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Aims and Scope of Acta Pharmaceutica Sciencia

Acta Pharmaceutica Sciencia is a continuation of the former "Eczacılık Bülteni" which was first published in 1953 by Prof. Dr. Kasım Cemal GÜVEN's editorship. At that time, "Eczacılık Bülteni" hosted scientific papers from School of Medicine-Pharmacy Branch, Istanbul University, Turkey.

Starting from 1984, the name of the journal was changed to "Acta Pharmaceutica Turcica" and became a journal for national and international manuscripts, in all fields of the pharmaceutical sciences in both English and Turkish. (1984-1995, edited by Prof. Dr. Kasım Cemal GÜVEN, 1995-2001, edited by Prof. Dr. Erden GÜLER, 2002-2011, edited by Prof. Dr. Kasım Cemal GÜVEN)

Since 2006, the journal has been publishing only in English with the name, "Acta Pharmaceutica Sciencia" that represents internationally excepted high level scientific standards.

The journal has been publishing quarterly per year except an interval from 2002 to 2009 which released its issues trimestral in a year. Publication was discontinued from the end of 2011.

With this latest published issue in 2016, Acta Pharmaceutica Sciencia will continue publication with the reestablished Editorial Board and also with support of you as precious scientists.

Yours Faithfully

Prof. Dr. Şeref DEMİRAYAK
Editor

Biography of Former Editor Prof. Dr. Kasım Cemal Güven

Prof. Dr. Kasım Cemal Güven was born in Trabzon, Beşikdüzü in 1925, graduated from Istanbul University College of Pharmacy in 1946 and became research assistant of pharmaceutical chemistry at the same college in 1947. He worked as a pharmacist in Elbistan between 1948 and 1950. He returned to Istanbul University in 1950. After receiving his PhD degree in 1953, he became Associate Professor in 1956 and Professor in 1965. Prof. Dr. Güven served as dean of Faculty of Pharmacy of Istanbul University between 1965 and 1969.

Prof. Dr. Güven conducted research at Munich University (Germany) with a DAAD fellowship in 1964 and at Münster University in 1984.

Prof. Dr. Güven's main research interest is galenical pharmacy / pharmaceutical technology. In addition to his main area of research interest, he has over 300 national and international publications and scientific papers covering topics such as synthesis and stability of drugs, chemistry of land plants, chemistry of sea animals (enzymes, insulin, heparin), chemistry of land and marine algae, and sea pollution. His publications have been cited in over 45 books and over 200 research papers. He also wrote many scientific and vocational books dealing with pharmaceutical technology and pharmaceutical sciences. During his academic career, he advised more than 50 doctoral and master's theses.

In 1953, Professor Güven initiated publication of *Acta Pharm.Sciencia* (formerly *Pharmaceutical Bulletin*), and as editor, he ensured continued publication of the journal until 2011. In addition, he was the editor of scientific journals such as *Journal of the Black Sea / Mediterranean Environment*.

After retiring from Istanbul University Faculty of Pharmacy Department of Pharmaceutical Technology in 1991, he gave doctorate lectures about chemical carcinogens at Istanbul University Oncology Institute, he served as a consultant for Istanbul Public Health Institute, and he continued his research at Istanbul University Institute of Marine Sciences and Management for many years.

Prof. Dr. Yıldız Özsoy

Beneficial Effects of Commonly Used Phytochemicals in Diabetes Mellitus

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ABSTRACT

Diabetes mellitus, a metabolic disorder, is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycemia. The prevalence of diabetes is increasing worldwide, especially in developing countries. The diabetes treatment has higher costs, limited efficacy and side effects. As a result of these factors, patients often have used alternative forms of therapy such as herbal medicines. Plants often contain various amounts of phenolics, flavonoids and tannins and most of the studies are focused on the antidiabetic effects of these phytochemicals due to their antioxidant properties. In this review, the role of oxidative stress on diabetes and the effects of different phytochemicals (limonene, sinnamic acid and ursolic acid) to diabetes mellitus therapy will be discussed.

Keywords: limonene, cinnamic acid, ursolic acid, diabetes

INTRODUCTION

Diabetes mellitus, a metabolic disorder, is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances of carbohydrate, lipid and protein metabolism[1]. Due to a higher incidence of the risk factors, the prevalence of diabetes is increasing worldwide, especially in developing countries[2]. 2.8% of world population suffer from diabetes and it is concluded that it may cross 5.4% by the year of 2025[3]. In Turkey, 7.4% of population suffer from diabetes and also it is estimated

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that the number of patients will be increased to 9.6% of population by the year of 2030[2].

The studies on diabetes therapy have gained interest due to its unwanted effects on human life e.g. changing lifestyles lead to reduced physical activity, and increased obesity[2]. In diabetes treatment, the current drugs can be divided into three groups: (i) Sulphonylureas such as glibenclamide, the glinides, insulin analogs, glucagon-like peptide 1 (GLP-1) agonists and dipeptidyl peptidase-IV (DPP-IV) inhibitors can increase endogenous insulin availability, (ii) Thiazolidinediones, agonists of the peroxisome proliferatoractivated receptor gamma (PPARγ) and the biguanide metformin can enhance the sensitivity of insulin, (iii) α-glucosidase inhibitors such as acarbose can reduce the digestion of polysaccharides and their bioavailability[4, 5]. All of these drugs have higher costs, limited efficacy and tolerability and/or significant side effects[6, 7].

As a result of these factors, patients have often used alternative forms of therapy such as herbal medicines[8]. Especially, the herbal medicine usage for diabetes treatment is common in West Africa, Central America and Asia[9, 10, 11]. According to an estimation published by the World Health Organization (WHO), approximately 80% of diabetic patients presently rely on herbal medicine for their successive treatments[12]. Traditional medicine is an accessible, affordable and culturally acceptable form of healthcare trusted by large numbers of people, which stands out as a way of coping with the relentless rise of chronic non-communicable diseases in the midst of soaring health-care costs and nearly universal austerity[13]. Unfortunately, pharmacological and toxicological evidences validating the safety and efficacy of these medicinal plants are not readily available[14].

Plants often contain various amounts of phenolics, flavonoids and tannins. Most of the studies are focus on the antidiabetic effects of these phytochemicals due to their antioxidant properties[15]. For example, epidemiological studies have associated a diet rich in isoflavones with a lower risk of diabetes and diabetes related complications[16, 17].

In this review, the role of oxidative stress on diabetes and diabetes mellitus therapy with different phytochemicals (limonene, cinnamic acid and ursolic acid) will be discussed.

DIABETES, OXIDATIVE STRESS and ANTIOXIDANTS

There are different types of diabetes. (i) Type 1 diabetes: This form of diabetes is also called insulin dependent diabetes mellitus (IDDM). When the pancreas produces insufficient amounts of insulin to meet the body's needs, this type of diabetes will occur. A trigger-either an illness or stress-causes the immune system

to attack and destroy the beta cells of the pancreas. As a result, pancreas stops producing insulin. Type 1 develops suddenly in childhood or in adolescence. (ii) Type 2 diabetes: This form of diabetes is also called Non-Insulin Dependent Diabetes Mellitus (NIDDM). When the pancreas produces insulin, but the cells are unable to use it efficiently; this effect is called "insulin resistance". Type 2 diabetes is far more common than Type 1 and approximately 90% of all diabetes cases are Type 2. There is a strong genetic predisposition. Age, obesity and sedentary lifestyle are also risk factors. (iii) Gestational diabetes mellitus: Glucose intolerance being recognized during pregnancy. It can complicate pregnancy leading to prenatal morbidity and mortality[3].

It is known that oxidative stress results from an imbalance between the generation of oxygen derived radicals and antioxidant system[18]. Numerous studies have shown that diabetes mellitus is associated with increased formation of free radicals and decrease in antioxidant potential. In both types of diabetes, oxidative stress is increased[19].

Multiple factors can cause oxidative stress in diabetes. The most important factor is glucose autoxidation leading to the production of free radicals. Other factors include cellular oxidation/reduction imbalances and reduction in antioxidant defenses (including decreased cellular antioxidant levels and a reduction in the activity of enzymes that dispose of free radicals). Levels of some prooxidants such as ferritin and homocysteine are elevated in diabetes. Another important factor is the interaction of advanced glycation end products (AGEs) with specific cellular receptors called AGE receptors (RAGE). Elevated levels of AGE are formed under hyperglycemic conditions. Their formation is initiated when glucose interacts with specific aminoacids on proteins forming a compound that then undergoes further chemical reactions. Glycation of protein alters protein and cellular function, and binding of AGEs to their receptors can lead to modification in cell signaling and further production of free radicals[20]. The other nonenzymatic factors are activation of NAD(P)H oxidases, nitric oxide synthase, and a specific enzyme activity, xanthine oxidase, which produces oxidant species and subsequent oxidative stress[21, 22, 23, 24].

Numerous reports have documented elevations in peroxide levels in plasma, red blood cells and tissues of animals with chemically-induced diabetes[25, 26]. Increases in blood peroxides or other indices of oxidative stress have also been reported in diabetic patients[27]. Both increases and decreases in the activities of key antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) have been reported[28]. In a pediatric study, antioxidant activity was found to be decreased in

relation to poor glycemic control[29]. It is also shown that oxidative stress exists in diabetic patients as evidenced by increased total antioxidant capacity in saliva and blood of patients[30].

The primary defense against oxidative stress in the cell, rests with antioxidants like vitamin E, glutathione and peroxidases[19]. Antioxidants show their effects with different mechanisms. These mechanisms are: enzymes that degrade free radicals, proteins (e.g. transferrin) bind metals which stimulate the production of free radicals and antioxidants like vitamin E and C scavenge free radicals[20].

Recently, there has been a growing interest in replacing synthetic diabetic drugs with natural antioxidants from plant materials. Studies have shown that plants contain a large variety of substances that possess antioxidant activity[31]. They can prevent the formation of advanced glycated end products (AGEs) and other diabetic complications associated with oxidative stress[32]. Phytochemicals with antioxidant effects include; cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes[33].

There are too many studies about the beneficial effects of phytochemicals on diabetes therapy. Arya *et al.* (2014) demonstrated that low dose quercetin and quinic acid showed protective effect on the degeneration in the liver, kidney and pancreas tissues of streptozotocin (STZ) induced diabetic rats[34]. In an other study, glucose tolerance significantly improved by two flavonoids, rutin and genistein, in STZ induced diabetic rats[35]. It is concluded that dietary soy isoflavones increased insulin secretion and prevented the diabetic cataracts in diabetic rats[36]. Similarly, Lee (2006) showed that soy protein and genistein were seemed to be beneficial for correcting hyperglycemia and preventing diabetic complications in diabetes induced rats[37]. Şakul *et al.* (2013) demonstrated that antioxidant pyridoindole reversed the effects of diabetes in rat brain and peripheral tissues[38]. The aqueous extract of *Anchusa strigosa* flowers (250 mg/kg and 500 mg/kg) caused a dose-dependent fall in blood glycose, cholesterol and triglyceride levels in STZ induced diabetic rats[39]. In a study with STZ induced diabetes rats, it is concluded that the extract of *Beta vulgaris* L. var cicla when administered by gavage may reduce glucose levels[40].

ANTIOXIDANT PHYTOCHEMICALS in DIABETES

Limonene

Limonene (p-Mentha-1,8-diene) is a major component of oils obtained from *Citrus* plants, orange, lemon and grape fruit[41, 42]. Limonene is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) for a flavoring agent[43]. It is commonly used as an additive in foods, soaps and perfumes[44]. Die-

tary intake of limonene varies depending on the intake of foods[43]. It is also shown that it has exerted antiproliferative effects in various cancer cell types[45, 46]. It has been clinically used to dissolve gallstones and also to prevent gastric diseases[43].

Glycation inhibitors possessing amino groups could compete to bind to glucose, scavenge dicarbonyls, and chelate metal ions and the structure of limonene precludes such an action. Joglekar *et al.* (2013) studied the antiglycative properties of limonene and also the interaction of limonene with bovine serum albumin (BSA) and the possible mechanism of inhibition of protein glycation[47]. They found that limonene functioned as a protein glycation inhibitor through a novel mechanism of stabilization of the native protein structure.

In a study with STZ induced diabetic rats, 50, 100 and 200 mg/kg doses of limonene and 600 μg/kg glibenclamide were administrated for 45 days. It was found that the antidiabetic effect of d-limonene was comparable with glibenclamide and the effect of d-limonene was more pronounced in the doses of 100 mg/kg body weight than the other two doses[48]. More *et al.* (2014) have demonstrated that 100 μM concentration of limonene demonstrated 85.61% inhibition of protein glycation while the positive control aminoguanidine demonstrated 88.02% inhibition at 1 mM concentration in STZ induced diabetic rats[49]. Administration of D-limonene to diabetic rats for 45 days also caused a significant reduction in the levels of lipid peroxidation by-products and an increase in the activities of antioxidant enzymes including SOD, CAT, GSH and glutathione S transferase, when compared with the untreated diabetic group[50].

Cinnamic Acid

Cinnamic acid and its derivatives possess a variety of pharmacologic properties such as antioxidant, hepatoprotective, antimalarial and antityrosinase activities[51, 52, 53, 54]. It is a phenolic acid that exist in many fruits, vegetables, and beverages including blueberry, kiwi, cherry, plum, apple, pear, chicory, artichoke, potato, cider and coffee[55]. Most of the studies have focused on the antidiabetic activities of cinnamic acid and its derivates.

Inhibition of α-glucosidase may be effective in diabetes therapy. Due to this effect, mammalian α-glucosidase inhibitors from natural sources can be beneficial in the prevention and treatment of diabetes mellitus. Adisakwattana *et al.* (2009) have demonstrated the α-glucosidase inhibitory activity of cinnamic acid derivates against intestinal sucrase inhibitors[56]. It is showed that Cinnamon extracts (50, 100, 150 and 200 mg/kg) which include cinnamic acid significantly, decreased the blood glucose and lipid levels in mice[57]. Ping *et al.* (2010) have studied the hypoglycemic effect of cinnamon oil which contains water soluble polyphenol type A polymer, cinnemaldehyde and cinnamic acid as active com-

pounds, in type 2 diabetic animal model[58]. They found that fasting blood glucose concentration was significantly decreased with the 100 mg/kg group compared to other groups. In addition, they found significant decreases in plasma C-peptide, serum triglyceride, total cholesterol and blood urea nitrogen levels while serum high density lipoprotein (HDL)-cholesterol levels were significantly increased after 35 days. Huang *et al.* (2009) have reported that caffeic and cinnamic acids improve glucose uptake in TNF-α-treated insulin-resistant FL83B[59]. Same group have treated the mouse FL83B cells with TNF- α to induce insulin resistance to evaluate the effect of caffeic and cinnamic acids on glucose metabolism. They found that caffeic and cinnamic acids increased expression of glycogen synthase, whereas the expression of glycogen synthase kinase and phosphorylation of glycogen synthase at Ser641 in insulin-resistant mouse hepatocytes was decreased. The compounds suppressed the expression of hepatic nuclear factor-4 in TNF-α-treated mouse FL83B hepatocytes. They concluded that caffeic and cinnamic acids ameliorated glucose metabolism by promoting glycogenesis and inhibiting gluconeogenesis in TNF-α-treated insulin-resistant mouse hepatocytes[60]. Rao and Rao (2001) have reported the antihyperglycemic effect of *Syzygium alternifolium* seeds which contain cinnamic acid[61]. The treatment with 50 mg of the fraction C (which includes cinnamic acid) kg b.w/day for 30 days resulted in a significant decrease in the fasting blood glucose levels of diabetic rats. The altered enzyme activities of carbohydrate metabolism in liver and kidney of diabetic rats were significantly reverted to near normal levels by the administration of fraction C[62].

Ursolic Acid

Ursolic acid (3β-hydroxy-12-urs-12-en-28-oic acid) is a well-known pentacylic triterpene which is commonly used in traditional Chinese medicine. *Malus pumila*, *Ocimum basilicum*, *Vaccinium* spp., *Vaccinium macrocarpon*, *Olea europaea*, *Origanum vulgare*, *Rosmarinus officinalis*, *Salvia* and *Thymus* plants are the main sources of ursolic acid[63]. In recent years, interest in ursolic acid has increased due to its many beneficial effects and low toxicity. Ursolic acid has been used against different diseases including osteoarthritis, rheumatoid arthritis, ulcer, cancer and diabetes[64]. Ursolic acid has been suggested to increase insulin level with the preservation of pancreatic β-cells and modulate blood glucose level in diabetic mice[65].

Yin and Chan (2007) have found that oleanolic acid and ursolic acid could inhibit in vitro formation of pentosidine and Nε-(carboxymethyl)lysine (CML) which have been implicated in the pathogenesis of diabetic nephropathy and other diabetic complications[66]. Wang *et al.* (2010) have demonstrated that ole-

anolic acid (0.1 and 0.2%) and ursolic acid (0.1 and 0.2%) markedly suppressed renal aldose reductase activity and enhanced glyoxalase I activity, which contributed to decrease renal AGEs formation and improve renal functions. The impact of these two triterpenes on mRNA expression of renal aldose reductase and glyoxalase I revealed that the effects of these agents occurred at transcription level. Low-dose ursolic acid (0.01% in food) administration in STZ induced diabetic mice with for three months, glomerular hypertrophy and type IV collagen accumulation in the kidneys were found to be markedly ameliorated[68]. It is concluded that, ursolic acid significantly inhibited sorbitol dehydrogenase activity as well as aldose reductase activity, and increased glucokinase activity. While decreasing glucose-6-phosphatase activity, it elevated the hepatic glycogen content and lowered the plasma total cholesterol, free fatty acid, and triglyceride concentrations compared with the diabetic control group. It also normalized hepatic triglyceride concentration in the livers of STZ induced diabetic mice[69]. In a study with STZ induced diabetic rats for 16 weeks, ursolic acid treatment prevented biochemical and histopathologic changes in the kidneys associated with diabetes such as alteration in renal function and increased oxidative stress, NF-κB activity, and P-selectin expression in the kidneys[70]. Similarly, it is found that ursolic acid (0.05% w/w) improved blood glucose levels, glucose intolerance, and insulin sensitivity compared to the diabetic group in diabetic rats[65] and at the doses of 0.01% w/w and 0.05% w/w, it improved blood glucose, glycosylated hemoglobin, glucose tolerance, insulin tolerance and plasma leptin levels as well as aminotransferase activity in diabetic mice[71].

CONCLUSION

Diabetes is affecting a significant proportion of the population worldwide. It affects many organs including pancreas, kidney and liver. The disease is associated with a reduced quality of life and increased risk factors for mortality and morbidity. In diabetes treatment, traditional herbal folk medicines are getting popular. Due to their antioxidant properties, herbal products give positive and promising results. In this review, we demonstrated the antidiabetic activity of different phytochemicals (limonene, cinnamic acid and ursolic acid). The studies about their antidiabetic activity have shown that these phytochemicals may be beneficial in diabetes therapy. But further *in vitro* and *in vivo* studies needed to clear up their efficacy, mechanism and toxicity on diabetes treatment.

Author Contributions

These authors contributed equally.

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Synthesis, Structure Elucidation and Determination of Acid Dissociation Constant, Tautomerism of Pyrido [1,2-a] benzimidazole-2,4-dione

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ABSTRACT

The first starting material, 1*H*-2-acetylbenzimidazole, 4, was synthesized by o-phenylenediamine, 1, and lactic acid solution, 2, in hydrochloric acid medium on the first step than, oxidation reaction was performed by CrO_3 in acetic acid medium on the second step. The last starting material, ethyl 2-(2-acetylbenzimidazol-1-yl) acetate, 6, was synthesized by using first starting material, 4, ethyl 2-bromoacetate, 5, and K_2CO_3 in acetone medium. The target compound, pyrido[1,2-*a*] benzimidazole-2,4-dione, 7, was synthesized by using the last starting material, 5, in sodium ethoxide medium. The compound, 7, can be found in two forms which are keto and enol which would be evaluated in this study. For the evaluation, it was performed some spectroscopic studies. In addition, it was evaluated experimentally obtained acidity constant, pK_a value and tautomeric equilibrium of the compound, 7, by using ultraviolet-visible (UV-Vis) spectrophotometer. All of these studies have shown that the valid form of the compound, 7, is keto form.

Keywords: Pyrido[1,2-*a*]benzimidazole, Acidity constant, pKa value, Tautomerism.

INTRODUCTION

The investigation of the proton tautomerism properties of heterocyclic compounds benefits the chemical and medicinal industry. The determination of acidity constants and tautomeric equilibrium are very important in understanding to predict reactions, ion transport behavior, binding to receptors and mechanisms of drug precursor compounds[1-8].

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It has been reported that pyrido[1,2-*a*]benzimidazole compounds have some biological activities which are antiviral, antimicrobial, analgesic, anti-inflammatory, anticancer and anti-HIV[9]. Pharmacological properties of the heterocyclic compounds are related to the determination of their acidity and tautomerism. Because of this, in this study, target compound, pyrido[1,2-*a*]benzimidazole-2,4-dione, 7, was synthesized and investigated its identification, acidity and tautomerism based on its spectral data.

The compound, 7, has two carbonyl carbon on 2 and 4 positions such as 1,3-dione compounds. The 1,3-diones attracted the attention of researchers due to their two characteristic features. One of them is that 1,3-diones have synthetic potential due to the presence of β-dicarbonyl moiety. The another is that a wide range of physicochemical properties such as tautomerism, proton transfer, quantum mechanical calculation are associated with 1,3-diones[10-12].

The infra red (IR) and nuclear magnetic resonance (NMR) spectroscopic techniques were employed for identification of pyrido[1,2-*a*]benzimidazole-2,4-dione, 7, and experimentally obtained acidity constant, pK_a value and tautomeric equilibrium of the compound, 7, were evaluated by using UV-Vis spectrophotometer in this pronounced study. We aimed to explain the tautomeric condition of the compound, 7, with all of the techniques. Previously, a research group published that some pyrido[1,2-*a*]benzimidazolone derivatives existed on enol form as dominated[13]. Another group reported that diverse pyrido[1,2-*a*]benzimidazolone derivatives existed on keto forms on the other hand their enol forms could be dominated in different solvents[6].

METHODOLOGY

Chemistry and Synthesis

The melting points were determined using WRS-2A Microprocessor. Spectroscopic data were recorded on the following instruments: UV-Vis, Shimadzu 1800 UV; IR, Shimadzu 8400 FTIR spectrophotometer; NMR, Bruker 500 MHz NMR Spectrometer. Analyses for C, H, and N were within 0.4% of the theoretical values. The 1*H*-2-(1-hydroxyethyl)benzimidazole, 3, and 1*H*-2-acetylbenzimidazole, 4, compounds used as starting materials were prepared according to the methods in the literature[14]. The reaction steps of syntheses in this study were shown in figure 1.

The synthesis of 1H-2-(1-hydroxyethyl)benzimidazole, 3

A mixture of o-phenylenediamine, 1, (185 mmol) and lactic acid solution, 2, (200 mmol) in 4N hydrochloric acid solution (75 mL) was refluxed for 120 h. The reaction medium was poured into cold water and kept for 24 h. The residue was fil-

tered and washed with water. The raw product was recrystallized from ethanol.

The synthesis of 1H-2-acetylbenzimidazole, 4

The 1*H*-2-(1-hydroxyethyl)benzimidazole, 3, (139 mmol) was completely dissolved in acetic acid (60 mL). The chromium trioxide solution, CrO_3 (104 mmol), was dissolved in water (70 mL) and gently and slowly dropped into the reaction medium while the 1*H*-2-(1-hydroxyethyl)benzimidazole, 3, solution was stirred in a hot water bath at 90°C. The mixture was refluxed for 2 h. The reaction medium was poured into water and chloroform in an extraction flask and kept for 15 min. The raw product in chloroform was extracted and evaporated by rotary evaporator, then recrystallized from toluene.

The synthesis of ethyl 2-(2-acetylbenzimidazol-1-yl)acetate, 6

A mixture of 1*H*-2-acetylbenzimidazole, 4, (30 mmol), ethyl 2-bromoacetate, 5, (30 mmol) and potassium carbonate (30 mmol) in acetone (40 mL) was stirred at room temperature for 4 h. The reaction medium was poured into cold water and kept for 24 h. The residue was filtered and washed with water. The raw product was recrystallized from ethanol.

The synthesis of pyrido[1,2-a]benzimidazole-2,4-dione, 7,

The sodium metal (18.3 mmol) was completely dissolved in ethanol (30 mL), then ethyl 2-(2-acetylbenzimidazol-1-yl)acetate, 6, (6.1 mmol) was added to this solution. The mixture was stirred in an ice bath for 1 h and then added to acetic acid (4 mL). The reaction medium was poured into cold water and kept for 24 h. The residue was filtered and washed with water. The raw product was recrystallized from chloroform-petroleum ether.

Determination of Acidity Constant and Tautomerism

Methanol, ethanol, glycine, KOH, H_2SO_4, HCl, CH_3COOH, CH_3COONa, NaOH, KH_2PO_4, Na_2CO_3, $NaHCO_3$, NaCl, methyl red indicator and standard buffer solutions were obtained from Merck and were not further purified for acidity studies. In addition, DMSO, ethanol, $CHCl_3$, C_6H_{12}, $(C_2H_5)_3N$, CF_3COOH were obtained from Merck and used for UV Studies. Spectroscopic data were recorded on the instrument: Unicam UV-2 UV-Vis spectrophotometer.

Determination of acidity constant

The acidity constant value was found by using the UV spectroscopic methods described in the literature[15-18].

The general procedure applied was as follows: a stock solution of compound under investigation was prepared by dissolving about (10 to 20) mg of compound

in alcohol in a volumetric flask. A liquid (1 mL) of this solution was transferred into 10 mL volumetric flask and diluted to the mark with buffers of various pH. The pH was measured before and after addition of the new solution. The optical density of each solution was then measured in 1 cm cells, against solvent blanks, using a constant temperature cell holder UV-Vis spectrophotometer. The scanning spectrophotometer was thermostated at 25°C (to within ±0.1°C). The wavelengths were chosen so that the fully protonated form of the substrate had a much greater or much smaller extinction coefficient than the neutral form. The analytical wavelengths, the half protonation values and the UV absorption maxima for substrate studied are given in Table 1.

Calculations of half protonation value was carried out as follows: the sigmoid curve of optical density or extinction coefficients at the analytical wavelengths (OD, l) was first obtained (figure 2).

The optical densities of the fully protonated molecule (ODca) and pure free base (ODfb) at acidity were then calculated by linear extrapolation of the arms of the curve. The following equation gives the ionization ratio where the optical density (OD) was converted into molar extinction (e) using Beer's Law of eqs 1 and 2.

$$OD = \varepsilon.b.c \qquad \text{eq 1}$$

b: cell width, cm

c: concentration, mol/dm^3

$$I = [BH^+] / [B] = (OD_{obs} - OD_{fb}) / (OD_{ca} - OD_{obs}) = (\varepsilon_{obs} - \varepsilon_{fb}) / (\varepsilon_{ca} - \varepsilon_{obs}) \qquad \text{eq 2}$$

OD_{ca}: optical density of conjugated acid

OD_{fb}: optical density of free base

The linear plot of log I against pH, using the values -1 < log I< 1 had slope m, yielding the half protonation value as $pH^{1/2}$ or more generally $H^{1/2}$ at log I=0 (figure 3). The acidity constant gives as follows eq 3[8].

$$pK_a = m\ pH^{1/2} \qquad \text{eq 3}$$

Determination of tautomerism

The keto percentage of pyrido[1,2-*a*]benzimidazole-2,4-dione, 7, was defined by measuring UV spectra at room temperature (25°C ±2) in four solvents of increasing polarity (i.e. cyclohexane, chloroform, ethanol and dimethylsulfoxide) were given in Table 2. The molecule concentration is 10^{-5} mol/L. The UV–Vis spectra of molecule were studied in polar and nonpolar solvents in both acidic (CF_3CO-OH) and basic (Et_3N) medium. The tautomerism of the compound, 7, was given in figure 4.

The parameters of the spectra for the molecule in polar and nonpolar solvents in both acidic and basic medium were given in Table 2. The calculated keto-enol tautomeric equilibrium of molecule was given in Table 3.

RESULTS AND DISCUSSION

Chemistry and Synthesis

The synthesis of 1H-2-(1-hydroxyethyl)benzimidazole, 3

The 1*H*-2-(1-hydroxyethyl)benzimidazole, 3, was prepared in a yield of 75%. Its melting point was determined as 178.7-180.6 °C. The melting point in the literature was given as 180.0-181.0 °C[14].

The synthesis of 1H-2-acetylbenzimidazole, 4

The 1*H*-2-acetylbenzimidazole, 4, was prepared in a yield of 59%. Its melting point was determined as 188.9-189.6 °C. The melting point in the literature was given as 189.0-191.0 °C[14].

The synthesis of ethyl 2-(2-acetylbenzimidazol-1-yl)acetate, 6

The ethyl 2-(2-acetylbenzimidazol-1-yl)acetate, 6, was prepared in a yield of 92%. Its melting point was determined as 91.4-92.9 °C. The melting point in the literature was given as 91.0-93.0 °C[19]. IR (potassium bromide): 1742, 1687 (C=O), 1604-1455 (C=C, C=N) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ (ppm): 1.22 (t, J=7.0 Hz, 3H), 2.72 (s, 3H), 4.17 (q, J=7.0 Hz, 2H), 5.41 (s, 2H), 7.39 (td, J=7.5 Hz, j=1.0 Hz, 1H), 7.47 (td, J=7.5 Hz, j=1.0 Hz, 1H), 7.81 (d, J=8.5 Hz, 1H), 7.88 (d, J=8.0 Hz, 1H); ^{13}C-NMR (125 MHz) δ (ppm): 14.61 (CH_3), 27.98 (CH_3), 47.39 (CH_2), 61.95 (CH_2), 112.63 (Ar-C), 122.51 (Ar-C), 124.94 (Ar-C), 127.20 (Ar-C), 137.82 (Ar-C), 142.16 (Ar-C), 147.17 (Ar-C), 169.65 (C=O), 194.30 (C=O).

The synthesis of pyrido[1,2-a]benzimidazole-2,4-dione, 7

The pyrido[1,2-*a*]benzimidazole-2,4-dione, 7, was prepared in a yield of 55%. Its melting point was determined as 142.2-145.1 °C. IR (potassium bromide): 1741, 1687 (C=O), 1582-1455 (C=C, C=N) cm^{-1}; ^{1}H-NMR (DMSO-d_6)d (ppm): 2.72 (s, 2H), 5.33 (s, 2H), 7.40 (t, J=8.0 Hz, 1H), 7.46 (t, J=7.5 Hz, 1H), 7.79 (d, J=8.0 Hz, 1H), 7.87 (d, J=8.0 Hz, 1H); ^{13}C-NMR (125 MHz)d (ppm): 45.91 (CH_2), 54.65 (CH_2), 111.23 (Ar-C), 122.36 (Ar-C), 123.46 (Ar-C), 126.92 (Ar-C), 137.63 (Ar-C), 141.81 (Ar-C), 148.37 (Ar-C), 195.36 (C=O), 204.73 (C=O).

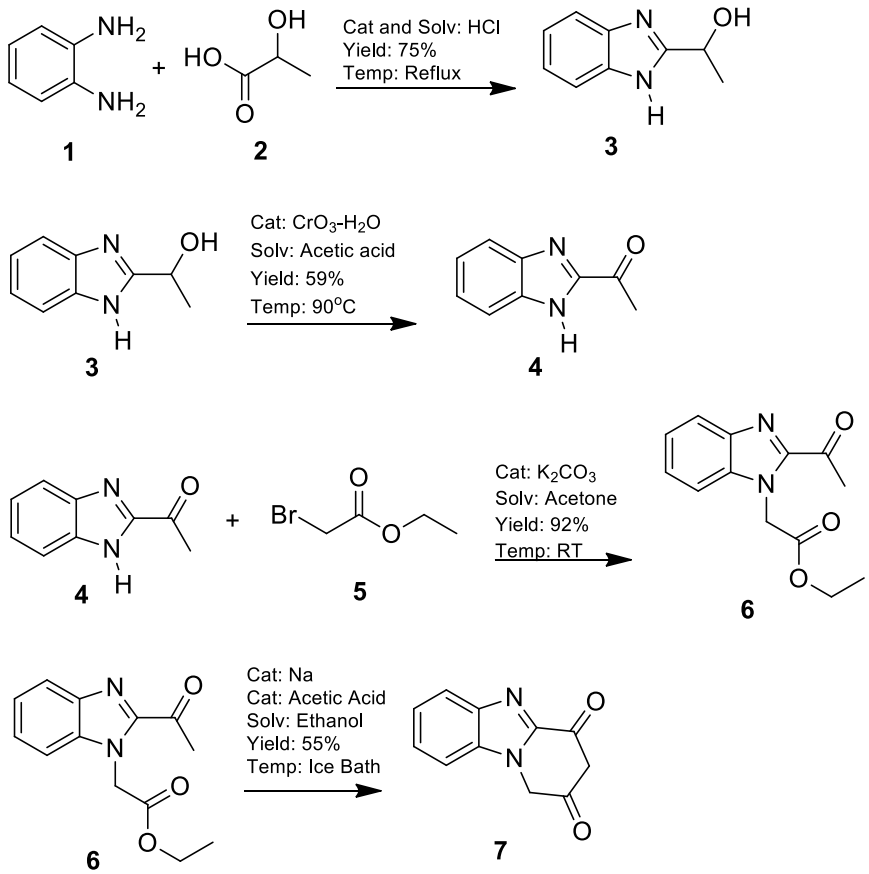

Figure 1: General representation of the reaction steps.

Determination of acidity constant

When the acidity constant value of 5,56[20] for the protonation of benzimidazole is taken into account, it might be said that the molecule acidity constant value is close enough. Therefore the protonation pattern of this molecule should be similar and had to be aza protonation. In addition, hydrogen bond provides stability (figure 5). This is supported by ab initio calculation (HF/6-31G (d,p), CPCM method, Gaussian 03 program[21]) which predict an interatomic bond distance between the protonated nitrogen atom and carbonyl of keto which was found as 2.70, sufficiently close for an intra molecular hydrogen bonding interaction. The UV spectra and protonation data for the molecule are given in Table 1.

Table 1: UV Spectral Data and Acidity constant values

Molecule	Spectral maximum l/nm		Acidity Measurements				
	species	monocation					
	(log ε_{max})[a]	(log ε_{max})[b]	λ /nm [c]	$H^{1/2}$ [d]	m	pK_a [e]	corr[f]
Benzimidazole	-	-	-	-	-	5.56[20]	-
The compound, 7	282 (5.00)	293 (6.19)	297	-8,35	-0.71	5.93±0.06	0.99

[a]Measured in pH=7 buffer solution for molecule 1.
[b]Measured in 1% H_2SO_4 for molecule 1.
[c]The analytical wavelength for pK_a determination.
[d]Half protonation value.
[e]Acidity constant value ± standard error.
[f]Correlation for log I as a function of pH graph.

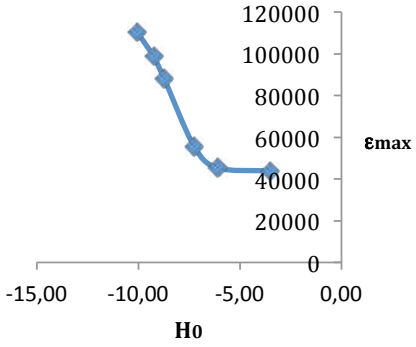

Figure 2: ε_{max} as a function of Ho (at 297 nm) plot for the protonation of the compound, 7

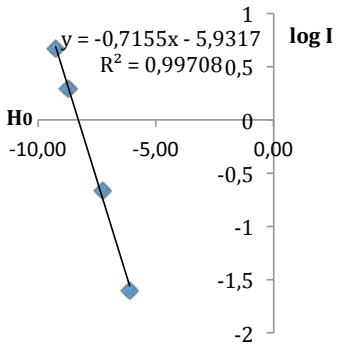

Figure 3: pH as a function of log I (at 297 nm) plot for the protonation of the compound, 7

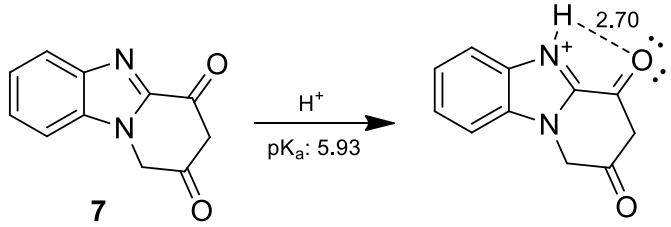

Figure 5: Possible protonation pattern of the compound, 7

Determination of tautomerism

For the pure solvent, acidic and basic medium, increasing the solvent polarity shifts the absorption maxima to red. Comparison of the correlations in Table 2 with the data in Table 3 indicates that in nonpolar solvent (cyclohexane) and polar solvents (DMSO, ethanol, $CHCl_3$) K>>1. The results showed the keto form which was predominant.

The high intensity of absorptions in the 200-265 nm range showed that diketone forms were present[22]. In all three medium (solvent, acidic and basic) the keto-enol tautomers (%) were measured for the compound, 7. In pure solvent medium, chloroform, the percent ratio of the keto-enol tautomers of the compound, 7, was higher than in DMSO, ethanol and cyclohexane. In acidic and basic solutions of cyclohexane, the percent ratio of keto-enol tautomer was observed as higher than DMSO, chloroform and ethanol solutions (figure 6).

Table 2. The acid and base effects in different solvent for tautomerizm (T=25°C ± 1)

Solvent	λ max. (nm) (Absorbance)		
	Neutral	**Acidic**	**Basic**
DMSO	232(A=0.697) 240(A=0.651) 257(A=0.806) 278(A=0.640) 285(A=0.647) 302(A=0.557)	228(A=0.850) 237(A=0.739) 244(A=0.874) 251(A=0,490) 256(A=0.731) 270(A=0.627) 279(A=0.600) 287(A=0.570) 301(A=0.578)	223(A=1,978) 253(A=1.015) 262(A=0.703) 277(A=0.368) 315(A=0.430)

EtOH	242(A=0.543) 276(A=0.466) 283(A=0.460) 308(A=0.429)	234(A=0.683) 240(A=0.657) 270(A=0.443) 278(A=0.465) 299(A=0.364)	217(A=0.863) 231(A=1.123) 284(A=0.339) 316(A=0.363)
$CHCl_3$	234(A=0.845) 241(A=0.788) 270(A=0.536) 285(A=0.534) 302(A=0.503)	230(A=0.633) 237(A=0,703) 274(A=0.480) 282(A=0.495) 300(A=0.467)	220(A=1.474) 236(A=0.862) 241(A=1.127) 280(A=0.865) 289(A=0.791) 317(A=0.401)
C_6H_{12}	213(A=0,159) 257(A=0.144) 278(A=0.146) 285(A=0.147)	211(A=0.650) 228(A=0.388) 298(A=0.151)	213(A=1.116) 223(A=1.027) 227(A=0.967) 248(A=0.766) 257(A=0.527) 310(A=0.193)

Table 3: Keto-enol tautomer (%) in solvent, acidic and basic medium for the compound, 7

Solvent	Keto-enol tautomer, %[a]			Equilibrium constant, K_{keto}[d]		
	Neutral	Acidic[b]	Basic[c]	Neutral	Acidic[b]	Basic[c]
DMSO	55.40	58.20	70.20	1.24	1.39	2.35
EtOH	53.80	58.55	75.57	1.16	1.41	3.09
$CHCl_3$	61.18	58.68	63.01	1.57	1.42	1.70
C_6H_{12}	51,96	81.14	84.18	1.08	4.30	5.32

[a]$\boldsymbol{Keto\ isomer\ (\%) = (A_2/A_2+A_1)\ x100}$ eq 4

A_1= the absorbance of enol form (π π^*)

A_2= the absorbance of keto form (π π^*)

[b]Acidic medium is attained by addition of CF_3COOH (~1 mL) to the given solution (molecule concentration $1x10^{-5}$ mol/L)

[c]Basic medium is attained by addition of Et_3N (~1 mL) to the given solution (molecule concentration $1x10^{-5}$ mol/L)

[d]$\boldsymbol{K_{keto} = keto\%/1\text{-}keto\%}$ eq 5[23]

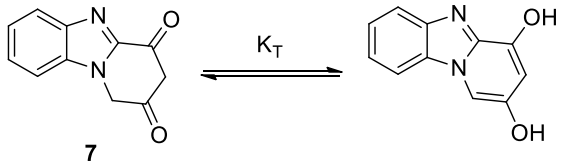

Figure 4: The tautomerism of compound, 7,

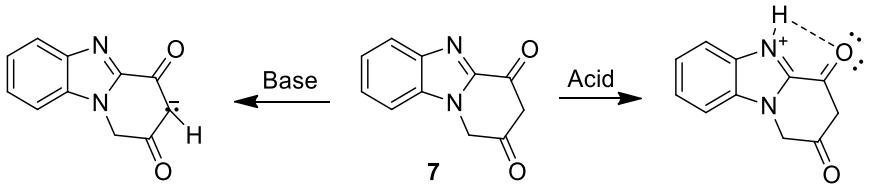

Figure 6. Possible protonation and deprotonation pattern of the compound, 7

CONCLUSIONS

It has been seen that keto form is dominated when the spectral data from IR and NMR techniques for identification studies was taken into consideration. The results of IR spectrums showed two carbonyl peaks and no hydroxyl peaks in addition to the results of ^{1}H-NMR spectrums explained that there are methylene peak between two carbonyls and the other methylene peak and no aromatic proton peaks for pyridine ring causing as a result of tautomerism. The results of ^{13}C-NMR spectrums showed also two peaks of carbons from carbonyls and no aromatic carbons peaks from the pyridine ring as well.

The acidity constant study ended up using by UV spectrophotometer shows, the possible form as keto form. In this pronounced study, benzimidazole results were employed to compare the compound, 7. On the other hand, the results of Gaussian 03 program were utilized to support this situation. When the tautomerism tests that performed in there mediums which are acidic, basic and neutral were searched, the keto form was decided as predominant.

All of these results have proved that the compound, 7, recrystallized as keto form. It has been reported that some pyrido[1,2-*a*]benzimidazolone derivatives existed on keto forms[6]. This situation has supported our study and results as well.

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Hepatoprotective Effects of Coriandrum Sativum Essential Oil Against Acute Hepatotoxicity Induced by Carbon Tetrachloride on Rats

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ABSTRACT

The aim of this study was to evaluate effect of *Coriandrum sativum (CS)* essential oil in rat model of carbon tetrachloride (CCl_4) induced liver toxicity. Experimental groups were formed as follows: isotonic saline solution (ISS), silibinin, CS-1 (0,3ml/kg), CS-2 (0,6 ml/kg). Agents were administered intraperitoneally. Blood and liver tissues were collected at the end of the study ended. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured. Liver tissues were evaluated histopathologically. One-way analysis of variance (ANOVA) was used for statistical analyses.As a result silibinin and CS-2 decreased blood AST and ALT levels of their groups and these biochemical results were supported by histopathological results. In conclusion this study has provided evidence that *Coriandrum sativum* essential oil has significant hepatoprotective effect on carbon tetrachloride induced liver toxicity in rats.

Keywords: *Coriandrum sativum*, hepatoprotective activity, carbon tetrachloride, rats, essential oil.

INTRODUCTION

Taking advantage of plants to treat diseases is becoming a popular and widespread topic. Also in Turkey, studying pharmacological and toxicological activity of plants is an increasing trend. Although Turkey has limited economic resources and drug production facilities through the synthesis could not come to an adequate level, it has a wide flora. It would be a rational approach for countries like Turkey to use natural sources for medicine development and encourage the society to utilize them[1].

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Coriandrum sativum L. (CS) (kişniş, aşuti) belongs to Apiaceace (Umbelliferae) family[2,3]. It is a herbaceous plant which grows annually and has a 20-60 cm height. Spice of CS contains volatile oil, tanin, resin and sugar. The volatile oil is colorless liquid with light-yellow color which is obtained by water vapor distillation with 0.3-0.4% yield. Major ingredients of the volatile oil are: 60-70% linalool, 6% γ-terpinen, α-pinene, camphor, geraniol, p-cymene, geranyl acetate, limonene, aldehydes, esters and alcohols. It is useful in food industry as spice, tincture and alcoholic/non-alcoholic beverages beside this perfumery and cosmetics industries use CS too[3]. It helps flatulence and indigestion[2]. In Turkish folk medicine, it is reported to be used as hepatoprotective and analgesic (head and tooth ache). Additionally the usage of this genus plants against dizziness, pharyngitis, glossitis, urinary tract infections, hemorrhoids, dysentery, urticaria and apht have been recorded[4].

According to literature CS is a very effective anxiolytic in mice[5], has antibacterial effect against *Escherichia coli*, *Bacillus megaterium* and *Salmonella choleraesuis*[6,7], can reduce cholesterol and triglyceride levels in rats[8]. In addition CS has a potent antioxidant activity (more potent than ascorbic acid) [9], effective in the treatment of inflammatory bowel diseases[10], has insulin-like activity in streptozotocin-induced diabetic rat model[11]. Lastly CS can cause abortus in pregnant rats related with the significant decrease in the progesterone levels in the 5th day of the pregnancy[12].

There is not sufficient data about hepatoprotective activity of CS in the literature. In current study CS was investigated for the potential hepatoprotective activities on carbon tetrachloride induced liver toxicity in rats.

METHODOLOGY

Plant materials

Coriandrum sativum L., was collected from different parts of Turkey. The taxonomic identification of the plants was confirmed by a botanist. Voucher specimens are kept in the laboratory (sample number: B-17). Seeds of the plant were boiled in the Clevenger apparatus. Essential oil which was collected from apparatus was stored in the laboratory tubes. Yield of the essential oil was 0.2%.

Chemicals

Carbon tetrachloride (CCl_4) obtained from Merck KgA (Darmstadt-Germany) and silibinin was provided from Sigma (Steinheim, Germany). CCl_4 was dissolved in the olive oil (v/v, 1:1) which was obtained from Fluka (Steinheim-Germany).

Animals

Male and female Sprague–Dawley rats (200-300 g) were used in this experiment and they were obtained from the Animal House. The animals were housed in standard plastic cages at room temperature (22±2 °C), with artificial light from 7.00 am to 7.00 pm, and provided with pelleted food and water *ad libitum*. The study protocol was approved by the Ethical Committee.

Hepatoprotective activity assay

Animal groups were designed as follow (n=6): Control group 1 received isotonic saline solution (ISS) 0.2 mL, Group 2 received CCl_4(0.8 mL/kg), Group 3 received silibinin (50 mg/kg) + CCl_4 (0.8 ml/kg), Group 4 received CS-1 (0.3 ml/kg)+CCl_4(0.8 mL/kg), Group 5 received CS-2 (0.6 ml/kg) + CCl_4(0.8 ml/kg) i.p. daily for seven days. Doses of CS were determined according to the study of Ozbek et al.[13]. Blood and liver samples were collected after seven days treatment and the serum was used for the assay of the marker enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Histopathological examination of the liver

The livers of the experimental animals were extracted after scarifying the animals and fixed in 10% neutral buffered-formalin prior to routine processing in paraffin-embedded blocks. Sections (4 μm thick) were cut and stained using Hematoxylin-eosin (HE). Histological damage was expressed using the following score system; 0:absent; +:mild; ++:moderate; +++:severe[14].

Statistical Analyses

Results are reported as mean±SEM (standard error of mean). One-way analysis of variance (One-way ANOVA; post-hoc Dunnett t ad LSD) was used for statistical analyses. Probability levels of less than 0.05 ($P<0.05$) were considered significant.

RESULTS AND DISCUSSION

Plasma AST and ALT levels of the groups were given in Table 1.

Histopathological examination results were exhibited in Table 2, Figure 1 and Figure 2.

This study provided evidence that CS essential oil has significant hepatoprotective effect on CCl_4induced liver toxicity in rats.

According to the Kumar et al. water-extract of CS leafs has hepatoprotective activity in mice model of profenofos induced liver toxicity[15]. Furthermore, in a study which was conducted by Pandey et al. ethanol extract of CS provided protective activity against carbon tetrachloride induced liver toxicity on rats[16]. Results of

these studies which were performed with different CS extracts supported our study which was conducted with CS essential oil. Beside these, Cioanca et al. stated that CS essential oil has antioxidant activity[17]. Hence, hepatoprotective activities can be related with the antioxidant properties of CS.

According to Samojlik et al. oral administration (0.03 g/kg) of CS essential oil to mice with CCl_4 induced liver toxicity did not produce hepatoprotective activity[18]. This result is in conflict with our findings and the findings of other studies mentioned above. This dilemma may be related with the species of the animals (Samojlik et al. used mice whereas we used rat). Samojlik et al. only administered a single dose which was 0.03 g/kg CS which may be inadequate for the activity. In accordance with this view, in our study although hepatoprotective activity in 0.3 mL/kg was not significant, the effective dose was 0.6 mL/kg. Additionally Samojlik et al. administered CS extract not intaperitoneally which may also change the results. Since, in oral route CS extract may be changed chemically in gastric acid, and also elimination in liver after duodenal absorption may be possible. However pharmacokinetics in i.p. is similar to i.v. route since there is no gastrointestinal absorption period and first pass effect.

Linalool, γ-terpinen, α-pinene, camphor, geraniol, p-cymene, geranyl acetate are reported as the major molecules of CS essential oil. Hepatoprotective effect of CS can be related with one or more molecules that are mentioned above. In further studies, all chemical molecules that are mentioned above should be studied separately to detect the molecule(s) which is/are responsible from the hepatoprotective effect.

Table 1: Effects of CS essential oil on serum levels of AST and ALT.

Uygulama	ALT		AST	
	Serum (U/L)	95 % CI	Serum (U/L)	95 % CI
Control (ISS)	48.8±2.9	41.43 – 56.24	164.5±10.8	136.67 – 192.33
CCl_4	[a] 1068.3±55.3	937.40 – 1199.10	[a] 1682.6±96.1	1455.29 – 1909.97
Silibinin	[ab] 406.5±56.5	261.21 – 551.79	[ab] 732.0±64.8	565.57 - 898.43
CS-1 (0.3 mL/kg)	[a] 992.2±294.4	235.32 – 1749.91	[a] 1619.7±456.8	445.43 – 2793.91
CS-2 (0.6 mL/kg)	[ab] 663.0±84.0	429.85 – 896.15	[ab] 765.0±58.4	602.93 – 927.07
F/p	9.983/0.001		10.125/0.001	

a: $p<0.05$ compared to control (ISS)
b: $p<0.05$ compared to CCl4

Table 2: Histopathological changes in the liver of rats.

Groups	Microscopic Observation			
	Ballooning degenerations and steatosis	Apoptosis and/ or necrosis	Bridging necrosis	Average score*
Control (ISS)	0	0	0	0/6=0.00
CCl_4	15	14	13	42/6=7.00
Silibinin	7	8	4	19/6=3.17
CS-1	12	12	10	34/6=5.67
CS-2	10	9	7	26/6=4.33

* Average score = Total score / n

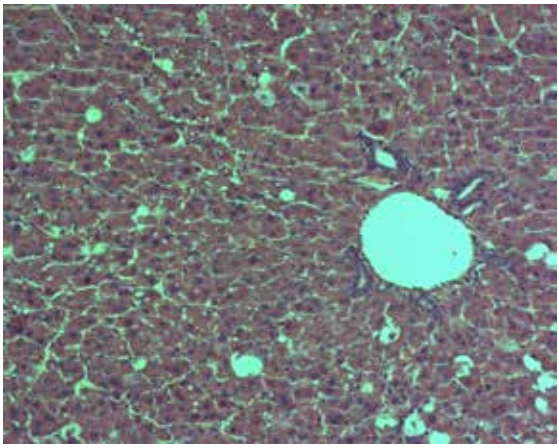

Figure 1: CS-1 0.3 mL/kg (HE x 20)

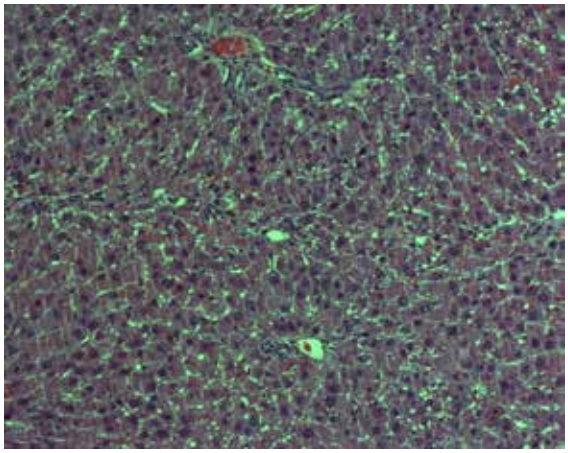

Figure 2: CS-2 0.6 mL/kg (HE x 20)

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GRI Application Level

Synthesis and Antimicrobial Activity Evaluation of Novel Nitrofuranthiazoles

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ABSTRACT

In this work, six novel 4-aryl-2-[2-((5-nitrofuran-2-yl)methylene)hydrazinyl] thiazole derivatives **(2a-f)** were synthesized starting from 5-nitro-2-furaldehyde diacetate by using Hantzsch thiazole synthesis. The antimicrobial activity of the title compounds were screened against five Gram positive bacteria *B. cereus*, *E. faecalis*, *S. aureus*, *S. epidermidis, L. monocytogenes* and two Gram negative bacteria *E. coli* and *S. typhi*. MIC and MBC were calculated and compared to standard drug nitrofurazone. Compounds bearing pyridine moiety (**2d-e**) exhibited significant antimicrobial activity which could be evaluated as new, potent antibacterial agents.

Keywords: nitrofurans, nitrofurazone, thiazole, antibacterial activity.

INTRODUCTION

The incidence of microbial infections have been increasing day by day in worldwide. Many antibacterial agents are in use against a wide range of infectious diseases[1,2]. However, the resistance to existing drugs is still a serious problem threat to global public health. This situation leads medicinal chemists to investigate newly synthesized more potent antimicrobial drugs[3]. Among many antibacterial agents, nitrofuran containing drugs (**Figure 1**. furazolidone, nitrofurantoin nitrofurazone and furaltadone) have been widely used for protection against microbial and protozoal infections especially associated with food contaminations[4]. The nitrofurans are characterized by a 5-nitro-2-furanyl group. First synthesized drug nitrofurazone, 5-nitro-2- furaldehyde semicarbazone, is still being used molecule due to its antitrypanosomal activity as well as antibac-

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terial potential to treat burns topically[5]. However, mutagenic/carcinogenic toxic effects of this molecule were detected for the antimicrobial and anti-protozoal applications[6]. Therefore, prodrug approach was suggested to increase biological activity and decrease toxicity along with improving the physico-chemical properties[7]. For this purpose, many 5-nitrofuryl derivatives have been extensively studied for the treatment of various microbial infections [8-12] and these compounds are determined to act via producing oxidative stress on the parasite which leads to death of the microbe[13].

Addition to all, nitrofuran agents have been developed through combining thiosemicarbazone moiety and different heterocyclic rings. Thiosemicarbazones and their derivatives are important compounds known with many biological properties especially as chemotherapeutic agents[14-18]. Their cyclization products, (4-aryl-thiazol-2-yl)hydrazines are also widely studied derivatives due to their numerous pharmacological applications and varied biological activities[19-21]. Also, thiazole containing compounds have attracted broad interest because of their synthesis ease of reaction and their capability to easily furnish valuable chemotherapeutics such as anticancer, antibacterial, antifungal, and antiprotozoal agents[22].

Considering the reported data, we have described six novel compounds combining (4-aryl-thiazol-2-yl)hydrazine and 5-nitrofuryl moieties which we based on well-known antibacterial agent, nitrofurazone. The antibacterial activity of the compounds have been investigated against various microorganisms compared with standard drug.

METHODOLOGY

Chemistry

Melting points were determined by MP90 digital melting point apparatus (Mettler Toledo, OH) and were uncorrected. Spectroscopic data were obtained by instruments: Bruker Tensor 27 IR spectrophotometer; ^{1}H NMR (nuclear magnetic resonance) Bruker DPX- 300 FT-NMR spectrometer, ^{13}C NMR, Bruker DPX 75 MHz spectrometer (Bruker Bioscience, Billerica, MA, USA); M+1 peaks were determined by Shimadzu LC/MS ITTOF system (Shimadzu, Tokyo, Japan).

Preparation of 5-nitrofuran-2-carbaldehyde thiosemicarbazone (1)

A mixture of thiosemicarbazide (0.27 g, 3 mmol) and 5-nitro-2-furaldehyde diacetate (3 mmol) in ethanol (20 mL) was refluxed for 3 h. After keeping the solution at 0 °C overnight, the precipitated raw product was filtered off and recrystallized from ethanol to afford corresponding thiosemicarbazone compound **(1)**.

Preparation of 4-aryl-2-[2-((5-nitrofuran-2-yl)methylene) hydrazinyl]thiazole derivatives (2a-f)

5-Nitro-2-furaldehyde thiosemicarbazone **(1)** (0.2 mmol) and appropriate α-bromoarylethanone derivative (0.16 g, 0.80 mmol) were stirred in ethanol at room temperature. The progress of the reaction was monitored by TLC. The mixture was filtered, the product was dried and recrystallized from ethanol, to give target compounds (**2a-f**).

4-(2-Hydroxyphenyl)-2-[2-((5-nitrofuran-2-yl)methylene) hydrazinyl]thiazole (2a)

72 % yield; mp 221 °C. IR ν_{max} (cm^{-1}): 3452 (OH), 3121 (amide N-H), 1574 and 1359 (NO_2), 1479-1454 (C=C, C=N), 1171-1014 (C-O, C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 6.84-6.92 (m, 2H, Ar-H), 7.13-7.19 (m, 2H, Ar-H), 7.52 (s, 1H, thiazole C_5-H), 7.79 (d, *J*=3.93 Hz, 1H, Ar-H), 7.85 (d, *J*=7.77 Hz, 1H, Ar-H), 7.99 (s, 1H, -CH=N), 10.84 (brs, 1H, OH), 12.79 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 106.54, 114.49, 115.58, 117.06, 119.63, 127.94, 129.40, 129.86, 152.08, 152.42, 155.53. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{10}N_4O_4S$ 331.32; found 331.05.

4-(3-Hydroxyphenyl)-2-[2-((5-nitrofuran-2-yl)methylene) hydrazinyl]thiazole (2b)

70 % yield; mp 188 °C. IR ν_{max} (cm^{-1}): 3450 (OH), 3112 (amide N-H), 1566 and 1315 (NO_2), 1512-1385 (C=C, C=N), 1198-1016 (C-O, C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 6.91-6.74 (m, 1H, Ar-H), 7.13 (d, *J*=3.99 1H, Ar-H), 7.21 (t, *J*=7.89 Hz, 1H, Ar-H), 7.28-7.33 (m, 3H, thiazole C_5-H and Ar-H), 7.78 (d, *J*=3.93 Hz, 1H, Ar-H), 7.98 (s, 1H, -CH=N), 9.49 (brs, 1H, OH), 12.75 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 105.22, 112.98, 114.19, 115.26, 116.88, 117.25, 129.31, 130.07, 130.98, 136.07, 151.22, 152.02, 152.59, 158.04, 167.42. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{10}N_4O_4S$ 331.32; found 331.05.

4-(4-Hydroxyphenyl)-2-[2-((5-nitrofuran-2-yl)methylene) hydrazinyl]thiazole (2c)

76 % yield; mp 210 °C. IR ν_{max} (cm^{-1}): 3454 (OH), 3120 (amide N-H), 1573 and 1317 (NO_2), 1471-1317 (C=C, C=N), 1201-1012 (C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 6.81 (d, *J*=8.64 Hz, 2H, Ar-H), 7.11-7.14 (m, 2H, thiazole C_5-H and Ar-H), 7.68 (d, *J*=8.61 Hz, 2H, Ar-H), 7.77 (d, *J*=3.96 Hz, 1H, Ar-H), 7.96 (s, 1H, -CH=N), 9.58 (s, 1H, OH), 12.70 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 102.26, 114.09, 115.64, 115.82, 126.13, 127.42, 129.18, 151.99, 152.68, 157.68, 167.43. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{14}H_{10}N_4O_4S$ 331.32; found 331.05.

4-(2-Pyridyl)-2-[2-((5-nitrofuran-2-yl)methylene)hydrazinyl] thiazole (2d)

76 % yield; mp 230 °C. IR ν_{max} (cm^{-1}): 3113 (amide N-H), 1516 and 1348 (NO_2), 1471-1313 (C=C, C=N), 1298-1024 (C-N, C-O). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 7.19 (d, *J*=3.96 Hz, 1H, Ar-H), 7.72-7.79 (m, 2H, Ar-H), 8.10 (d, *J*=4.14 Hz, 2H, Ar-H), 8.29 (d, *J*=7.89 Hz, 1H, Ar-H), 8.38 (t, *J*=8.76 Hz, 1H, Ar-H), 8.72 (d, *J*=4.74 Hz, 1H, Ar-H), 11.85 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 114.56, 114.95, 114.91, 115.48, 123.08, 123.85, 124.98, 130.76, 143.97, 144.84, 145.48, 147.62, 152.09, 152.16, 168.74. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{13}H_9N_5O_3S$ 316.31; found 316.05.

4-(3-Pyridyl)-2-[2-((5-nitrofuran-2-yl)methylene)hydrazinyl] thiazole (2e)

71 % yield; mp 240 °C. IR ν_{max} (cm^{-1}): 3115 (amide N-H), 1568 and 1352 (NO_2), 1475-1315 (C=C, C=N), 1302-1016 (C-N, C-O). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 7.77-7.79 (m, 1H, Ar-H), 8.39-8.48 (m, 3H, Ar-H), 8.64 (s, 1H, thiazole C_5-H), 9.23-9.34 (m, 2H, Ar-H), 9.80 (s, 1H, -CH=N), 13.50 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 110.11, 115.33, 116.16, 126.95, 130.73, 132.92, 138.79, 143.39, 144.99, 147.00, 152.74, 152.88, 169.11. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{13}H_9N_5O_3S$ 316.31; found 316.05.

4-(4-Pyridyl)-2-[2-((5-nitrofuran-2-yl)methylene)hydrazinyl] thiazole (2f)

69 % yield; mp 236 °C. IR ν_{max} (cm^{-1}): 3115 (amide N-H), 1556 and 1346 (NO_2), 1481-1323 (C=C, C=N), 1247-1012 (C-N, C-O). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 7.18 (d, *J*= Hz, 1H, Ar-H), 7.78 (d, *J*= Hz, 1H, Ar-H), 8.03 (s, 1H, thiazole C_5-H), 8.27-8.30 (m, 3H, -CH=N and Ar-H), 8.86 (d, *J*=6.40 Hz, 2H, Ar-H), 12.96 (brs, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 114.96, 115.48, 116.30, 122.23, 130.56, 144.11, 146.82, 147.95, 152.08, 147.00, 152.16, 168.62. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{13}H_9N_5O_3S$ 316.31; found 316.05.

Antibacterial activity

Antibacterial activity against *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Salmonella typhi* ATCC 14028, and *Listeria monocytogenes* ATCC 7644 was determined by the microbroth dilutions technique using the Clinical Laboratory Standarts Institute (CLSI) recommendations[23].

The minimum inhibitory concentrations (MIC) was defined as the lowest concentration of compound giving complete inhibition of visible growth and the

minimum bactericidal concentration (MBC) was defined as the lowest concentration of the compound to completely kill bacteria. Nitrofurazone was used as positive control. According to values of the controls, the results were evaluated. Each experiment was replicated twice.

RESULTS AND DISCUSSION

Chemistry

In this study, 4-aryl-2-[2-((5-nitrofuran-2-yl)methylene)hydrazinyl]thiazole derivatives **(2a-f)** were synthesized with a two-step synthetic procedure as shown **Scheme 1**. Compound **1** was synthesized by the reaction of 5-nitro-2-furaldehyde diacetate and thiosemicarbazide in ethanol at reflux conditions. The gained 5-nitrofuran-2-carbaldehyde thiosemicarbazone is a previously reported molecule with a melting point of 163-165 ^{o}C[24]. In second step, six new derivatives (**2a-f**) were synthesized via Hantzsch thiazole synthesis. 2-/3- or /4-Hydroxyphenyl-α-bromoethanone and 2-/3- or 4-pyridyl-α-bromoethanones were used as α-haloketone compounds. In this way, six novel 4-aryl-2-[2-((5-nitrofuran-2-yl) methylene)hydrazinyl]thiazole derivatives **(2a-f)** were obtained with the 68-76 % yield.

Characteristic infra-red stretching bands belong to amine (NH) and nitro (NO_2) groups were detected at 3121-3112 cm^{-1} and about 1550, 1340 cm^{-1}, respectively. In ^{1}H NMR spectra, peaks of azomethine (-CH=N) and amine protons were detected in range 7.99-7.89 ppm and 11.85-12.89 ppm, respectively. OH protons of **2a-c** were detected at about 9.49-10.84 ppm as broad singlet peaks. In the ^{13}C NMR spectra, all carbons were observed at 102.26-169.11 ppm range, correctly. Molecular ion peaks were also determined in agreement with molecular weights of the compounds.

Biology

Final compounds (**2a-f**) were screened to determine their antimicrobial activities against totally seven Gram negative and Gram positive bacteria; *E. coli*, *S. typhi, E. faecalis*, *S. aureus*, *S. epidermidis, L. monocytogenes* and *B. cereus*. MIC and MBC were identified for standart drug nitrofurazone and all compounds. As were represented in **Table 1**, The MIC and MBC values were found between 0.5-2 mg/mL and higher for the **2a-c**, whereas both of the values were found as 1 mg/mL and higher for nitrofurazone.

Among the hydroxylated compounds (**2a-c**), compound **2a** exhibited the highest activity against *B. cereus* (MIC=0.5 mg/mL, MBC=1 mg/mL). Besides, it showed same potency to nitrofurazone (MIC: 1 mg/mL) against *E. coli* and *E. faecalis*. Compound **2b** did not show prominent antibacterial activity which had

MIC and MBC values 2 mg/mL and higher against all tested bacteria. Another hydroxyl containing compound **2c** showed same antibacterial potency to standard drug against three bacteria, *S. aureus, S. typhi* and *L. monocytogenes*.

Regarding to compounds **2d-e** with pyridine moiety, they exhibited significant activity with a higher potency than hydroxylated compounds (**2a-c**). Compound **2d** including 2-pyridyl moiety showed two-fold antibacterial potency against *B. cereus* (MIC and MBC: 0.5 mg/mL) compared to nitrofurazone. It exhbited same potency to standard drug against *E. coli* and *S. epidermis*. Also, the lowest concentrations of compound **2d** caused inhibition growth of bacteria (MIC) were found as 1 mg/mL whereas the lowest concentrations of the compound caused completely death of bacteria (MBC) were found higher than the highest tested concentration (>2 mg/mL) against *E. faecalis, S. aureus, S. typhi* and *L. monocytogenes*. MIC values for compound **2e** were found between 0.5-1 mg/mL whereas MBC values were found as 2 mg/mL. Additionally, compound **2e** containing 3-pyridine moiety could be declared as the most active compound with the lowest MIC values among the other compounds. Also, sixth compound **2f** bearing 4-pyridyl moiety showed remarkable activity. MIC and MB values were calculated as 0.5 mg/mL against the most susceptible bacteri *B. cereus*.

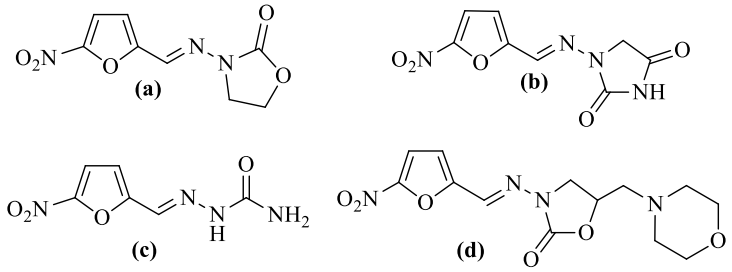
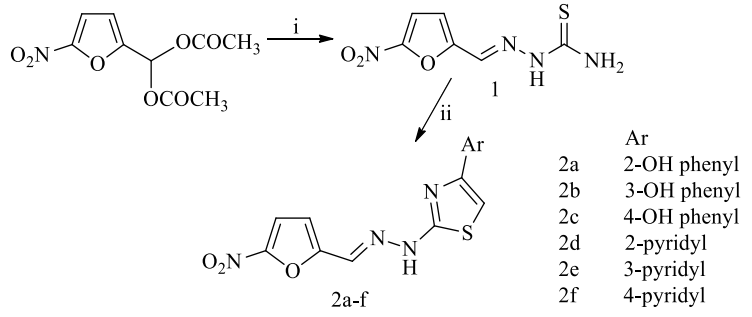


Figure 1: Nitrofuran drugs. (**a**) Furazolidone, (**b**) Nitrofurantoin, (**c**) Nitrofurazone, (**d**) Furaltadone.



Scheme 1: Synthesis of the compounds (**2a-f).** Reagents: (i) thiosemicarbazide, ethanol, reflux, 3h; (ii) α-bromoarylethanone, ethanol, rt.

Table 1: Antimicrobial activity of the compounds (mg/mL)

		Compounds						
Microorg.		**2a**	**2b**	**2c**	**2d**	**2e**	**2f**	**Ref***
A	**MIC**	0.5	2	2	0.5	0.5	0.5	1
	MBC	1	2	2	0.5	>2	0.5	1
B	**MIC**	1	2	2	1	1	1	1
	MBC	2	2	2	1	>2	2	1
C	**MIC**	1	2	2	1	1	0.5	1
	MBC	>2	2	2	>2	>2	>2	1
D	**MIC**	2	2	1	1	0.5	0.5	1
	MBC	2	2	>2	1	2	1	1
E	**MIC**	2	2	1	1	0.5	1	1
	MBC	>2	2	1	>2	>2	>2	1
F	**MIC**	2	2	1	1	0.5	1	1
	MBC	>2	>2	1	>2	>2	>2	1
G	**MIC**	2	2	1	1	0.5	1	1
	MBC	>2	>2	1	>2	>2	>2	>1

***Reference :** Nitrofurazone; **MIC:** Minimum inhibition concentration; **MBC:** Minimum bactericidal concentration

A : *Bacillus cereus* ATCC 14579; **B** : *Escherichia coli* ATCC 25922; **C** : *Enterococcus faecalis* ATCC 29212; **D** : *Staphylococcus epidermidis* ATCC 12228; **E** : *Staphylococcus aureus* ATCC 25923; **F** : *Salmonella typhi* ATCC 14028; **G** : *Listeria monocytogenes* ATCC 7644

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DNA Damage in Gasoline Station Workers Caused by Occupational Exposure to Petrol Vapour in Turkey

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ABSTRACT

The refueling of vehicles is a major source of volatile organic compounds generated by used and evaporated fuel. Gasoline workers are directly exposed to various fuel products via many routes in the ambient air. The aim of this study is to determine the potential DNA damage in the peripheral blood samples of the gasoline station workers in Istanbul, Turkey by using the alkaline comet assay. Blood samples were collected from exposed workers (n= 25) and healthy controls (n= 14) with no history of occupational exposure. Significant difference in the mean total comet scores (TCS) of gasoline station workers (89.24 ± 30.83) were observed as compared with the control group (15.64 ± 16.17) ($p < 0.001$). Results from our study indicate that exposure to petrol vapour induce genotoxic effects, confirming that the gasoline station workers have a high risk of cancer due to their daily occupational exposure.

Key words: gasoline station workers, fuel exposure, comet assay, DNA damage

INTRODUCTION

Gasoline is a mixture of various hydrocarbons; its vapour consists of 95% aliphatic and alicyclic compounds and aromatic compounds. According to the International Agency of Research on Cancer (IARC), gasoline vapour is included in class of human carcinogens[1, 2]. Benzene is a volatile aromatic hydrocarbon solvent used extensively in industry in the past. It continues to be used, in low concentration, in some occupational processes. Benzene is a cytotoxic, hematoxic, immunotoxic and genotoxic aromatic hydrocarbon that is used in many fields in industry. It is also a common environmental contaminant and component of

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cigarette, gasoline and automobile emissions. It has long been known that benzene causes leukemia, although there are still some ambiguities regarding its mechanism and concentration-response correlation. Chronic exposure to low benzene concentrations in the workplace is associated with blood diseases like aplastic anemia and leukemia[3]. Benzene, toluene and xylene are key aromatic compounds in gasoline. Benzene is listed as a carcinogen for humans and the Association Advancing Occupational and Environmental Health (ACGIH) sets the recommended threshold value of occupational exposure to benzene at 0.5 ppm. Exposure to toluene and xylene can contribute to neurological effects including headaches, dizziness, tiredness, tremors, coordination disorders, anxiety disorders, memory insufficiency, even if they are not listed as carcinogens[4, 5].

Benzene is also considered to be a reason for acute myelogenous leukemia (AML) and its derivatives, but it is not yet clear whether benzene contributes to lymphocytic hematologic neoplasms such as non-hodgkin lymphoma, acute and chronic lymphatic leukemia, or multiple myeloma[6]. Observing gasoline station workers demonstrates that average exposure level for benzene is 3.9 times more than normal; for toluene it is 5.5 more than normal. Risks caused by chronic exposure to harmful volatile organic solvents, and mixed vital cancer risks caused by benzene, ethylbenzene, formaldehyde and acetaldehyde indicate include cancer as well as other chronic health effects for exposed workers[7].

Volatile organic solvents belong to a heterogeneous group of chemicals characterized by high vapour pressure. Volatile organic compounds have come under significant scrutiny of late in gasoline stations that emit these compounds. Environmental studies that assess related air quality have also become important[8]. It is thought that some petrol-derivative compounds are formed from a mixture of complex chemicals well-known to be genotoxic agents. It is reported by the IARC that exposure to some well-known compounds like benzene and its vapours have a carcinogenic effect[9]. Petrol derivatives are chemical mixtures that also contain well-known genotoxicants like benzene, and chronic occupational exposure to these derivatives create a genotoxic risk[10].

According to a recent study, the urine of gasoline-exposed workers contains a high percentage of phenol[11], which can also transform into potential toxic metabolites such as catechol and hydroquinone. They can oxidize to benzoquinones by myeloperoxidase enzyme (MPO) in bone marrow. Benzoquinones, quinones and other benzene metabolites can create a reactive oxygen species that can damage important macromolecular targets including DNA, proteins and lipids. Reactive products can bind to cellular molecules, proteins and DNA covalently, creating an alternative path, that potentially induces breakages of DNA bonds, sister chromatid exchange (SCE), micronucleus (MN) and chromosomal aberra-

tions (CA). Single breakages of DNA bonds, SCE, MN and CA are reported in the leukocytes of workers who are occupationally exposed to benzene. It is suggested that benzene also plays a role in mutagenesis because of an indirect mechanism that causes oxidative DNA damage by inducing the formation of hydroxyl radicals from hydrogen peroxidase[3].

The use of appropriate biomarkers in evaluating exposure or genotoxicity-dependent disorders is important in order to interpret the relevant data correctly[12]. The Comet (single cell gel electrophoresis, SCGE) technique is a rapid, sensitive and cheap method that is used for assessing DNA damage[13]. Due to its high sensitivity, this assay is more advantageous than other methods of determining DNA-damage[14]. With this technique, the degree of damage can also be visually observed by evaluating nuclei as tailless, short tail (where the occurrence of stretches in DNA strands is more likely than individual fraction migration at low damage levels) and long tail (with an increase in breakage numbers, DNA fractions migrate freely from the nucleus, creating a 'comet' like appearance)[15].

The objective of this study was to evaluate the level of DNA damage in fuel filling station workers using the alkaline Comet technique, and for this purpose peripheral blood samples were collected from exposed workers and the control group.

METHODOLOGY

Study and Control Groups

The research was performed on 25 workers between the ages of 20 and 57 employees at various busy gasoline stations in Istanbul as well as on 14 control subjects. A short survey containing questions about demographical characteristics and factors that enhance DNA damage is given to all participants. Ethical approval for this study was obtained from Marmara University's Institute of Health Sciences (30.03.2011 - 20).

Analysis

Blood sampling and lymphocyte preparation: Approximately 0.5 ml of peripheral blood samples were collected from volunteers in sterile disposable syringes, and transferred into heparinized tubes. Unstimulated lymphocytes were isolated by Histopaque 1077 density gradient centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in ice-cold PBS at $5x10^3$ to $1x10^4$ cells/ml, respectively. Viability was tested by trypan blue exclusion. The number of dye-excluding cells always exceeded 90%.

Comet assay

Chemicals: Unless otherwise stated, all chemicals were purchased from Sigma

Chemical (Steinhelm, Germany). Superfrost 1.0-1.2 mm thick microscope slides were obtained from Menzel (Braunschweig, Germany).

***Slide preparation**:* The alkaline comet assay was performed using an adaptation of the method of Singh *et al*[16]. Fully frosted microscope slides were dipped briefly into (60°C), 0.7% normal melting agarose (NMA) prepared in PBS. The slides were dried overnight at room temperature and then stored at 4°C until examined. Prepared cells (50.000) were mixed with 0.7% low melting point agarose (LMA) and placed on microscope slides. After adding the cell-containing layer, a second layer of LMA was added to fill in any residual holes in the second agarose layer, and to increase the distance between the cells and the gel surface. The slides were maintained on an ice-cold flat tray for 15 min to solidify. The slides were then carefully immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10) for at least 1 h at 4°C.

Electrophoresis: The slides were removed from the lysing solution, drained and placed in a horizontal electrophoresis tank. The tank was filled with fresh electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH 13) to a level just covering the slides. Before electrophoresis, the slides were left in the solution for 20 minutes to allow for the unwinding of the DNA and the expression of alkali-labile damage. Subsequently, the DNA was electrophoresed for 30 minutes at 300 mA and 15 V. To prevent additional DNA damage, all of the steps described above were conducted in the dark at 4°C. After electrophoresis, the slides were taken from the tank and washed three times (5 min each) with 0.4 M Tris buffer, pH 7.5 to neutralize the excess alkali. After the neutralization process, slides washed with varying concentrations of cold ethanol (50%, 75%, 100%) respectively, in 5 minutes intervals. Then the slides were lined on paper to dry, and kept in a cold, dry environment before the dyeing process.

Staining: 50 ml ethidium bromide (EtBr - 20 ml/ml) was added to each slide. The slides were covered with a cover-slip, stored in a humidified box at 4°C, and analyzed using a fluorescence microscope within 3-4 hours.

Scoring: The analysis of 100 randomly selected cells per subject was kept at 40× magnification, under a fluorescence microscope (Olympus, BX51). Cells were scored visually into five classes according to tail size, in order to facilitate the management of the data. The mean total comet scores (mean TCS) were calculated as follows: TCS = 0× no migration + 1× low migration + 2× medium migration + 3× high migration + 4× extensive migration, following Collins[17].

Statistical Analysis

SPSS 17 statistical software (Statistical Package for Social Sciences Inc, Chicago,

IL, USA) was used for the statistical analyses of the results. All results were expressed as means ± Standart Deviation (SD); data are assigned a number or percentage where a p-value less than 0.05 was defined as statistically significant. A Shapiro Wilk test was conducted to determine whether the results of the analysis were in accordance with a normal distribution. The differences between the two independent groups at variance with a normal distribution were measured by the Mann-Whitney U test. The mean values among the three groups were compared using the Kruskal–Wallis test. The correlation between continuous variables was evaluated by spearman's correlation. Qualitative variables were expressed as a percentage of the respective groups and the differences were tested with the Chi square ($\chi 2$) test.

RESULTS AND DISCUSSION

Gasoline is a colorless, pale brown or pink fluid. Increased use of gasoline products in automobiles and industry has had a negative impact on human health and air quality. Some products contain carcinogenic compounds[4]. Benzene is a natural component of crude oil that is mixed with toluene and xylene in petrol[18, 5].

Significant DNA damage can result from occupational exposure. The level of exposure depends on shift length, job rotation, individual precautions and workplace conditions[19].

The results of the analysis were in accordance with the normal distribution as evaluated by the Shapiro Wilk test, and this difference was statistically significant (p=0.014). The correlation between continuous variables evaluated by Spearman's correlation, such as the effect of age on TCS; the effect of age was not significant on TCS in workers (p=0.263). The results of comparing the control group with the exposed group with respect to demographical characteristics (age, smoking habit) are given in Table 1. There was no significant difference between groups where demographic properties are such that $p > 0.05$.

The TCS frequency observed in workers (89.24 ± 30.83) was higher than the TCS of the control group (15.64 ± 16.17) (Table 2).

As for the duration of exposure, the groups were categorized as follows; less than 5 years, 5-20 years, and more than 20 years of exposure. The effect of occupational exposure on TCS was determined by a Kruskal-Wallis test and there were no significant differences ($p = 0.398$). [Duration of exposure < 5 years TCS (92.60 ± 26.77); 5-20 years TCS (82.29 ± 41.63); > 20 years TCS (88.67 ± 30.55)]. Comparisons between the TCS frequency according to duration of occupational exposure are shown in Figure 1. Although none of the smokers in either the exposed or control groups were heavy smokers (the number of cigarettes currently

smoked per day was 1-8 cigarettes) and although the sample size is small, DNA damage was more apparent in exposed smokers. Taken together, meta-analysis on the basis of 38 studies (from 37 publications), with 803 smokers and 959 nonsmokers confirms that smoking has a DNA damaging effect on peripheral blood cells as measured by the comet assay[20]. Seven of our previously published papers appear in the above meta-analysis; they contain results similar to those observed in this study, indicating that DNA damage levels were higher in occupationally exposured smokers.

Basal DNA damage is considered to be influenced by extended periods of work in polluted environments. Although the exposed subjects who worked less than five years or between 5-20 years had elevated DNA damage, no significant increase in DNA damage was observed in workers exposed for more than twenty years. However, Sardas et al. (2010) supports our observation by indicating the relation between the DNA damage and DNA repair[19].

The photograph in Figure 2 shows comet cells from a healthy control, and Figure 3 shows comet cells from a gasoline station worker. Changes in the values of the blood parameters of workers chronically exposed to benzene were reported by Qu et al. in 2002[21]. Similar observations by Ray et al. in 2007 indicated decreased erythrocyte, hemoglobin, lymphocyte and thrombocyte levels, whereas neutrophil, band cell, and target cell levels were increased in gasoline workers who use materials with benzene and in workers employees at car service stations[22]. On the other hand, studies of gasoline workers exposed to petrol products reveal multiple myeloma risks[23], exocrine pancreatic cancer development[24], increased prostate cancer incidence[25] at levels significantly higher than those of control groups. Reviews by Smith, M.T., *et al* and Vlaanderen. J. *et al* summarize the case-control and cohort evidence studies that evince an association between cancer incidence and occupational benzene exposure[26, 27].

Analysis of buccal cells revealed that micronucleus frequencies in gasoline station workers were significantly higher than in control subjects; as well the average urine phenol levels of station workers were significantly higher than those in the control group[28]. In a study employing the Comet technique on age-matched workers, Andreoli *et al* showed that significant DNA damage in lymphocytes resulting from exposure to low benzene levels was higher than that of the control group[29]. A similar observation was reported in a 2008 study of Indian gasoline workers where workers were subject to higher exposure conditions to p-benzocaine, a metabolite of gasoline, than the control group[11]. In a similar study, workers from gas stations and a refinery plant who were occupationally exposed to benzene were evaluated by blood benzene concentrations; median occupational benzene exposure was lower in the morning after a shift than at end of shift[30].

In conclusion, total comet scores in randomly selected workers in gasoline stations were significantly higher than in control subjects. Since benzene is a relatively common environmental and occupational contaminant, the genotoxic effect on human health status remains a matter of concern, and the biomarkers of such effects are valuable predictors in assessing potential risks. This preliminary study needs to continue by monitoring more detailed risk assessment parameters such as ambient air, exposure levels, blood and urine concentration measurements, and clinical neurological effects such as findings related to headaches, sleeping disorders, memory loss, general tiredness, with long term follow-up in order to provide important public health information regarding fuel exposure.

Author Contributions

These authors contributed equally.

Table 1: The comparison of control and gasoline station workers with respect to demographical characteristics (age, smoking).

	Control (n=14)	Gasoline station workers (n=25)	p
Age; mean±SD (median)	39.90 ± 13.30 (40)	32.12 ± 11.29 (28)	[a] **p =0.092**
Non Smoker	9 (64.29%)	12 (48%)	[†] **p = 0.520**
Smoker	5 (35.71%)	13 (52%)	

n: *number* ***n (%):*** *percentage of group* [a]***:*** *Mann–Whitney U test* [†]***:*** *Chi square test*

Table 2: Comparisons between the Total comet score in gasoline station workers and unexposed controls expressed as Mean ± SD.

		n	TCS				p[a]
			Mean	±	SD	median	
Control		14	15.64	±	16.17	12	**0.001**
Gasoline station workers		25	89.24	±	30.83	83	
Control	Smoker	5	13.6	±	8.44	13	**0.738**
	Nonsmoker	9	16.77	±	19.62	11	
Gasoline station workers	Smoker	13	92.92	±	36.54	77	**0.978**
	Nonsmoker	12	85.25	±	24.15	84.5	
Nonsmoker	Control	9	16.77	±	19.62	11	**0.001**
	Gasoline Station workers	13	85.25	±	24.15	84.5	

n: *number* ***SD:*** *standard deviation* [a]***:*** *Mann–Whitney U test*

TCS: Total Comet Score

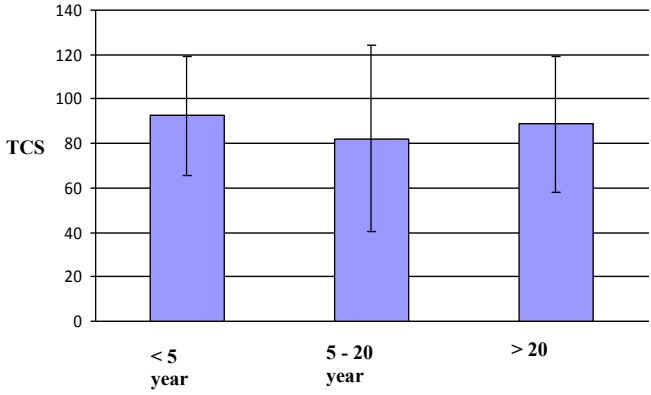

Figure 1: The comparison of Total Comet Scores with respect to duration of occupational exposure

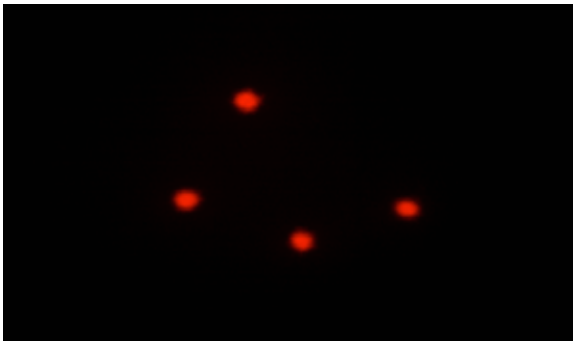

Figure 2: Comet cells from a healthy control.

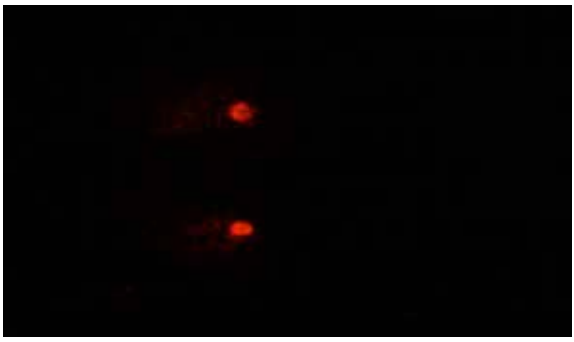

Figure 3: Comet cells from a gasoline station worker.

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Design and Synthesis of Stable N-[2-(aryl/heteroaryl substituted)ethyl] propanamide Derivatives of (S)-Ketoprofen and (S)-Ibuprofen as Non-Ulcerogenic Anti-Inflammatory and Analgesic Agents

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ABSTRACT

The carboxylic acid groups of (*S*) ketoprofen and (*S*) ibuprofen were brought into reaction with substituted ethylamine derivatives to form (*S*)-2-(4-isobutylphenyl)- and (*S*)-2-(3-benzoylphenyl)-*N*-[2-(aryl/heteroaryl substituted) ethyl]propanamide derivatives. Then, these sets were evaluated in terms of their in vivo anti-inflammatory and analgesic properties using the carrageenan-induced paw edema and *p*-benzoquinone-induced writhing models. Among the synthesized compounds, (*S*)-2-(4-isobutylphenyl)-*N*-[2-(pyrrolidin-1-yl)ethyl]propanamide (4f) showed the highest activity at the 100mg/kg dose inducing no gastric lesions when compared to the parent compound, ibuprofen. In vitro studies on chemical stability revealed that the amide derivative with the highest activity (4f) was chemically stable in simulated gastric (pH 1.2) and intestinal fluids (pH 7.4). In 80% v/v human plasma, the amide derivative was found to be stable against plasma hydrolases over the experimental period. The most active compound, (*S*)-2-(4-isobutylphenyl)-*N*-[2-(pyrrolidin-1-yl)ethyl]propanamide, was also studied in 10% rat liver homogenate (pH 7.4) to identify its release pattern as a prodrug.

Keywords: Ketoprofen, Ibuprofen, anti-inflammatory, NSAIDs

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most frequently prescribed medication in the management of pain, inflammation, and fever. They exert their therapeutic activity by non-selectively inhibiting cyclooxygenase-derived prostaglandin synthesis[1-2]. This mechanism of action is inherently responsible for their gastrointestinal (GI) [3-7], renal [8-10] and hepatic [11-13] side effects observed in

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long-term treatments. To improve the GI safety profile of NSAIDs, the following four strategies have been identified: (a) development of selective cyclooxygenase-2 (COX-2) inhibitors; (b) co-administration of a proton pump inhibitor with the NSAID; (c) linking a nitrate-based nitric oxide (NO)-releasing moiety to classical NSAIDs (NO-NSAIDs); and (d) preparing ester or amide derivative as prodrugs. The former three strategies have different advantages and limitations. For example, despite the relatively safe profile of COX-2 inhibitors in the GI tract, their adverse cardiovascular effects reported in some patients undergoing chronic treatment have attracted considerable attention, which resulted in the withdrawal of rofecoxib from the market[14-15]. Organic nitrate-based NO-NSAIDs such as NCX-4016[16], nitronaproxen[17], NCX-2216[18-19] and NO-diclofenac (5)[20] suppress prostaglandin synthesis as effectively as the parent NSAIDs [21-23] but have been shown not to impair the GI tract both in animals and humans. However, an important drawback to this design is the fact that production of NO from nitrate esters requires a three-electron reduction, and this metabolic activation can decrease the efficiency of these drugs when they are used continuously, thus increasing nitrate tolerance[24-26].

The fourth strategy is based on the fact that GI mucosal injury is caused by two different mechanisms[27-29]. The primary mechanism involves a local action comprising a direct contact mechanism, and an indirect effect on the GI mucosa. The direct effect can be attributed to a combination of local irritation produced by the acidic group of the NSAIDs and local inhibition of prostaglandin synthesis in the GI tract. The indirect effect is associated with a combination of an ion-trapping mechanism of NSAIDs in mucosal cells and back diffusion of hydrogen ions from the lumen into the mucosa. The subsequent mechanism is based on a generalized systemic action occurring after absorption, which can be demonstrated following intravenous dosing. These direct and indirect effects can be altered by producing amide and ester derivatives of these structures as prodrugs[30-35].

Ketoprofen and ibuprofen are aryl propionic acid derivatives with known GI side effects of the prolonged use of frequently prescribed NSAIDs. In this present study, we synthesized *N*-[2-(aryl/heteroaryl substituted)ethyl]propanamide derivatives of ketoprofen and ibuprofen (compounds 3a-f and 4a-f) to investigate their anti-inflammatory properties, GI ulceration, and their potential as analgesics and prodrugs.

The idea of using an ethylene linker between the amide and R functional moieties is substantiated by our recent report and other studies using similar derivatives of naproxen[36]. Although compounds 3a, 4c, 4d and 4e had been previously synthesized using different methods and investigated in other subjects[37-41], they were resynthesized and included in pharmacological and kinetic studies.

METHODOLOGY

Chemistry

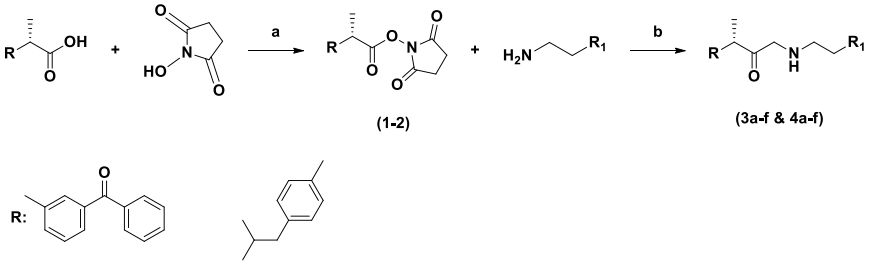
All chemicals were purchased from Aldrich Chemical Co. (Steinheim, Germany). Melting points were detected with a Thomas Hoover capillary melting point apparatus (Philadelphia, PA, USA) and uncorrected. IR spectra (KBr) were recorded on a Perkin Elmer 1720X FT-IR spectrometer (Beaconsfield, UK) and ^{1}H-NMR spectra were obtained by Bruker DPX-400, 400 MHz High Performance Digital FT-NMR using DMSO-d6 and tetramethylsilane as internal standard. All chemical shift values were recorded as δ (ppm). Mass spectra were recorded using an Agilent 1100 series LC/APCI/MS 1946 G spectrometer in the negative ionization mode. The purity of the compounds was checked by thin-layer chromatography on silica gel-coated aluminum sheets (Merck, 1.005554, silica gel HF254–361, Type 60, 0.25 mm; Darmstadt, Germany). The elemental analyses were performed with a Leco CHNS 932 analyzer (Leco Corp., MI, USA). Elemental analysis for C, H and O were within ± 0.4 % of the theoretical values. ^{1}H-NMR spectra and elemental analysis were performed at the Instrumental Analysis Laboratory of the Scientific and Technical Research Council of Turkey in Ankara.

The HPLC analyses of ibuprofen and the (*S*)-2-(4-isobutylphenyl)-*N*-[2-(pyrrolidin-1-yl)ethyl]propanamide (4f) derivative were performed on an Agilent 1100 series LC spectrometer containing a quaternary pump, multiple wave length UV detector equipped with a C-18 reverse phase column (μ-Bondapak). HPLC-grade solvents were used for HPLC analyses. The mobile phase was prepared by dissolving 500 mg of NaH_2PO4 in 150mL of water and 850 mL of methanol in a one-liter volumetric flask and filtered through 0.2 μm Whatmann filter prior to use. The flow rate was 1mL/min and the eluent was monitored at 275 nm using the detector. Naproxen was used as an internal standard.

General synthesis for amide derivatives

To the ice-cooled solution of (*S*)-ketoprofen or (*S*)-ibuprofen (22 mmol) and *N*-hydroxysuccinimide (28 mmol) in tetrahydrofuran (THF) (20 mL) were added equimolar (28 mmol) *N*,*N'*-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) in 20 mL dichloromethane (DCM). The mixture was stirred for 2 hours at 0°C, and refrigerated overnight for the total precipitation of dicyclohexylurea (DCU). After filtering DCU, the solution was concentrated in a vacuum. The leftover was washed with a $NaHCO_3$ solution, extracted with DCM and the separated DCM phase was evaporated. The residue was solidified with ether and crude precipitates of either (*S*)-2,5-dioxopyrrolidin-1-yl 2-(3-benzoylphenyl)propanoate **(1)** or (*S*)-2,5-dioxopyrrolidin-1-yl 2-(4-isobutylphenyl)propanoate **(2)** crystallized from ethanol-water. Then equimolar appropriate ami-

nes were added to the solution of compounds 1 or 2 (0.01 mol) dissolved in the mixture of THF and DCM (20:10 mL), and refluxed for 30 min. The precipitates of the derivatives were filtered directly or the mixture was evaporated and triturated with diethyl ether, crystallized from 1,4-dioxane-water.



R_1: Phenyl, pyridin-2-yl, piperidin-1-yl, morpholin-4-yl, 1-methylpyrrolidin-2-yl, pyrrolidin-1-yl

Scheme 1: Synthetic pathway followed for the preparation of (*S*)-2-(4-isobutylphenyl) and (*S*)-2-(3-benzoylphenyl)-*N*-[2-(aryl/heteroaryl substituted)ethyl]propanamide derivatives (Compounds 3a-f &4 a-f)

Reagents and conditions: (a) DCC, DMAP, DCM, 0 °C; 2h, 4 °C; overnight; conc. $NaHCO_3$ (b) THF/DCM, reflux

(*S*)-2-(3-benzoylphenyl)-*N*-phenethylpropanamide (Compound 3a)

Yield 89%, m.p. 150-151 °C. IR (KBr) ν (cm^{-1}): 1655 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.28 (d, 3H, CH_3), 2.74 (t, 2H, CH_2-Ph.), 3.21 (t, 2H, CH_2-NH), 3.52 (q, 1H, CH-CO), 7.1-7.8 (m, 14H, Ph. H), 8.2 (s, 1H, NH). MS 357.2 (M^+). Anal. calcd for $C_{24}H_{23}NO_2$; C, 80.64; H, 6.49; N, 3.92 Found; C, 80.68; H, 6.45; N, 3.96.

(*S*)-2-(3-benzoylphenyl)-*N*-[2-(pyridin-2-yl)ethyl]propanamide (Compound 3b)

Yield 86 %, m.p. 135-136 °C. IR (KBr) ν (cm^{-1}): 1651 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.28 (d, 3H, CH_3), 3.14 (t, 2H, CH_2-Py.), 3.46-3.53 (m, 3H, CH-CO and CH_2-NH), 7.20-8.00 (m, 12H, Ph. H and Py. H), 8.2 (s, 1H, NH), 8.45 (d, 1H, Py. H). MS 358.2 (M^+). Anal. calcd for $C_{23}H_{22}N_2O_2$; C, 77.07; H, 6.19; N, 7.82 Found; C, 77.04; H, 6.15; N, 7.85.

(*S*)-2-(3-benzoylphenyl)-*N*-[2-(piperidin-1-yl)ethyl]propanamide (Compound 3c)

Yield 85 %, m.p. 147-148 °C. IR (KBr) ν (cm^{-1}): 1653 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.28 (d, 3H, CH_3), 1.53-1.59 (m, 6H, Pip. H), 2.45 (t, 4H, Pip. H), 2.62 (t, 2H, CH_2-Pip.), 3.30 (t, 2H, CH_2-NH), 3.52 (q, 1H,

CH-CO), 7.40-7.80 (m, 9H, Ph. H), 8.0 (s, 1H, NH). MS 364.2 (M^+). Anal. calcd for $C_{23}H_{28}N_2O_2$; C, 75.79; H, 7.74; N, 7.69 Found; C, 75.76; H, 7.77; N, 7.67.

(*S*)-2-(3-benzoylphenyl)-*N*-[2-(morpholin-4-yl)ethyl]propanamide (Compound 3d)

Yield 78 %, m.p. 143-144 °C. IR (KBr) ν (cm^{-1}): 1650 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.28 (d, 3H, CH_3), 2.41 (t, 4H, Mor. H), 2.53 (t, 2H, CH_2-Mor.), 3.30 (t, 2H, CH_2-NH), 3.52 (q, 1H, CH-CO), 3.65 (t, 4H, Mor. H), 7.3-7.60 (m, 9H, Ph. H), 8.0 (s, 1H, NH). MS 366.2 (M^+). Anal. calcd for $C_{22}H_{26}N_2O_3$; C, 72.11; H, 7.15; N, 7.64 Found; C, 72.09; H, 7.11; N, 7.66.

(2*S*)-2-(3-benzoylphenyl)-*N*-[2-(1-methylpyrrolidin-2-yl)ethyl] propanamide (Compound 3e)

Yield 89 %, m.p. 159-160 °C. IR (KBr) ν (cm^{-1}): 1653 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.28 (d, 3H, CH_3), 1.40-1.60 (m, 6H, CH_2-Pyr and Pyr. H), 2.20-2.30 (m, 3H, Pyr. H), 2.32 (s, 3H, N-CH_3), 3.20 (t, 2H, CH_2-NH), 3.52 (q, 1H, CH-CO), 7.30-7.60 (m, 9H, Ph. H), 8.0 (s, 1H, NH). MS 364.2 (M^+). Anal. calcd for $C_{23}H_{28}N_2O_2$ Calc; C, 75.79; H, 7.74; N, 7.69 Found; C, 75.77; H, 7.78; N, 7.71.

(*S*)-2-(3-benzoylphenyl)-*N*-[2-(pyrrolidin-1-yl)ethyl]propanamide (Compound 3f)

Yield 80 %, m.p. 158-159 °C. IR (KBr) ν (cm^{-1}): 1650 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 1.28 (d, 3H, CH_3), 1.68 (t, 4H, Pyr. H), 2.25 (t, 4H, Pyr. H), 2.62 (t, 2H, CH_2-Pyr.), 3.30 (t, 2H, CH_2-NH), 3.52 (q, 1H, CH-CO), 7.30-7.60 (m, 9H, Ph. H), 8.2 (s, 1H, NH). MS 350.2 (M^+). Anal. calcd for $C_{22}H_{26}N_2O_2$; C, 75.40; H, 7.48; N, 7.99 Found; C, 75.37; H, 7.46; N, 8.02.

(*S*)-2-(4-isobutylphenyl)-*N*-phenethylpropanamide (Compound 4a)

Yield 91 %, m.p. 148-149 °C. IR (KBr) ν (cm^{-1}): 1651 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 0.84 (d, 6H, CH_3), 1.28 (d, 3H, CH_3), 1.82 (m, 1H, $(CH_3)_2$-CH), 2.43 (d, 2H, CH-CH_2), 2.74 (t, 2H, CH_2-Ph.), 3.21 (t, 2H, CH_2-NH), 3.52 (q, 1H, CH-CO), 7.0–7.3 (m, 9H , Ph. H), 7.9 (s, 1H, NH). MS 309.2 (M^+). Anal. calcd for $C_{21}H_{27}NO$; C, 81.51; H, 8.79; N, 4.53. Found; C, 81.48; H, 8.77; N, 4.56.

(*S*)-2-(4-isobutylphenyl)-*N*-[2-(pyridin-2-yl)ethyl]propanamide (Compound 4b)

Yield 88 %, m.p. 133-134 °C. IR (KBr) ν (cm-1): 1652 (C=O, amide). ^{1}H-NMR (DMSO-d6, 400 MHz), δ (ppm): 0.87 (d, 6H, CH_3), 1.28 (d, 3H, CH_3), 1.82 (m, 1H, $(CH_3)_2$-CH), 2.43 (d, 2H, CH-CH_2.), 3.14 (t, 2H, CH_2-Py.), 3.21 (t, 2H, CH_2-

NH), 3.52 (q, 1H, CH-CO), 7.1–7.24 (m, 4H , Ph. H), 7.26-7.30 (m, 2H, Py. H), 7.58 (t, 1H, Py. H), 8.0 (s, 1H, NH), 8.4 (d, 1H, Py. H). MS 310.2 (M^+). Anal. calcd for $C_{20}H_{26}N_2O$; C, 77.38; H, 8.44; N, 9.02 Found; C, 77.36; H, 8.41; N, 9.04.

(*S*)-2-(4-isobutylphenyl)-*N*-[2-(piperidin-1-yl)ethyl]propanamide (Compound 4c)

Yield 84 %, m.p. 145-146 °C. IR (KBr) ν (cm^{-1}): 1654 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 0.87 (d, 6H, CH_3), 1.28 (d, 3H, CH_3), 1.52-1.55 and 2.24 (m and t, 6H and 4H, Pip. H), 1.82 (m, 1H, $(CH_3)_2$-CH), 2.43 (d, 2H, CH-CH_2), 2.62 (t, 2H, CH_2-Pip.), 3.30 (t, 2H, CH_2-NH, 3.52 (q, 1H, CH-CO), 7.1–7.3 (m, 4H, Ph. H), 8.0 (s, 1H, NH). MS 316.3 (M^+). Anal. calcd for $C_{20}H_{32}N_2O$; C, 75.90; H, 10.19; N, 8.85 Found; C, 75.92; H, 10.17; N, 8.83.

(*S*)-2-(4-isobutylphenyl)-*N*-[2-(morpholin-4-yl)ethyl]propanamide (Compound 4d)

Yield 75 %, m.p. 141-142 °C. IR (KBr) ν (cm^{-1}): 1653 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 0.87 (d, 6H, CH_3), 1.28 (d, 3H, CH_3), 1.82 (m, 1H, $(CH_3)_2$-CH), 2.41 (t, 4H, Mor. H), 2.43 (d, 2H, CH-CH_2), 2.62 (t, 2H, CH_2-Mor.), 3.30 (t, 2H, CH_2-NH), 3.52 (q, 1H, CH-CO), 3.65(t, 4H Mor. H), 7.1–7.3 (m, 4H, Ph. H), 8.0 (s, 1H, NH). MS 318.2 (M^+). Anal. calcd for $C_{19}H_{30}N_2O_2$; C, 71.66; H, 9.50; N, 8.80 Found; C, 71.65; H, 9.53; N, 8.81.

(2*S*)-2-(4-isobutylphenyl)-*N*-[2-(1-methylpyrrolidin-2-yl)ethyl] propanamide (Compound 4e)

Yield 86 %, m.p. 154-155 °C. IR (KBr) ν (cm^{-1}): 1654 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 0.87 (d, 6H, CH_3), 1.28 (d, 3H, CH_3), 1.40-1.60 (m, 6H, CH_2-Pyr. and Pyr. H), 1.82 (m, 1H, $(CH_3)_2$-CH), 2.20-2.30 (m, 6H, N-CH_3 and Pyr. H), 2.43 (d, 2H, CH-CH_2.), 3.20 (t, 2H, CH_2-NH), 3.52 (q, 1H, CH-CO), 7.1–7.3 (m, 4H, Ph. H), 8.0 (s, 1H, NH). MS 316.3 (M^+). Anal. calcd for $C_{20}H_{32}N_2O$; C, 75.90; H, 10.19; N, 8.85 Found; C, 75.87; H, 10.14; N, 8.88.

(2*S*)-2-(4-isobutylphenyl)-*N*-[2-(pyrrolidin-1-yl)ethyl]propanamide (Compound 4f)

Yield 82 %, m.p. 156-157 °C. IR (KBr) ν (cm^{-1}): 1650 (C=O, amide). ^{1}H-NMR (DMSO-d_6, 400 MHz), δ (ppm): 0.87 (d, 6H, CH_3), 1.28 (d, 3H, CH_3), 1.68 (t, 4H, Pyr. H), 1.82 (m, 1H, $(CH_3)_2$-CH), 2.43 (d, 2H, CH-CH_2), 2.50 (t, 4H, Pyr. H), 2.62 (t, 2H, CH_2-Pyr.), 3.30 (t, 2H, CH_2-NH, 3.52 (q, 1H, CH-CO), 7.1–7.3 (m, 4H, Ph. H), 8.0 (s, 1H, NH). MS 302.3 (M^+). Anal. calcd for $C_{19}H_{30}N_2O$; C, 75.45; H, 10.00; N, 9.26 Found; C, 75.41; H, 9.97; N, 9.30.

Pharmacological Screening

Animals

Male Swiss albino mice (25–30 g) and male Wistar rats weighing 160-200 g. were purchased from the animal breeding laboratories of Refik Saydam Hıfzısıhha Institute in Ankara, Turkey. For acclimatization, the animals were housed in a room with controlled temperature (22± 1 °C), humidity (55 ± 10%) and photoperiod (12:12 h) for one week. They were maintained on a standard pellet diet and water ad libitum throughout the experiment. The food was withheld on the day before the experiments only allowing free access to water. A minimum of six animals was used in each group for the examination of anti-inflammatory, analgesic and gastric ulcerogenic effects and only two animals were sacrificed for liver extracts. All the animal manipulations and experiments were carried out according to the rules and approval of the Ethical Committee for the use and care of laboratory animals of Gazi University, Ankara, Turkey.

Anti-inflammatory activity

The carrageenan-induced hind paw edema model was used to determine the anti-inflammatory activity[42]. Each group contained a minimum of six animals. Sixty min after the subcutaneous administration of a test sample (100 mg/kg body weight suspended in 0.5 % sodium carboxymethyl cellulose (CMC)) or dosing vehicle, each mouse was injected with a freshly prepared suspension of carrageenan (0.5 mg/25 µL) in physiological saline into the sub-plantar tissue of the right hind paw. As a control, 25 µL of saline was injected into the same tissue on the left side. Paw edema was measured 90, 180, 270, and 360 min after the induction of inflammation. The difference between the thicknesses of the right and left hind paws were measured using a caliber compass (Ozaki Co., Tokyo, Japan). The mean values obtained for each study group were compared with the control group and analyzed using statistical methods.

Analgesic activity

Analgesic activity was measured using phenyl-*p*-benzoquinone (PBQ)-induced writhing (abdominal constriction) test in mice[43]. According to the protocol, 30 min after the subcutaneous administration of a test sample (100 mg/kg body weight), the mice were intraperitoneally injected with 0.1 mL/10 g body weight of 2.5% (w/v) PBQ solution in distilled water. The control animals received an appropriate volume of the dosing vehicle. The mice were then kept individually for observation, and the total number of abdominal contractions (writhing movements) was counted for the next 15 min starting on the 5th minute after the PBQ injection. The data represent the average of the total number of writhing

movements observed. Analgesic activity was then expressed as the change in percentage compared to the writhing controls.

Gastric ulcerogenic effect

The ulcerogenic effect was investigated as described in a previous publication[44]. The animals were sacrificed with an overdose of diethyl ether 270 min after the administration of the compounds. Following abdominal dissection, the stomachs of the animals were taken out. Then, the esophagus was tied in a knot near to the cardia by a surgical suture. From the duodenum side, 2.5 mL of a 10% formalin solution was injected into the stomach. The distended stomach was immediately tied to the pyloric sphincter using another surgical suture to avoid leakage of the formalin solution. Finally, the stomachs were removed from the abdominal cavity and immersed in the same solution to fix the outer layer of the stomach. Each stomach was then dissected along the greater curvature, rinsed with tap water to remove the gastric contents and examined under a dissecting microscope to assess the formation of ulcers. Lesions and bleeding points were counted and documented.

Statistical analysis

The data were expressed as means ± SEM. The significance of differences between the treatment and control groups were determined using one-way ANOVA with Bartlett's test following a post hoc Student-Newman-Keuls multiple comparison test for analgesic activity, and two-way ANOVA following a post hoc Bonferroni test for anti-inflammatory activity. Values of $p<0.05$ were considered statistically significant.

Hydrolysis Studies

The most active compound, 4f, was analyzed for its hydrolysis behavior in an acidic buffer (simulated gastric fluid, pH 1.2), basic buffer (simulated intestine fluid, pH 7.4), 80% human plasma and 10% rat liver homogenate.

Acidic and basic buffers (pH 1.2 and 7.4)

In a 10 mL capacity volumetric flask, accurately weighed amount of compound 4f (10 mg) was dissolved in 5 mL methanol and kept in a bath at a constant temperature of 37°C for 10 min. The contents were then transferred to a vessel of dissolution apparatus containing 995 mL of 0.1N hydrochloric acid buffer (pH 1.2) or phosphate buffer (pH 7.4). The vessels were stirred continuously at 100 rpm and aliquots of 10 mL were withdrawn at selected time intervals of 5, 30, 60, 120, 180, 240, 300, 360, 420, 480, 560 and 600 minutes, immediately followed by the addition of an equal aliquot of fresh 0.1N HCl (pH 1.2) or phosphate

buffer (pH 7.4). The aliquots withdrawn were extracted thrice with 5 mL chloroform. The organic phases were mixed and washed thrice with distilled water (3 mL). The water extracts were discarded. The organic phase was evaporated to dryness. The residue was dissolved and diluted with the mobile phase. 20 μL of this solution was directly injected into HPLC for analysis.

80% v/v human plasma (pH 7.4)

In a 10 mL capacity volumetric flask, accurately weighed amount of compound 4f (10 mg) was dissolved in 5 mL methanol and kept in a bath at a constant temperature of 37 °C for 10 min. The content was transferred to a 250 mL beaker containing 95 mL of 80% v/v human plasma (pH 7.4) and stirred continuously. Aliquots of 2 mL were withdrawn at various time intervals, immediately followed by the addition of equal aliquots of 80% v/v human plasma (pH 7.4). The samples were shaken and centrifuged for 10 min. The amount of compound in the supernatant liquid was determined by HPLC.

10% w/v rat liver homogenate (pH 7.4)

The Wistar rats were sacrificed by cervical dislocation, and the liver was removed, washed and chopped. A 10% w/v suspension of the liver was prepared in a phosphate buffer (pH 7.4). The liver was homogenized using a tissue homogenizer to be used for hydrolysis. Compound 4f (10 mg) was dissolved in 5mL methanol in a 10 mL volumetric flask and kept in a bath at a constant temperature of 37°C for 10 min. Then, the content of the flask was transferred to a 250 mL beaker containing 95 mL of 10% w/v rat liver homogenate (pH 7.4). The beaker was kept on a rotating shaker (60 rpm) at 37 °C, and aliquots of 2 mL were withdrawn at various time intervals, immediately followed by the addition of equal aliquots of 10% w/v rat liver homogenate. The samples were shaken and centrifuged for 10 min. The amount of compound in the supernatant liquid was determined by HPLC.

RESULTS AND DISCUSSION

The proposed *N*-(2-substitutedethyl)propanamide derivatives of (*S*) ketoprofen and (*S*) ibuprofen were successfully synthesized using the conventional DCC/DMAP method giving yields between 75-91%. In the IR spectra, all the compounds had a strong C=O stretching band at 1650–1655 cm^{-1}, which was accepted as an evidence for the formation of amide bond. The ^{1}H-NMR spectra of compounds showed that the phenyl protons belonging to ketoprofen and ibuprofen have been exhibited at δ 7.10–7.70 ppm as a multiplet and sometimes in aromatic region together with other aromatic groups attached to the other side of the molecules. The protons of the third carbon forming the propanamide moiety for

ketoprofen and ibuprofen are at δ 1.28 and 1.52 ppm, respectively as doublets. All the other protons were observed according to the expected chemical shift and integral values.

The mass spectroscopic fragmentation of the compounds was studied under electron ionization. Molecular ion peaks (M^+) confirmed the molecular weights of the compounds examined. The fragmentation pattern was essentially identical. In the mass spectra of the compounds, the following were detected; basic fragmentation peaks for parent ketoprofen such as m/z 209, 105, 77 with the loss of functional groups such as amide, phenethyl, carbonyl moieties and for parent ibuprofen, m/z 189, 175, 148, 133, 134 with the loss of functional groups such as amide moieties, terminal methyl remaining in the propionyl, terminal methyls in isobutyl, isobutyl with the ring system, and the isobutyl itself remained.

The parent NSAIDs and their corresponding ester amide derivatives (3a-f and 4a-f) were also evaluated for their in vivo systemic anti-inflammatory activity using carrageenan-induced paw edema in mice at a dose of 100 mg per kg body weight. During this evaluation, the compounds with similar functional groups such as 3b, 3d-f and 4b, 4d-f were found to be equal in terms of potency or more potent than parent compounds in the same time-dependent manner. These compounds were further evaluated to investigate their analgesic activity and gastric ulcerogenic effect (Table 1).

Animals were administered the selected test compounds at a dose of 100 mg per kg body weight. Table 2 presents the percentage of analgesic activity by means of inhibition of writhing movements in comparison with parent NSAIDs. Compound 3f had similar analgesic activity to ketoprofen whereas compounds 4e-f demonstrated significantly better activities than their parent compound. These results indicate that both parent NSAIDs showed a measurable ulcerogenic index in at least one animal after the subcutaneous administration of 100 mg per kg doses. None of the amide prodrugs caused any gastric mucosal lesions nor bleeding points in the gastric mucosa.

Compound 4f with the highest anti-inflammatory and analgesic activity was further evaluated for its chemical hydrolysis behaviors in simulated gastric fluid (acidic buffer, pH 1.2), simulated intestine fluid (basic buffer, pH 7.4). In addition, to explore its potential as a prodrug in various biological systems, the enzymatic hydrolysis behaviors of this compound were observed in 80% v/v human plasma and 10% w/v rat liver homogenate. The expected experimental response was the release of its parent compound as evident by the HPLC analysis. Negligible hydrolysis was observed in acidic (pH 1.2) and basic buffers, and in 80% v/v human plasma resembling a linear decrease similar to the first-order kinetic

model. The rate of conversion to the parent drug was between 8 to 15 %, which indicated that the synthesized ester and amide derivatives were sufficiently stable throughout the period of experiments.

A similar but sharper linear decrease was observed during the enzymatic hydrolysis experiment performed with 10% w/v rat liver homogenate. The rate of conversion to the parent drug ranged from 52 to 86 % after 120 and 600 min, respectively. The results of the experiments clearly show that the hydrolyzing enzymes of the liver play a much more significant role than plasma enzymes in changing these amide derivatives to their parent compounds.

Another significant result was that all the active compounds were able to inhibit the change in paw volume after carrageenan injection, which demonstrates their anti-inflammatory action. In addition, most compounds were found to be more active than the parent drugs, indicating that amidification with these NSAIDs maintains or even improves the analgesic activity.

Compared to the chemical stability studies, the synthesized amide derivative was sufficiently stable at different pH levels of the stomach and intestine. Furthermore, it was stable against enzymatic hydrolysis of plasma constituents, releasing the parent drug with degradation in the liver.

It is well evidenced that direct contact or indirect mechanisms play a major role in the production of gastrointestinal lesions following the administration of NSAIDs, and designing amide and ester derivatives can be a solution for these lesions. Furthermore, these derivatives may lead to the development of new and potent non-ulcerogenic anti-inflammatory and analgesic agents with potential clinical applications.

Table 1: Effect of compounds 3a-f and 4a-f at a dose of 100 mg per kg dose against carrageenan-induced hind paw edema in mice.

Compound	Swelling in thickness [$\times 10^{-2}$ mm] (Inhibitory percentage)			
	90 min	180 min	270 min	360 min
Control	47.6±5.4	55.0±5.4	58.6±4.1	64.8±4.0
3a	35.0±4.0 (26.5)	42.8±4.1 (22.2)	43.5±2.6 (25.8)	51.8±2.4 (20.1)*
3b	33.3±6.9 (30.0)	39.8±7.0 (27.6)	42.3±7.4 (27.8)	44.5±5.6 (31.3)*
3c	33.8±3.5 (28.9)	39.8±3.7 (27.6)	42.5±3.9 (27.5)	46.5±2.7 (28.2)
3d	31.3±4.1 (34.2)	35.5±4.2 (35.5)	37.8±4.8 (35.5)*	42.5±5.6 (34.4)**
3e	32.0±5.0 (32.8)	36.3±4.8 (34.0)	40.5±5.6 (30.9)	46.5±5.2 (28.2)*
3f	29.0±5.3 (38.4)	33.0±3.8 (40.0)*	37.0±3.5 (36.9)*	41.3±4.9 (36.3)**
4a	35.3±3.9 (25.8)	40.0±3.8 (27.3)	45.5±3.5 (22.4)	51.0±3.0 (21.3)*
4b	33.8±3.5 (28.9)	38.3±3.6 (30.4)	40.5±5.1 (30.9)**	45.0±5.6 (30.6)*
4c	34.3±4.8 (27.9)	38.3±4.8 (30.4)	42.0±4.4 (28.3)	46.3±4.0 (28.5)*
4d	26.8±5.5 (43.7)	33.0±4.8 (40.0)*	35.5±3.9 (39.4)*	38.5±3.7 (40.6)**
4e	32.5±4.0 (31.7)	37.0±3.9 (32.7)	40.0±4.1 (31.7)	44.8±4.5 (30.9)*
4f	25.3±3.4 (46.8)	30.0±3.3 (45.5)**	34.3±3.6 (41.5)	38.8±3.9 (40.1)**
Ketoprofen	34.1±4.0 (28.3)	38.4±4.3 (29.8)	38.6±3.8 (34.1)	40.4±3.1 (37.5)*
Ibuprofen	29.5±2.9 (38.2)	33.6±3.3 (38.9)	35.4±2.9 (39.5)*	37.4±2.8 (42.2)**

Table 2: Analgesic effects of compounds 3a-f and 4a-f against PBQ-induced writhings in mice and ulcer scores.

Compound	Number of writhing ± SEM	% Inhibition	Ulcer score
Control	44.2±1.8		
3b	22.3±3.0**	49.5	0/6
3d	22.8±2.1**	48,1	0/6
3e	21.1±2.3**	52.2	0/6
3f	18,9±1.3***	57.2	0/6
4b	22.3±1.3***	49.7	0/6
4d	23.3±3.0**	47.3	0/6
4e	14.0±1.6***	68.3	0/6
4f	12.3±1.9***	72.2	0/6
Ketoprofen	17.5±2.6***	60.4	1/6
Ibuprofen	16.0±1.5***	63.8	1/6

**p<0.01, ***p<0.001 significant from the control

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Synthesis of Novel 3, 5, 6-trisubstituted triazine Derivatives and Their Biological Activity Evaluation as Potential Antitumor and Anti-inflammatory Agents

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ABSTRACT

In this study, new 3, 5, 6-trisubstituted 1, 2, 4-triazine derivatives (**1-9**) were synthesized and their structures were determined by using NMR, IR and Mass spectroscopic methods. *In vitro* antitumor activities against MCF-7 breast adenocarcinoma and C6 rat glioma cell lines were evaluated via MTT colorimetric assay. Among the compounds, compound **4** (IC_{50}=21.0 μg/mL) was found as the most active one against C6 cell line, whereas compound **5** (IC_{50}=9.5 μg/mL) was found the most potent compound against MCF-7 cell line and both of compounds had higher activity than cisplatin in their line. Furthermore, IC_{50} value of compound **6** was found as 26.0 μg/mL against C6 which was very close to cisplatin potency (IC_{50}=23.5 μg/mL). Besides, all compounds were tested to determine their lipoxygenase (LOX) inhibitory activity. Compounds **1** and **6** showed LOX inhibition with percentages of 43.35% and 38.79% at 100 μg/mL concentration, respectively. The obtained results on cell lines inspire to synthesise new and more potent molecules compounds as anticancer agents.

Keywords: Triazine, cytotoxicity, antitumor, lipooxygenase (LOX) inhibition

INTRODUCTION

Together cardiovascular diseases, cancer is one of the common causes of death. By the discovery of mechanisms of cancer, efforts focused on different targets to treat disease. Besides the new techniques like hyperthermia, photodynamic therapy and stem cell transplation; old techniques radiotherapy, hormonal and non-hormonal chemotherapy, immunotherapy and surgery methods is still be-

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ing used[1]. In chemotherapy, alkylation agents, mitotic inhibitors and antimetabolites are used either *alone* or in *combination* with each other. In simple terms, a selective drug must be active on cancer cells but inactive in normal cells. But the differences between normal and cancer cells is quite little and not qualitative so that its hard to find an exploitable mechanism[2].

Purine and pyrimidine analog drugs such as 5-fluorouracil and 6-mercaptopurine have been widely used in succesful treatment of cancer disease[3]. Many nitrogen containing heterocylic compounds such as 1,2,4-triazines have been constitutes medicinal chemists' interest area in cancer theraphy[4,5]. The potential anticancer-cytostatic effects of 1,2,4-triazine derivatives were widely studied and reported to have promising activity[6-12].

5-Lipoxygenase (5-LOX) is a crucial enzyme which catalyses biosynthesis of leukotrienes in the arachidonic acid (AA) cascade. Variable leukotriene levels were reported in different diseases like cardiovascular diseases and certain types of cancer as well as asthma, allergic rhinitis[13]. In particular, 5-LOX have been found to be up-regulated in many cancer cell lines, which results in promoting the development of carcinogenesis. Inhibition of LOX enzyme could be a subsidiary mechanism for down-regulation of tumors accordingly this approach enables a rational concept for the design of more effective antitumor agents[14].

Accordingly, we aimed to synthesize 1-[4-(5,6-bis-(4-substituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1*H*-(benz)imidazol/thiazole/oxazole-2-yl)thio] ethan-1-one derivatives (**1-9**) for evaluating anticancer activity by following a study reported before a number of derivatives with similar structures[15]. The structure elucidation was carried out by spectroscopic techniques and *in vitro* anticancer activities of compounds were evaluated using MTT technique on MCF-7 and C6 tumor cell lines. The lipooxygenase (LOX) enzyme inhibitory activiy of the compounds were also studied.

METHODOLOGY

Chemistry

Synthesis and characterization

All needed chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, MO, USA). All melting points (m.p.) were determined by MP90 digital melting point apparatus (Mettler Toledo, OH) and were uncorrected. All the reactions were monitored by thin-layer chromatography (TLC) using Silica Gel 60 F254 TLC plates (Merck KGaA, Darmstadt, Germany). Spectroscopic data were recorded with the following instruments: a Bruker Tensor 27 IR spectrophotometer; ^{1}H NMR (nuclear magnetic resonance) Bruker DPX- 300 FT-NMR

spectrometer, ^{13}C NMR, Bruker DPX 75 MHz spectrometer (Bruker Bioscience, Billerica, MA, USA); M+1 peaks were determined by Shimadzu LC/MS ITTOF system (Shimadzu, Tokyo, Japan). The synthesis of three intermediate products, 2-chloro-1-[4-(5,6-bis-(4-substituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl] ethanones (**IIIa-c**) was carried out by the following study of Demirayak and co-workers[15].

General procedure for the synthesis of the final compounds (1-9)

Equal moles of halogenated compounds (**IIIa-c**), 2-mercapto(benz)imidazole/ thiazole/oxazole and potassium carbonate were stirred in acetone for 5 h. At the end of the reaction, the solvent was evaporated and the residue was treated with water. The obtained precipitation was filtered and it recrystallized from ethanol after dryness.

To realize the characterization and identification of compounds, Infrared-^{1}H-NMR, ^{13}C-NMR and mass spectroscopy methods were used. Melting points were determined by using stuart melting apparatus. Yields and elemental analysis also calculated for each compound.

1-[4-(5,6-Bis-(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzimidazol-2-yl)thio]ethan-1-one (1):

75 % yield; mp 248 °C. IR ν_{max} (cm^{-1}): 1639 (C=O), 1684 (amide C=O), 1525-1379 (C=C, C=N), 1269-1051 (C-N). ^{1}H-NMR (300 MHz, DMSO-$d_{6,}$ ppm) δ 2.31 (s, 6H, CH_3), 3.67 (brs, 2H, piperazine CH_2), 3.78 (brs, 2H, piperazine CH_2), 3.92 (brs, 2H, piperazine CH_2), 4.04 (brs, 2H, piperazine CH_2), 4.50 (s, 2H, CH_2CO), 7.09-7.13 (m, 2H, Ar-H), 7.17 (d, J= 8.07 Hz, 2H, Ar-H), 7.27 (d, J= 8.13 Hz, 2H, Ar-H), 7.37 (d, J=8.13 Hz, 2H, Ar-H), 7.44 (brs, 2H, Ar-H), 12.58 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-$d_{6,}$ ppm) δ 21.28 (CH_3), 21.38 (CH_3), 35.59 ($COCH_2$), 41.78 (CH_2), 43.38 (CH_2), 43.68 (CH_2), 45.53 (CH_2), 121.83, 129.21, 129.35, 129.41, 129.87, 133.76, 133.96, 138.11, 140.68, 148.95, 150.22, 155.64, 159.60, 166.46 (C=O). For $C_{30}H_{29}N_7OS$ HRMS (*m/z*): $[M+H]^+$ calcd: 534.67; found 536.22.

1-[4-(5,6-Bis-(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzoxazol-2-yl)thio]ethan-1-one (2):

72 % yield; mp 135 °C. IR ν_{max} (cm^{-1}): 1651 (C=O), 1533-1381 (C=C, C=N), 1219-1068 (C-N, C-O). ^{1}H-NMR (300 MHz, DMSO-$d_{6,}$ ppm) δ 2.32 (s, 6H, CH_3), 3.69 (brs, 2H, piperazine CH_2), 3.77 (brs, 2H, piperazine CH_2), 3.94 (brs, 2H, piperazine CH_2), 4.06 (brs, 2H, piperazine CH_2), 4.66 (s, 2H, CH_2CO), 7.17 (d, J= 7.71 Hz, 4H, Ar-H), 7.26-7.35 (m, 6H, Ar-H), 7.63-7.7.68 (m, 2H, Ar-H). ^{13}C-NMR (75 MHz, DMSO-$d_{6,}$ ppm) δ 21.29 (CH_3), 21.38 (CH_3), 36.98 ($COCH_2$), 41.89 (CH_2),

43.34 (CH_2), 43.63 (CH_2), 45.46 (CH_2), 110.68, 118.68, 124.75, 125.11, 129.22, 129.36, 129.42, 129.87, 133.76, 133.95, 138.13, 140.69, 141.76, 148.98, 151.67, 155.66, 159.60, 164.56, 165.54 (C=O). For $C_{30}H_{28}N_6O_2S$ HRMS (*m/z*): $[M+H]^+$ calcd: 537.65; found 537.20.

1-[4-(5,6-Bis-(4-methylphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzothiazol-2-yl)thio]ethan-1-one (3):

74 % yield; mp 130 °C. IR ν_{max} (cm^{-1}): 1647 (C=O), 1525-1379 (C=C, C=N), 1238-1001 (C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 2.32 (s, 6H, CH_3), 3.69 (brs, 2H, piperazine CH_2), 3.80 (brs, 2H, piperazine CH_2), 3.94 (brs, 2H, piperazine CH_2), 4.06 (brs, 2H, piperazine CH_2), 4.64 (s, 2H, CH_2CO), 7.17 (d, J= 8.22 Hz, 4H, Ar-H), 7.27 (d, J= 8.13 Hz, 2H, Ar-H), 7.34-7.7.40 (m, 3H, Ar-H), 7.46 (t, J=7.10 Hz, Ar-H), 7.85 (d, J=7.83 Hz, 1H, Ar-H), 8.02 (d, J=7.86 Hz, 1H, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 21.29 (CH_3), 21.38 (CH_3), 37.14 ($COCH_2$), 41.89 (CH_2), 43.41 (CH_2), 43.71 (CH_2), 45.55 (CH_2), 121.52, 122.29, 124.93, 126.84, 129.21, 129.36, 129.42, 129.88, 133.75, 133.96, 135.20, 138.13, 140.70, 148.97, 153.09, 155.64, 159.60, 165.75, 166.69 (C=O). For $C_{30}H_{28}N_6OS_2$ HRMS (*m/z*): $[M+H]^+$ calcd: 553.72; found 553.18.

1-[4-(5,6-Bis-(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzimidazol-2-yl)thio]ethan-1-one (4):

78 % yield; mp 243 °C. IR ν_{max} (cm^{-1}): 1647 (C=O), 1608-1325 (C=C, C=N), 1244-1024 (C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 3.67 (brs, 2H, piperazine CH_2), 3.77 (brs, 8H, OCH_3 and piperazine CH_2), 3.91 (brs, 2H, piperazine CH_2), 4.03 (brs, 2H, piperazine CH_2), 4.50 (s, 2H, CH_2CO), 6.91-6.96 (m, 4H, Ar-H), 7.10-7.13 (m, 2H, Ar-H), 7.32 (d, J= 8.79 Hz, 2H, Ar-H), 7.44-7.48 (m, 4H, Ar-H), 12.57 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 35.58 ($COCH_2$), 41.79 (CH_2), 43.39 (CH_2), 43.70 (CH_2), 45.55 (CH_2), 55.61 (OCH_3), 55.79 (OCH_3), 114.25, 114.34, 121.85, 128.63, 129.26, 130.57, 131.64, 148.65, 150.22, 154.98, 159.52, 159.73, 161.45, 166.46. For $C_{30}H_{29}N_7O_3S$ HRMS (*m/z*): $[M+H]^+$ calcd: 568.67; found 568.21.

1-[4-(5,6-Bis-(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzoxazol-2-yl)thio]ethan-1-one (5):

78 % yield; mp 215 °C. IR ν_{max} (cm^{-1}): 1649 (C=O), 1529-1377 (C=C, C=N), 1244-1026 (C-N, C-O). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 3.69 (brs, 2H, piperazine CH_2), 3.78 (brs, 8H, OCH_3 and piperazine CH_2), 3.92 (brs, 2H, piperazine CH_2), 4.04 (brs, 2H, piperazine CH_2), 4.66 (s, 2H, CH_2CO), 6.92-6.96 (m, 4H, Ar-H), 7.30-7.35 (m, 4H, Ar-H), 7.47 (d, J= 8.85 Hz, 2H, Ar-H), 7.63-7.68 (m, 2H, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 36.96 ($COCH_2$), 41.88 (CH_2),

43.69 (CH_2), 45.44 (CH_2), 55.80 (OCH_3), 110.68, 114.26, 114.34, 118.67, 124.75, 125.11, 128.63, 129.25, 130.57, 131.64, 148.67, 155.0, 159.51, 159.74, 161.46, 165.54 (C=O). For $C_{30}H_{28}N_6O_4S$ HRMS (*m/z*): $[M+H]^+$ calcd: 569.65; found 569.20.

1-[4-(5,6-Bis-(4-methoxyphenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzothiazol-2-yl)thio]ethan-1-one (6):

71 % yield; mp 189 °C. IR ν_{max} (cm^{-1}): 1647 (C=O), 1527-1377 (C=C, C=N), 1244-1008 (C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 3.68 (brs, 2H, piperazine CH_2), 3.77 (brs, 8H, OCH_3 and piperazine CH_2), 3.93 (brs, 2H, piperazine CH_2), 4.04 (brs, 2H, piperazine CH_2), 4.63 (s, 2H, CH_2CO), 6.91-6.96 (m, 4H, Ar-H), 7.31-7.49 (m, 6H, Ar-H), 7.84 (d, J= 7.80 Hz, 2H, Ar-H), 8.02 (d, J=7.32 Hz, 1H, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 37.14 ($COCH_2$), 41.88 (CH_2), 43.41 (CH_2), 43.71 (CH_2), 45.59 (CH_2), 55.61 (OCH_3), 55.80 (OCH_3), 114.25, 114.34, 121.52, 122.29, 124.93, 126.84, 128.63, 129.25, 130.57, 131.64, 148.67, 153.09, 154.98, 159.52, 159.74, 161.46, 165.74, 166.69 (C=O). For $C_{30}H_{28}N_6O_3S_2$ HRMS (*m/z*): $[M+H]^+$ calcd: 585.21; found 585.17.

1-[4-(5,6-Bis-(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzimidazol-2-yl)thio]ethan-1-one (7):

78 % yield; mp 175 °C. IR ν_{max} (cm^{-1}): 1647 (C=O), 1525-1379 (C=C, C=N), 1240-1014 (C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 3.67 (brs, 2H, piperazine CH_2), 3.79 (brs, 2H, piperazine CH_2), 3.94 (brs, 2H, piperazine CH_2), 4.06 (brs, 2H, piperazine CH_2), 4.50 (s, 2H, CH_2CO), 7.10-7.13 (m, 2H, Ar-H), 7.39-7.50 (m, 10H, Ar-H), 12.61 (s, 1H, NH). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 35.56 ($COCH_2$), 43.41 (CH_2), 43.72 (CH_2), 45.51 (CH_2), 122.03, 129.02, 131.19, 131.83, 133.74, 135.23, 135.32, 135.80, 147.80, 150.19, 154.93, 159.63, 166.49 (C=O). For $C_{28}H_{23}Cl_2N_7OS$ HRMS (*m/z*): $[M+H]^+$ calcd: 577.50; found 577.11.

1-[4-(5,6-Bis-(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzoxazol-2-yl)thio]ethan-1-one (8):

78 % yield; mp 242 °C. IR ν_{max} (cm^{-1}): 1651 (C=O), 1527-1379 (C=C, C=N), 1240-1014 (C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 3.69 (brs, 2H, piperazine CH_2), 3.78 (brs, 2H, piperazine19 CH_2), 3.95 (brs, 2H, piperazine CH_2), 4.07 (brs, 2H, piperazine CH_2), 4.66 (s, 2H, CH_2CO), 7.31-7.35 (m, 2H, Ar-H), 7.39-7.42 (m, 4H, Ar-H), 7.46-7.49 (m, 4H, Ar-H), 7.64-7.66 (m, 2H, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 36.95 ($COCH_2$), 41.84 (CH_2), 43.39 (CH_2), 45.30 (CH_2), 110.68, 118.67, 124.75, 125.12, 129.0, 131.19, 131.82, 133.75, 135.22, 135.31, 135.81, 141.76, 147.83, 159.62, 165.58 (C=O). For $C_{28}H_{22}Cl_2N_6O_2S$ HRMS (*m/z*): $[M+H]^+$ calcd: 578.48; found 578.10.

1-[4-(5,6-Bis-(4-chlorophenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1H-benzothiazol-2-yl)thio]ethan-1-one (9):

78 % yield; mp 124 °C. IR ν_{max} (cm^{-1}): 1647 (C=O), 1525-1379 (C=C, C=N), 1240-1014 (C-N). ^{1}H-NMR (300 MHz, DMSO-d_6, ppm) δ 3.69 (brs, 2H, piperazine CH_2), 3.80 (brs, 2H, piperazine CH_2), 3.95 (brs, 2H, piperazine CH_2), 4.07 (brs, 2H, piperazine CH_2), 4.64 (s, 2H, CH_2CO), 7.36-7.39 (m, 2H, Ar-H), 7.42-7.49 (m, 10H, Ar-H), 7.85 (d, J=7.56 Hz, 1H, Ar-H), 8.02 (d, J=8.25 Hz, 1H, Ar-H). ^{13}C-NMR (75 MHz, DMSO-d_6, ppm) δ 37.12 ($COCH_2$), 41.84 (CH_2), 43.42 (CH_2), 43.76 (CH_2), 45.49 (CH_2), 121.53, 121.69, 122.30, 124.94, 126.85, 129.0, 131.19, 131.83, 133.75, 135.32, 135.82, 147.82, 154.92, 159.63, 165.78 (C=O). For $C_{28}H_{22}Cl_2N_6OS_2$ HRMS (*m/z*): $[M+H]^+$ calcd: 594.55; found 594.07.

Biochemistry

Cytotoxicity

For measuring the cytotoxic activity of compounds, MTT method (tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was applied on MCF-7 breast adenocarcinoma and C6 rat glioma cell lines. The instruction in literature was followed to carry out experiments. To produce the cells, cell lines incubated in 96 well-plates for 24 h at 37 °C. Then, 20 μL of MTT solution (5 mg/mL MTT in PBS) was added for each well and incubation was continued for more 2 hours at 37 °C [16,17]. Before measuring the absorbance by ELISA reader (OD 570 nm), dissolving of crystals using DMSO (200 μL) was needed. Absorbance values were read and percentage of survival cells was calculated compared medium. All measurements were measured triplicate[18].

In vitro lipoxygenase (LOX) inhibition assay

LOX inhibition activity was measured by a modified the spectrophotometric method developed by Baylac and Racine[19]. LOX (1.13.11.12, type I-B, Soybean), linoleic acid and all required chemicals were purchased from Sigma-Aldrich. Potassium phosphate buffer (1,94 mL; 100mM; pH 9.0), 40 μL of test compound solution and 20 μL of lipoxygenase solution were mixed and incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 μL linoleic acid solution, the change of absorbance at 234 nm was followed for 10 min. Test compounds and positive control Nordihydroguaiaretic acid (NDGA) were dissolved in methanol. All spectrum measurements were carried out in quartz cuvette avoiding material absorbance interference.

The concentration of test compounds which provided 50% inhibition (IC_{50}) of LOX enzyme was calculated and the experiments were performed triplicate for each concentration. For eliminating faults and non-enzymatic hydrolysis, blanks

were compared without test compounds. Alteration in absorbance was recorded comparingly between with and without test compounds for determining enzyme inhibition activity and the results were given in percentages and standard deviations (SD+) were performed using Microsoft Office Excel 2013 program.[20]

RESULTS AND DISCUSSION

Chemistry

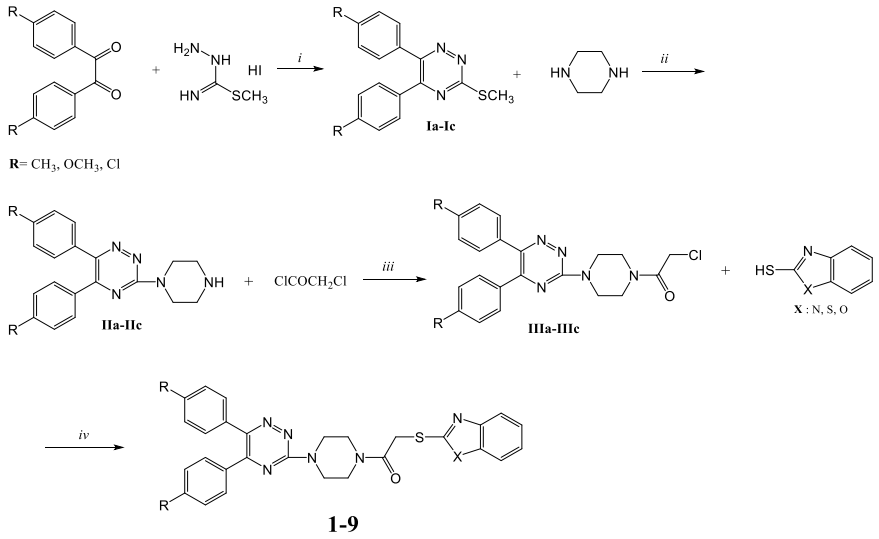
The synthesis of title compounds were carried out by a multi-step synthetic procedure according to previously reported study of us[10]. New 1-[4-(5,6-bis-(4-substituted phenyl)-1,2,4-triazin-3-yl)piperazin-1-yl]-2-[(1*H*-(benz)imidazol/thiazole/oxazole-2-yl)thio]ethan-1-one derivatives (**1-9**) were acquired with the reaction of 2-mercapto(benz)imidazole/thiazole/oxazoles and the intermediates (**IIIa-c**) which were already synthesized in early step. Nine final compounds were yielded in a range of 72%-78% range and melting points were found between 124 °C and 248 °C.

In IR spectra of the compounds characteristic streching bands were observed at 1647-1680 cm^{-1}, 1325-1608 cm^{-1} and 1001-1269 cm^{-1} belong to C=O double bond, C=C and C=N double bonds and C-N and C-O single bonds. According to ^{1}H-NMR results, four methylene groups of piperazine rings resonated as broad singlets at about 3.67-4.07 ppm. The other methylene protons vicinal to carbonyl group were observed with a chemical shift at 4.50-4.68 ppm range. The aromatic protons of heterocyclic ring and phenyl rings were seen in between 7.0-8.80 ppm. In the ^{13}C-NMR spectra of the compounds, signals belonging to aliphatic carbon atoms were assigned at about 21.28-55.80 ppm; and signals for aromatic carbon atoms were observed at 101.68-165.75 ppm. The carbonyl carbon of amide group was seen at about 166 ppm. In mass spectroscopy, $[M+H]^+$ peaks were established in accordance with the molecular weights of the compounds.

Biology

The cytotoxic activity of nine triazine compounds (**1-9**) were determined against C6 rat glioma and MCF-7 breast adenocarcinoma cell lines and results were summarized in **Table 1.** IC_{50} values were calculated in between 9.5-500 μg/mL. Compound **4** (IC_{50}=21.0 μg/mL) was found as the most active compound even more than cisplatin against C6 cell line. Compound **6** was also exhibited good antiproliferative activity with a IC_{50} value of 26.0 μg/mL which is very close to cisplatin (IC_{50}=23.5 μg/mL). The IC_{50} values could not be calculated for compounds **1, 3, 8** and **9** even if at the highest tested concentration which is 500 μg/mL against C6 tumor cells. Against MCF-7 cell line, compound **5** showed the strongest cytotoxicity (IC_{50}=9.5 μg/mL) which was higher than cisplatin. Com-

pound **2** and **8** also showed good activity with IC_{50} values of 58.33 and 51.67 μg/mL, respectively. Two active compounds against C6 cells, compound **4** and **6** did not exhibite enough cytotoxic activity against MCF-7 cell line. Moreover, the lipooxygenase (LOX) inhibitory activity of the compounds were studied. None of the compounds showed LOX inhibition as much as standard drug nordihydroguaiaretic acid, even if IC_{50}, the half maximal inhibitory concentration could not be calculated. At 100 μg/mL concentration, LOX inhibition percentages were identified for compounds **1** and **6** as 43.35% and 38.79%, respectively. The results revelaed that 1,2,4-triazine derivatives have considerable cytotoxic activity. As a follow up study from past to present studies, antiproliferative activity of the compounds are rational and it worths to design new compounds based on this substructure.



Scheme 1: The synthesis of the compounds. Reactants and reagents ; *i*: $NaHCO_3$, CH_3OH, reflux 3 h; ii: pyridine, reflux 6 h; *iii*: Et_3N, DMF, r.t., 45 min; *iv*: K_2CO_3, Acetone, r.t., 5 h.

Table 1: IC_{50} values (μg/mL) against C6 and MCF-7 tumor cell lines and % LOX enzyme inhibition of the compounds

Comp.	C6	MCF-7	% LOX inhb. (100 μg/mL)
1	>500	>500	43.35±3.08
2	150.0±26.46	58.33±2.89	---
3	>500	>500	---
4	21.0±3.61	>500	---
5	490.0±14.14	9.5±0.50	---
6	26.0±1.73	85.0±5.0	38.79±0.86
7	445.0±7.07	>500	---
8	>500	51.67±10.41	---
9	>500	>500	---
Cisplatin	23.5±2.12	11.67±2.89	---
NDGA*	-	-	3.35±0.07

Not determined : ---

*NDGA : Nordihydroguaiaretic acid, IC_{50} value is represented in the corresponding line.

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