

Synthesis, anti-microbial and anti-mutagenic activities of some Schiff bases derivatives containing thiophene group

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Abstract: The aim of this study is to investigate for the first time *in vitro* antimicrobial and antimutagenic activities of Schiff bases included the azomethine group. Antimutagenic activity was evaluated by micronucleus (MN) assay. These group have been examined for antibacterial activity against pathogenic strains, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* H, *Brucella abortus*, *Micrococcus luteus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and antifungal activity against *Candida albicans* and *Saccharomyces cerevisiae*. The results of MN showed that Schiff bases ((E)-N-(4-chlorophenyl)-1-(5-nitrothiophen-2-yl)methanimine ; (E)-N-(2,4-dichlorophenyl)-1-(5-nitrothiophen-2-yl) methanimine) different concentrations decreased the toxic effects of Aflatoxin B₁. Especially, high concentration (20µM) of (E)-N-(4-chlorophenyl)-1-(5-nitrothiophen-2-yl)methanimine (compound 1) has strong antimutagenic activity. In our *in vitro* test systems, it was observed that Schiff bases had antimutagenic effects on human lymphocytes. On the other hand these compounds were also found to possess antimicrobial activity against some test bacteria and yeast. The antimicrobial test results of these Schiff bases included the azomethine group exhibited better activity than some known antibiotics. In particular, Compound 1 were more potent bactericides than all of the substances synthesized. In conclusion, this Schiff bases included the azomethine group can be use pharmacy industries as recognized with their noncytotoxic, antimicrobial and antimutagenic features.

Keywords: Anti-mutagenic, anti-microbial activity, micronuclei, Schiff bases.

INTRODUCTION

One important class of organic compounds is Schiff bases include the azomethine group which are prepared by the condensation between amine and aldehyde or ketone (-RC=N-). On account of similarity of imine group with natural biological systems, appearing in synthetic organic processes and organometallic chemistry, possessing a variety of substituents with different electron-donating, electron withdrawing groups Schiff bases are novel ligands of recent times (Schiff, 1864; Metzler *et al.*, 1954; Snell and Jenkins, 1959; Cordes and Jencks, 1962; Moffett, 1963). Schiff bases can soluble in many solvents and also exhibits excellent properties such as high thermal stability, biological activities, facility to form complexes (Dhar and Taploo, 1982; Bringmann *et al.*, 2004; Zabolica *et al.*, 2013). Schiff bases have been used in medicinal, agricultural and pharmaceutical fields due to catalytic, biological, fluorescence properties (Tanak *et al.*, 2014; Anar *et al.*, 2016; Poonia *et al.*, 2016). However, so far, no report has shown a antimutagenic and antimicrobial effects of these compounds. Therefore, in this study, first of all Schiff bases were synthesized using the condensation methods. Then, antimicrobial and antimutagenic activity was tested using complexes the well diffusion method and the micronuclei (MN) assay (Nartop *et al.*, 2012; Altundas *et al.*, 2016).

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MATERIALS AND METHODS

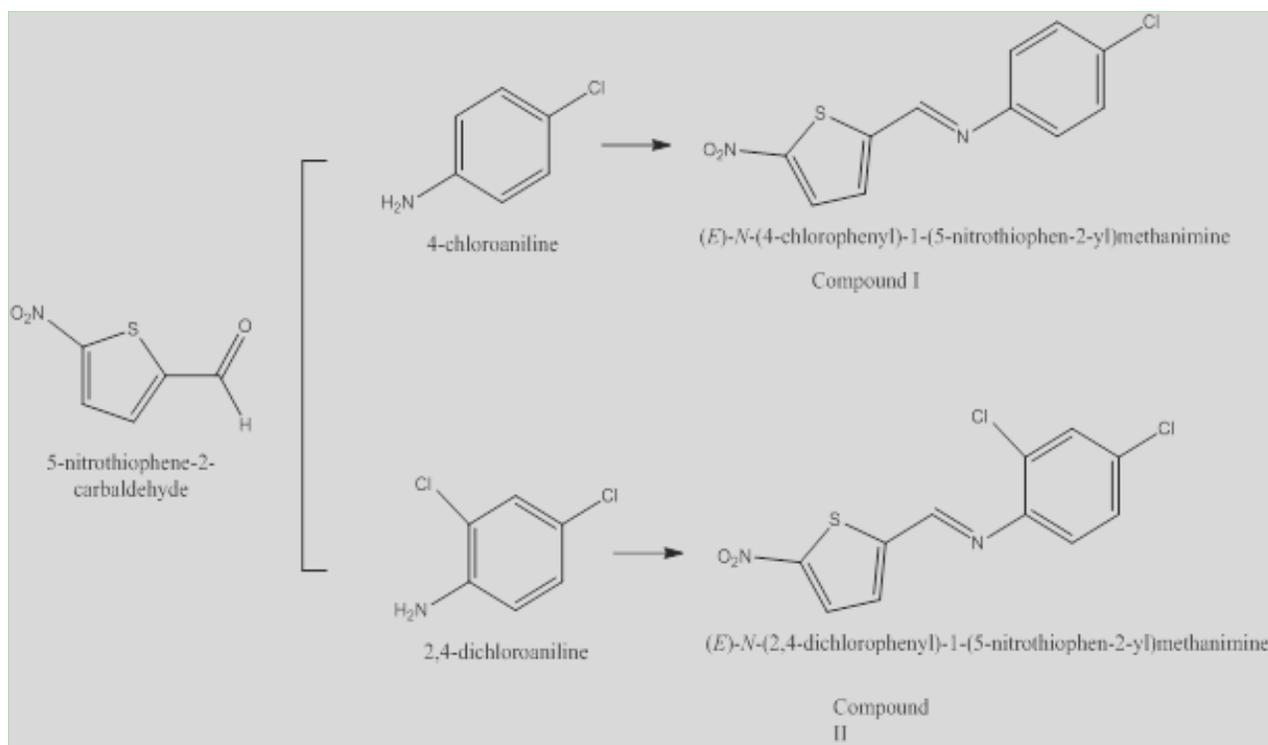
Synthesis of title compounds

Schiff bases (Schiff, 1864; Moffett, 1963) were prepared by refluxing a mixture of solution containing 5-nitro-2-thiophene carboxyaldehyde (0,09 mmol) in ethanol (20 mL) and a solution containing a primary amine (4-chloroanilin, 2,4-dichloroaniline 0,09 mmol) in ethanol (20mL). The reaction mixture was stirred for 5 hour under reflux. Synthesized compounds were evaporated at room temperature (m.p.416-419 K; 430-432 K respectively) (Gumus, 2014). (E)-N-(4-chlorophenyl)-1-(5-nitrothiophen-2-yl)methanimine (Compound I) was synthesized with 67% yield and melting point is 416-419 K. (E)-N-(2,4-dichlorophenyl)-1-(5-nitrothiophen-2-yl) methanimine (Compound II) was synthesized with 72% yield and melting point is 430-432 K.

Antimicrobial activity

Test microorganisms

The bacterial cultures chosen were; *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC1280, *Salmonella typhi* H NCTC901.8394, *Pseudomonas aeruginosa* ATCC19115, *Brucella abortus* RSKK03026, *Staphylococcus epidermis sp.*, *Micrococcus luteus* ATCC9341, *Bacillus cereus* RSKK-863 and yeast were used *Candida albicans* Y-1200-NIH and *Saccharomyces cerevisiae* sp.



Scheme 1: Synthesised of title compounds

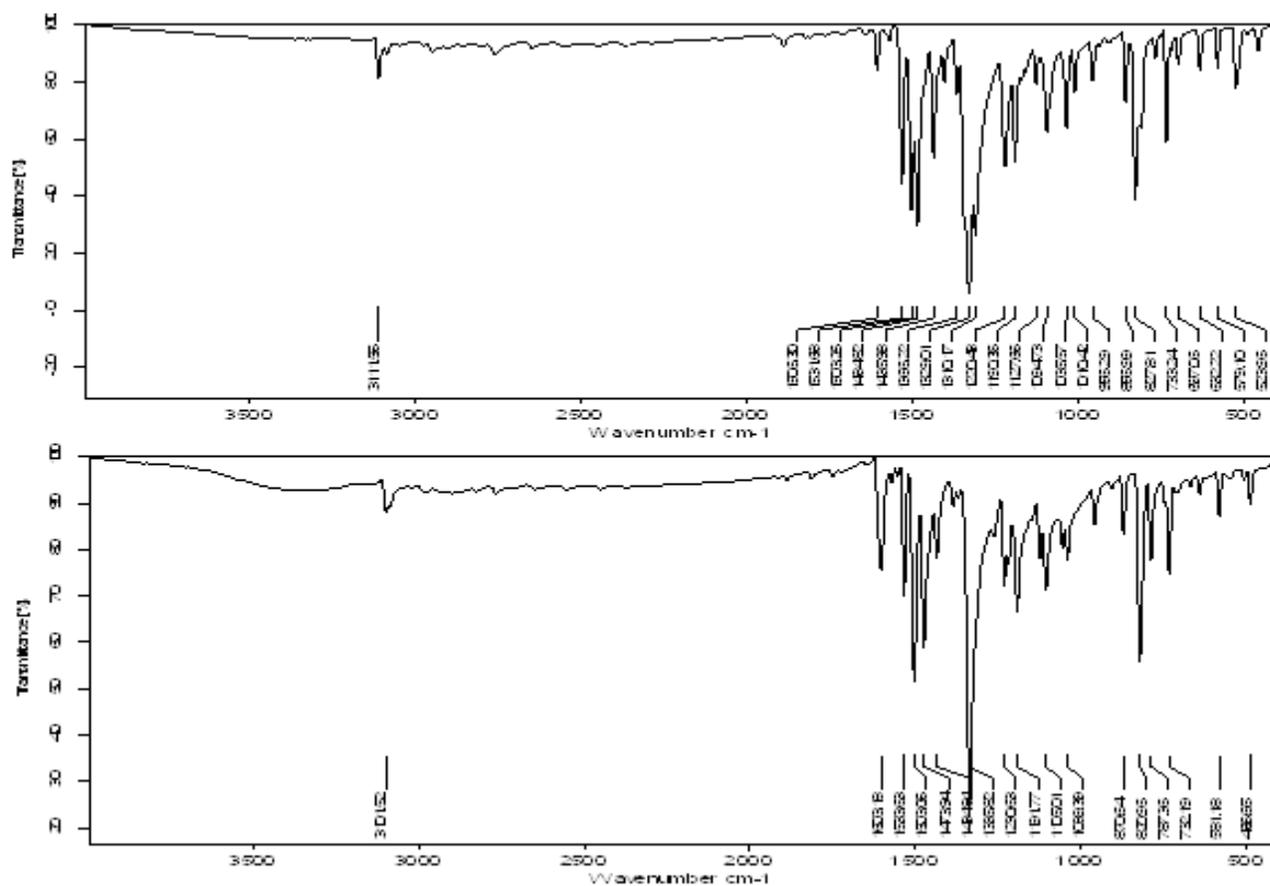


Fig. 1: FT-IR spectrum of Compound I and Compound II

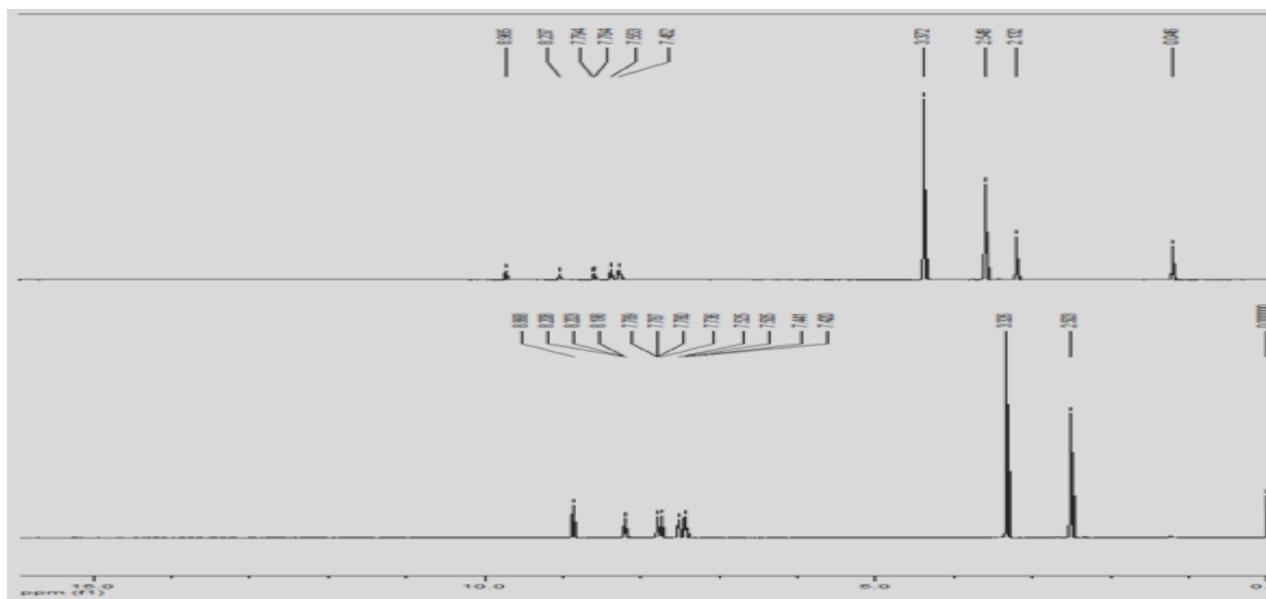


Fig. 2: ¹H-NMR spectrum of Compound I and Compound II

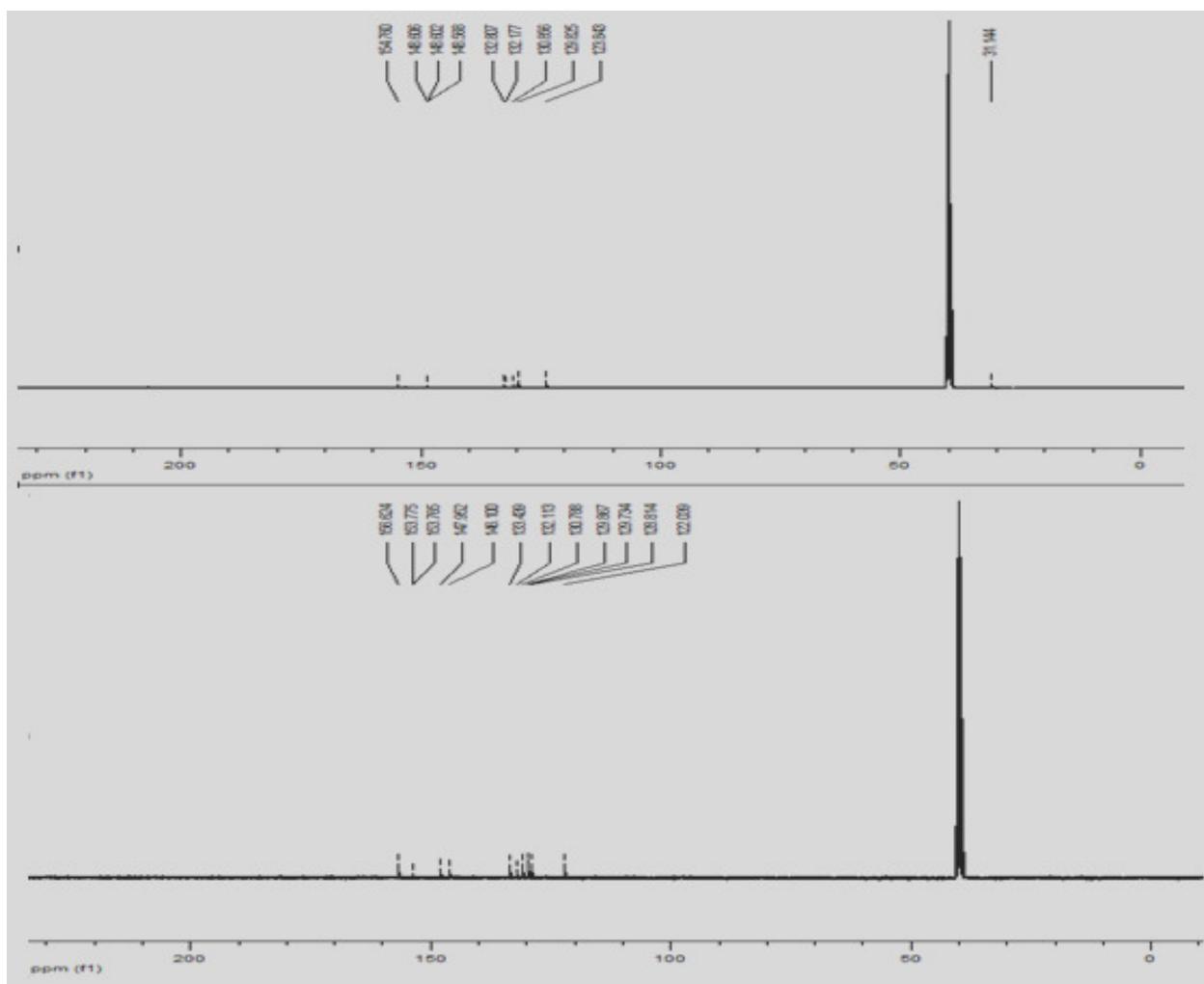


Fig. 3: ¹³C NMR spectrum of Compound I and Compound II

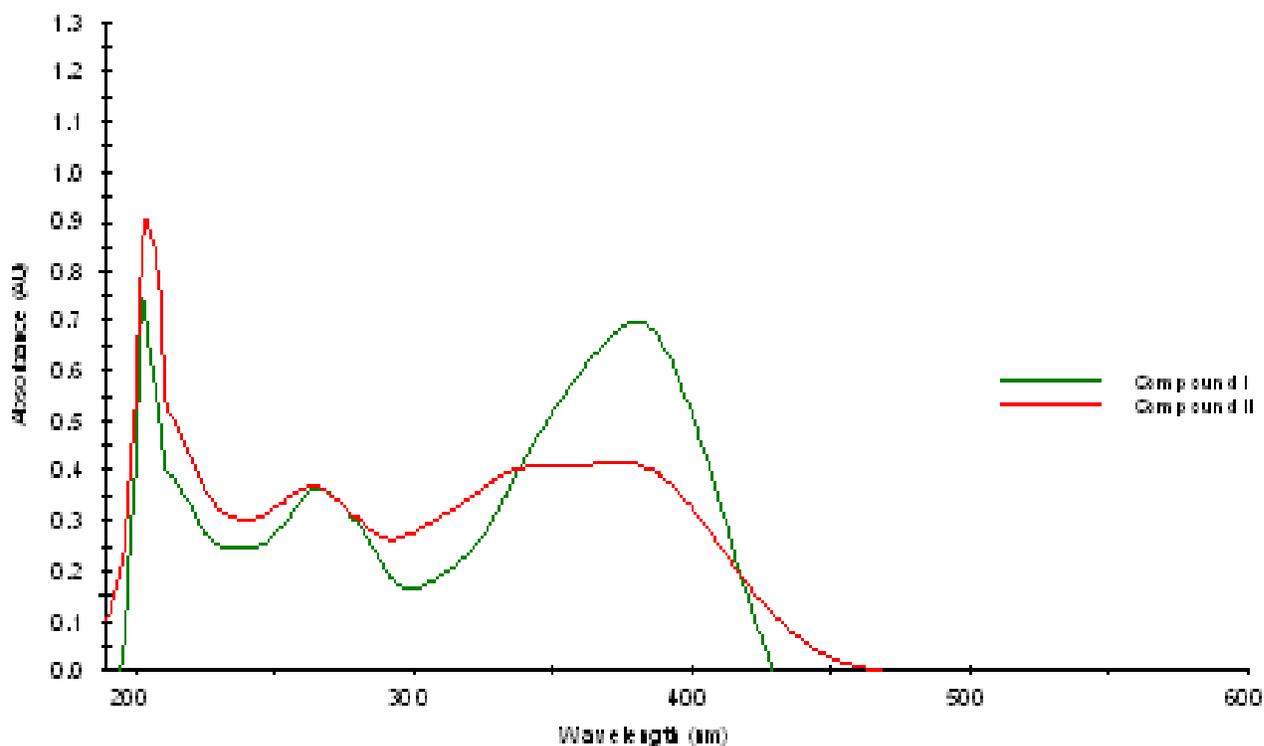


Fig. 4: Electronic spectrum of title compounds

Table 1: Elemental analytical data of title compounds.

Elemental (%)			
	C	H	N
Compound I	47.67 (49.54)	2.76 (2.65)	10.29 (10.50)
Compound II	45.72 (43.87)	2.33 (2.01)	9.95 (9.30)

Table 2: Biological activity of compounds (compound I and II) and standard reagents (diameter of zone inhibition (mm)).

Compound and Positive control Microorganisms		Compounds 1	Compounds 2	K30	SXT 25	AMP 10	AMC 30	NYS 100
Gram (-)	<i>P.aeruginosa</i>	20	15	14	18	8	15	-
	<i>S.typhi H</i>	20	16	20	17	11	19	-
	<i>Br. abortus</i>	17	18	-	-	-	-	-
	<i>E.coli</i>	-	-	25	18	10	14	-
Gram (+)	<i>S.aureus</i>	17	13	25	24	30	30	-
	<i>S.epidermis</i>	20	15	-	-	-	-	-
	<i>M.luteus</i>	18	22	-	-	-	-	-
	<i>B.cereus</i>	20	23	-	-	-	-	-
Yeast	<i>C. albicans</i>	32	22	-	-	-	-	20
	<i>S. cerevisiae</i>	20	15	-	-	-	-	-
Control	DMSO	-	-	-	-	-	-	-

K30, Kanamycin 30µg; SXT25, Sulphamethoxazol 25µg; AMP10, Ampicillin 10µg; AMC30, Amoxycillin 30µg; NYS100, Nystatin 100µg.

Table 3: The effects of AFB₁ and Comp I on MN and oxidative stress parameters (SOD, CAT and MDA).

Test Items	Concentrations	MN numbers ± S. E	CAT ± S. E (k/g protein)	SOD ± S. E (U/mL)	MDA ± S. E (n mol/mL)
Control		3.62 ± 0.23 ^a	1854.2 ± 38.4 ^a	6,24 ± 0,14 ^a	2,62 ± 0,264 ^a
AFB ₁	5 µM	6.15 ± 0.37 ^c	1547.5 ± 13.6 ^c	4,12 ± 1,02 ^c	6,12 ± 0,32 ^c
Comp I	10 µM	3.83 ± 0.26 ^a	1760.7 ± 19.4 ^b	5,93 ± 0,34 ^{ab}	3,02 ± 0,18 ^a
AFB ₁ + Comp I	5 µM + 5 µM	5.24 ± 0.12 ^{cd}	1653.1 ± 14.4 ^{cd}	5,52 ± 0,29 ^b	3,42 ± 0,14 ^b
AFB ₁ + Comp I	5 µM + 10 µM	4.18 ± 0.62 ^b	1701.3 ± 24.3 ^c	5,84 ± 0,23 ^{ab}	3,20 ± 0,38 ^{ab}
AFB ₁ + Comp I	5 µM + 20 µM	3.96 ± 0.40 ^{ab}	1784.2 ± 18.7 ^{ab}	6,17 ± 0,62 ^a	2,98 ± 0,18 ^a

Table 4: The effects of AFB₁ and Comp II on MN and oxidative stress parameters (SOD, CAT and MDA).

Test Items	Concentrations	MN numbers ± S. E	CAT ± S. E (k/g protein)	SOD ± S. E (U/mL)	MDA ± S. E (n mol/mL)
Control		3.62 ± 0.23 ^a	1854.2 ± 38.4 ^a	6,24 ± 0,14 ^a	2,62 ± 0,264 ^a
AFB ₁	5 µM	6.15 ± 0.37 ^c	1547.5 ± 13.6 ^c	4,12 ± 1,02 ^c	6,12 ± 0,32 ^c
Comp II	10 µM	3.66 ± 0.45 ^a	1708.6 ± 22.6 ^c	5,96 ± 0,12 ^a	3,24 ± 0,32 ^{ab}
AFB ₁ + Comp II	5 µM + 5 µM	5.38 ± 0.57 ^d	1614.2 ± 03.6 ^d	4,94 ± 0,13 ^{cd}	4,35 ± 0,03 ^{cd}
AFB ₁ + Comp II	5 µM + 10 µM	4.72 ± 0.83 ^c	1665.6 ± 10.2 ^{cd}	5,21 ± 0,29 ^{bc}	4,08 ± 0,47 ^c
AFB ₁ + Comp II	5 µM + 20 µM	4.60 ± 0.72 ^{bc}	1716.1 ± 09.4 ^{bc}	5,62 ± 0,28 ^{ab}	3,46 ± 0,05 ^b

Aflatoxin B₁ (AFB₁) was used as positive controls for human blood cells.

a, b, c, d, e, f Statistically significant differences in the same column are indicated by the different superscripts ($\alpha = 0.05$).

Detection of antimicrobial activity

The synthesized compounds (compound I and II) were examined for their antimicrobial activity by the well-diffusion method (Altundas *et al.*, 2010) against four Gram-positive bacteria (*S.aureus*, *S.epidermis*, *M. luteus*, *B.cereus*) and four Gram-negative bacteria (*P.aeruginosa*, *S. typhi* H, *Br. abortus*, *E.coli*) and two yeast (*C. albicans*, *Saccharomyces cerevisiae*). Bacterial subcultures and yeast were tested for resistance to five antibiotics produced by Oxoid Lt., Basingstoke, UK. These were: Ampicillin (prevents the growth of Gram-negative bacteria), Nystatin (binds to sterols in the fungal cellular membrane and alters the permeability allowing leakage of the cellular contents), Kanamycin (used in molecular biology as an agent in isolating bacteria), Sulphamethoxazol (a bacteriostatic antibacterial agent that interferes with folic acid synthesis in susceptible bacteria), Amoxycillin (a β -lactam antibiotic used to treat bacterial infections caused by sensitive microorganisms).

Antimutagenic activity (In vitro micronuclei assay)

Whole blood samples from four healthy nonsmoking donors between the ages of 23 and 25 were used for MN assay (the study was approved by Ataturk University, Medical Faculty Ethical Review Board). For the MN assay, the method used by Orhan *et al.*, (2016) was applied. Aflatoxin B₁ (AFB₁) and the agents to be tested were dissolved in 0.5% dimethyl sulfoxide (DMSO). AFB₁ (5µM) and different concentrations of compound 1 (comp-1) (5µM, 10µM, 20µM) were added to the prepared

medium and cultured at 37°C for 72 h in a 5% CO₂ moist atmosphere. Same protocols were done for compound 2 (Comp-2)

Oxidative Stress Parameters

Superoxide dismutase (SOD) assay

Cu-Zn-SOD activity of the whole blood cell culture supernatant was evaluated by the method of Sun *et al.*, (1988). In the assay, 2.45mL of assay reagent [0.3mM xanthine, 0.6mM Na₂EDTA, 0.15mM nitroblue tetrazolium (NBT), 0.4M Na₂CO₃, and 1g/L bovine serum albumin] was combined with equal amount of protein from each experimental group and 50µL xanthine oxidase was added to initiate the reaction. The reduction of NBT by superoxide anion radicals was determined by measuring the absorbance at 560 nm. Cu, Zn-SOD activity was expressed in units of SOD per mg protein, where 1 U was determined as the amount of enzyme causing half-maximal inhibition of NBT reduction.

Catalase (CAT) assay

Catalase activity was determined by measuring the decrease in absorbance at 240 nm (Aebi, 1984). The reaction mixture contained 0.5ml of enzyme extract and 2.0ml of 0.1M sodium phosphate buffer (pH 6.8) and the reaction was started by the addition of 0.5ml of 10mM hydrogen peroxide. The decrease of absorbance was recorded. Decrease of absorbance was recorded in every 15 sec up to 3 min. The values of the 1-minute linear absorbance reduction are based on the calculations.

Malanoaldehyde (MDA) assay

MDA levels in the whole blood cell culture supernatant were determined spectrophotometrically according to the method described by Ohkawa *et al.* (1979). A mixture of 8.1% sodium dodecyl sulphate, 20% acetic acid and 0.9% thiobarbituric acid was combined with equal amount of protein from each experimental group (Paglia and Valentine, 1967). Distilled water was added to the mixture to make the total volume 4mL. This mixture was incubated at 95°C for 1h. After incubation, the samples were left to cool under cold water, 1mL distilled water and 5mL n-butanol/pyridine (15:1, v/v) were added to the solution and mixed thoroughly. The samples were centrifuged at 4000 rpm for 10 min. The supernatants were separated and measured at 532 nm. The level of MDA was calculated from a standard graph made by using different concentrations (1-10 nmol) of 1,1,3,3-tetramethoxypropane and was expressed as μmol of formed MDA in one mL of serum.

STATISTICAL ANALYSIS

For statistical analysis, we used SPSS for Windows 18.0 (SPSS Inc., Chicago, USA). Statistical significances of the MN frequencies and oxidative stress parameters were determined using a one-way ANOVA analysis followed by a high range statistical domain using Turkey's test. Results are presented as mean \pm standard error (SE) and values of $p < 0.05$ were regarded as statistically significant. All experiments were performed in three replicates and data was compared for reproducibility (Orhan *et al.*, 2016).

RESULTS

Chemistry

Schiff bases (Schiff, 1864; Metzler *et al.*, 1954; Cordes and Jencks, 1962; Moffett, 1963) were obtained by condensation of aldehyde and primary amine in ethanol solution. Structures of Schiff bases were analysed with elemental analysis, FT-IR, UV-vis, ^{13}C and ^1H NMR spectroscopy. Data of elemental analysis for title compounds are given table 1 and data are agreeable with predicted structures as shown in Scheme 1.

The C = O bond became a C = N bond by attack of the amine group to the carbonyl group of the thiophene derivative (5-nitro-2-thiophene carboxyaldehyde) and then removal of a water molecule. Compound I and II synthesised with about 70% yield in mild conditions but Compound II have two electronegative atoms (Cl) and so yield is higher than Compound I due to more reactive ring. In FT-IR spectrum, the C=N stretching vibrations which usually are expected in 1600-1700 cm^{-1} region (Anar *et al.*, 2016; Poonia *et al.*, 2016) and are characteristic peaks for Schiff bases. For synthesised all compounds the C=N peaks were observed at 1603-1606

cm^{-1} . In addition sharp peak at 1650-1750 cm^{-1} which indicate C=O bond and at 3400 cm^{-1} amin peaks were not observed, this may be evidence for synthesise of Schiff base. The absorption band at 3110-3098 cm^{-1} were attributed to the aromatic C-H vibrations which are shown 3000-3100 cm^{-1} region (Nartop *et al.*, 2012) and in addition at 1339-1355 and 1531-1501 cm^{-1} two sharp peaks indicated the asymmetric and symmetric vibrations of NO_2 group of thiophene moiety for compound I and II. C-Cl vibration peak appeared at 826 and 819 cm^{-1} respectively.

The imine proton of Schiff bases in ^1H NMR spectrum was determined at 8.867 for Compound I and at 8.919 ppm for Compound II (s, 1H). The aromatic protons exhibited signal at about 7.406-7.789 ppm for all compounds. As well the peaks at 8.191-8.207 ppm was due to proton of thiophene ring.

In ^{13}C NMR spectrum, imin carbon was appeared at 148 ppm for Compound I and 152 ppm for Compound II. Aromatic phenyl carbons were observed in the range of 148-122 ppm and carbons of thiophene also appeared in the range of 156-129 ppm for all compounds.

Electronic spectra of compounds were taken in ethanol solutions. Two absorption bands were observed for all compounds due to similar functional groups. The peak was attributed π - π transition of imine for compound I at 265 nm and at 264 nm for Compound II. The peaks at 380 and 390 nm were due to n- π transitions of imine for Compound I and II, respectively.

Biological activity

The synthesized compounds (compound I and II) were screened for *in vitro* antimicrobial and concentrations in DMSO solution (0.25 $\mu\text{g}/\mu\text{L}$). All the synthesized compounds and antibiotics exhibited varying degrees of inhibitory effects on the growth of different tested strains (table 2).

The results of antibacterial screening indicated Compound I showed activity against most of the strains both gram-positive and gram negative bacteria (*S.typhi* H, *S.epidermis*, *P.aeruginosa*- *S. aureus*, 20mm-17mm respectively) and yeast (*C.albicans* (32mm) and *S. cerevisiae* (20mm). Salmonella serovars cause very diverse clinical symptoms, from asymptomatic infection to serious typhoid-like syndromes in infants or certain highly susceptible animals. Furthermore, Compound I and II were potent growth inhibitors against *Br. abortus* with a zone value of 17 and 18mm respectively. *Br. abortus* is a gram-negative bacterium that causes premature abortion of cattle fetus (Halling *et al.*, 2005), in addition it is a human pathogen which is a very serious, debilitating and sometimes chronic disease that may affect a variety of organs (Sauret and Vilissova, 2002; Nartop *et al.*, 2012).

The antimicrobial activity of these compounds was also compared with seven commercial antibiotics, namely, Kanamycin, Sulphamethoxazol, Ampicillin, Chloroamphenicol, Ciprofloxacin, Amoxicillin, Sulbactam and Nystatin. It was seen that the synthesized compounds were effective as the antibiotics mentioned table 2.

Antimutagenic and antioxidative effects

In the present study, different concentrations of compound 1 and 2 were performed with the MN test system which is widely used as a short-term test system. In MN test, the used positive control (AFB₁) significantly increased the MN frequency on peripheral lymphocytes when compared with the control (tables 3-4). Such an increase was found to be statistically significant ($p < 0,05$). It was observed that treatment group with different concentrations (5 μ M, 10 μ M, 20 μ M) of compound 1 and 2 together with AFB₁, decreased the MN frequency, compared to the one, intoxicated with AFB₁. Especially, the highest concentration (20 μ M) was determined as the effective concentration. The results showed that compound 1 more effective than compound 2.

DISCUSSION

One important class of organic compounds is Schiff bases include the azomethine group which are prepared by the condensation between amine and aldehyde or ketone (-RC=N-). Schiff bases are popular ligands in coordination chemistry because of their ease of synthesis and their ability to be readily modified both electronically and sterically (Naik *et al.*, 2014). In this study we derived some chemical compounds including azomethine group. In the literatures, researchers showed that some chemical compounds in including azomethine group has antioxidant activity.

AFB₁, which has a strong mutagenic effect on DNA, is the most potent of naturally occurring mycotoxins. By metabolizing AFB₁ by cytochrome P450 enzymes in the liver, more harmful byproducts are produced. These byproducts may bind to nuclear DNA, leading to nuclear damage due to electrophilic attack on the N7 position of guanine of DNA and RNA. Consequently, adduct formation in vivo may occur, leading to the transformation of cells, or even cell death (Sarı *et al.*, 2013; Anar *et al.*, 2016). In this study; our compounds with azomethine group have been shown to exhibit antimutagenic properties against AFB₁ and reduce the strong mutagenic effect of AFB₁ on DNA. This determined antimutagenic effect is thought to be related to the effects of the compounds on the enzymatic activation system. This effect shown by the compounds under investigation can be attributed primarily to their antioxidant effects or to their function as cofactors for enzyme systems known to protect DNA and other cellular components from damage by oxygen radicals. According

to the findings; the compounds studied were found to be active inhibitors of the mutagenic effect of AFB₁.

The antibacterial activity of Schiff bases depends on substitution type, structural backbone complexity and substitution type. These properties affect the effect of Schiff bases on the cell wall of bacteria (Okoli and Modise 2018). Chlorine is an attractive group of electron, therefore carbon gets partial positive charge and more polar. Compound II has more chlorine atom unlike compound I, as a result polarity of compound II is greater than other one. So that compound II is less effective in respect to antimicrobial property due to hydrophilic character of molecule.

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