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Full Length Research Paper

Imbalance of the oxidant - antioxidant status by aspartame in the organs of immune system of Wistar albino rats

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Aspartame (L- aspartyl- L-phenylalanine methyl ester) is one of the most widely artificial sweeteners consumed in so many products worldwide in various countries which added to a large variety of food, most commonly found in low calorie beverages. On metabolism in humans and experimental animals, aspartame is rapidly and completely metabolized to aspartic acid (40%), phenylalanine (50%) and methanol (10%). Methanol, a toxic metabolite is primarily metabolized by oxidation to formaldehyde and then to formate; these processes are accompanied by the formation of superoxide anion and hydrogen peroxide. This study focus is to understand whether the oral administration of aspartame (40 mg/kg b.w) for 15, 30 and 90 days have any effect on the antioxidant status (enzymatic and non-enzymatic) in immune organs such as the spleen, thymus, lymph nodes and bone marrow of rats. To mimic human methanol metabolism, folate deficient rats were used. After 15 days of aspartame administration, animals showed a significant increase in free radical production as indicated by the increase in both enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (reduced glutathione and vitamin C) antioxidant level along with the marked increase in lipid peroxidation and nitric oxide level. However, after repeated long term administration (30 and 90 days), the generation of reactive free radicals overwhelmed the antioxidant defense as indicated by an increase in lipid peroxidation with the decrease in antioxidants level. This study concludes that administration of aspartame even at the Food and Drug Administration permitted level its repeated exposure causes oxidative stress by altering the oxidant/antioxidant balance in immune organs of the rats and its effects also reflected in the histology of the spleen and lymph nodes

Key words: Aspartame, folate- deficient, immune organs, oxidative stress.

INTRODUCTION

Aspartame also marketed as nutra sweet, candrelel or equal was serendipitously discovered in 1965 by James Schlatter, a chemist working for G.D. Searle & company (Garriga and Metcalfe, 1988). Aspartame (L- aspartyl- L-phenylalanine methyl ester) is one of the most widely artificial sweeteners consumed in so many products

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worldwide in various countries (Magnuson et al., 2007) which added to a large variety of food most commonly found in low calorie beverages, desserts and table top sweeteners added to tea or coffee (Butchko and Stargel, 2001). After oral administration to humans and experimental animals, aspartame is rapidly and completely metabolized to 40% aspartic acid, 50% phenylalanine and 10% methanol (Karim and Burns, 1996; Stegink and Filer, 1996). It forms methanol when the methyl group of aspartame encounters the enzyme chymotrypsin in the small intestine (Stegink et al., 1981). A relatively small amount of aspartame can significantly increase plasma methanol levels (Davoli, 1986). In an update on the safety in use of aspartame, the European Union Scientific Committee on Food maintained the established acceptable daily intake (ADI) of aspartame in humans at 40 mg/kg of body weight (European Food Safety Authority (EFSA), 2006).

According to Garriga (1988), consumption of 40 mg aspartame (ASP)/kg body weight would result in ingestion of 4 mg methanol/kg body weight (10% of ASP by weight is methanol), which is less than the amount of methanol formed during consumption of many foods including fruits and vegetables. Based on these acceptable dosages the aspartame dosage was fixed at 40 mg/kg. Oxidative stress is defined as an imbalance between higher cellular levels of reactive oxygen species (like superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen, nitric oxide, peroxynitrite) and the cellular antioxidant defense (Ilhan et al., 2005). Methanol is being increasingly recognized as a substance that damages the liver cells where it is oxidized to formaldehyde and later to formate (Oppermann et al., 1984). These processes are accompanied by elevation of nicotinamide adenine dinucleotide (NADH) level and the formation of superoxide anion, which may be involved in lipid peroxidation (Parthasarathy et al., 2006). However, accumulating evidence has implied that the production of free radicals plays a critical role in oxidative stress (Liu and Mori, 1999).

Cells of the immune system are particularly susceptible to changes in the antioxidant status because they carry out essential functions through the generation of a high number of oxygen free radicals (Pieri et al., 1993). This antioxidant-oxidant balance is an important determinant of immune cell function, including maintenance of the integrity and functionality of membrane lipids, cellular proteins, and nucleic acids and control of signal transduction of gene expression in immune. The secondary lymphoid organ (spleen and lymph node) is considered the draining site of toxic substance and is therefore considered an important organ to evaluate for any immune toxic and immune modulatory compounds treatment.

Due to the presence of B and T lymphocytes, the immune toxic effects of xenobiotic or their metabolites on these cell populations may be reflected in the spleen and lymph nodes and the routine histology of lymphoid organs such as spleen and lymph nodes could reveal the modifications occurring at cellular level. Hence the focus of the study is to investigate lipid peroxidation and antioxidant status and histology of the immune organ of wistar albino male rats on exposure of aspartame (40 mg/kg bw).

MATERIALS AND METHODS

Chemicals

Pure aspartame powder and methotrexate was purchased from sigma Aldrich chemical, st.louis, USA and all other chemical used were of analytical grade obtained from sisco research laboratory, Mumbai, India.

Animal model

Animal experiments were carried out only after getting clearance from the Institutional Animal Ethical Committee (IAEC No: 02/03/11) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The experimental animals were healthy; inbreed adult male Wistar albino rats, weighing approximately 200 to 220 g. The animals were maintained under standard laboratory conditions and were allowed to have food and water *ad libitum* (standard rat feed pellets supplied by M/s. Hindustan Lever Ltd., India) for control animals and for folate deficient, and aspartame treated rat had given special folate deficient diet (Andrew and Rosemaryl, 1988) for 37 days. All the rats were housed under condition of controlled temperature (26 ± 2°C) with 12 h light and 12 h dark exposure.

Experimental design

Group-I was saline control animals, in order to make animals folate deficient the folate deficient diet was given to them on a special dietary regime for 37 days and after that methotrexate (MTX) in sterile saline were administered by intra peritoneal every other day for two weeks (Ming et al., 1989) before euthanasia. After that, MTX folate deficiency was confirmed by estimating the urinary excretion of formaminoglutamic acid (FIGLU) (Rabinowitz and pricer, 1956) prior to the experiment and in these confirmed animals the rest of the study were conducted, and these folate deficient animals were further divided into 4 groups consisting of 6 animals each. Group-II is folate deficient control, Group-III is folate deficient rats treated with aspartame for 15 days, Group-IV is folate deficient rats treated with aspartame for 30 days and Group-V is folate deficient rats treated with aspartame for 90 days. Animals of control and folate deficient animals (Groups I and II) received daily normal saline orally (by means of lavarge needle) throughout the experimental protocol, prior to the experiment. Animals of aspartame treated group were daily administered aspartame (40 mg/kg) (EFSA, 2006) dissolved in normal saline orally (by means of lavarge needle) for 15, 30 and 90 days.

Sample collection

At the end of the experimental period (15, 30 and 90 days) all the animals were sacrificed under deep anesthesia using pentothal sodium (40 mg/kg). Blood samples and isolation of immune organs were performed between 8 and 10 a.m. to avoid circadian rhythm induced changes. Stress-free blood samples were collected as per the technique described earlier (Feldman and Conforti, 1980).

Table 1. Effect of aspartame on plasma corticosterone level (µg of corticosterone/dl of plasma).

Control	FD	15 days	30 days	90 days
40.82±1.72	42.89±2.31	48.71±1.77*a,*b	54.99±2.29*a,*b	92.96±1.9*a,*b,*c,*d

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group. FD – Folate deficient, D – Number of days.

Table 2. Effect of aspartame on lipid peroxidation level [MDA (n moles/mg protein)].

Organs	Control	FD	15 days	30 days	90 days
Spleen	2.55±0.37	2.82±0.23	5.91±0.58*a,*b	8.84±0.49*a,*b,*c	12.16±1.00*a,*b,*c,*d,
Thymus	3.58±0.36	3.85±0.22	7.93±0.26*a,*b	10.87±0.50*a,*b,*c	14.94±0.53*a,*b,*c,*d
Lymphnode	2.93±0.333	3.09±0.21	6.70±0.45*a,*b	8.78±0.53*a, *b,*c	12.86±0.45*a,*b,*c,*d
Bonemarrow	3.71±0.42	3.89±0.37	5.68±0.52*a,*b	7.82±0.59*a, *b,*c	13.94±0.73*a,*b,*c,*d

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with control, *b - compared with FD group, *c- compared with 15 D group, *d compared with 30D group. FD – Folate deficient, D – Number of days.

Handling of sample

After administrating long acting anesthesia (penrtathol sodium), the animal was then perfused with ice-cold phosphate buffered saline (PBS), the immune organs (spleen, thymus, lymph nodes and bone marrow) were removed immediately, washed with the PBS solution to remove blood cells, blotted on filter paper, quickly weighed and homogenized by using Teflon glass homogenizers in (1/10 weight per volume) ice cold phosphate buffer (0.1 M, pH 7.0) and centrifuged. The supernatant was used for estimation of lipid peroxidation and various enzymatic and non-enzymatic antioxidants. A separate set of animals were used for the histological study.

Biochemical determinations

Estimation of plasma cortisol was determined by the procedure of Clark (1955). Lipid peroxidation (LPO) was determined by the method of Ohkawa et al. (1979). Protein estimations were carried out according to the method of Lowry et al. (1951). Nitric oxide (NO) levels were measured as total nitrite + nitrate levels with the use of the Griess reagent as previously described by Bradford (1976).

Determination of the activities of enzymatic antioxidants

Superoxide dismutase ((EC 1.15.1.1) (SOD) was assayed according to the method of Marklund and Marklund (1974). The activity of Catalase (EC.1.11.1.6) (CAT) was assayed by the method of Sinha (1972). Glutathione peroxidase (EC.1.11.1.9) (GPx) activity was estimated by the method of Rotruck et al. (1973).

Estimation of non-enzymatic antioxidants

Reduced glutathione (GSH) in the immune organs were estimated by the method of Moron et al. (1979). Ascorbic acid was assayed by the method of Omaye et al. (1979).

Histology

For light microscopic study, spleen and lymph nodes were processed for routine paraffin sectioning and stained with Haematoxylin and Eosin (Bancroft and Gamble, 2002).

Statistical analysis

All data were analyzed with the statistical package for social sciences (SPSS) statistical package for Windows (version 20.0, SPSS Institute Inc., Cary, North Carolina). Data are expressed as mean ± standard deviation (SD) and was analyzed by one way-analysis of variance (ANOVA). The significance was fixed at $p < 0.05$. If the data showed a significant difference, it was followed by Tukey's multiple comparison tests.

RESULT

Effect of aspartame on plasma corticosterone level

The data are presented in Table 1 as mean ± SD. The corticosterone level was similar in folate deficient group when compare to control group. The rat treated with aspartame showed a marked increase in corticosterone level irrespective of the duration of exposure (15, 30 as well as 90 days) when compared to the control as well as folate deficient groups. There was marked increases in the corticosterone level of 30 and 90 days aspartame treated animals when compare to control, folate deficient animals as well as 15 days treated animals, moreover this increase was more marked in 90 days aspartame exposed rats than the 30 days exposed rats indicating that aspartame may act as a chemical stressor.

Effect of aspartame on LPO Level

The result of lipid peroxidation in the spleen, thymus, popliteal lymph nodes and bone marrow are summarized in Table 2 as mean ± SD. The LPO level of folate

Table 3. Effect of aspartame on Nitric oxide (µmoles /L).

Organs	Control	FD	15 days	30 days	90 days
Spleen	8.68±0.43	9.02±0.35	10.64±0.37*a,*b	12.08±0.54*a,*b,*c	16.84±0.41*a,*b,*c,*d
Thymus	4.93±0.41	5.09±0.64	6.41±0.49* a, *b	8.07±0.58*a, *b,*c	12.76±0.66*a,*b,*c,*d
Lymphnode	7.08±0.35	7.12±0.53	8.70±0.37* a, *b	9.80±0.34*a, *b,*c	13.90±0.60*a,*b,*c,*d
Bonemarrow	9.22±0.46	9.65±0.53	11.69±0.50*a,*b	13.67±0.39*a,*b,*c	17.75±0.84*a,*b,*c,*d,

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group. FD – Folate deficient, D – Number of days.

Table 4. Effect of aspartame on (organ weight/animal weight ratio)

Organs	Control	FD	15 days	30 days	90 days
Spleen	3.82±0.23	3.79±0.22	3.73±0.19*a,*b	3.19±0.38*a,*b,*c	1.69±0.10*a,*b,*c,*d
Thymus	1.53±0.07	1.52±0.08	1.50±0.06* a, *b	1.06±0.19*a,*b,*c	0.46±0.06*a,*b,*c,*d
Lymphnode	0.10±0.02	0.09±0.01	0.08±0.04*a,*b	0.06±0.007*a,*b,*c	0.02±.009*a,*b,*c,*d

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group. CONT – Control, F.D – Folate deficient, D – Number of days.

deficient animals was similar to the control animals. In animals treated with aspartame, the LPO levels were significantly elevated irrespective of the duration of exposure (15, 30 as well as 90 days). This increase was more marked in 30 days as well as 90 days aspartame exposed rats when compare to control, folate deficient as well as 15 days treated animals. Moreover, this increase was more marked in 90 days aspartame exposed rats than the 30 days exposed rats clearly indicating the generation of free radicals by aspartame.

Effect of aspartame on nitric oxide level

The results of nitric oxide level in spleen, thymus, popliteal lymph nodes and bone marrow are summarized in Table 3 as mean ± SD. The nitric oxide level was similar in control animals as well as in the folate deficient animals. In rat treated with aspartame, the nitric oxide level was significantly increased irrespective of duration of exposure (15, 30, as well as 90 days) from control as well as folate deficient animals. Moreover the nitric oxide level was found to be more elevated in the 30 days, as well as 90 days aspartame treated animals when compare to control, folate deficient, as well as 15 days treated animals. Moreover this increase was more marked in 90 days aspartame exposed rats than the 30 days exposed rats clearly indicating the generation of free radicals by aspartame.

Effect of aspartame on enzymatic and non-enzymatic antioxidant level

The result of enzymatic and non-enzymatic antioxidant level in spleen, thymus, popliteal lymph nodes and bone marrow are summarized in Figures 1 to 5, as bar diagram with mean ± SD. All enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH and Vit C) antioxidants level did not get altered in the folate deficient animal when compare to control animal. Though the rat treated with aspartame for 15 days showed a significant increase in all enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH and Vit C) antioxidants level, when compare with control as well as folate deficient animals. However, all the enzymatic and non-enzymatic antioxidant levels were significantly decreased in 30 and 90 days aspartame treated animal when compared to the control, folate deficient, as well as 15 days aspartame treated animals. Furthermore, this enzymatic and non-enzymatic level decrease showed more marked decrease in 90 days aspartame treated animals when compared to the 30 days aspartame treated animals.

Effect of aspartame on organ-weight to animal-weight ratio

The results of organ weight ratio are summarized in Table 4 as mean ± SD. The organ weight ratio of folate deficient animal did not deviate from the control animals. Though the organ weight ratio of the 15 days aspartame treated animals remained similar to controls as well as folate deficient animals. The 30 and 90 days aspartame treated animals showed a marked decrease from control, folate deficient animals and also 15 days aspartame treated animals. This decrease was more marked in 90 days aspartame treated animals when compared to 30 days treated animals.

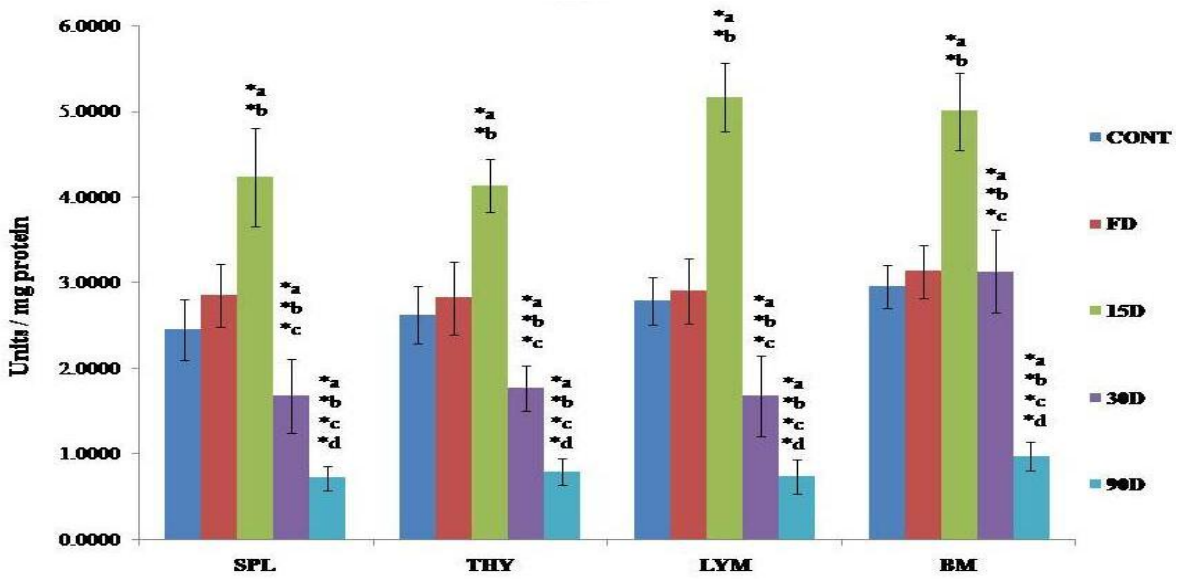

Figure 1. Effect of aspartame on SOD level in spleen, thymus , lymph node, and bone marrow of Wistar albino rats . Each value represents mean ± SD. Significance at *$p < 0.05$*, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group.
CONT – Control, F.D – Folate deficient, D – Number of days. SPL- Spleen, THY – Thymus, LYM – Lymphnode, BM – Bone Marrow.

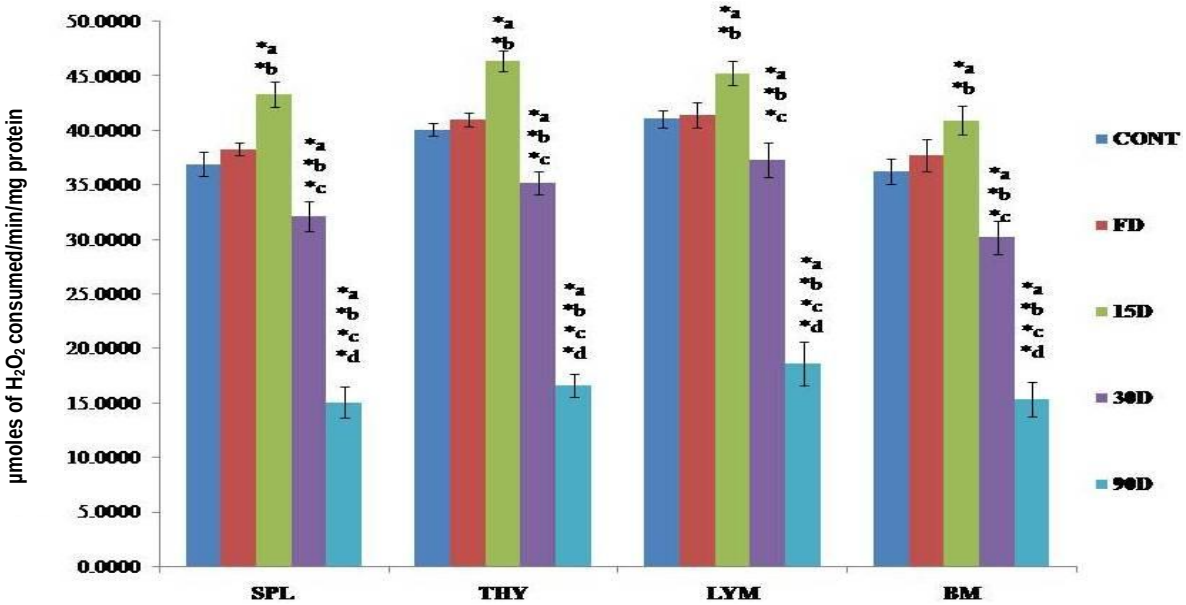

Figure 2. Effect of aspartame on Catalase level in spleen, thymus, lymphnode, and bone marrow of Wistar albino rats. Each value represents mean ± SD. Significance at *$p < 0.05$*, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group.
CONT – Control, F.D – Folate deficient, D – Number of days. SPL- Spleen, THY – Thymus, LYM – Lymph node, BM – Bone Marrow.

Effect of aspartame on histology of Spleen and Lymph node

The histology of spleen and lymph nodes are given in Figures 6a, b, c and 7a, b, c. There was no variation in the histology of spleen and lymph node of folate deficient animal observed when compared to control animals. In90 days aspartame treated animal's spleen, the boundary between white and red pulp started to disappear. There were cellular disruption and degeneration of the white

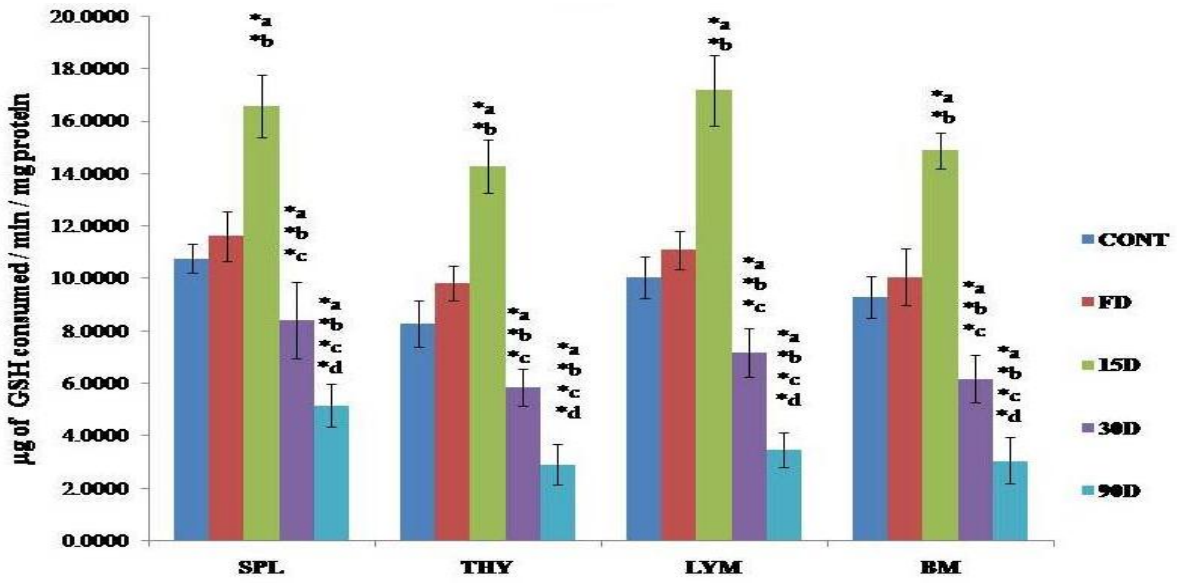

Figure 3. Effect of aspartame on GPX level in spleen, thymus, lymph node, and bone marrow of wistar albino rats. Each value represents mean ± SD. Significance at *$p < 0.05$*, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group.
CONT – Control, F.D – Folate deficient, D – Number of days. SPL- Spleen, THY – Thymus, LYM – Lymph node, BM – Bone Marrow.

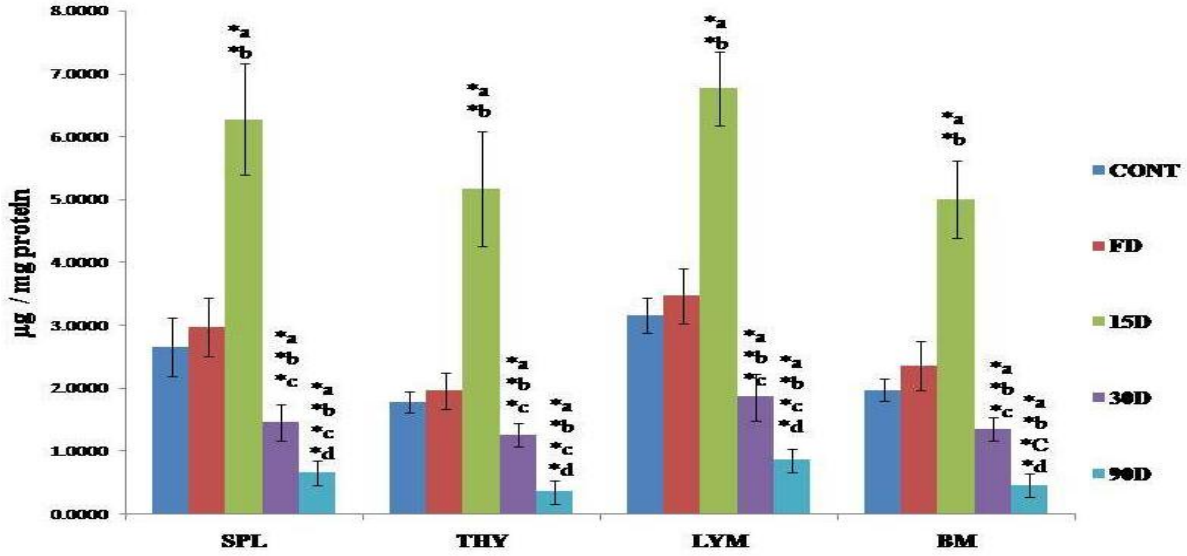

Figure 4. Effect of aspartame on GSH level in spleen, thymus, lymph node, and bone marrow of wistar albino rats. Each value represents mean ± SD. Significance at *$p < 0.05$*, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group.
CONT – Control, F.D – Folate deficient, D – Number of days. SPL- Spleen, THY – Thymus, LYM – Lymphnode , BM – Bone Marrow .

pulp and there was little absence of germinal centers. This was also associated with a slight increase in the thickness of the capsule and trabeculae. Moreover, there was also infiltration of neutrophils and lymphocytes observed with a significant number of giant cells increase in the parenchyma of spleen. In 90 days aspartame treated animal's lymphnode, there was little absence of germinate centers in cortex. There was infiltration of neutrophils and lymphocytes, and a proliferation of macrophages with vacuolated cytoplasm within the medullary region was observed in the 90 days aspartame treated animals.

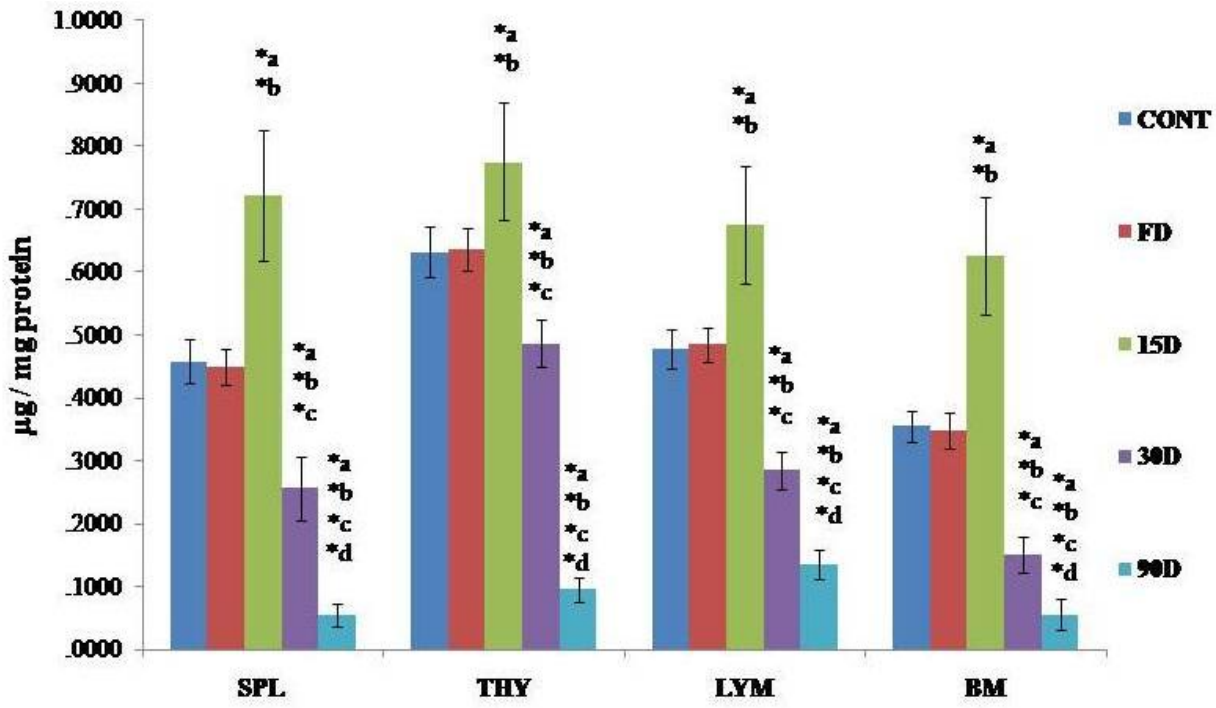

Figure 5. Effect of aspartame on Vitamin- C level in spleen, thymus, lymph node, and bone marrow of wistar albino rats. Each value represents mean ± SD. Significance at *$p < 0.05$*, *a - compared with control, *b - compared with FD group, *c- compared with 15D group, *d compared with 30D group.
CONT – Control, F.D – Folate deficient, D – Number of days. SPL- Spleen, THY – Thymus, LYM – Lymph node, BM – Bone Marrow.

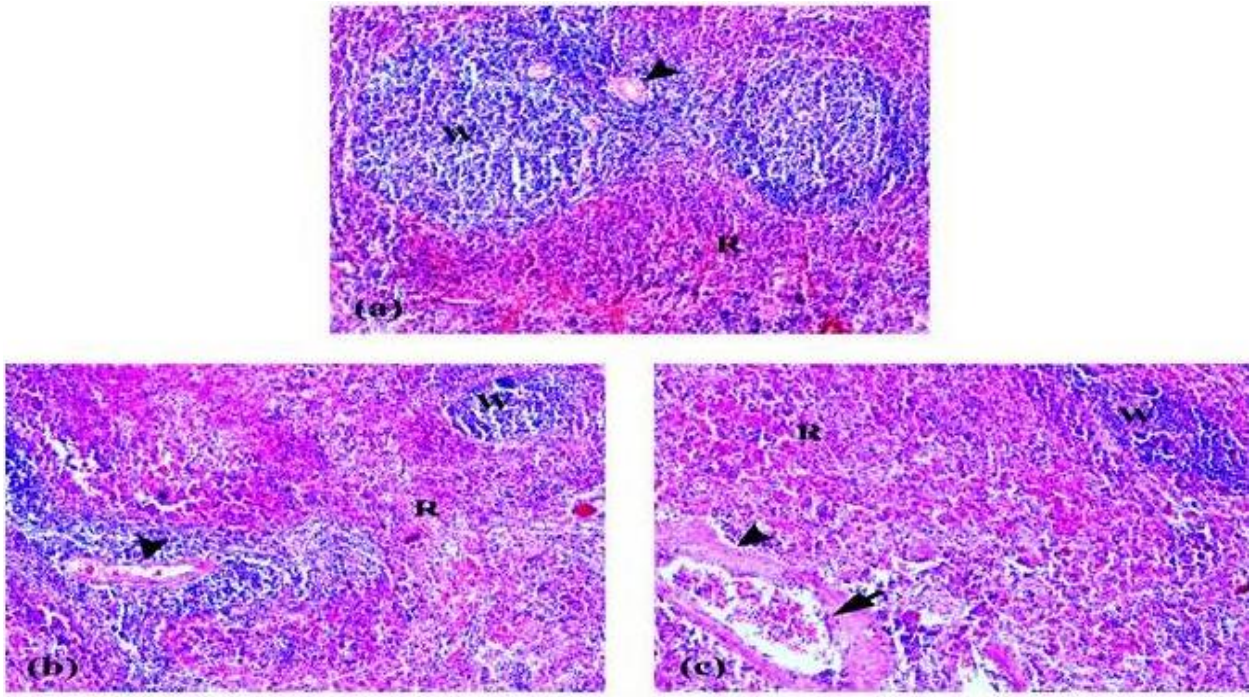

Figure 6. Photomicrograph showing histological changes in spleen of (a) Control, (b) Folate deficient and (c) Aspartame treated (90D) groups, stained with H&E depicting the central arteriole (arrow heads), white pulp (W), red pulp (R). Arrow indicating leukocytes infiltration in the arteriole of Aspartame treated group. Magnification: ×10.

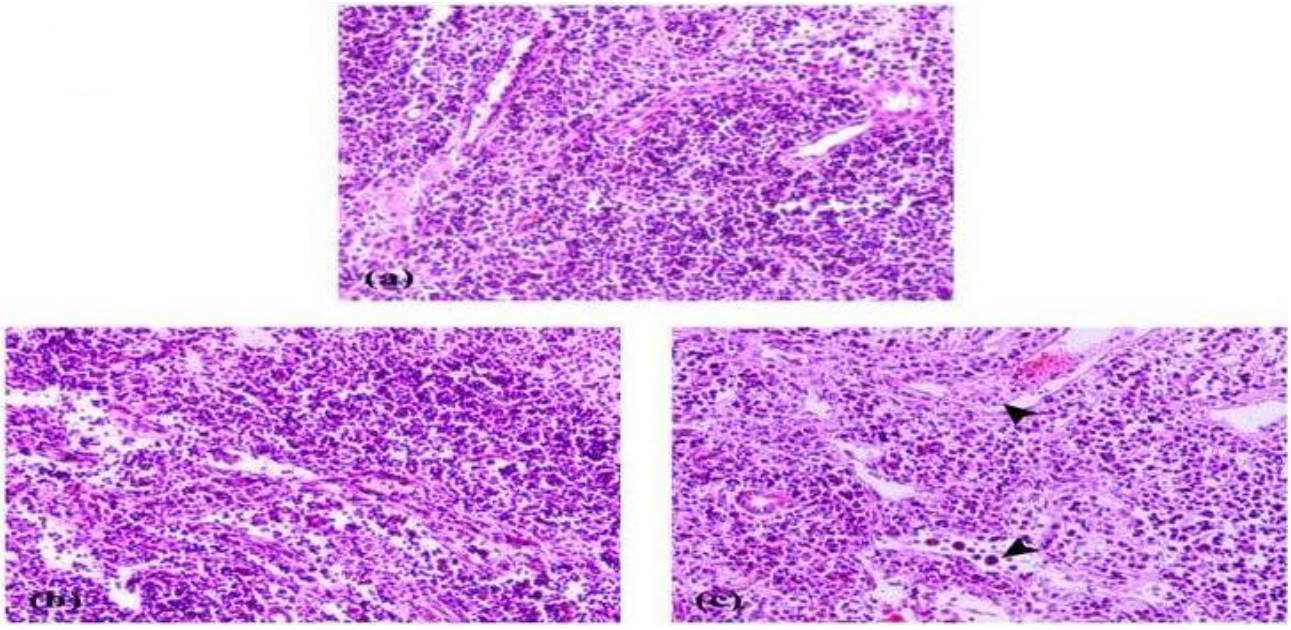

Figure 7. Photomicrograph showing histological changes in lymph nodes of (a)Control, (b) Folate deficient and (c) Aspartame treated (90D) groups, stained with H&E. Arrow heads indicating leucocytes infiltration. Magnification: ×10.

DISCUSSION

In this study, the folate deficient animals were used to mimic the human methanol metabolism. However the folate deficient animals did not show any significant changes in the parameters studied and remained similar to controls. The present study clearly confirms that aspartame can act as chemical stressor as indicated by the elevated corticosteroid level in the entire aspartame group studied, irrespective of duration of exposure. However it is not clear at what level the aspartame/its products are interfering with the HPA axis. According to Britton et al. (1992), it may be due to methanol, a metabolite of aspartame which stimulates norepinephrine to act on corticotrophin releasing factor (CRF) neurons in the paraventicular nucleus of hypothalamus to directly stimulate CRF release to act on pituitary to release adrenocorticotropic hormone (ACTH). Then ACTH acts on adrenal gland to release corticosterone secretion. Parthasarathy et al. (2006) reported a similar increase in plasma corticosterone level in rats after methanol administration for day 1 and 15 days. However after 30 days of methanol intoxication it showed considerable decrease in corticosterone level. This difference may be due to the higher dosage of methanol they have used.

The modified enzymatic and non-enzymatic free radical scavenging system with an elevated LPO level after aspartame administration clearly indicated the generation of free radicals in present study. The status of lipid peroxidation as well as altered levels of certain endogenous radical scavengers is taken as direct evidence for oxidetive stress (Khan, 2006). This alteration after aspartame administration may be attributed to its metabolite methanol. Methanol is primarily metabolized by oxidation to formaldehyde and then to formate, these processes are accompanied by the formation of superoxide anion and hydrogen peroxide (Parthasarathy et al., 2006). Oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species (O_2^-, H_2O_2, and OH^-) generated exceeds the antioxidant capability of the cell (Sies, 1991). Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotic (Hernanz et al., 1990).

LPO is initiated by the abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane (Bergendi et al., 1999). LPO is an auto catalytic mechanism leading to oxidative destruction of cellular membranes (Cheese-man, 1993). LPO in cellular membranes damages polyunsaturated fatty acids tending to reduce membrane fluidity, which is essential for proper functioning of the cell. Hence, the elevated level of LPO in the immune organs after aspartame administration in this study could not be ignored as it could affect the organ functions. This alteration could have been due to the methanol released during aspartame metabolism and the formaldehyde formed during methanol metabolism. This is well supported by the report of Parthasarathy et al. (2006) who observed an increase LPO level after methanol administration in the lymphoid organs. Similarly, Zararsiz et al. (2007) recorded a significant increase in LPO level in the kidney of rats after treatment with formaldehyde.

Aspartame administration to rats induces excess free

radical generation obviously, which is also again substantiated by the elevated nitric oxide level in this study. Nitric oxide is thought to react with superoxide anion to gain a radical property, which is a potent source of oxidative injury (Jaeschke, 2003). NO in excess can cause organ damage either directly or by reacting with superoxide anion to yield per- oxy- nitrate (Blough and Zafiriou, 1995). Elevated NO during stress reported to trigger the lipid peroxidation reactions probably due to its conversion to per-oxy-nitrite radical (Matsumoto et al., 1999). One of the reasons for enhancement of free radicals during stress may be due to the elevation of nitric oxide (NO) production (Matsumoto et al., 1999).

SOD constitutes an important link in the biological defense mechanism through dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 and O^{2-}, which are deleterious to polyunsaturated fatty acids and proteins (Fridovich, 1975). Catalase further detoxifies H_2O_2 into H_2O and O_2 (Murray et al., 2003). Glutathione peroxidase also functions in detoxifying H_2O_2 similar to catalase. Thus, SOD, catalase and glutathione peroxidase act mutually and constitute the enzymatic anti oxidative defense mechanism against reactive oxygen species (Bhattacharjee and Sil, 2006).

In this study, there was a marked increase of SOD, catalase and GpX enzyme activity after aspartame 15 days administration. The free radical slowly increases due to methanol metabolite of aspartame. To remove the free radical there is increase in both enzymatic and non-enzymatic level initially in order to prevent oxidative cell damage (Vidyasagaret et al., 2004) and justifying the findings of this study. However, repeated administration for 30 and 90 days could markedly inhibit these enzyme activities, and methanol may be the cause behind this. This is in agreement with earlier report that methanol administration could decrease the enzymatic antioxidant (SOD, CAT and GPx) in the lymphoid organs (Parthasarathy et al., 2006).

Zararsiz et al. (2007) demonstrated that in renal tissue of formaldehyde treated rats and Mourad (2011), in liver tissue of aspartame treated rats, SOD was significantly decreased when compared with the control animals. The decline in the activities of these enzymes might be due to their inactivation caused by excess ROS production (Pigeolet et al., 1990). Normally, the antioxidant enzymes catalase and GPx protect SOD against inactivation by H_2O_2. Reciprocally, SOD protects catalase and GPx against superoxide anion. However, over load of free radical could have been these regulations. Furthermore, the decrease in SOD and CAT activities may be due to the formation of formaldehyde from the methanol. This is in accordance with Gulec et al. (2006) who indicated that formaldehyde exposure led to a decrease in SOD and CAT activities in the liver tissue compared to the control. Also, Chang and Xu (2006) recorded a decrease in SOD activity and there was a dose-response relationship between formaldehyde concentration and SOD activity.

Usually, GSH non-enzymatically reacts with superoxide, NO (Clancy et al., 1994), hydroxyl radical (Bains and Shaw, 1997) and per-oxy-nitrite radicals (Koppal et al., 1999). Though during 15 days of aspartame administration it could elevate GSH level, but after 30 and 90 days of administration the drastic decrease in GSH may be a contributing factor for the nitric oxide level increase. The decrease in GSH activity observed in the present study could be caused by methanol, because methanol metabolism depends upon GSH (Pankow and Jagielki, 1993). The decrease in cellular glutathione content increases cell vulnerability to oxidative stress (Oyama et al., 2002).

GSH reduction can also explain the decreased concentration of Vit C, which enters the cell mainly in its oxidized form where it is reduced by GSH (Briviba and Sies, 1994). Vitamin C is a hydrophilic reducing agent which directly reacts with superoxides, hydroxyls and various lipid hydro peroxides more effectively than any other water soluble antioxidant (Niki, 1991). Vit C is a nutrient that regulates the immune system, and because of its antiviral and antioxidant properties, it plays a role in the phagocytic function (Hernanz, 1990). Therefore the decrease in the vitamin C could not be overlooked as it is essential for the immune regulations.

In the present study, though 15 days did not alter the organ weight/animal weight ratio, but later after 30 and 90 days aspartame administered animals showed a marked decrease in organ weight/animal weight ratio. The significant reduction in organ weight may be due to oxidative damage which is studied by Skrzydlewksa and Szynaka (1996) who reported that oxidative damage caused marked organ weight loss in albino rats upon methanol intoxication. This is also reported by Parthasarathy et al. (2006). Formaldehyde, the first metabolite of methanol, increases the population of shrunken cells, dead cells and hydolipid cells (Nakao et al., 2003). This reduction was also associated with the histological changes in the 90 days aspartame administered animals. Normal spleen was composed of white and red pulps surrounded by a capsule of dense connective tissue. In 90 days, in aspartame treated animals spleen, the boundary between white and red pulp started to disappear and in these animals, there was little absence of germinal centers in cortex. There were cellular disruption and degeneration of the white pulp and there was little absence of germinate centers indicating the cellular loss could be the source behind the decrease in the organ weight body weight ratio.

Farshid et al. (2002) reported that peroxynitrite, a biological oxidant and cytotoxic anion, induced inflammation and injury to the lung parenchyma (Mageid, 1994). The possible causative agent behind the cellular changes includes the free radicals that are generated by aspartame. Then next possibility is the methanol metabolite formaldehyde. Menezes et al. (2005) reported that all extensive injuries were repaired with collagen fibers

(scar) irrespective of its cause. Therefore the increased thicknesses of the capsule and of trabeculae in our experiment may be due to increase in collagen fibers deposition induced by injury resultant after aspartame administration. Most et al. (1997) reported that monocytes migrated at the site of inflammation coalesce with macrophages to form multinucleated giant cells. Therefore, it could be considered that infiltration of neutrophils, lymphocytes and increase in the number of giant cells observed in the present study might be due to the inflammation in the immune organs studied. The proliferation of macrophages with vacuolated cytoplasm within the medullary region in the 90 days aspartame treated animals strongly recommend further studies with aspartame on the cells involved in immunity.

Conclusion

The present study clearly points out that aspartame can increase the excess free radicals where by it inactivates the scavenging system as the days of administration increased that results in oxidative stress. It is essential to note that the dosage used in this study is Food and Drug Administration (FDA) permitted level and humans are consuming more than this without knowing its consequences. Aspartame metabolite methanol and also its metabolite formaldehyde may be the causative factors behind the changes observed. Moreover, such induced oxidative stress and the resultant inflammation in the immune organs along with the elevated corticosteroid level could be immune suppressive.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Short Communication

Antibacterial and antifungal activities of the crude extracts from the stem of *Chenopodium ambrosioides* Linn., an indiginous medicinal plant

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The crude methanolic extract along with the n-hexane, ethyl acetate, dichloromethane, n-butanol and aqueous fractions from the stem of *Chenopodium ambrosioides* Linn was studied against human pathogenic bacterial strains of *Escherichia coli, Klebsiella pneumoniae* (Gram negative bacteria), *Staphylococcus aureus, Bacilus subtilis* and *Staphylococcus epidermidis* (Gram positive bacteria) while against the fungal strains of *Aspergillus niger, Aspergillus parasiticus, Trycophyton horzianum, Rhizopus tolenapur* and *Aspergillus flavus.* The tested bacterial strains were taken from Center for Phytomedicine and Medicinal Organic Chemistry (CPMMOC) University of Peshawar, Pakistan which were previously collected from hospital patients of Khyber Teaching Hospital, University road Peshawar, Pakistan while the antifungal strains were collected from Center for Biotechnology and microbiology (CBM) University of Peshawar, Pakistan which were also in advance collected from hospital patients of Khyber Teaching Hospital, University road Peshawar. The selected strains were tested against crude extract and its fractions. Zones of inhibition were measured by using National Committee for Clinical Lab Standards (NCCLS) method in which for antibacterial activities, streptomycine while meconozole was used as standard drugs for antifungal activities. Dimethyl sulphoxide (DMSO) was used as negative control in both cases. The n-hexane, ethyl acetate, dichloromethane as well as n-butanol fractions showed moderate to significant activities against all bacterial strains especially against *B. subtilus, K. pneumoniae* and *S. epidermidis.* The fractions showed low antifungal activities against these strains.

Key words: Antibacterial, antifungal, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Klebsiella pneumonia*, *Aspergillus niger, Aspergillus Parasiticus, Trycophyton horzianum, Rhizopus tolenapur* , *Aspergillus flavus,* moderate, significant.

INTRODUCTION

Chenopodium ambrosioides Linn. is used traditionally as antihelmintic and antiparasitic agent in America, Asia and Europe (Monzote et al., 2009; Gadano et al., 2006). The roadsides and river banks in Pakistan, at Peshawar, Baluchistan, Dir, Swat, Kohala, Kashmir and Rawalpindi are widely covered by this plant (Stewart, 1972; Nisar et al., 2010). The important medicinal plant family of Chenopodiaceae, consisting of 102 genera and 1400

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Table 1. Criteria for determination of antibacterial activity.

Entry	Diameter (mm)	Activity
1	Below 9	No activity
2	9-12	Non-significant
3	13-15	Low
4	16-18	Good
5	Above 18	Significant

species (Marie, 1965), having an elevated importance for phytochemical investigation and medicinal evaluation, also provide space to this specie and thus increases the curiosity of researcher for antimicrobial potential and phytochemical studies of this plant. Due to a characteristic smell of the plant, the essential oil from this plant, known as 'Baltimore oil', has remained a main focus for the researchers and from the beginning of 19th century, was used for curing the patients with worms (Monzote et al., 2009).

The essential oil obtained from this plant has been reported for antifungal (Jardim et al., 2008; Kumar et al., 2007) and insecticidal (Cloyd and Chiasson, 2007) activities. The main component of the whole plant is ascaridol (45 to 70%). Its content is very variable and depends on the environment and the time of harvesting. Because of its toxicity, the content should not exceed 62 to 65% for pharmaceutical preparations. Ascaridole is the main constituent of the essential oil of this plant along with carvacrole and cryophyllene oxide and the toxic effects of these compounds on mitochondria has been reported (Monzote et al., 2009). Other components isolated from the essential oil of this plant are limonene, transpinocarveol, ascaridole-glycol, aritasone, β-pynene, myrcene, phelandrene, alcanphor and α-terpineol (De Pascual et al., 1980; Sagrero-Nieves and Bartley, 1995).

However, after a thorough literature survey it was found that no medicinal or phytochemical evaluations were carried out using whole plant or parts of the plant. Major studies are carried out on the essential oil of this plant through steam distillation along with the leaves extracts. Due to this very reason the plant stem methanolic extract was used to study antimicrobial potential of this medicinal plant.

MATERIALS AND METHODS

Plant collection

The plant was collected from Peshawar, Pakistan in June and the plant stem was separated from other parts. The well known Botanist of Peshawar University, Dr. Abdur Rashid, identified the plant. After getting shade dried in a closed room, the plant stem was grinded to powder form. The powdered plant stem was extracted with methanol three times by maceration for one week each time and thus methanolic crude extract was obtained. The crude methanolic extract was concentrated at 40°C through vacuum distillation by using rotary evaporator. This methanolic extract was further concentrated till complete dryness in water bath at 25°C temperature. The dried methanolic crude extract was further dissolved in distilled water and was further fractionated using n-hexane, ethyl acetate, dichloromethane, and n-butanol solvent systems leaving at the end the aqueous fractions. All these fractions along with crude methanolic extract were tested for antibacterial and antifungal potential.

Antibacterial bioassay

The antibacterial activity was checked by the agar well diffusion method (Nisar et al., 2010, 2013). The solid nutrient agar was prepared by dissolving 28 g nutrient agar in distilled water and making the solution up to 1 ml. The bacterial culture was kept on stock nutrient cultural agar. A bacterial colony was inoculated in broth and incubated at 37 ± 1°C for 24 h. After a day, soft agar was melted and cooled up to 40°C and was added by 100 µl bacterial culture, shaken and poured in plates containing nutrient agar. After rotating to cause even distribution of culture, the plates were further allowed for solidification. Wells were dug in the medium with the help of sterile metallic cork borer (6 mm diameter) in each plate at least 24 mm apart with their centers. Stock solution of the test samples in the concentration of 22 mg/ml were prepared in the dimethyl sulphoxide (DMSO) and 150 micro liter dilutions were added in their respective wells. The antibacterial activity of samples was compared with standard drug, streptomycin. The concentration of streptomycin was 2 mg/ml in DMSO. The standard drug streptomycin and DMSO were used as positive and negative control. Sample solutions (22 mg/ml in DMSO) were added in their respective wells by sterilized dropping pipettes. Two wells were filled with DMSO and streptomycin as negative and positive control. Plates were incubated at 37 ± 1°C for 24 h. The inoculums with two to eight hours bacterial strains containing approximately 10^4 to 10^6 colony forming units/ml (CFU) was spread on the surface of Mueller-Hinton agar plates with the help of a sterile cotton swab. All the agar surface of each plate was streaked for three times each with swabbed cotton turning the plate 60° between each streaking. All the solutions along with positive and negative control were added in their respective wells. The plates were again incubated for 14 to 19 h or more at 37°C. The antibacterial activity of samples was compared with standard drug, streptomycin showing inhibition in mm. Antibacterial potential of samples was then determined as per criteria mentioned in Table 1. Growth inhibition was calculated with reference to positive control.

Antifungal bioassay

The antifungal activity was determined by the agar well diffusion method (Nisar et al., 2010, 2013). In this method, Sabouraud dextrose agar (SDA) was prepared by mixing and dissolving mycological peptone, 10 g, glucose, 40 g, agar 15 g in approximately 900 ml of deionized water while the pH was maintained at 5.6 with hydrochloric acid and the total solution was made up to 1 L. The mixture was heated for at least 10 min and was then sterilized in autoclave at 121°C and 15 lb/in^2 pressure for a total of 15 min. The culture of organisms was maintained at SDA. Miconazole was used as the standard drug while DMSO was used as negative control. The extracts were dissolved in DMSO (24 mg/ml). Sterile Sabouraud dextrose agar medium (7 ml) was placed in a test tube and inoculated in a sample solution (40 µg/ml) kept in slanting position at room temperature overnight. Each tube was inoculated with a piece of inoculums of 4 mm diameter. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 30°C and growth inhibition was observed. The percent growth inhibition was calculated with reference to the negative control by applying the formula:

Table 2. Criteria for determination of antifungal activity.

Entry	Percent inhibition	Activity
1	30-40	Low
2	50-60	Moderate
3	61-70	Good
4	Above 70	Significant

Table 3. Diameter of zone of inhibition (mm) of samples against bacterial strains.

Sample	*Escherichia coli*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Bacilus subtilis*	*Staphylococcus epidermidis*
Crude methanolic extract	10	12	10	08	12
n-hexane fraction	14	18	13	14	15
Ethyl acetate fraction	13	20	12	14	11
Dichloromethane fraction	00	13	12	00	12
n-butanol fraction	16	12	14	12	10
Aqueous fraction	00	00	00	00	11
DMSO	00	00	00	00	00
Streptomycin	26	28	26	30	30

% Inhibition = (Linear growth of the negative control - Linear growth of sample) × 100

Growth in medium containing crude extract and fractions was determined. The results were evaluated by comparing with Table 2.

RESULTS AND DISCUSSIONS

Antibacterial bioassay

The antibacterial activity was studied against various human pathogens including *Escherichia coli, Klebsiella pneumonia* (Gram negative bacteria), *Staphylococcus aureus, Bacilus subtilis* and *Staphylococcus epidermidis*. The tested bacterial strains were taken from Center for Phytomedicine and Medicinal Organic Chemistry (CPMMOC) University of Peshawar, Pakistan which were previously collected from hospital patients of Khyber Teaching Hospital, University road Peshawar, Pakistan. The diameter of zone of inhibition (mm) samples against the bacteria is given in Table 3.

The crude extract showed non significant activity against bacterial strains. The n-hexane fraction showed low to good activities against all bacterial strains. It showed good activity against *K. pneumoniae* and low against four other bacterial strains. The ethyl acetate fraction showed non significant, low and significant activities. This fraction showed significant activity against *K. pneumoniae,* low against *E. coli* and *B. subtilis* while non significant against *S. aureus* and *S. epidermidis.* Dichloromethane fraction showed no activity against *E. coli* and *B. subtilis,* non significant against *S. aureus* and *S. epidermidis* while low activity against *K. pneumoniae*. n-Butanol fraction showed non significant activities against *K. pneumoniae, B. subtilis* and *S. epidermidis*, low activity against *S. aureus* and good activity against *E. coli* while the aqueous fraction remained non significant against *S. epidermidis* and non active against all other bacterial strains. Streptomycin used as a standard drug showed zone of inhibition (mm) 26, 28, 26, 30 and 30 against *E. coli, K. pneumoniae, S. aureus, B. subtilis* and *S. epidermidis,* respectively.

Antifungal bioassay

Antifungal activities were performed against the five fungal strains including *Aspergillus niger, Aspergillus parasiticus, Trycophyton horzianum, Rhizopus tolenapur* and *Aspergillus flavus* which were collected from Center for Biotechnology and microbiology (CBM) University of Peshawar, Pakistan and were also in advance collected from hospital patients of Khyber Teaching Hospital, University road Peshawar. The % inhibition of samples against fungal strains is shown in the Table 4. The crude as well as all the fractions showed from none to low activities against all fungal strains. The best activities are of ethyl acetate fraction against *A. niger* and *R. tolenapur* with % inhibition of 40 and 35, respectively.

Conclusion

The results confirm the antimicrobial strength of the crude extracts of the stem of this plant which supports the traditional medicinal use of this plant extracts. The results

Table 4. Percentage inhibition of samples against fungal strains.

Sample	*Aspergillus niger*	*Aspergillus parasiticus*	*Trycophyton horzianum*	*Rhizopus tolenapur*	*Aspergillus flavus*
DMSO	100	100	100	100	100
Crude methanolic extract	10	08	12	00	00
n-hexane fraction	25	20	21	18	19
Ethyl acetate fraction	40	09	27	35	00
Dichloromethane fraction	11	18	24	00	00
n-butanol fraction	00	28	22	10	27
Aqueous fraction	24	13	00	18	22

also go in favor of the importance of screening plants as a potential source of bioactive compounds. However further studies are required to investigate this important medicinal plant for isolation of novel compounds.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Hypolipidemic and antithrombotic evaluation of *Myrtus communis* L. in cholesterol-fed rabbits

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Herbs have been a great source of natural substances used to prevent many cardiovascular diseases (CVD). Blood lipid levels and coagulation parameters are probably the major determinant for producing CVDs. The present study was undertaken to evaluate the hypo-lipidemic and anti-thrombotic activities of aqueous extract of *Myrtus communis* L. fruit (AEM) in cholesterol-fed rabbits. Hyper-lipidemia was induced following administration of cholesterol for 45 days. Animals of treated group were administered AEM daily for 30 and 45 days at dose of 50 mg/kg. Biochemical tests were performed at the end of dosing that is, on 31st day and again on 46th day. The administration of AEM (50 mg/kg/day) revealed reduction in serum triglycerides and low density lipoprotein; there was also an increase in thrombin and fibrinogen time. Results of present study indicate that the extract exhibited hypolipidemic effects and has also an effect on blood coagulation parameters which may be of value in CVDs. However, further studies are necessary to explore the precise mechanism of action of these effects.

Key words: Herbs, lipid profile, blood coagulation, rabbits.

INTRODUCTION

Traditional medicine based on herbal remedies has always played a vital role in the health care systems of many countries (Verma et al., 2007) owing to their lesser side effects than synthetic drugs (Srivastava et al., 2006). There are many plants which have been listed in the traditional systems of medicine, and the individuals suffering from cardiovascular diseases (CVD), particularly hyperlipidemia and ischemic heart disease, get relief from these medicines (Mahmood et al., 2010). The World Health Organization (WHO) reports that approximately 80% of the world's population are using herbal drugs for their primary health care (Azaizeh et al., 2003; Mahmood et al., 2010).

Myrtus communis L. (Myrtaceae) is widely distributed all over the Mediterranean region and the Middle East. It is an evergreen shrub, about 1 to 5 m high, h as small

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deep-green leaves, white flowers and small dark fruits (Asif et al., 2011). In folk medicine, the decoction of leaves and fruits of myrtle are used orally for stomach aches, hypoglycemia, cough, constipation as well as externally for wound healing (Serce at al., 2010). The volatile oil (Myrtii Oleum) obtained from leaves are used to lower blood glucose (Jung et al., 2006). Similarly, in Italian folk medicine, the fruit of this plant is used in the treatment of many types of infectious disease, including diarrhea and dysentery (Gortzi et al., 2008).

Lipoprotein abnormalities are considered as a highly modifiable risk factor for CVD (Allen at al., 1996). Hyperlipidemia constitutes a foremost etiopathological factor for atherosclerosis (Banerjee and Maulik, 2002) and is often associated with myocardial infarction and cerebrovascular disorders (Ross, 1999). Hence, alteration in plasmalipid levels, coagulation proteins, platelets and fibrinolytic factors may reduce the chance of atherosclerosis (Eitzman et al., 2000). The use of herbal drugs is well-established in many countries. However, despite the increasing scientific interest in this field, there is a lack of summarized data on composition of herbal medicines and therapeutic applications (Aleksic and Knezevic, 2013). Hence, present study has been planned to evaluate the effects of aqueous extract *M. communis* (AEM) on lipid profile and blood coagulation in hyperlipidemia induced rabbits.

MATERIALS AND METHODS

Myrtus communis extract

Myrtus communis L. is a medicinal plant endemic to the Mediterranean area and has been used by locals for its food and medicinal properties since ancient times (Atzei, 2003). *Myrtus* species have been reported to be very rich in volatile oils (Satrani et al., 2006; Shikhiev et al., 1978), phenolic acids and flavonoids (Romani et al., 1999), tannins (Diaz and Abeger, 1986), anthocyanin pigments (Martin et al., 1990) and fatty acids (Cakir, 2004). The fruit of *M. communis* was grinded and aqueous extract was prepared by decoction. The grinded fruit was boiled in water (10 parts of water and 1 part of herb) for 3 h followed by filtration. The filtrate was further concentrated by boiling to obtain the aqueous extract. The extract so obtained was kept at -20°C until further use

Animals

Rabbits were selected as experimental animals in the present study since biochemical changes produced in rabbits are relatively similar as observed in humans; rabbits are easily obtainable, easy to handle and cost-effective. The study was conducted on 14 healthy white rabbits of both sexes (1100 to 1600 g), housed at the animal house, Department of Pharmacology, University of Karachi, under controlled temperature condition of 22±2°C, and humidity (50 to 60%) in an alternating 12-h of light/dark cycle. The animals were kept in plastic cages and were given green leafy diet and water regularly. The use of animals in this experiment was in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the Ethical Committee of University of Karachi.

Animal's treatment protocols

The aqueous extract of *Myrtus communis* L. (AEM) was initially tested in the dose of 20 mg/kg for 30 days in a group of 5 animals, but no significant response was observed. New rabbits were then uniformly divided into 2 groups of 7 animals each that is, control and treated. Animals of either group received high cholesterol diet (HCD) regularly for 45 days, 0.125 g/kg cholesterol supplied by Merck in 0.5% corn oil. After 45 days, animals of treated group were administered AEM at the dose of 50 mg/kg (Jung et al., 2006) for 30 days during the first phase of study. Animals of control group were given saline equal to the volume of respective doses according to their body weight. During the second phase of study, animals of treated group were further administered AEM for more15 days making a total period of 45 days and compared with control for the same period. All substances were administered through oral route. Blood sam-ples were collected thrice from the ear vein of animals, first after 45 days of HCD then again after 30 and 45 days dosing of AEM.

Estimation of lipid profile

Blood sample of about 5 ml were collected in gel tube. Serum was immediately separated out by centrifuging blood samples on 14K Humax centrifuge at 3000 rpm for 15 min. Lipid profile were analyzed on Humalyzer 3000 (semi-automatic chemistry analyzer, Model #16700) (Human Germany) using standard kits supplied by Human. Total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) was estimated by CHOD-PAP method; triglyceride (TG) by GPO-PAP methods (Trinder, 1969), and high density lipoprotein cholesterol (HDL-C) by the method of Friedwald et al. (1972).

Estimation of coagulation parameters

Blood sample of about 3 ml were collected in coagulation tubes containing 3.2% sodium citrate. Plasma was separated by centrifuging blood samples on 14K Humax centrifuge at 3000 rpm for 15 min. Thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen were measured by Humaclot duo (Human Germany), using standard reagent kits supplied by Human (Chan et al., 2007).

Mortality rate

Mortality rates were observed in animals receiving HCD and AEM during the total period of experiment. The number of animals that died during these intervals was also noted.

Statistical analysis

All values were compared with the control by taking mean and standard error to the mean using t-test, values of $P < 0.05$ were considered as significant and $P < 0.01$ as highly significant. All statistical procedures were performed according to the method of Alcaraz and Jimenez (1989).

RESULTS

Mortality rate

No death was observed in any group of animals during

Table 1. Effects of *Myrtus communis* on lipid profile after 30 days.

Animal group	Parameter (mg/dl)			
	Cholesterol	Triglyceride	HDL-C	LDL-C
Control	132.4±22	234.0±17	4.49±0.75	176.7±7.4
Myrtus communis	76.0±6.5	**149.9±7.5	*2.68±0.26	132.7±14.0

n = 7. Average value±SEM *P < 0.05 significant as compared to control. **P < 0.01highly significant as compared to control

Table 2. Effects of *Myrtus communis* on lipid profile after 45 days.

Animal group	Parameter (mg/dl)			
	Cholesterol	Triglyceride	HDL-C	LDL-C
Control	85.2±18	115.4±17	1.743±0.16	141.4±12
Myrtus communis	44.4±3.4	*61.2±9.7	1.72±0.08	**58.11±1.3

n = 7. Average value ± SEM. *P < 0.05 significant as compared to control. **P < 0.01highly significant as compared to control

the total period of experiment.

Lipid profile

Table 1 gives the comparison of serum total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C) levels in animals of control and treated groups after 30 days, while a similar comparison between the same groups of animals after 45 days is presented in Table 2. Animals which received AEM for 30 days showed highly significant and significant decrease in the levels of TG and HDL-C that is, 149.9 ± 7.5 mg/dl and 149.9 ± 7.5 mg/dl in comparison to control values that is, 234.0 ± 17.0 mg/dl and 4.49 ± 0.75 mg/dl, respectively. Conversely there was no significant change in the levels of TC and LDL-C levels at the completion of dosing. Animals which received AEM for 45 days showed highly significant and significant decrease in the level of LDL-C and TG that is, 58.11 ± 1.3 mg/dl and 61.2 ± 9.7 mg/dl in comparison to control values that is, 141.4 ± 12.0 mg/dl and 115.4 ± 17 mg/dl. However the other parameters were not altered significantly at the end of dosing.

Coagulation parameters

Table 3 discloses the comparison of thrombin time (TT), partial thrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen time in animals of control and treated animals, while a similar comparison between the same groups of animals after 45 days is presented in Table 4. Animals given AEM for 30 days showed significant increase in fibrinogen time that is, 57.3 ± 13 s as compared to control that is, 26.01 ± 2.9 s. However there were no significant changes in TT, aPTT and PT at the completion of dosing. Animals given AEM for 45 days showed highly significant increase in TT that is, 24.7 ± 1.9 s as compared to control, that is, 16.56 ± 1.4 s. However the other parameters were not altered significantly.

DISCUSSION

AEM have been found to reduce LDL-C and increase fibrinogen time that is, decrease fibrinogen level which seems to be of clinical importance since there are studies which shows that increased fibrinogen has been associated with cardiovascular risks. Fibrinogen increases cardiovascular risk by several mechanisms since it specifically binds to activated platelets via glycoprotein IIb/IIIa, contributing to platelet aggregation, promotes fibrin formation and increase plasma viscosity (James et al., 2000).

Hypercholesterolemia is a strong risk factor for producing atherosclerosis. There is a strong relationship between blood lipids and coagulation parameters since acute changes in plasma lipids appear to have significant effects on factors affecting thrombosis (Eitzman et al., 2000). Coagulation abnormalities are usually present in critically ill patients and may contribute to morbidity and mortality; hence requires rapid examination to establish the underlying cause and to initiate corrective and supportive treatment (Marcel and Steven, 2006). Thus in the present study, highly significant decrease in LDL-C after 45 days and significant increase in the fibrinogen time after 30 days seems to be very important from clinical point of view since there has been a connection between augmented LDL-C and atherosclerosis. High plasma LDL-C concentration is one of the major risk factor for

Table 3. Effects of *Myrtus communis* on blood coagulation after 30 days.

Animal group	Parameter (seconds)			
	TT	PT	aPTT	Fibrinogen
Control	17.84±1.7	7.11±0.6	30.5±6.2	26.01±2.9
Myrtus communis	30.5±4.0	6.2±0.26	41.0±4.2	*57.3±13

n = 7. Average value ± SEM. *P < 0.05 significant as compared to control.

Table 4. Effects of *Myrtus communis* on blood coagulation after 45 days.

Animal group	Parameter (seconds)			
	TT	PT	aPTT	Fibrinogen
Control	16.56±1.4	5.871±0.22	33.04±3.5	26.41±2.3
Myrtus communis	**24.7±1.9	5.60±0.13	39.3±3.6	38.7±5.9

n = 7. Average value±SEM. **P < 0.01 highly significant as compared to control.

atherosclerosis (Ross, 1999).

There are studies which suggest that reducing LDL-C decreases the risk of CVD (Aghasadeghi et al., 2008). The decrease in LDL-C by AEM, may be due to the presence of myrtle oil (Jung et al., 2006), while natural compounds semi myrtucommulone and myrtucommulone A may also be responsible for potential anti-atherogenic effect of *Myrtus communis* (Rosa et al., 2008). Hypercholesterolemia induces oxidative stress, since it increases the formation of reactive oxygen species from membrane phospholipids during prostaglandin synthesis. Thus antioxidants and hypolipidemic agents suppress the development of hypercholesterolemic atherosclerosis and induce regression of atherosclerosis (Paul and Kailash, 2003). Therefore suppression of atherosclerosis is associated with decrease in oxidative stress and serum lipids (Kabiri et al., 2011). Hence it may be concluded that *M. communis* may have some role in the prevention of atherosclerosis due to its hypolipidemic and anti-inflammatory effects.

Present study also revealed significant increase in TT that indicates deficiency of fibrinogen or inhibition of thrombin (Lane et al., 2005). Hence prolonged TT may be the results of reduced activity of coagulation factors because factors IX and X (Di Cera, 2008), XI and XII are essentially required for thrombin generation (Gailani et al., 2007). Present study also revealed significant increase in fibrinogen time that is, decreased fibrinogen level after 30 days at 50 mg/kg dose. There are studies which show that increases in the fibrinogen levels are a strong risk factor for the development of CVD (Barazzoni et al., 2000; Zhao et al., 2011). Hence, AEM reduces the risk of vascular diseases by reducing the fibrinogen level.

Conclusion

The present study was conducted to explore the effects of AEM on lipid profile and blood coagulation. The overall results of the study reveal AEM to be effective as hypolipidemic agent in the dose of 50 mg/kg and also have an effect on blood coagulation parameters which may be of value in CVD.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Nature and frequency of prescription modifications: An evaluation from the community pharmacy

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Medication errors can occur at any point in the medication use process. The present study was undertaken to investigate the frequency and nature of prescription modifications and pharmacist's interventions outcomes at the community pharmacy. A descriptive and prospective study was conducted and data were structured by all prescriptions that were modified by the pharmacy during the study. All medicines were classified into therapeutic groups using the Anatomical Therapeutic Chemical classification. A total of 20,205 prescriptions were processed during the study and the overall incidence of modifications by the community pharmacy was 10.9 % (2216 prescriptions). The majority (1676; 75.6%) of the reasons for the medications concerned the clarification of an insufficiently specified prescription. Drug-drug interaction (32.5%), contraindication (6.5%) or double medications (40.6%) were prevalent. The findings of this study reinforce the importance of prescription screening and interventions by pharmacists in reduce preventable adverse events attributed to medication errors. It also emphasizes the necessity of interdisciplinary communication and cooperation in identifying and resolving prescribing errors and irregularities in order to achieve optimal therapeutic outcomes for the patient.

Key words: Community pharmacy, medication error, pharmacist intervention.

INTRODUCTION

Patient safety has become a major concern since the November 1999 release of the Institute of Medicine (IOM) report, *To Err Is Human*. Health care practitioners may have been surprised to learn from this report that errors involving prescription medications are responsible for up to 7,000 American deaths per year and that the financial costs of drug-related morbidity and mortality may cost nearly $77 billion a year (Grissinger et al., 2003).

Medication errors can occur at any point in the medication use process. The prescribing step of the medication use process involves clinical decision making, selecting a treatment or drug regimen, documenting information in the medical record, and ordering the selected drug

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treatment (IOM, 2007). Some of the reasons that errors occur during this stage of the medication use process are because prescribers do not use current available treatment evidence or available patient information, (i.e. allergy information, other medications, other conditions), do not follow set policies or procedures, fail to document appro-priate information in the patient chart, or do not communi-cate the prescription appropriately (Giampaolo and Pietro, 2009; Ross et al., 2012). The dichotomous nature of community pharmacy practice is a critical dilemma for the profession. The role of community pharmacists has been traditionally characterized by dispensing prescrip-tion medicines, selling over-the-counter medication and offering healthcare advice. Community pharmacists are often not viewed as a core part of the primary healthcare team. Perceptions around being a retailer and healthcare provider create uncertainty in the minds of the medical profession, funders and consumers. Pharmacy is the only health profession that is reimbursed for its sale of a product rather than provision of a service (Rigby, 2010). In contrast; pharmacists are placed in an excellent posi-tion to promote rational use of medicines (for example, prescribing, dispensing, and use of drugs).

The literature on prescribing errors is gaining momen-tum, and the data so far suggests that the problem is not limited to any specific health care environment or defined practice setting. For example, a study developed in Galway (Ireland) to estimate the seriousness and level of prescribing errors that occurred in general practice reported 12.4% prescribing errors identified (Sayers, 2009). Similarly, pharmacists' interventions effectiveness have been demonstrated to come up with interventions that are most effective for impacting prescribing practice including audit and feedback, reminders, educational outreach visits, and patient-mediated interventions (Grindrod et al., 2006).

According to Hopper, though there is evidence pub-lished so far on prescribing errors, there is still a paucity of research reporting the role of pharmacists in identifying these errors and the prevalence of near-miss incidents in the prescribing process (Hopper et al., 2009). Therefore, the present study was undertaken to investigate the frequency and nature of prescription modifications and pharmacist's interventions outcomes at the community pharmacy.

METHODS

Setting and design

The study was conducted over a 4-month period (February 5- and June 15, 2011) at an urban community pharmacy in Madrid (Spain). The Community pharmacy is a shift of 12 hours, attached to Ambulatory Health Center, which dispenses about 4000 prescrip-tions each month. Like all community pharmacy in Spain, this is a private community pharmacy. In Spain, the Pharmacy Office (Community Pharmacy) is a private health establishment run for public interest, wherein autonomous communities are subject to health planning, with which the owner-pharmacist works through aides or assistants. The pharmacies dispense drugs to patients covered by the National Health System under the conditions set forth in the regulations.

The professional functions of pharmacists have changed from a passive to a more active role; now pharmacists personally follow up with patients (Bosch, 2000). The pharmacy technician assists the pharmacist in the dispensing of pharmaceutical products; controls inventory and the organization of pharmaceutical products; and evaluates the user's physiological parameters and vital signs under the pharmacist's supervision (Martínez-Sanchez, 2012). Ethical approval was obtained from the local research ethics committee.

The community pharmacy offers services like compounding, weight and blood pressure measurement, and cholesterol and glucose testing. A population of about 2000 inhabitants is served. Pharmacists and pharmacy technicians who worked there were invited to participate (3 pharmacists and 2 pharmacy technicians); eventually, 2 pharmacists and 2 pharmacy technicians agreed to partake. All participants received a pretested study protocol with definitions used, objectives and the methods to use during the period of the study. Each participating pharmacy had to collect all modified prescriptions (cases) during this period.

Selection of cases

All prescriptions for other health care products (such as dressings, incontinence materials, syringes and needles) that were dispensed in the predetermined period to the community pharmacy by the patient were excluded. The data were structured by all prescriptions that were modified by the pharmacy during the study. Reasons for including a prescription modification as a case were defined in the protocol and in the registration form for cases. If there were two or more reasons for modifying a prescription, the pharmacist had to select the one he/she considered most relevant. The protocol excluded the following modifications because of their lack of potential impact on patient care: incorrect or absent address, no or incorrect insurance data, product not in stock. In this study, a prescription error is defined as a result of a prescribing decision or prescription writing process where there is an unintentional significant reduction in the probability of treatment being timely and effective or increase in the risk of harm.

During the data management process the nature of prescription medications were divided into three groups. In the first group a clarification was needed to carry out the prescription order. In most cases, an essential administrative feature of the prescription was missing or obviously incorrect. In fact, the pharmacy could not have dispensed the drug without clarification. In the second group for items identified as `Correction prescription error', the prescription was administratively correct but could potentially have had clinical consequences if not altered. Those identified as `wrong dose' is an important example, for which there are several reasons like too high/low dose according to standard references or in-conflict with the patient's own records. The third group included reasons for medication not covered by the first two categories. Classifications of reported causes of the errors and types of error were adapted from Ashcroft et al., (2005).

All medicines were classified into therapeutic groups using the Anatomical Therapeutic Chemical (ATC) classification of the WHO Collaborating Centre for Drug Statistics Methodology (Anonymous, 1999). After inspection, data from the registration forms were entered in a Microsoft Access database and statistically analyzed

Table 1. Characteristics of the modified prescriptions according to the distribution of ATC classes.

Class	Number (%)
ATC class Blood and blood forming organs	271 (12.2)
ATC class Antiinfectives for systemic use	425 (19.1)
ATC class Nervous system	356 (16.0)
ATC class Cardiovascular system	309 (13.9)
ATC class Musculo-skeletal system	293 (13.2)
Other ATC clases	562 (25.3)

using SPSS 9.0.

All interventions and their outcomes were later reviewed by one member from the research team, who also categorized the intervention as per the Pharmaceutical Care Network of Europe (PCNE) Classifications in Broad Drug Related Problem (DRP) classes (van Mil, 1999). The outcome of the modification (on pres-criber or patient level) was recorded as intervention; a) approved and prescription changed, b) approved and no prescript-tion was changed, c) rejected, information only. The community pharmacy anonymised patients and healthcare providers.

RESULTS

A total of 20,205 prescriptions were processed during the study and the overall incidence of modifications by the community pharmacy was 10.9 % (2216 prescriptions). Modifications of prescriptions were most frequently found in the following therapeutic domains: (B) Blood and blood forming organs, (C) Cardiovascular system, (J) Anti-infectives for systemic use, (N) Nervous system, (M) Musculo-skeletal system (Table 1).

Table 2 shows the nature of the prescription modifications. The majority (1676; 75.6%) of the reasons for the medications concerned the clarification of an insufficiently specified prescription (e.g. no specification, insufficient patient data, wrong strength or strength not specified), whereas in 123 cases (5.5%) a prescription error was corrected that might have had clinical conse-quences (`Correction Prescription Error'). Drug - drug interaction (32.5%), contraindication (6.5%) or double medication (40.6%) were more prevalent in this latter group than other intervention, for example, dose cor-rections (20.3 %). In Table 3 some individual examples of modifications are presented.

At the prescriber's level, 1,551prescriptions (70%) of all modifications made were accepted and prescription modified. Other outcomes in this category were des-cribed as follows: prescriber asked for clarification (5%), prescriber informed only (10%), and intervention not accepted (15%). At the patient level, written information was provided to the patient in over 70% of the modifIca-tions made, and medication counseling (over and above the routine instructions given at the dispensing window) took place in 20% of all interventions in this category.

DISCUSSION

Our study reports an incidence of 10.9% for prescription modifications at the community pharmacy. This incidence would translate to about 70% pharmacist´ interventions made during the period of the study. Prescribing errors were the most frequent type of error (75.6%), related to clarifications needed to carry out the prescription order. Correction prescription error represented the second prescription modification causes (5.5%). Wrong patient data, double medication, interaction with other medicines, contraindication pregnancy or children, and contraindication allergy were significantly higher (92.4%). The prescribing error incidence is comparable to those reported in other studies (Taylor, 2005). In an Ireland-based study by Sayers et al., (2009) from a total of 3,948 prescriptions, 491 (12.4%) contained one or more errors, and from a total of 8,686 drug items, 546 (6.2%) contained one or more errors. In a UK-based study developed at the primary care Sandars and Esmail (2003) revealed that prescribing and prescription errors occur in up to 11% of all prescriptions, mainly related to errors in dose. In a Taiwan-based study, identified pres-cription errors in 18.3% (n = 560) of prescriptions at the community setting; potential prescribing errors included errors of omission (25.5%), errors of commission (53.4%), and others (21.1%). The top three errors were incorrect do-sage (27.5%), missing indication (23.6%), and insufficient or unavailable drug information (18.9%) (Ho et al., 2012). Similarly, pharmacist´s intervention is comparable. Hopper et al. (2009) found prescription error in 0.71% of the total 82,800 prescriptions received at the primary health care. The intercepted prescriptions generated 890 drug-related problems (DRPs)-related interventions, and the prescriber accepted intervention in 53% of all interventions, and the treatment was changed accordingly (Hopper et al., 2009). In a Canada-based study by Young et al., (2012) 2.8% of pharmacist´s interventions were reported with the prescriber contacted for 69% of the interventions, seventy-two percent of prescriptions changed and 89% of the problems resolved (Young et al., 2012).

Interventions that were more likely to be accepted by

Table 2. Nature of prescription modifications.

Cause of modification	Number (%) n= 2216
Clarification needed to carry out the prescription order	**1676 (75.6%)**
No or insufficient patient data	358 (21.3)
Confusion of similar names	269 (16.0)
Dose wrong by multiple of 10	227 (13.5)
stated Strength of preparation not	123 (7.3)
Medicine, strength or dosage form not on the market	162 (9.6)
Dose not specified	158 (9.5)
Dosage form not specified	136 (8.1)
Number of tablets, capsules, etc. not specified or incorrect	122 (7.2)
Wrong strength	121 (7.2)
Correction prescription error	**123 (5.5%)**
Wrong patient data	27 (21.9)
Interaction with other medicines	22 (17.8)
Double medication*	22 (21.9)
Wrong dose	18 (14.6)
Contraindication pregnancy or children	13 (10.5)
Contraindication allergy	11 (8.9)
Medicine obsolete	10 (0.8)
Other causes	**417 (18.9%)**
Controlled drug regulations not followed	232 (55.6)
Missing Information about the prescriber	157 (37.6)
Various	28 (6.7)

*double medication is a combination of the same substance or different substances from the same therapeutic group.

Table 3. Some examples of modifications of prescription

Original Prescription	Modified prescription
Ibuprofen (600 mg)	not dispensed because may reduce the diuretic's effectiveness of furosemide (40 mg) prescribed by other doctor
Syrup (Diphenhydramine (5 mg) + chlorpheniramine (0.75 mg) + phenylephrine (5 mg))	not dispensed because dose undetermined in infants
Prednisone (10 mg)	not dispensed because of contraindication in glaucoma
Amoxicillin 250mg/5ml (syrup 60ml)	First prescription of Amoxicillin 250mg/5ml (syrup 60ml) for 7 days (5.5ml/day) instead of Amoxicillin 250/5ml (syrup 120 ml) Amoxicillin changed to ciprofloxacin because of hypersensitivity

the prescribing physicians were those involving dosage errors, duplicate therapy, and questions for patient´s prescribing clarification. We have not attempted to trace the fate of rejected interventions in this study. In the

absence of a structured validation process, we were unable to investigate the basis of rejected interventions. Our findings only refer to actual modifications of the prescriptions presented on the study day as our protocol did not ask for the recording of other potentially relevant interventions such as the modification or discontinuation of an already dispensed drug or an instruction to the patient to avoid certain drug problems.

At the prescriber level similar results were described by Hopper et al. (2009) prescriber asked for clarification (3%). Nature of pharmacist interventions reported are comparable to those described in other studies. In a US-based study by Warholak et al., the most common reason for pharmacists' interventions was to supplement omitted information (31.9%), especially missing directions. Dosing errors were also quite common. The most common response by pharmacists was to contact the prescriber (64.1%) (Warholak et al., 2009). In most cases (56%), the prescription order was changed and the pres-cription was ultimately dispensed. Other Malaysia-based study reported that 24.2% of the pharmacist' intervention carried out were related to contacting the prescribers and clarifying with the patient or his/her representative (19.4%) (Chua et al., 2003). In this study, at the patient level, the most frequent pharmacist' intervention were providing information to patients about prescribing modifications and medication counseling. Similar findings have been described in recent studies carried out in the US by Kuo et al. (2013) and Carole and Kimberlin (2011).

In fact, our findings are consistent with other studies, related to pharmacist´ intervention and prescribing problems; showing that in a primary care setting, the focus is most often prescription problems (Ekedahl, 2010; Mandt et al., 2009; Leemans et al., 2003). At the same time, to conduct a descriptive study, we have been cautious in comparing these results with other studies, due to varying methodology and definitions of interventions that characterizes these studies (Pottegard et al., 2011). Likewise, from the perspectives of the causes of prescribing errors reported in the scientific literature (Chen et al., 2005b; Lewis et al., 2009; Chen et al., 2005a) two basic considerations could be made. First, despite the computer revolution, much prescription continues to be handwritten and this is a reality in the Spanish health system (Rodríguez-Vera et al., 2002). A number of European countries such as Britain or Spain are still struggling to implement an integrated digitized module (Heise, 2011). Some studies show the association between handwritten prescriptions and the incidence of errors (Al Shahabi et al., 2012; Gandhi et al., 2005; Yuosif, 2011; Tully, 2012). This topic must be taking into consideration in future researches to evaluate the nature of prescription related to prescribing errors at the community pharmacy.

Second, pharmacist-physician communication is a vital component of quality health care. Enhanced communication can reduce costs, promote patient safety, and prevent medical errors (Schenkel, 2000). However, the community pharmacist and the physician play separate roles in the delivery of prescription drugs to patients, a protocol to pharmacist-physician communication, and standardized process to manage the patient´s pharmacotherapy. From our point of view, the outcomes of the study reinforce the importance of prescription screening and interventions by pharmacists in minimizing preventable adverse events attributed to medication errors. At the same time, the impact of the interdisciplinary communication and cooperation in identifying and resolving prescribing errors and irregularities in order to achieve optimal therapeutic outcomes for the patient should be taken into account in future researches.. Professional cooperation between pharmacist and physician should combine the unique knowledge of both professions and thereby achieve optimal drug therapy for the patient (Saanum and Mellbye, 1996).

Conclusion

The findings of this study reinforce the importance of prescription screening and interventions by pharmacists in reducing preventable adverse events attributed to medication errors. It also emphasizes the necessity of interdisciplinary communication and cooperation in identi-fying and resolving prescribing errors and irregularities in order to achieve optimal therapeutic outcomes for the patient. A systematic and more uniform registration of medication errors in community pharmacy will strengthen the quality of the data and help optimize the possibilities to learn from the described incidents and, hence, improve patient safety.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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