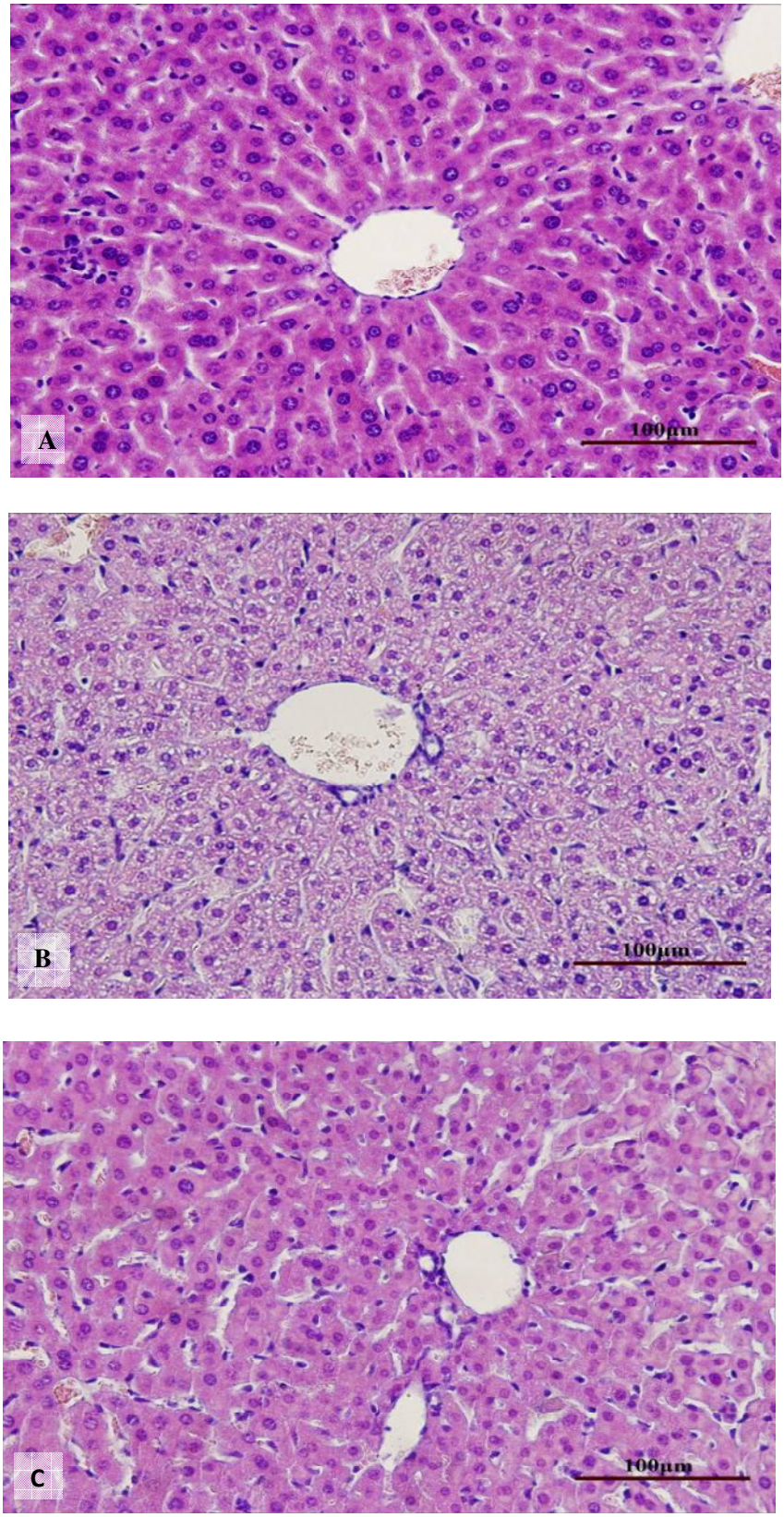

Figure 1. Hematoxylin-eosin-stained liver sections (400x). A, Control group showed normal liver histopathology; B, ethanol-treated group showed steatosis; C, OSR125mg/kg treatment showed examples of alleviation of steatosis and inflammation.

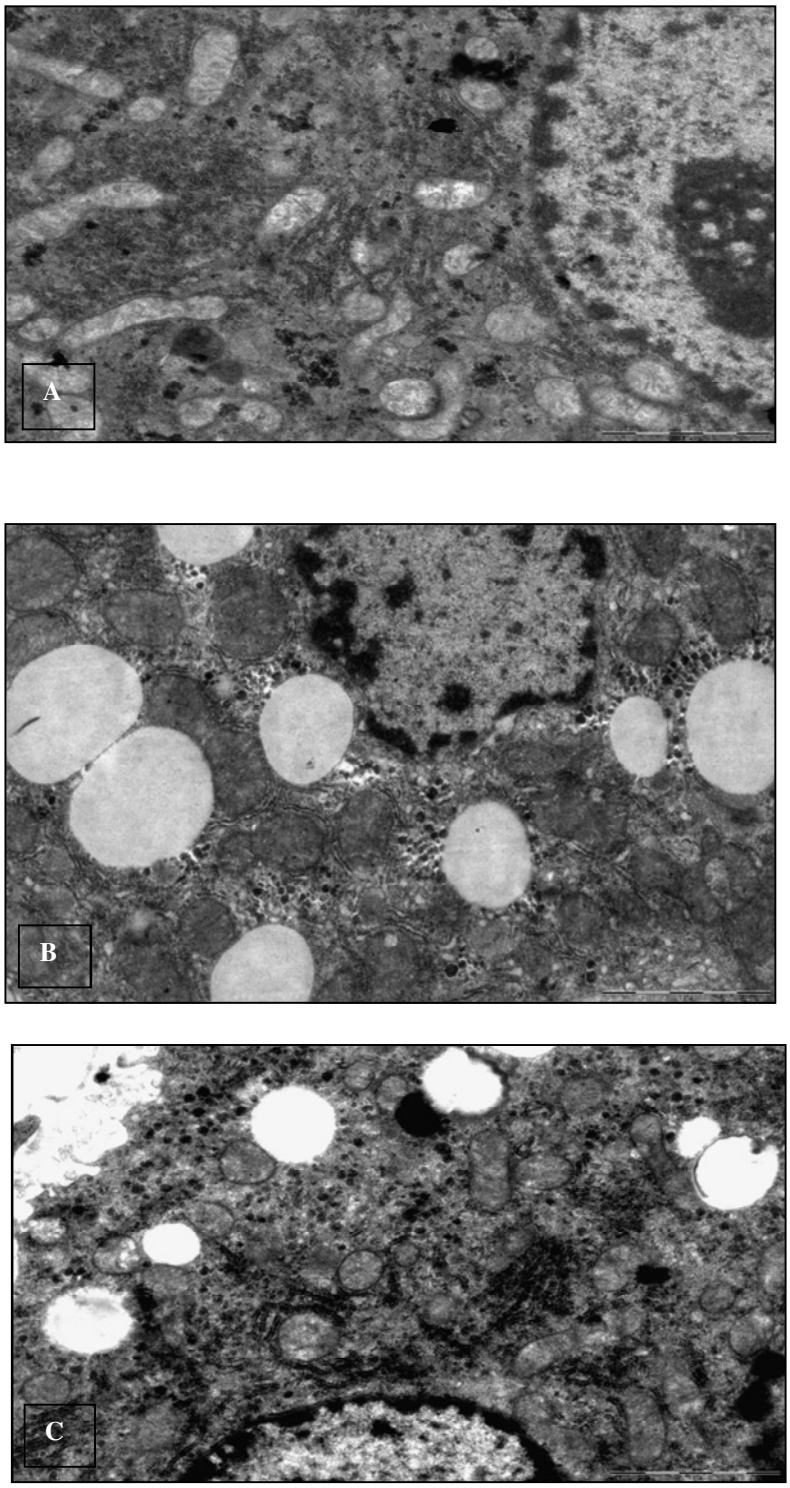

Figure 2. Electron micrograph of the liver (3000x). A, Control group showed normal liver histopathology; B, ethanol-treated group showed steatosis; C, ethanol + OSR 125 mg/kg treatment showed examples of alleviation in steatosis and inflammation.

lipid droplets in the hepatocytes, along with introcession of nuclear membrane, enlargement of mitochondria, disappeared mitochondrial crista, and dilated alteration of the rough endoplasmic reticulum (Figure 2B). The pathological changes were improved in OSR pretreated mice, which exhibited shape of normal liver ultrastructure and

organelle quantity (Figure 2C).

DISCUSSION

Alcohol abuse is a public health issue which has received wide attention. An abrupt increase of alcohol consumption in China was found in an epidemiological survey since the 1980s, accompanied by increasing problems due to alcohol abuse (Min et al., 2010). An accelerated tendency is observed in both morbidity and mortality of alcoholic liver disease, which was second compared to viral hepatitis.

To examine ethanol effects on the liver, most studies applied gastric intubation or intraperitoneal injection in rodents (Siegmund et al., 2003). Interestingly, the most commonly used amount of ethanol to mimic so called human binge drinking was 5–6 g/kg body weight, which approximately resembles 0.75 l of whiskey (40% v/v) in a 75 kg human (Sören et al., 2005). Our experiment chose to dilute the 50% ethanol solution from anhydrous ethanol every day, with a dose of 5g/kg body weight for 10 days. It caused alterations in liver morphology such as hepatocyte swelling and a decrease in sinusoidal fenestrae as seen by electron microscopy in mice.

The liver contains many kinds of enzymes. ALT is a useful screening factor for the detection of liver disease. AST and ALT are reliable markers of a liver function. Increased level of serum enzymes such as AST and ALT indicate increased permeability as well as damage and/or necrosis of hepatocytes (Hou et al., 2010). In our study, we have found that ethanol consumption caused a marked increase in the activities of ALT and AST due to severe damage of the tissue membrane. Decreased activities of these enzymes indicate the hepatoprotective effect of OSR.

The histological features of true microvesicular steatosis include hepatocyte enlargement, flocculent alteration of the cytoplasm and deep stained nucleus (Matthew and Elizabeth, 2008). In our study, the AC group cell showed alcoholic foamy degeneration and signs of hepatocyte ballooning. These results are consistent with several previous reports showing increased lipid peroxidation in other experiments (Min et al., 2010). Changes were improved in OSR and tiopronin pretreated mice, which exhibited areas of normal liver architecture and patches of necrotic hepatocytes, which indicates that pretreatment of OSR may protect liver cells from ethanol damage.

These changes were confirmed at the ultrastructural level. The hepatocytes from the ethanol model group showed large lipid droplets, along with introcession of nuclear membrane, enlargement of mitochondria, disappearance of mitochondrial crista, and dilated alteration of the rough endoplasmic reticulum. Compared with the AC group, pathological changes showed a noticeable improvement in the oxysophoridine group at varied degrees accordingly, which indicates that OSR may improve pathological changes of ultrastructure in hepatocytes, such as decrease of lipid droplets and recovery of the nuclei shape.

Many pathways are thought to be involved in ALD, including oxidative stress and mitochondrial damage (Stewart et al., 2001). Ethanol administration has been shown to induce oxidative stress (Dupont et al., 2000). Ethanol metabolism generates free radicals that result in degeneration of hepatic cells, due to alcohol-induced lipid peroxidation (Lieber, 1997). Lipid peroxidation serves as a marker of cellular oxidative stress and has long been recognized as a major causative factor of oxidative damage in chronic diseases (Son et al., 2007). Lipid peroxidation causes apoptosis and necrosis of hepatocytes, with the release of substances as MDA that triggers inflammatory and immune-mediated mechanisms of hepatocyte injury (Chikako et al., 2004). The elevation of MDA indicates increased production of lipid peroxide, suggesting that the content of MDA may reflect the extent of the cell being attacked by free radicals (Li et al., 2004). In our study, we found significant increase in serum MDA concentration in the AC group. Therefore, low levels of MDA in the experimental groups are indicative that OSR scavenges free radical.

Free radical scavenging enzymes, such as SOD and GPx, are the first line of defense against oxidative injury. The inhibition of antioxidant system may cause the accumulation of H_2O_2 or its decomposition products (Halliwell, 1994). Consistent with these reports, our results also showed decreased activities of SOD and GPx in tissues in the ethanol model group. Therefore, high level of these enzymes in the experimental groups suggests that the OSR helps eliminate free radicals in liver tissue. Administration of OSR restored the activities of enzymatic antioxidant in liver.

Conclusion

In conclusion, the results of functional tests together with histological observations suggest that OSR can play a significant role in preventing lipid peroxidation, and can be protective against alcohol-induced hepatotoxicity and liver damage. Furthermore, it enhances the antioxidant system of the body by elevating the activity of SOD and GPx.

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to development and implementation of this study.

ABBREVIATIONS

ALD, alcohol-related liver disease; **OSR,** oxysophoridine; **ALT,** alanine transferase; **AST,** aspartate transaminase; **MDA,** malondialdehyde; **SOD,** superoxide dismutase; **GPx,** glutathione peroxidase ; **NC,** normal control; **AC,** alcohol control; **PC,** positive control; H and E, hematoxylin and eosin; **TCA,** trichloroacetic acid; **EDTA,** ,ethlenediaminetetracetic acid; **NBT,**nitroblue tetrazolium; **DNPH,** 2,4-dinitro-phenylhydrazine .

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Full Length Research Paper

The neuroprotective role of *Nigella sativa* extract on ciprofloxacin and pentylenetetrazole treated rats

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This study aimed to investigate the *Nigella sativa* seed (NS) neuroprotective effect on ciprofloxacin (CFX) antibiotic with suggesting neurotoxic effect in rats through the determination of monoamines levels and acetylcholinesterase (AChE) activity in different brain areas. We used valporic acid as a reference antiepileptic drug and pentylenetetrazole (PTZ), a drug used for induction of epileptic model in rats. The present data revealed that the daily oral administration of NS (350 mg/kg b.wt.) for 14 days caused significant increase in the monoamines contents with no statistical difference in AChE as compare to control values. While the administration of CFX (500 mg/kg b.wt.) and/or PTZ (60 mg/kg b.wt.) was found to produce significant decrease in the concentration of monoamines and the activity of AChE in cerebral cortex, cerebellum, striatum and hippocampus after 7 and 14 days. Moreover, the pre- and post-treatment with NS in CFX and/or PTZ treated rats was found to ameliorate most of the side effects induced by these drugs. It could be concluded that the treatment with the therapeutic dose of CFX 14 days could lead to the development of seizures through the reduction of monoamines level and decreasing the activity of AChE in the tested brain areas. The administration of *N. sativa* pre- and the post treatment to CFX and/or PTZ treated rats were found to ameliorate their side effects. Suggesting that *N. sativa* seeds with antiepileptic activity and its administration could alleviate ciprofloxacin neurotoxicity.

Key words: Ciprofloxacin, Pentylenetetrazole, *Nigella sativa*; monoamines, acetylcholinesterase.

INTRODUCTION

Epilepsy is a common chronic neurological disorders affecting ~1–2% of the population worldwide and characterized by the repeated occurrence of seizures (McNamara, 1999; Blume et al., 2001; Loscher, 2002; Berg et al., 2010). Epilepsy may be developed as a result of the imbalance between excitatory and inhibitory neuro-transmission, alterations in neurotransmitter expression and function (Hirose et al., 2000). Animal models have played an important role for detecting the pathophy-siology of human epilepsies (Avoli et al., 2005). Pentylenetetrazole is considered the most useful experimental model which can reveal the changes associated with epilepsy (da Silva et al., 1998). Pentylenetetrazole (PTZ) exerts its action by binding to the picrotoxin-recognition site and benzodiazepine-binding site of the post-synapticgamma-aminobutyric acid A (GABAA) receptor. Thus, PTZ reduces the effects of endogenous GABA and other inhibitory transmitters, which renders the system in a hyperexcitable state. In the case of a convulsive dose, PTZ induces generalized

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tonic–clonic seizure activity within seconds (Huang et al., 2001). Also PTZ induced oxidative stress results in disturbance of the antioxidant enzyme status accompanied by neuronal injury and the development of epilepsy in rats (Sharma et al., 2010).

Ciprofloxacin belongs to the class of 4-fluoroquinolone antibiotics a commonly used therapy of many bacterial infections. Its antimicrobial activity is based on the inhibition of bacterial DNA gyrase. Ciprofloxacin have few reports of serious reactions over a period of 15 years of use and more than 340 million prescriptions (Ball et al., 1999; Segev et al., 1999). Ciprofloxacin-associated seizures occur most commonly in patients with special risk factors that may cause accumulation of drug (high doses of the drug, old age, renal insufficiency, drug interactions). Several mechanisms are thought to be responsible, the involvement of gamma-aminobutyric acid (GABA) and excitatory amino acid (EAA) neurotransmission and the kinetics of quinolones distribution in brain tissue are discussed (De Sarro and De Sarro, 2001). Extensive toxicological and biochemical experiments have been performed to explain the central nervous system (CNS) effects observed under therapeutic conditions (Akahane et al., 1993; De Sarro et al., 1999; De Sarro and De Sarro, 2001). Seizure activity is associated with a wide range of local biochemical changes, affecting various neurotransmitters (monoamines, amino acids) (Freitas et al., 2004; Cavalheiro et al., 2006). Green and Halliwell (1997) contributed the excitatory action of ciprofloxacin to its selective antagonistic effect on GABA (A) receptors and proconvulsion activity (Dodd et al., 1988; Kawakami et al., 1997). A number of antibiotics, including ciprofloxacin, have been demonstrated to stimulate the production of reactive oxygen species (ROS) in bacterial cells (Becerra and Albesa, 2002; Albesa et al., 2004). Ciprofloxacin previously reported for induction of oxidative stress in cerebral and hepatic tissues of rat (Gürbay and Hincal, 2004; Gürbay et al., 2007) and DNA damage in astrocytes (Gürbay et al., 2006) and the involvement of oxidative stress in tendenopathy related classic side effect of ciprofloxacin (Pouzaud et al., 2004).

Valproate (VPA) is one of the conventional antiepileptic drugs that possess a broad spectrum of antiepileptic activity (Loscher, 1993). Its pharmacological effects involve a variety of mechanisms, including increased GABA-ergic transmission, reduced release and/or effects of excitatory amino acids, blockade of voltage-gated sodium channels and modulation of dopaminergic and serotoninergic transmission (Perucca, 2002). Herbal remedies and alternative medicines are used throughout the world and in the past, herbs often represented the original sources of most drugs (Cooper et al., 2004; Tsao et al., 2005). *N. sativa* Linn., belonging to the family Ranunculaceae, commonly known as black seed or black cumin (Ali and Blunden, 2003), provide a highly nutritional product that has been used extensively as a supplement to help maintain good health and well-being (Cheikh-Rouhou et al., 2008). Several pharmacological properties have been documented such as antidiabetic (Kanter et al., 2004), antibacterial (Kanter et al., 2003), hepatoprotective (Nagi et al., 1999), nephroprotective (Yaman and Balikci, 2010), antitumor (Khan et al., 2003; Salem, 2005; Yi et al., 2008) antiepileptic (Akhondian et al., 2007) and neuroprotective (Ismail et al., 2008; Ezz et al., 2011; Akhtar et al., 2012).

Therefore, the aim of the current work was to evaluate whether the treatment with the therapeutic dose of ciprofloxacin for 14 days can induce convulsions or not and the efficacy of *N. sativa* seeds supplementation pre- or post-treatment to alleviate ciprofloxacin side effects as compared to the effect on PTZ induced seizure model through the estimation of monoamines and the activity of acetylcholinesterase in different brain areas in adult male albino rats.

MATERIALS AND METHODS

Adult male albino rats (Rattus Norvigicus) weighing 140±20 g were used for the experiment purchased from the Egyptian Institution of Serum and Vaccine (VACSERA) Cairo, Egypt. Animals were kept under normal conditions throughout the experiment and allowed to adapt to laboratory conditions for 10 days before the beginning of the experiment. The animal care conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). Animals had free access to food and water ad libitum throughout the experimental period and were divided into 9 groups (n=24/group) randomly as shown in Table 1.

Drugs

Ciprofloxacin manufactured by Bayer healthcare pharmaceuticals, Germany and *N. sativa* extract was purchased from the Arab company for pharmaceuticals and medicinal plants (MEPACO), Egypt. Pentylenetetrazole was obtained from Sigma-Aldrich, MO, USA while sodium valproate (Depakine) was obtained from Sanofi-aventis, Paris, France.

Tissue sampling

Animals of this study were scarified by sudden decapitation after 7 and 14 days post-treatment, except PTZ group which decapitated after 24 h. Brains were rapidly dissected into four areas of cerebral cortex, cerebellum, striatum and hippocampus and used for the determination of monoamines by high performance liquid chromatography (HPLC) according to Pagel et al. (2000) and previously detailed in Tousson et al. (2012), and acetylcholinesterase (AChE) activity by the method of Ellman et al. (1961) and modified by Gorun et al. (1978).

Statistical analysis

Reported values represent means ± SE. Statistical analysis was evaluated by one-way ANOVA. Once a significant F test was obtained, least significant difference (LSD) comparisons were performed to assess the significance of differences among various treatment groups. Statistical Processor System Support "SPSS" for Windows software, Release 17.0 (SPSS, Chicago, IL) was used.

Table 1. Animals'groups used in the present study.

Group	Treatment daily/duration
Control	Normal saline (P.O)/14 days
CFX	Ciprofloxacin (500mg/kg) (P.O)/ 7and 14 days (Gürbay and Hincal, 2004)
PTZ	Pentylenetetrazole single dose (60mg/kg) (IP) (Quintans-J̊nior et al., 2009)
NS	*Nigella sativa* (350mg/kg) (P.O)/ 7 and 14 days.
NSandCFX	*Nigella sativa* (P.O) as in NS/ 7 and 14 days, followed by ciprofloxacin as in CFX group/ 7 and 14 days.
CFXandNS	Ciprofloxacin (P.O) as in CFX group for 7 and 14 days, followed by *Nigella sativa* as in NS group/ 7 and 14 days.
NSandPTZ	*Nigella sativa* (P.O) as in NS group/ 7 and 14 days, followed by single PTZ (IP) (60mg/kg).
PTZandNS	Single PTZ (IP) (60mg/kg), after 24h received daily *Nigella sativa* (P.O) as in NS/ 7 and 14 days.
PTZandVPA	Single PTZ (IP) (60mg/kg), after 24h received daily valproate (200mg/kg) (P.O)/ 7 and 14 days (Chen et al., 2009).

RESULTS

The animal behavior was observed throughout the experimental period diurnally, since it has been recorded that PTZ treated rats developed seizures, these seizures ranged from ear and facial twitching, clonic and myoclonic convulsions with the animal falling on its side and lethal convulsions in some animals. Meanwhile, no seizures were observed in ciprofloxacin treated rats. In addition, no seizures was manifested in pretreated animals with *N. sativa*, while the post treatment with *N. sativa* prolonged the onset of seizures and reduce the duration of seizures in PTZ post treated animals. Data in Tables 2 and 3 represented the mean monoamines (noradrenaline, dopamine and serotonin) and AChE activities values ± SE, respectively, in all tested groups, recording the significance at 0.05 level according to one-way ANOVA. Figures 1-4) represented the percentage change in monoamines levels and AChE activities, respectively, in *N. sativa* seed (NS) and PTZ, PTZ and NS and PTZ and valproic acid (VPA) groups from PTZ group values and NS and ciprofloxacin (CFX) and CFX and NS from CFX group values. In CFX group, the three monoamines decreased significantly throughout the experimental periods as compared to control values, with the exception of no change in striatal noradrenaline level at the 14th day and not statistically different decrease in hippocampus dopamine at the 14th day and cerebellar and hippocampal serotonin at the 7th day of CFX administration. AChE activities decreased significantly as compared to the control value except in cerebellum at 7 days. Pentylenetetrazole administered group showed dramatic significant decrease in monoamines levels as well as AChE activities in all tested areas throughout the two experimental durations as compared to control values. Nigella extract orally administered group (NS) exhibited after 14 days significant increase in noradrenaline and serotonin in both cortex and hippocampus and cortex and striatum dopamine levels increased significantly as compared to the control values. The AChE values recorded in NS group showed no statistically significant difference as compared to control group recorded values. Nigella extract orally administered before and after PTZ i.p administration in group (NS and PTZ) and (PTZ and NS) reflected significant increase in all monoamines levels as compared to that of PTZ group in the tested areas throughout the two tested periods. Also, AChE activities increased significantly in hippocampus at 7 days and in all tested areas at 14 days in NS and PTZ group as compared to the corresponding PTZ group values while AChE activities significantly increased in all areas throughout the two periods in PTZ and NS group except in cerebellum as compared to the corresponding PTZ group values. Valproate treatment after PTZ i.p administration in PTZ and VPA group, recorded significant increase in all monoamines levels and AChE activities in all tested areas throughout the experimental duration as compared to the corresponding values recorded in PTZ group. Also, in PTZ and VPA, cortex and striatum noradrenaline levels at 14th day decreased significantly as compared to their corresponding values in PTZ and NS group. But dopamine increased significantly in cortex throughout the two periods, in cerebellum in 7 days and in hippocampus at the 14th day as compared to values in PTZ and NS group. Results about Nigella extract orally administered as prophylactic before ciprofloxacin administration or administered after ciprofloxacin groups (NS and CFX) or (CFX and NS), respectively, showed significant increase in all monoamines levels throughout the two periods as compared to CFX group values. In the same manner, AChE activities increased significantly as compared to CFX group values except at the 1st duration in NS and CFX in all areas and in cerebellar AChE activity at the 1st duration in CFX and NS group.

DISCUSSION

The study extended to the brain areas as the cortex and hippocampus areas appeared to be important in the expression of early convulsive seizures (Kelly et al., 1999; Ang et al., 2006) in addition to the important functional association between cortical regions and the hippocampus in seizure propagation (Cavalheiro et al., 1991; Kelly et al., 2002). The cortex and hippocampus

Table 2. Effect of *Nigella sativa* extract on monoamines content in brain areas of male rats treated with either pentylenetetrazole or ciprofloxacin for 7 or 14 days.

Area / Group	Period (days)	Cerebral Cortex	Cerebellum	Striatum	Hippocampus
Norepinephrine					
Control		0.65 ± 0.01	0.69 ± 0.03	0.32 ± 0.008	0.53 ± 0.03
CFX	7	0.51 ± 0.01[a]	0.54 ± 0.02[a]	0.18 ± 0.01[a]	0.31 ± 0.03[a]
	14	0.47 ± 0.03[a]	0.32 ± 0.01[a]	0.32 ± 0.01	0.20 ± 0.02[a]
PTZ		0.17 ± 0.01[a]	0.14 ± 0.01[a]	0.07 ± 0.01[a]	0.14 ± 0.01[a]
NS	7	0.68 ± 0.03	0.63 ± 0.03	0.32 ± 0.01	0.51 ± 0.03
	14	0.76 ± 0.04[a]	0.69 ± 0.02	0.34 ± 0.02	0.61 ± 0.02[a]
NS & PTZ	7	0.49 ± 0.03[ac]	0.44 ± 0.03[ac]	0.18 ± 0.01[ac]	0.42 ± 0.01[ac]
	14	0.59 ± 0.05[c]	0.53 ± 0.01[ac]	0.20 ± 0.01[ac]	0.49 ± 0.02[c]
PTZ & NS	7	0.54 ± 0.03[ac]	0.49 ± 0.01[ac]	0.19 ± 0.01[ac]	0.30 ± 0.01[ac]
	14	0.60 ± 0.03[c]	0.57 ± 0.01[ac]	0.28 ± 0.01[c]	0.45 ± 0.02[ac]
PTZ & VPA	7	0.57 ± 0.003[c]	0.50 ± 0.02[ac]	0.21 ± 0.01[ac]	0.32 ± 0.01[ac]
	14	0.45 ± 0.01[acd]	0.55 ± 0.02[ac]	0.23 ± 0.01[acd]	0.49 ± 0.02[c]
NS & CFX	7	0.60 ± 0.01[b]	0.75 ± 0.01[b]	0.37 ± 0.01[b]	0.45 ± 0.02[ab]
	14	0.68 ± 0.02[b]	0.81 ± 0.03[ab]	0.35 ± 0.01	0.57 ± 0.02[b]
CFX & NS	7	0.62 ± 0.01[b]	0.71 ± 0.04[b]	0.30 ± 0.01[b]	0.46 ± 0.02[b]
	14	0.66 ± 0.03[b]	0.79 ± 0.03[b]	0.30 ± 0.01	0.50 ± 0.02[b]
Dopamine					
Control		0.35 ± 0.01	0.53 ± 0.01	0.85 ± 0.03	0.79 ± 0.03
CFX	7	0.25 ± 0.01[a]	0.31 ± 0.01[a]	0.58 ± 0.04[a]	0.58 ± 0.03[a]
	14	0.17 ± 0.01[a]	0.31 ± 0.01[a]	0.54 ± 0.05[a]	0.68 ± 0.02
PTZ		0.09 ± 0.01[a]	0.12 ± 0.01[a]	0.32 ± 0.01[a]	0.19 ± 0.01[a]
NS	7	0.33 ± 0.01	0.57 ± 0.01	0.91 ± 0.03	0.81 ± 0.03
	14	0.43 ± 0.01[a]	0.58 ± 0.02	1.05 ± 0.06[a]	0.89 ± 0.02
NS & PTZ	7	0.30 ± 0.01[c]	0.41 ± 0.01[ac]	0.67 ± 0.02[ac]	0.32 ± 0.02[ac]
	14	0.33 ± 0.01[c]	0.46 ± 0.02[c]	0.77 ± 0.04[c]	0.60 ± 0.01[ac]
PTZ & NS	7	0.24 ± 0.01[ac]	0.37 ± 0.01[ac]	0.71 ± 0.01[ac]	0.62 ± 0.02[ac]
	14	0.29 ± 0.01[ac]	0.51 ± 0.01[c]	0.79 ± 0.08[c]	0.72± 0.01[c]
PTZ & VPA	7	0.32 ± 0.01[cd]	0.52 ± 0.01[cd]	0.77 ± 0.03[c]	0.57 ± 0.02[ac]
	14	0.38 ± 0.01[cd]	0.56 ± 0.01[c]	0.80 ± 0.03[c]	0.60 ± 0.02[acd]
NS & CFX	7	0.40 ± 0.02[b]	0.55 ± 0.01[b]	0.74 ± 0.03[b]	0.85 ± 0.02[b]
	14	0.41 ± 0.01[ab]	0.52 ± 0.01[b]	0.85 ± 0.01[b]	0.81 ± 0.02[b]
CFX & NS	7	0.38 ± 0.01[b]	0.48 ± 0.01[b]	0.80 ± 0.02[b]	0.82 ± 0.01[b]
	14	0.33 ± 0.01[b]	0.57 ± 0.05[b]	0.78 ± 0.03[b]	0.79 ± 0.02[b]
Serotonin					
Control		0.53 ± 0.01	0.44 ± 0.01	1.38 ± 0.05	6.40 ± 0.34
CFX	7	0.45 ± 0.02[a]	0.42 ± 0.01	1.19 ± 0.06	4.85 ± 0.15[a]
	14	0.36 ± 0.02[a]	0.43 ± 0.02	0.94 ± 0.02[a]	3.97 ± 0.28[a]
PTZ		0.04 ± 0.01[a]	0.04 ± 0.01[a]	0.40 ± 0.05[a]	0.97 ± 0.08[a]
NS	7	0.56 ± 0.02	0.46 ± 0.01	1.27 ± 0.05	6.89 ± 0.26
	14	0.67 ± 0.02[a]	0.48 ± 0.02	1.42 ± 0.06	8.05 ± 0.18[a]
NS & PTZ	7	0.37 ± 0.01[ac]	0.36 ± 0.02[ac]	0.74 ± 0.05[ac]	5.42 ± 0.29[ac]
	14	0.48 ± 0.03[c]	0.31 ± 0.01[ac]	0.98 ± 0.04[ac]	5.87 ± 0.27[c]
PTZ & NS	7	0.42 ± 0.02[ac]	0.38 ± 0.002[c]	1.09 ± 0.04[ac]	5.28 ± 0.09[ac]
	14	0.46 ± 0.01[c]	0.41 ± 0.01[c]	1.23 ± 0.05[c]	5.97 ± 0.13[c]
PTZ & VPA	7	0.38 ± 0.01[ac]	0.37 ± 0.02[ac]	0.75 ± 0.04[ac]	4.42 ± 0.25[acd]
	14	0.35 ± 0.01[acd]	0.44 ± 0.02[c]	1.06 ± 0.04[ac]	5.38 ± 0.26[ac]

Table 2. Contd.

NS & CFX	7	0.55 ± 0.01[b]	0.49 ± 0.01[b]	1.32 ± 0.04	6.44 ± 0.30[b]
	14	0.57 ± 0.01[b]	0.50 ± 0.01[b]	1.31 ± 0.04[b]	6.04 ± 0.35[b]
CFX & NS	7	0.60 ± 0.02[b]	0.42 ± 0.01	1.19 ± 0.02	6.35 ± 0.11[b]
	14	0.55 ± 0.03[b]	0.46 ± 0.005	1.46 ± 0.04[b]	6.90 ± 0.27[b]

Data expressed as mean ± standard error. n=12. One way analysis performed between groups. Multiple range Duncan test with significance level 0.05. Within the same column superscripts represent significance. a, Control; b, ciprofloxacin (CFX) group; c, pentylenetetrazole (PTZ) group; d, from pentylenetetrazole and *Nigella sativa* (PTZ and NS) group; VPA, valproic acid.

Table 3. Effect of *Nigella sativa* extract on acetylcholinesterase (AChE) activities in brain areas of male rats treated with either pentylenetetrazole or ciprofloxacin for 7 or 14 days.

Area / Group	Period (days)	Cerebral cortex	Cerebellum	Striatum	Hippocampus
Control		15.24 ± 1.11	12.55 ± 0.84	11.85 ± 0.71	17.58 ± 1.00
CFX	7	12.36 ± 0.60[a]	11.38 ± 0.64	9.39 ± 0.34[a]	14.29 ± 0.97[a]
	14	11.75 ± 0.53[a]	9.85 ± 0.44[a]	9.13 ± 0.31[a]	13.14 ± 0.62[a]
PTZ		10.19 ± 0.58[a]	8.59 ± 0.67[a]	7.27 ± 0.24[a]	11.21 ± 0.48[a]
NS	7	13.47 ± 0.90	14.01 ± 0.77	11.14 ± 0.28	18.24 ± 1.22
	14	14.68 ± 0.89	13.58 ± 0.81	12.54 ± 0.97	16.92 ± 0.95
NS and PTZ	7	11.42 ± 0.72[a]	9.65 ± 0.34[a]	8.81 ± 0.16[ac]	13.11 ± 0.89[ac]
	14	13.49 ± 0.65[c]	11.13 ± 0.57[c]	10.91 ± 0.55[c]	15.81 ± 0.71[c]
PTZ and NS	7	12.48 ± 0.49[ac]	9.52 ± 0.48[a]	9.01 ± 0.17[ac]	13.25 ± 0.90[ac]
	14	13.78 ± 0.87[c]	11.72 ± 0.53[c]	11.51 ± 0.38[c]	16.75 ± 0.83[c]
PTZ and VPA	7	12.34 ± 0.85[ac]	10.12 ± 0.33[ac]	9.29 ± 0.13[ac]	14.12 ± 0.78[ac]
	14	13.44 ± 0.85[c]	10.82 ± 0.24[c]	10.79 ± 0.36[c]	16.22 ± 0.81[c]
NS and CFX	7	13.88 ± 0.45	12.22 ± 0.45	10.18 ± 0.23	15.95 ± 0.72
	14	14.16 ± 0.88[b]	12.82 ± 0.65[b]	12.28 ± 0.43[b]	17.25 ± 0.92[b]
CFX and NS	7	14.52 ± 0.67[b]	11.78 ± 0.52	11.47 ± 0.29[b]	16.79 ± 0.86 [b]
	14	14.47 ± 0.71[b]	13.14 ± 0.38[b]	12.52 ± 0.39[b]	15.89 ± 0.91[b]

Data expressed as mean ± standard error. n=12. One way analysis performed between groups. Multiple range Duncan test with significance level 0.05. Within the same column superscripts represent significance, a, Control; b, ciprofloxacin (CFX) group; c, pentylenetetrazole (PTZ) group; d, pentylenetetrazole and *Nigella sativa* (PTZ and NS) group; valproic acid (VPA).

suggested playing a role in inducing convulsions by quinolones (Motomura et al., 1991) and there are direct anatomical connections between the hippocampus and the striatum (Voorn et al., 2004). In addition, there are connections between striatum and hippocampus via the entorhinal and prefrontal cortex (Hyman et al. 1990; Christakou et al., 2004). The sole output of the cerebellum is inhibitory Purkinje cell projections to deep cerebellar nuclei in brainstem. Cerebellar pathways subsequently project to widespread frontal lobe and subcortical structures. The Purkinje cell inhibitory output and widespread cortical projections support the possible role of cerebellar stimulation to reduce epileptogenic activity (Krauss and Koubeissi, 2007). The role of monoamines in epileptogenesis and seizure activity is well documented. Many studies have shown role in unravelling the pathophysiology of human epilepsies (Pitkanen et al., 2006). Pentylenetetrazole provoked clonic convulsions as recorded in Safar et al. (2010). Several studies focus on the role of oxidative stress both as a consequence and a cause of epileptic seizures (Patsoukis et al., 2004; Ilhan et al., 2006). In PTZ group, acetylcholinesterase activities as well as monoamines levels intensely decreased in tested brain areas which is in line with Visweswari et al. (2010) and Chimakurthy and Talasila (2010) results reflecting their roles in the PTZ induced seizure. Paciaa et al. (2001) demonstrated that a marked NE depletion in the temporal neocortex temporal lobe epilepsy (NTLE) patients, this depletion has been shown to enhance the frequency, intensity and spread of seizures (Ferrendelli, 1986; Browning et al., 1989). Chimakurthy and Talasila (2010) found that a decrease in NE levels was observed in the hippocampus and hypothalamus, also a significant decrease in DA levels was observed in the cortex, hippocampus, hypothalamus, and pons in PTZ-treated rats. el-Hamdi et al. (1992)

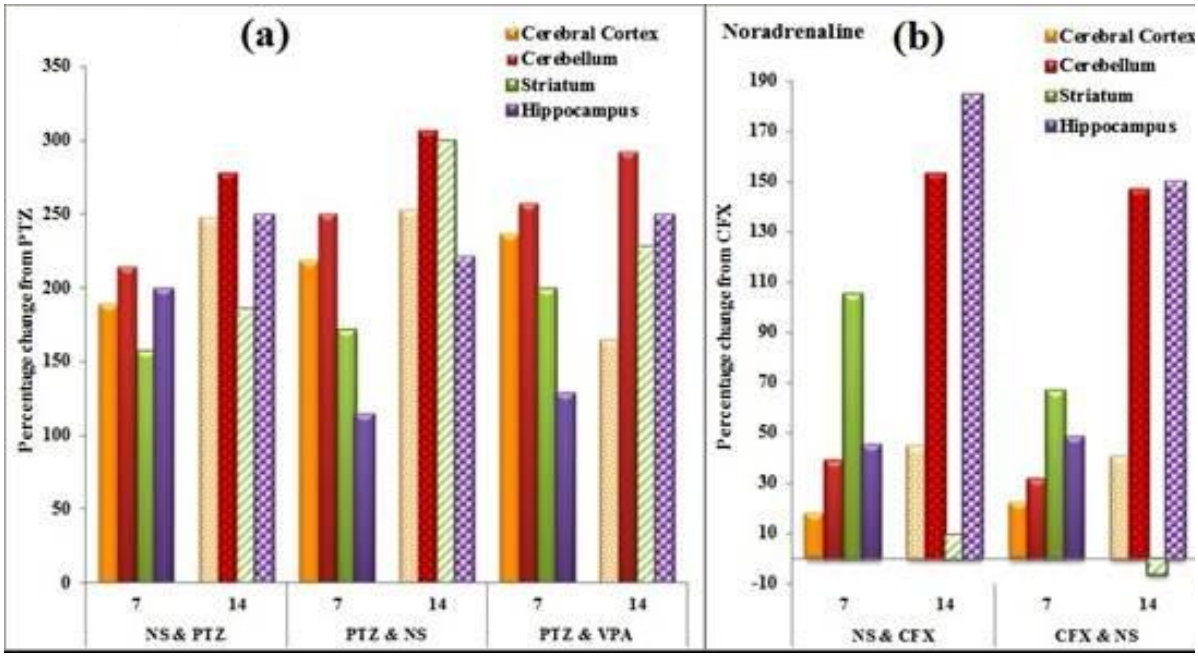
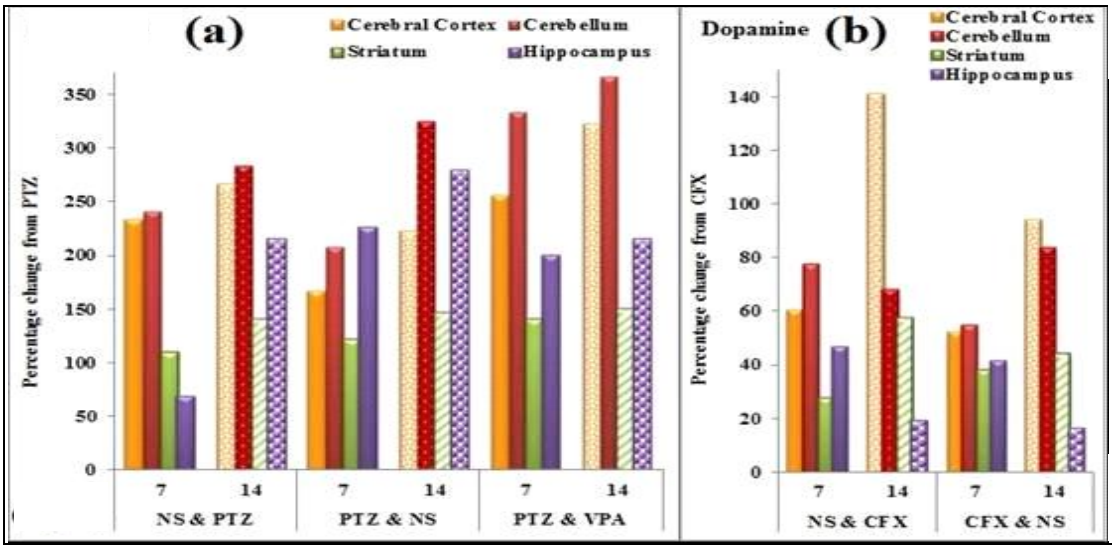


Figure 1. Percentage change of noradrenaline levels in (a) *Nigella sativa* pre-or post-administration to PTZ administered groups (NS and PTZ), (PTZ and NS) and valproate administered post PTZ administration (PTZ and VPA) as compared to the value in PTZ group. In (b) *Nigella sativa* pre- or post-administration to CFX groups (NS and CFX) (CFX and NS) as compared to the value in CFX group.



Figures 2. Percentage change of dopamine levels in (a) *Nigella sativa* pre-or post-administration to PTZ administered groups (NS and PTZ), (PTZ and NS) and valproate administered post PTZ administration (PTZ and VPA) as compared to the value in PTZ group. In (b) *Nigella sativa* pre- or post-administration to CFX groups (NS and CFX) (CFX and NS) as compared to the value in CFX group.

found that the tissue levels of DA concentration were markedly reduced in the cerebral cortex and striatum of PTZ treated rats. Serotonin is a well-recognized modulator of cortical excitability which reduces susceptibility to seizures. In a model of generalized epilepsy, a decrease in serotonin concentration, synaptosomal 5-HT uptake, and tryptophan hydroxylase activity (measured in vivo and in vitro) in most regions of the forebrain and in selected regions of brainstem rat brain tissue was recorded (Statnick et al., 1996). It has been demonstrated

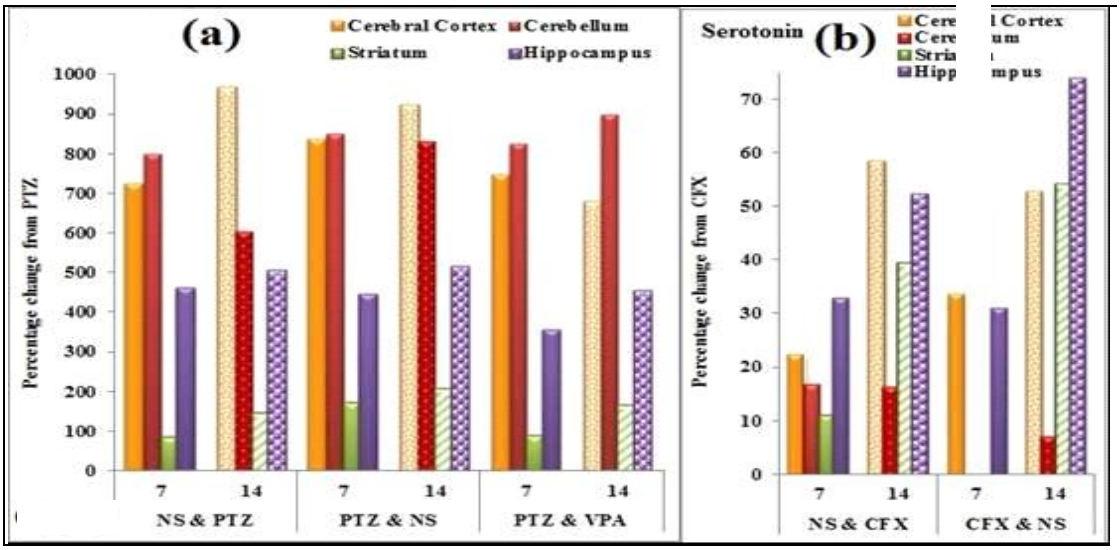


Figure 3. Percentage change of serotonin levels in (a) *Nigella sativa* pre-or post-administration to PTZ administered groups (NS and PTZ), (PTZ and NS) and valproate administered post PTZ administration (PTZ and VPA) as compared to the value in PTZ group. In (b) *Nigella sativa* pre- or post-administration to CFX groups (NS and CFX) (CFX and NS) as compared to the value in CFX group.

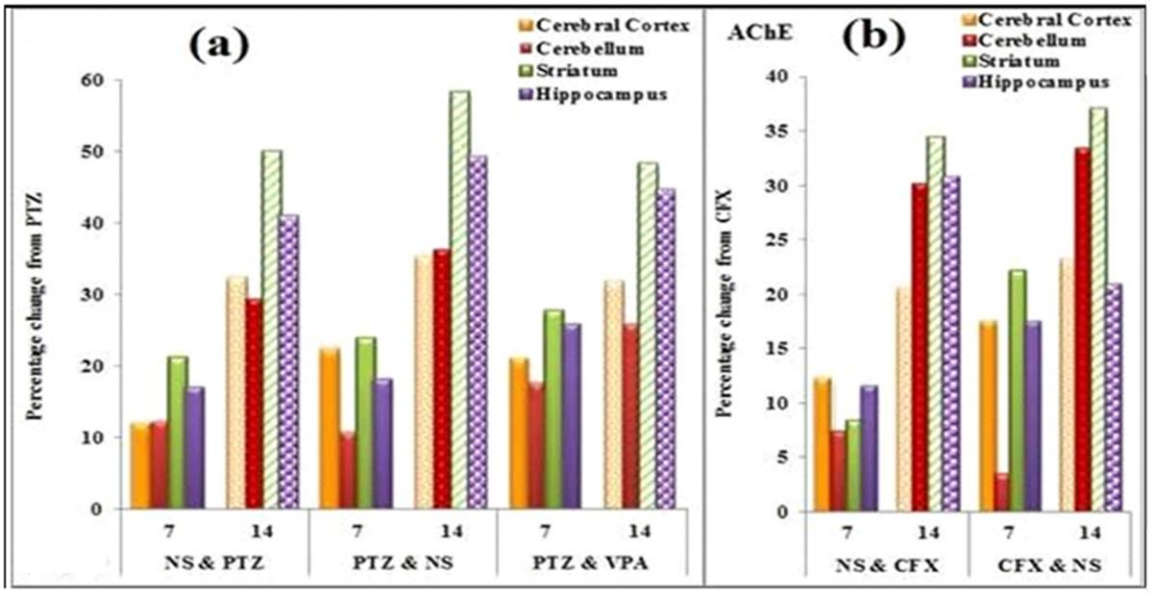


Figure 4. Percentage change of AChE in (a) *Nigella sativa* pre-or post-administration to PTZ administered groups (NS and PTZ) (PTZandNS) and valproate administered post PTZ administration (PTZ and VPA) as compared to the value in PTZ group. In (b) *Nigella sativa* pre-or post-administration to CFX groups (NS and CFX) (CFX and NS) as compared to the value in CFX group.

that the reduction of brain serotonin concentrations leads to an increase in seizure susceptibility in animal models of epilepsy (Wenger et al., 1973; Lazarova et al., 1983). In vivo microdialysis experiments have confirmed the increased release of NE during seizures in normal animals, in addition, changes in NE synthesis and release that occur after seizures may affect the rate and severity of recurring seizures. So, the decrement in the content of the studied neurotransmitters in the present study may be due in part to increase in their release or turnover. The impairment of the brain redox status after PTZ administration reflecting oxidative stress involved in seizure formation previously reported (Hosseinzadeh and Parvardeh, 2004; Silva et al., 2009; Safar et al., 2010).

Ciprofloxacin administration in a less vigorous extent as regard with PTZ results also decreased the acetylcholinesterase activities and monoamines levels in the investigated brain areas which support the proconvulsant effect of the quinolones previously discussed in Smolders et al. (2002) and Rawi et al. (2011). Ciprofloxacin may decrease the threshold of epileptogenic activity (Agbaht et al., 2009) where, ciprofloxacin induced seizures in healthy patients (Darwish, 2008). Extensive toxicological and biochemical experiments have been performed to explain the CNS side effects observed under therapeutic conditions (Stahlmann and Lode, 1999; Rawi et al., 2011). Bidziński et al. (1998) concluded that there is a functional interaction between brain serotonin and GABA systems, due to the effect of serotonin depletion in GABAA receptor down-regulation. Quinolones was found to have a Mg+2 chelating properties which led to prolong opening time of the calcium ion channel, thus increasing intracellular Ca+2 ions concentration (Davies and Maesen, 1989; Stahlmann et al., 1997), this increase in intracellular Ca+2 ions concentration led to the rupture of the vesicles in the presynaptic terminals and increase the release of the neurotransmitters (Bullock et al., 1995), as a result the content of catecholamine is decreased. This is in agreement with the present results where the administration of CFX caused a decrease in monoamines concentration in different tested brain areas which may lead to the initiation of seizures. Also data recorded about monoamines in the tested antibiotic may be a supplement data to the previously mentioned seizure inducing activity of quinolones (Moorthy et al., 2008; Agbaht et al., 2009). The results of Rawi et al. (2011 and 2011a) suggested a shift in the balance of antioxidant markers towards the oxidative stress in cortex, hippocampus and striatum through elevation in malondialdehyde, nitric oxide contents and superoxide dismutase enzyme activity and reduction of glutathione, glutathione peroxidase and Na+, K+, adenosine triphosphatase enzymes activity. The effect tested through histopathological examinations showed focal gliosis, hemorrhagic areas in cerebral cortex and neuronal degeneration, oedema and astrosytosis in hippocampus. Also focal gliosis with congested blood vessels in striatum in ciprofloxacin treated rats under dose equivalent to the human therapeutic onewas noted. Recently, Abdel-Zaher et al. (2012) suggested that elevation of brain glutamate levels with consequent oxidative stress and increase in the expression and activity of brain inducible NO synthase may play a pivotal role in ciprofloxacin-induced convulsive seizures. Delgado-Escueta (1984) demonstrated that the increased activity in noradrenergic, dopaminergic, and serotonergic systems are believed to reduce cortical excitability and decrease seizure activity.

Moreover, it has been implicated in the onset and perpetuation of many seizure disorders, many experimental procedures designed to increase monoaminergic activity have proven to posses antiepileptic properties (McIntyre and Edson, 1989; Yan et al., 1995). The seizure induction through the assumption about the pharmacological treatments that lowering monoamine levels in the brain generally increase the susceptibility to seizures, while treatments that increase monoamines decrease the susceptibility (Kiyofumi Kobayashi and Akitane Mori, 1977). In animal models, treatments that increase serotonin decrease seizure susceptibility. Conversely, decreasing serotonin function increases seizure susceptibility (Bagdy et al., 2007). According to unanimous opinion, elevated levels of NE, 5-HT, and DA in the brain exert an anticonvulsant activity (Starr, 1996; Jobe et al., 1999). It was previously reported that the efficacy of antioxidants lies in the management of convulsive disorders (Sudha et al., 2001; Ilhan et al., 2005). The data about sodium valproate as established anticonvulsant drug is mediated by alteration in monoamine levels in rat brain areas (Baf et al., 1994; Löscher and Hönack, 1996; Ichikawa and Meltzer, 1999) as the enhancement of monoaminergic transmission reduced seizure threshold (Wahnschaffe and Loscher, 1991; Wada et al., 1993). This may be explained through valproate mechanism via activation of monoamine oxidase (MAO) the key enzyme that degrades a number of monoamine neurotransmitters as recently cited by Wu and Shih (2011). Also valproate post PTZ administration revealed antioxidant potential (Safar et al., 2010). The present study revealed that, the pre- and post-treatment with *N. sativa* alleviate the changes in monoamines (NE, DA and 5-HT) and the activity of AChE in PTZ and CFX treated rats in most investigated brain areas throughout the experimental days, these findings reflect the potent antiepileptic efficiency of *N. sativa* as suggested by Guha et al. (2005), Ilhan et al. (2005) and Ezz et al. (2011), where, Ilhan et al. (2005) reported that the neurotransmitter receptor-mediated activity may be involved in the mechanism of action of *N. sativa* oil in preventing PTZ kindling seizures. Moreover, the pre administration of *N. sativa* restored the AChE activity in cerebral cortex, cerebellum and hippocampus in propoxur-treated rats (Mohamadin et al., 2010). Furthermore, studies carried out by Perveen et al. (2008 and 2009) stated that the administration of *N. sativa* oil increased the 5-HT, tryptophan levels and decreased levels of 5-HIAA in the rat brain suggesting a decreased 5-HT turnover supporting its anti-anxiety effect, these may explain the increase in monoamines content in the present study. Thymoquinone, the major constituent of *N. sativa* seeds prolonged the onset of PTZ-induced seizures and reduced the duration of myoclonic seizures and postulated thymoquinone anticonvulsant activity in the petit mal epilepsy through an increase in GABAergic tone (Hosseinzadeh and Parvardeh, 2004). O'Donnell et al. (2010) stated that the inhibition of AchE leads to a build-up of extracellular ACh and a series of toxic consequences including hypersecretion, tremor, convulsion/

seizure,coma, and death. In addition, the findings of de Sales et al. (2010) reported that seizures caused a decrease in AChE activity, expecting that the constant inhibition of this enzyme by seizures might increase ACh levels. In addition to the antioxidant effect of *N. sativa*, oil and thymoquinone have been demonstrated to prevent oxidative injury during cerebral ischemia-reperfusion injury in rat (Hosseinzadeha et al., 2007) and in a rat model of subarachnoid hemorrhage (Ersahin et al., 2011). Abdel-Zaher et al. (2011) results provided evidence about the therapeutic potential of *N. sativa* oil in tramadol tolerance and dependence through blockade of nitric oxide overproduction and oxidative stress induced by the drug.

From the aforementioned study results, it could be concluded that the treatment with ciprofloxacin for 14 days can behave to a lesser extent like PTZ side effects by decreasing the content of monoamines; this may be due to the increase in their release or turnover, the activity of AChE is also decreased which may lead to a build-up of extracellular Ach and a series of toxic consequences including tremor and seizures. On the other hand, the administration of *N. sativa* pre- and the post treatment to PTZ and CFX treated rats were found to ameliorate their side effects suggesting that *N. sativa* with antiepileptic activity and its administration could alleviate ciprofloxacin neurotoxicity.

ABBREVIATIONS

GABAA, Gamma-aminobutyric acid A; **PTZ**, pentylenetetrazole; **EAA**, excitatory amino acid; **CNS**, central nervous system; **ROS**, reactive oxygen species; **VPA**, valproate; **AChE**, acetylcholinesterase; **NS**, *N. sativa* seed; **CFX**, ciprofloxacin; **MAO**, monoamine oxidase.

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Full Length Research Paper

Influence of a *Ginkgo biloba* extract on the binding of [F-18]-fluorodeoxyglucose (18F-FDG) on blood constituents

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Radiopharmaceuticals are used in procedures of nuclear medicine for the diagnosis and treatment of illnesses. Fluorine-18(^{18}F) is a positron emitter produced in cyclotron. It is used to get ^{18}fluorodeoxyglucose (^{18}F-FDG) that is a radiopharmaceutical utilized in the positron emission tomography scan. The aim of this work was to evaluate the *in vitro* effect of an extract of *Ginkgo biloba* extract (EGb) on the distribution in blood cells (BC) and plasma (P) compartments and on the binding to the blood constituents of the ^{18}F-FDG using precipitation with trichloroacetic acid (TCA). EGb was not capable to interfere on the distribution of the ^{18}F-FDG on the BC and P compartments. However, this extract was capable of interfering significantly ($p<0.05$) on the fixation of the ^{18}F-FDG on IF-P (in all the concentrations tested, $P<0.05$) and IF-BC in 1% TCA concentration from 14.04±1.13 to 10.23±1.92 (40mg/ml, EGb) and to 9.35 ±1.57 (400mg/ml/EGb), in 5% TCA concentration from 14.83±3.78 to 11.15±1.64 (40mg/ml, EGb) and to 10.23±1.6 (400mg/ml, EGb). In conclusion, the analysis of the results indicates that the EGb was not capable to interfere on the distribution of the ^{18}F-FDG on P and BC compartments, however, alter the fixation of the 18FDG on IF-P and IF-BC.

Key words: ^{18}Fluorodeoxyglucose, blood compartments, radiopharmaceutical, *Ginkgo biloba,* medicinal plants.

INTRODUCTION

Radiopharmaceuticals or radiobiocomplexes are employed in nuclear medicine for diagnostic and/or treatment of diseases or to study blood flow, morphology of organs, bioavailability and metabolism of drugs (Saha, 2010). An important step to understand the mechanism of localization of radiopharmaceuticals in a specific target, as well as they are cleared from blood or eliminated from the body or the rate at which their excretions occur, it is

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the determination of their binding to the blood proteins. The secure determination of the binding of radiopharmaceuticals to the plasma (P) and blood cell (BC) constituents can aid to understand the interference of various conditions on the distribution of radiopharmaceuticals in the body (Saha, 2010). To investigate the radiobiocomplexes-protein binding, these complexes of protein-bound-radiobiocomplex must be separated from the free radiobiocomplex. This has been accomplished by precipitation of the proteins with precipitating agents, as trichloroacetic acid (TCA) (Freitas et al., 2007) or ethanol (Fernandes et al., 2007).

Fluorine-18 (^{18}F) is the positron emitter radionuclide (half life of 109.77 minutes) produced in cyclotron mostly used to positron emission tomography (PET) scan. It is utilized to get ^{18}fluorodeoxyglucose (^{18}F-FDG), that is a radiolabeled glucose analogue (Aus et al., 2005; Saha, 2010; Velasques de Oliveira et al., 2010a). ^{18}FDG-PET provides insight into the biological behavior of tumors rather than their morphological appearance (Velasques de Oliveira et al., 2010b). PET is useful to determine *in vivo* physiological and biochemical processes of non-invasive character (Phelps, 2000). PET can target several biological features of tumors including glucose metabolism, cell proliferation, tissue perfusion, and hypoxia (Rohren et al., 2004; Zhang et al., 2007). Following malignant transformation, a range of tumors can be characterized by elevated glucose consumption and subsequent increased uptake of the radiolabeled glucose analogue FDG (Rohren et al., 2004; Zhang et al., 2007).

Normal distribution of ^{18}F-FDG includes high uptake in the brain, in the kidneys and bladder because of renal clearance (Jager, 2005). Some authors have already reported that in mice, dogs, and man, the ^{18}F-FDG clears from the other organs and it is excreted to a large extent in the urine. Reymond et al. (2007) have observed that the ^{18}F-FDG on the blood decreases rapidly with the time.

Jeghers et al. (1990) have reported that the reversible and non-reversible interactions of small molecules with macromolecules, such as proteins. These bindings can interfere with the bioavailability, the rate of elimination, the access to the action site/target, and with the metabolism (Jeghers et al., 1990). Moreover, authors have pointed out that in nuclear medicine, it is essential the understanding and quantification of this phenomenon in order to anticipate the behavior *in vivo* of radiotracers (Velasques de Oliveira et al., 2010b). In addition, authors have reported that several synthetic (Nigri et al., 2002) and natural medications (Moreno et al., 2007; Moreno et al., 2008a; Moreno et al., 2008b; Moreno et al., 2008c; Souza et al., 2011) are capable of interfering with the biodistribution and/or on the radiolabeling of blood constituents. These considerations show the importance of the studies about the effect of medications on the behavior of the radiopharmaceuticals. This fact has an additional importance if the natural medications are considered, due to the consumption of natural products, as food, additives or medication, has been growing all over the world (Simões et al., 2007; Steenkamp et al., 2013).

Ginkgo biloba extract (EGb) is a medicinal herb, which comes from leaves of the ginkgo tree, one of the oldest living plant species (Simões et al., 2007). This extract has several effects, including, increases the blood flow, acts as platelet activating factor antagonism and prevents the membrane against the damage caused by free radicals (Simões et al., 2007). Moreover, EGb scavenges free radicals such as hydroxyl radicals and superoxide anions (Moreno et al., 2004; Simões et al., 2007). The redox properties of this extract are probably due to the presence of the flavonoids (Moreno et al., 2004). Considering the publications in the PubMed (http//: wwwnobi.nlm.nih.gov/sites/entrez, at april 18, 2013) there are no references on the effect of natural drugs on the fixation of ^{18}F-FDG on blood constituents, and this fact has stimulated the evaluation of the effect *in vitro* of a *G. biloba* extract on the distribution of the ^{18}F-FDG on the blood compartments (plasma and blood cells) and on the blood proteins (plasma and cellular) with TCA.

MATERIALS AND METHODS

Ethical guidelines

All the experimental procedures followed the Ethical Guidelines of the Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro with the protocol number CEA/129/2006. The experiments were carried out with heparinized whole blood withdrawn from Wistar rats (male, 3 months of age, about 300 g).

Radiopharmaceutical production

^{18}F-FDG was obtained through the synthesis module Tracer Lab MX of GE Medical System, Benelux SA–Belgium. The production and all the controls were performed by the the Departamento de Radiofarmácia, Instituto de Engenharia Nuclear, Comissão Nacional de Energia Nuclear, Rio de Janeiro, Brazil.

Preparation of the extract

An aqueous extract was prepared mixing 4 gram of G. biloba (Herbarium Laboratório Botânico LTDA, lot number 535036) in 10 ml of 0.9% NaCl (saline). The mixture was centrifuged (clinical centrifuge, 1500 rpm, 5 min). The supernatant was considered to be 400 mg/ml and denominated 100% solution. Dilutions were performed with saline that was also used as a control. A spectrophotometric analysis (Analyser, 800M, São Paulo, Brazil) of the undiluted extract was carried out and the absorbance was determined to each 20 nm in the range 400-700 nm. The absorbance at 440 nm was considered the marker of the quality control of preparation of this extract. All the prepared extracts to be used in the experiments must have the optical density of 0.162 (Figure 1).

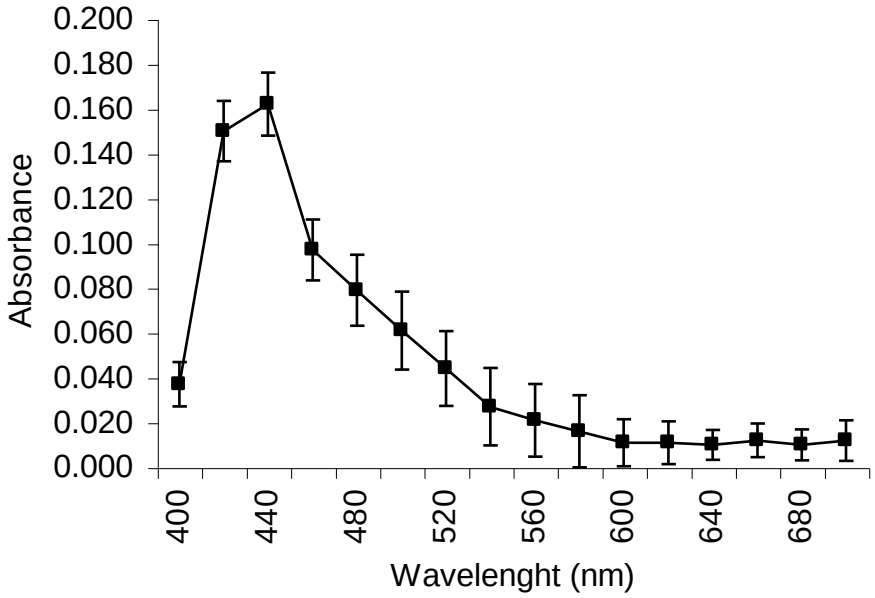

Figure 1. Absorption spectrum of the extracts of *Ginkgo biloba* used in the experiments. The pattern of the absorption spectrum of the extracts of *Ginkgo biloba* used in the experiments. It presents the highest measure of the optical density (0.162 ± 0.014) at 440 nm. This condition has permitted to control the conditions to prepare the extracts and has been used as a marker.

Protein-binding

Fresh anticoagulated whole blood (3 ml) was incubated for 1 h with 300 μl of the *G. biloba* extract solution in the concentrations of 40 or 400 mg/ml at room temperature. NaCl 0.9% was used as control. After that, 100 μl of ^{18}F-FDG (3.7 MBq) was added and incubated for more 20 min. Blood preparations were centrifuged and plasma (P) and blood cells (BC) were isolated. Aliquots (25 μl) of P and BC were also precipitated with 1 ml of freshly prepared solution of TCA in various concentrations (0.1, 0.5, 1.0, 5.0, 10.0 and 20.0%). In this condition, soluble (SF) and insoluble (IF) fractions from plasma and blood cells were separated. All samples, (P, BC, IF-P, SF-P, IF-BC and SF-BC) were counted in a well counter with NaI(Tl) crystal (Clinigamma, gamma counter, Packard Instrument Company, mod C5002, USA).

Percentage of radioactivity determination

The percentage of radioactivity (%ATI) (i) in P was determined dividing the counts in P by sum of the counts in P plus BC, (ii) in BC was determined dividing the counts in P by sum of the counts in P plus BC, (iii) in IF–P was determined dividing the counts in IF–P by the sum of the counts in IF–P plus SF–P and (iv) in IF–BC was determined dividing the counts in IF–BC by the sum of the counts in IF–BC plus SF–BC. The values found were multiplied by 100. The values are mean of 5 isolated experiments.

Statistical analysis

Statistical analysis (ANOVA test, with significance level $P<0.05$, $n = 5$) was utilized to compare the %ATI of radiopharmaceutical in the blood constituents and the various TCA concentrations.

RESULTS AND DISCUSSION

Figure 1 shows the pattern of the absorption spectrum of an EGb used in the experiments. It presented the highest measure of the optical density (0.162 ± 0.014) at 440 nm. This condition has permitted to control the conditions to prepare the extracts used in the assays and it was used as a marker.

Table 1 shows the distribution of the ^{18}F-FDG on plasma and cellular compartment of the whole blood from Wistar rats that were incubated with the *G. biloba* extract. The %ATI for ^{18}F-FDG was found mainly in the plasma compartment. This extract, in the two concentrations, was not capable to alter distribution of the ^{18}F-FDG in both compartments. Table 2 shows the fixation of the radioactivity in insoluble fractions obtained from plasma (IF-P) samples precipitated with different TCA concentrations. These samples of plasma were obtained from whole blood incubated with ^{18}F-FDG and with an EGb or saline. The extract, in the two used concentrations (40 and 400 mg/ml), was capable to increase the fixation of the ^{18}F-FDG in the IF-P. The fixation of the radioactivity in IF-P in the control, in general, has increased with the concentration of TCA used. The % of radioactivity that was found in the IF-P with the various TCA concentrations from whole blood treated with the *G. biloba*

Table 1. Distribution of the radioactivity of the ^{18}F-FDG in the plasma and cellular compartment of the blood from Wistar rats treated with *G. biloba* extract.

Samples	Cellular compartment	Plasma compartment
^{18}F-FDG (control)	16.83 ± 4.92	83.17 ± 4.92
^{18}F-FDG + extract 40mg/ml	15.32 ± 3.18	84.68 ± 3.18
^{18}F-FDG + extract 400mg/ml	17.61 ± 2.91	82.39 ± 2.91

Fresh whole blood (3 ml) was incubated for 1 h with 300 µl of the vegetable extract of *G. biloba* in the concentrations of 40 and 400 mg/ml. NaCl 0.9% was used as control. After incubation 100 µl of ^{18}F-FDG (100µCi; 3.7 MBq) was added and incubated for additional 20 min. After that, the blood preparations were centrifuged and plasma (P) and blood cells (BC) were isolated. Statistical analysis with ANOVA test, significance level $P < 0.05$, $n = 5$, was utilized. Note that the difference between the %ATI of treated samples with the extract (40 and 400 mg/mL) and controls is not significant (p>0.05).

Table 2. Distribution of the radioactivity of the ^{18}F-FDG in the plasma (IF-P) of the blood from Wistar rats treated with *Ginkgo biloba* extract.

TCA concentration (%)	Control (Mean ± S.D.)	Insoluble fraction (Mean ± S.D.)	
		Extract concentration (40 mg/ml)	Extract concentration (400 mg/ml)
0.1	2.29 ± 1.15	6.23 ± 1.08*	5.32 ± 0.94*
0.5	1.92 ± 0.68	5.12 ± 0.91*	4.26 ±1.23*
1.0	1.98 ± 0.48	4.89 ± 1.11*	4.11 ± 0.82*
5.0	2.16 ± 0.41	5.23 ± 0.62*	5.01 ± 1.30*
10.0	2.72 ± 1.01	5.04 ± 0.92*	4.12 ± 0.93*
20.0	3.92 ± 0.44	6.09 ± 0.73*	5.75 ± 0.89*

Fresh whole blood (3 ml) was incubated for 1 h with 300 µl of the vegetable extract of *G. biloba* in the concentrations of 40 and 400 mg/ml. NaCl 0.9% was used as control. After incubation 100 µl of ^{18}F-FDG (100µCi; 3.7 MBq) was added and incubated for more 20 min. After that the blood preparations were centrifuged and plasma (P) and blood cells (BC) were isolated. Aliquots (25 µl) of P and BC were also precipitated with 1 ml of solution of trichloroacetic acid (TCA) in various concentrations (0.1, 0.5, 1.0, 5.0, 10.0 and 20.0%). Statistical analysis with ANOVA test, significance level $P < 0.05$, $n = 5$, was utilized. *Note that the difference between the %ATI of treated samples with the extract (40 and 400mg/mL) and controls is significant (p<0.05).

extract was statistically (p<0.05) higher than in the control.

Table 3 shows the fixation of the radioactivity in insoluble fractions obtained from blood (IF-BC) samples precipitated with TCA concentrations. These samples of blood cells were obtained from whole blood that was incubated with ^{18}F-FDG and with an EGb or saline. It was observed that the fixation of the radioactivity in IF-BC in the control depends on the TCA concentration. The presence of the extract influenced the results and has statistically (p<0.05) decreased the %ATI on IF-BC in almost all the concentrations of the TCA.

The purpose of this investigation was to verify the action of an extract of *G. biloba* extract on the distribution of the ^{18}F-FDG on the blood constituents. Although the EGb was not capable to interfere on the distribution of this radiopharmaceutical on the BC and P compartments, it was capable to interfere significantly (p<0.05) on the fixation of the ^{18}F-FDG in IF-P and in IF-BC. These findings are worthwhile due to this interference might bring some complications in the interpretation of examinations done with ^{18}F-FDG patients that are using an extract of *G. biloba.* Our investigation with an extract of *G. biloba* is related to the fact that there is considerable evidence that the bioavailability of radiopharmaceuticals may be also altered by disease states, but also by a variety of drugs (natural and synthetic) (Bernardo-Filho et al., 2005). Moreover, the distribution of a radiopharmaceutical depends on its fixation on the blood constituents (Gano et al., 2009). If these factors are unknown, this fact may lead to the deficient visualization of organ, being necessary to repeat the procedure resulting in unnecessary exposure to the radiation or even misdiagnosis (Bernardo-Filho et al., 2005; Saha, 2010).

Authors have reported that the biodistribution, the rate of elimination, the access to the target organ and the metabolism depends on the interaction of the radiopharmaceutical with plasma proteins (Freitas et al., 2007). Although the introduction of ^{18}F-FDG has provided a valuable tool for the study, the glucose metabolism in

Table 3. Distribution of the radioactivity of the ^{18}F-FDG in the blood cell (IF-BC) of the blood from Wistar rats treated with *G. biloba* extract.

TCA concentration (%)	Control (Mean ± S.D.)	Insoluble fraction (Mean ± S.D.)	
		Extract concentration (40mg/ml)	Extract concentration (400mg/ml)
0.1	5.30 ± 2.01	5.17 ± 1.51	4.91 ± 0.97
0.5	11.91 ± 2.62	9.25 ± 1.37	8.12 ± 1.33
1.0	14.04 ± 1.13	10.23± 1.92*	9.35± 1.57*
5.0	14.83 ± 3.78	11.15± 1.64*	10.23± 1.6*
10.0	10.02 ± 2.57	10.36± 1.71	8.94 ±2.43
20.0	7.38 ± 3.09	6.25± 1.39	5.23 ± 1.38

Fresh whole blood (3 ml) was incubated for 1 h with 300 μl of the vegetable extract of *G. biloba* in the concentrations of 40 and 400 mg/ml. NaCl 0.9% was used as control. After incubation, 100 μl of ^{18}F-FDG (100μCi; 3.7 MBq) was added and incubated for additional 20 min. After that, the blood preparations were centrifuged and plasma (P) and blood cells (BC) were isolated. Aliquots (25 μl) of P and BC were also precipitated with 1 ml of solution of trichloroacetic acid (TCA) in various concentrations (0.1, 0.5, 1.0, 5.0, 10.0 and 20.0%). Statistical analysis with ANOVA test, significance level $P < 0.05$, $n = 5$, was utilized. *Note that the difference between the %ATI of treated samples with the extract (40 and 400 mg/mL) and controls is significant ($p<0.05$).

normal and in disease tissue in conjunction with PET for brain, heart and tumors studies as well as research, there are no publications in the PubMed about studies of possible interactions of the ^{18} F-FDG with natural drugs. The pharmacokinetic behavior of radiopharmaceuticals also depends on the fixation of the radiotracer on the blood proteins.

Moreover, a part of the radiopharmaceutical that reaches the blood is bound to the plasma proteins (Gano et al., 1989) and to blood proteins (Freitas et al., 2007). The central idea of this study is to verify if the EGb extract is capable to interfere in the fixation of the 18F-FDG in the IF-P and IF-BC due to the impact of this alteration on the bioavailability of the radiopharmaceutical and the undesirable consequences. It is known that the radiopharmaceutical uptake in organs may depend on its biochemical characteristics as well as the binding to blood constituents.

The correct determination of the binding of radioactivity on blood elements would be worthwhile for several reasons (i) to better understand how a drug is capable of modifying the biodistribution of radiopharmaceuticals, (ii) to evaluate the specific characteristics of the binding of each radiopharmaceutical to its targets in the blood, (iii) to avoid misdiagnosis, (iv) to avoid the repetition of examinations, (v) to avoid erroneous visualization and elucidation of the organ and (vi) to reduce the radiation dose to patients (HladikIII et al., 1987; Santos-Oliveira and Machado, 2011).

According to Jeghers et al. (1990) are necessary new methods for the determination of protein binding for each class of radiopharmaceuticals (Jeghers et al., 1990). The findings obtained in this study indicated in Tables 1, 2 and 3 may aid to understand the rapid elimination of the ^{18}F-FDG.

Some authors have demonstrated that the natural products *G. biloba* (Moreno et al., 2004), *Uncaria tomentosa* (Moreno et al., 2007) and *Paullinia cupana* (Freitas et al., 2007*)* are able to interfere with the labeling of red blood cells with ^{99m}Tc and alter the fixation of the sodium pertechnetate to the precipitated blood proteins (plasma and cells proteins).

According to Aleixo et al. (2012), the extract of *G. biloba* interferes on the distribution of the sodium [^{123}I] iodide (Na^{123}I) on the compartments as well as on the fixation on the plasma and blood cells proteins (Aleixo et al., 2012). The findings presented in Table 1 have revealed that *G. biloba* extract was not capable of interfering on the distribution of the ^{18}F-FDG in the plasma and cellular compartments. However, it was capable to interfere on the fixation of the ^{18}F-radio-pharmaceutical on the insoluble fraction obtained from plasma and cellular proteins (Tables 2 and 3) isolated from whole blood incubated with the *G. biloba* extract. It is possible to suggest that the chemical compounds present in *G. biloba* extract may alter these bindings, increasing or decreasing the radioactivity on the IF of the blood constituents.

In conclusion, as the biodistribution also depends on the fixation of the radiopharmaceuticals on the blood proteins and although, the experiments were carried out with animals, it is necessary to have precaution and to think about unexpected consequences in the bioavailability of the ^{18}F-FDG in patients that are undergoing *G. biloba*.

This conclusion is due to EGb altering the fixation of

the ^{18}F-FDG on the blood proteins.

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ABBREVIATIONS

^{18}F-FDG, ^{18}Fluorodeoxyglucose; **P,** plasma; BC, blood cell; **IF,** insoluble fraction; **SF,** Soluble fraction; **TCA,** thricloroacetic acid; **PET**, positron emission tomography; **EGb,** *Ginkgo biloba* extract.

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Short Communication

Gabapentin as an adjuvant treatment in renal colic: A randomized double-blind clinical trial

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Acute renal colic probably is the most painful event a person can endure and pain relief with minimal side effects is a humane approach. Gabapentin is an antiepileptic drug, also used in the treatment of neuropathic pain but it's efficacy and safety in treating renal colic has not been demonstrated. We studied patients with renal colic to assess the efficacy and safety of single dose of 600 mg oral Gabapentin compared to placebo as an analgesic in renal colic. In this randomized double-blind controlled clinical trial, 100 patients aged 20-40 y/o with renal colic, admitted to Vali Asr hospital of Arak city, Iran, were randomized to receive 0.5 mg/kg of Pethidin hydrochloride and single-dose of 600 mg Gabapentin (n=50) or Pethidin hydrochloride 0.5 mg/kg and placebo (n=50). Clinical signs and symptoms, abdominal pain intensity (based on visual analog pain score (VAS)) were recorded prior to, and 1,2,3,6 h after drug administration and compared between groups. The significant pain score reduction was reported in Gabapentin group (p<0.001). Nausea severity score, mean vomiting frequency and additional Pethidin hydrochloride administration after intervention in Gabapentin group was significantly lower than placebo group(p<0.001). According to the result of present study, the use of Gabapentin can effectively reduce the pain intensity, nausea, vomiting and the need of analgesics among patients with renal colic.

Key words: Abdominal pain, gabapentin, pain, renal colic.

INTRODUCTION

Acute renal colic probably is the most painful event a person can endure. Renal colic affects approximately 1.2 million people each year, and accounts for approximately 1% of all hospital admissions (Wolf and Howes, 2012). Pain relief is one of the most essential aspects of the successful management of renal colic. In recent years, great emphasis has been laid on ensuring that adequate pain relief is coupled with minimal side effects (Helfand and Freeman, 2009).

Non-steroidal anti-inflammatory drugs (NSAIDs) and opioids have been used as a pain relief in renal colic (Curry and Kelly, 1995; Smally, 1997). Opioids have some advantages such as cheapness, titratability and potency but there are concerns of dependency and drug seeking behavior presenting as renal colic, which may limit their usefulness (Reich and Hanno, 1997). NSAIDs act directly on the main cause of pain; prostaglandin release, and have been shown to be effective, particularly when used intravenously (Tramer et al., 1998) but NSAIDS are generally not titratable with some severe adverse effects including renal failure and gastrointestinal bleeding and may be less immediate and potent in their action.

Gabapentin as an antiepileptic drug is used for epilepsy, neurodegenerative disorders, and pain (Vedula et al., 2011; Moore et al., 2011). A number of animal studies

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and clinical trials have shown that compounds related to Gabapentin are useful for treating neuropathic pain (Rosner et al., 1996; Segal et al., 1996; Khan et al., 1998; Solaro et al., 1998). Placebo-controlled clinical trials also have indicated a therapeutic role of Gabapentin in diabetic neuropathy (Backonja et al., 1998; Seidl et al., 1999). Some recent publications also have used Gabapentin as an analgesic in established acute postoperative pain (Straube et al., 2010), implicating even broader use of Gabapentin in variety of pain states. There are some non-controlled, open-labeled clinical studies supporting the of Gabapentin role in reducing spontaneous pain and allodynia regardless of the etiology (Mao et al., 2000), so the mechanism of Gabapentin effect on pain control is not clear (Mao et al., 2000). The aim of present study was to assess the efficacy and safety of single dose of 600 mg oral Gabapentin compared with placebo as an adjuvant analgesic in renal colic.

MATERIALS AND METHODS

In this randomized double-blind controlled clinical trial, 100 patients' aged 20-40 years old with renal colic, admitted to Vali Asr hospital of Arak city, were enrolled in the study. Simple randomization was applied based on the patients' code (even: control, odd: treatment). Exclusion criteria were: Cardio-vascular disease or hypertension, analgesic consumption or psychotropic medication, epilepsy, chronic vertigo, ataxia and pregnancy or breast feeding.

This study was ethically approved by the research ethical committee of the Arak University of medical Sciences, Iran. Written informed consent was obtained from all patients or their relatives before the trial. Due to the lack of availability of intravenous diclofenac sodium in our country, Iran, Pethidin hydrochloride is routinely used for pain management in renal colic.

All of the enrolled patients were randomized to receive 0.5 mg/kg of Pethidin hydrochloride and single-dose of 600 mg Gabapentin (n=50) or 0.5 mg/kg Pethidin hydrochloride and placebo (n=50). The patients and clinicians who were responsible for visiting the patients remained blind during the trial. Clinical symptoms, physical signs including nausea and vomiting and pain intensity (based on visual analog pain score (VAS)) were recorded prior to and 1, 2, 3 and 6 h after drug administration. Nausea severity (was scored 0-3), frequency of vomiting and drug adverse effects including headache, vertigo and sleepiness were recorded 1, 2, 3 and 6 h after intervention too. Additional Pethidin hydrochloride was administered based on patients´ request and recorded. All statistical analysis were performed with the Statistical Package for the Social Sciences (SPSS) software 16 (t-test, paired t-test, chi-square) and statistical significance was considered at $P \leq 0.05$.

RESULTS AND DISCUSSION

In this study, there were a total of 100 patients with renal colic. The mean age of studied population was 32.31(±5.87) years old and 71% of them were men. There was no significant difference between two groups regarding to age, gender, weight ($p>0.05$, Table 1) and initial clinical evaluation and VAS score (Table 2). The VAS scores upon admission to the hospital and after intervention were measured and is shown in Table 2. The significant pain scores reduction was reported in Gabapentin group ($p<0.001$) and the difference between VAS score of two groups increased during the hours.

Nausea severity score in first evaluation in Gabapentin group was 2.68±0.68 vs. 2.74±0.44 in the control group (P: NS) meanwhile after drug administration, Gabapentin group was significantly lower than placebo group (6.36±3.16 vs. 7.68±2.03, respectively). Mean vomiting frequency in intervention and control group were 0.00 vs. 0.38±0.77 which was respectively, and significantly different ($p<0.001$). Mean additional patients´ requested Pethidine hydrochloride in Gabapentin group was 0.8±0.23 mg/kg which was significantly less than control group (1.11±0.42 mg/kg). Dizziness as a drug adverse effect was seen in 3(6%) patients in Gabapentin group compared with control group which was significantly different ($P<0.001$). Any other adverse effect was not seen in both treatment and control group.

Based on the results of present study, Gabapentin can effectively reduce the pain intensity, nausea, vomiting and the need of analgesics among patients with renal colic. According to Mao et al. (2000) Gabapentin is effective in reducing neuropathic pain associated with diabetic neuropathy, post herpetic neuralgia, multiple sclerosis and cancer-related neuropathic pain.

Several mechanisms such as modulation of gamma-aminobutyric acid (GABA) synthesis, peripheral effect on abnormal sodium channel activation and a specific binding site (the $\alpha_2\delta$ subunit of voltage dependent calcium channels), are considered for Gabapentin analgesic effect but there is little information to support these hypotheses (Mao et al., 2000). Angello (2005) noticed GABA analogs work both peripherally and centrally to relieve pain. GABA analogs have interactions with calcium channels which are considered having potential role in renal colic. Calcium channel antagonists like nifedipine have vasodilatory effect on ureter smooth muscles and can relieve the pain and help passage of the renal calculi. Based on this possible central and peripheral interference with pain pathways and potential ureter smooth muscle relaxation, Gabapentin can provides superior pain relief for renal colic but optimal dose and how to use of Gabapentin is unclear yet (Imani, 2012).

Straube (2010) showed that 250 mg Gabapentin is significantly effective than placebo in postoperative pain relief in participants having pain following dental or orthopedic surgery but the clinical value of it is limited and inferior to commonly used analgesics. There are some clinical trials which has been shown the suppressant effect of Gabapentin in postoperative nausea and vomiting (Pandey, 2006), chemotherapy induced nausea (Guttuso et al., 2003) and some other medical situations. In the present study, the nausea severity score and the mean vomiting frequency in Gabapentin group was less than control group. In patients with renal colic, the nausea and vomiting is due to severe pain (Kehlet et al, 2003), so it seems that

Table 1. Baseline characteristics upon admission to the hospital.

Parameter	Gabapentin Group	Control Group
Age(years)	68.18±9.26	66.98±7.62
Weight(kg)	71.62±10.30	69.92±7.98
Male/Female(n)	36/14	35/15
Opacity in KUB(n)	32	38

Table 2. Abdominal pain scores (VAS).

Groups/ Time	Gabapentin	Control(placebo)	P value
First evaluation	9.32±0.79	9.28±0.8	0.804
Hour 1	7.66±1.13	8.76±0.93	<0.001*
Hour 2	5.82±1.62	7.94±0.01	<0.001*
Hour 3	4.02±2.12	7.24±1.11	<0.001*
Mean pain score	2.36±1.83	5.88±1.11	<0.001*

* p<0.05, statistically significant.

effective pain relief can reduce the frequency and intensity of nausea and vomiting. In this double blind placebo-controlled clinical trial, we used Gabapentin as an adjuvant analgesic and it demonstrated that administration of 600 mg Gabapentin reduced the intensity of pain among patients with renal colic compared to control group without any severe side effects (p<0.001). Gabapentin has few interactions with major classes of drugs since it is not metabolized in the body. It seems that the nontoxic nature of Gabapentin and its suitable analgesic effects in renal colic proposes the drug as an adjuvant treatment in renal colic.

ABBREVIATIONS

NSAIDs, Non-steroidal anti-inflammatory drugs; **VAS,** visual analog pain score; **GABA,** gamma-aminobutyric acid.

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