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SYNTHESIS AND CHARACTERIZATION OF NEW 3-SUBSTITUTED 2(3H)-BENZOXAZOLONE DERIVATIVES AS CYTOTOXIC AND ANTIMICROBIAL AGENTS

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LIST OF ABBREVIATIONS

EUCAST : European Committee on Antimicrobial Susceptibility Testing

DAB : 3,3'-Diaminobenzidine

DMF : Dimethylformamide

DMSO : Dimethyl sulfoxide

FT-IR : Fourier Transform Infrared Spectroscopy

MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NADP: Nicotinamide Adenine Dinucleotide Phosphate

NMR : Nuclear Magnetic Resonance

PPA: Polyphosphoric acid

TEA: Triethylamine

THF : Tetrahydrofuran

TLC : Thin Layer Chromatography

Sitotoksik ve Antimikrobiyal Ajanlar Olarak Yeni 3-Sübstitüe-2(3H)-Benzoksazolon Türevlerinin Sentezi ve Karakterizasyonu

Öğrencinin Adı : Emine Erdağ (Uzm.Ecz.)

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ÖZET

Amaç : Bu tez çalışmasında 2(3H)-benzoksazolon türevlerinin yeni Mannich bazları sentezlenmiş ve bu türevlerin metastatik olmayan MCF-7 ve metastatik M4A4 meme kanseri hücre hatlarına karşı sitotoksisiteleri, olası apoptoz mekanizmaları ve farklı suşlar üzerindeki antimikrobiyal etkilerinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Yeni sentezlenen moleküllerin karakterizasyon testleri Nükleer Manyetik Rezonans spektroskopisi, Kızılötesi spektroskopisi ve elemental analiz kullanılarak yapılmıştır. Bileşiklerin sitotoksik aktiviteleri, MTT deneyleri ile test edilmiştir. Aktivite gösteren benzoksazolon türevlerinin apoptotik özellikleri, antikorlar (kaspaz-3, sitokrom-c ve FasL) ve TUNEL testi kullanılarak immünositokimya ile belirlenmiştir. Antimikrobiyal duyarlılık testi, Mueller-Hinton agar kullanılarak EUCAST Disk Difüzyon yöntemi ile bileşikler üzerinde denenmiştir.

Bulgular: MTT ve immünositokimya sonuçları, bileşik **1** ve bileşik **2**'nin, MCF-7 hücrelerine karşı hücre canlılığının azaltılması açısından etkili olduğunu ve farklı apoptotik yolaklar üzerinden etki gösterdiklerini, ancak test edilen bileşiklerin hiç birinin M4A4 hücrelerine karşı etkili olmadığını göstermiştir. Test edilen tüm bileşikler için inhibisyon bölgesi sonuçlarına göre, hem gram negatif *E.coli* hem de gram pozitif *S. aureus*'a karşı en etkili bileşiğin Bileşik **10** olduğunu göstermektedir.

Sonuç : Elde edilen sonuçlar, sentezlenen bazı bileşiklerin yeni terapötik maddeler olarak potansiyel bir antikanser etkisine sahip olabileceğini ve bazılarının özellikle gram-negatif bakteri suşlarına karşı antimikrobiyal etkilere sahip olduğunu göstermektedir.

Anahtar Kelimeler: 2(3H)-Benzoksazolon, piperazin, sitotoksisite, Mannich reaksiyonu, meme kanseri, disk difüzyon, antimikrobiyal duyarlılık

Synthesis and Characterization of New 3-Substituted 2(3H)-Benzoxazolone Derivatives As Cytotoxic and Antimicrobial Agents

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Department: Pharmaceutical Chemistry

ABSTRACT

Objective: Novel Mannich bases derived from 2(3H)-benzoxazolone and 5-chloro-2(3H)-benzoxazolone were synthesized and it was aimed to determine cytotoxic activities of these derivatives against non-metastatic MCF-7 and metastatic M4A4 breast cancer cell lines, to illustrate possible apoptosis mechanisms and antimicrobial effects on different strains.

Materials and Methods: The chemical structures of synthesized molecules were characterized by Infrared spectroscopy, Nuclear Magnetic Resonance spectroscopy and elemental analysis techniques. Cytotoxic activities were tested using MTT assays and possible apoptotic properties of active benzoxazolone derivatives were evaluated by immunocytochemical staining using antibodies in order to bind caspase-3, cytochrome-c and FasL antigens and their apoptosis was supported by TUNEL test as an evidence. The antimicrobial susceptibility of target compounds was tested using Mueller-Hinton type of agar by EUCAST Disk Diffusion method.

Results : MTT assay and immunocytochemistry results showed that compound 1 and compound 2 were effective in terms of reducing cell viability against MCF-7 cells by using different apoptotic pathways, but none of the compounds tested were effective against M4A4 cells. According to the zone of inhibition results, none of the compounds were effective against *Candida albicans* and compound 10 is the most effective compound against gram negative *E. Coli.*

Conclusion: The results show that, some of the synthesized compounds may have a potential anticancer effect as novel therapeutic agents and some have antimicrobial effects, especially against gram-negative bacterial strains.

Keywords: 2(3H)-benzoxazolone, piperazine, cytotoxicity, Mannich reaction, breast cancer, disk diffusion, antimicrobial susceptibility

1. INTRODUCTION AND AIM

Benzoxazolone nucleus is very important heterocyclic system in the design of various pharmacological probes [1]. Since it is possible to do modifications at various positions on the core structure, many different derivatives can be synthesized that have the potential to show biological activity. Heteroatomic nitrogen in position 3 of this pharmacore is especially of interest because it allows various important chemical transformations to take place [1]. Therefore, this molecule has attracted great attention in the design of new drug candidates [1-3]. Some of the reported activities of 2(3H)-Benzoxazolone derivatives include antimicrobial [4], analgesics [5-8], anti-inflammatory [9-12], anti-nociceptive [13], anticonvulsant [14], dopaminergic [15], and immuno-deficiency virus (HIV) reverse transcriptase [16] activities.

Antimicrobials and cytotoxic agents are in the group of essential drugs needed worldwide. Within the large increase in the number of cancer cases worldwide, the need to design selective anti-cancer drugs has become important [17, 18]. To develop effective cytotoxic drugs, it is essential to study the mechanism of how cell death is controlled and or induced.

Mannich bases of 2(3H)-benzoxazolone derivatives were prepared which have different piperazine substituents on 3rd position of benzoxazolone core structure. All of the compounds were analysed using Infrared and Nuclear Magnetic Resonance spectroscopy techniques as well as elemental analysis.

These Mannich bases of 2(3H)-benzoxazolones were screened for their cytotoxicity against nonmetastatic MCF-7 and metastatic M4A4 breast cancer cell lines using MTT assays. Apoptotic properties of active benzoxazolone derivatives were determined by immunocytochemical labelling of antibodies for caspase-3, cytochrome-c and FasL antigens. TUNEL assay was used to support possible apoptotic mechanism. The antimicrobial susceptibility was tested on compounds using Mueller-Hinton agar using EUCAST disk diffusion method.

Nowadays, due to the problem of increased bacterial and fungal resistance and a high increase in the microbial infections, current antimicrobial drug therapy requires synthesis of new chemicals by derivatization of current antimicrobial agents to improve their efficacy [19-22]. The goal of this thesis is to synthesize new 3-substituted benzoxazolone derivatives as potential anticancer agents to screen their cytotoxicity by elucidating the apoptosis mechanism and to screen their possible antimicrobial effects against different strains of microorganisms without any bacterial resistance.

2. GENERAL KNOWLEDGE

2.1 2(3H)-Benzoxazolone

2.1.1 Chemistry of 2(3H)-Benzoxazolone

2(3H)-Benzoxazolone (also known as 2-benzoxazolinone) is heterocyclic aromatic ring system formed by fusion of benzene ring to carbamate functional group. Benzoxazolone is a light brown powder with a molecular formula of $C_7H_5NO_2$ and molecular weight of 135,12 $gmol^{-1}$. The pKa value of benzoxazolone is 8,7 so it is weak acid in aqueous solution [1]. The numbering of benzoxazolone ring is given in figure 2.1.

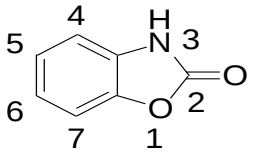


Figure 2.1 Structure and numbering of 2(3H)-Benzoxazolone [1]

2.1.2 Synthesis of 2(3H)-Benzoxazolone

One of the preparation method for 2(3H)Benzoxazolone was reporetd in 1946 by Cornforth which involves the reaction between o-aminophenol dissolved in benzene and phosgene [3] as shown in figure 2.2.

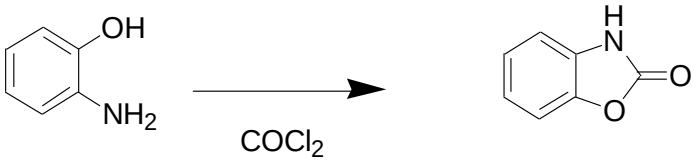


Figure 2.2 Synthesis of 2(3H)-benzoxazolone with phosgene [3]

Srikanth *et al* synthesized benzoxazolone by addition of urea to ortho-amino-phenol in benzene under reflux conditions and then heating for 15 minutes for ring closure as shown in figure 2.3, then pure benzoxazolone crystals were obtained after recrystallization process with methanol [22].

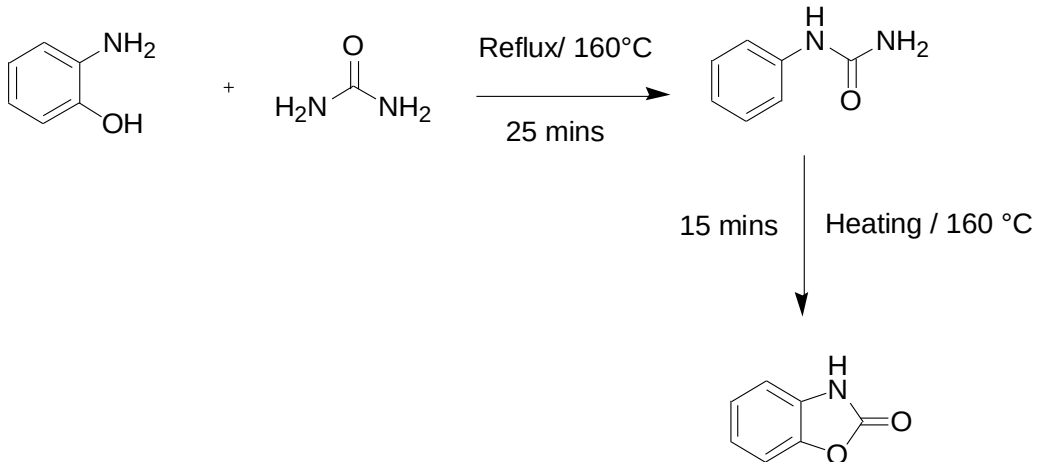


Figure 2.3 Synthesis method of 2(3H)-benzoxazolone with urea under reflux [22]

2.1.3 Reactivity of 2(3H)-Benzoxazolone

2.1.3.1 Tautomerization of Benzoxazolone

2(3H)-benzoxazolonene undergoes tautomerization to form keto and enol forms due to enolizable character of amide moiety[1] which allows useful modifications to occur on the heteroatomic nitrogen at 3rd position shown in figure 2.4.

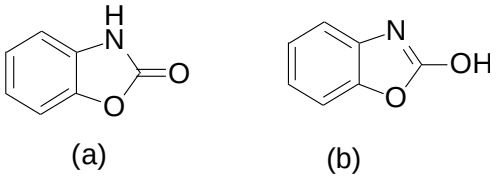
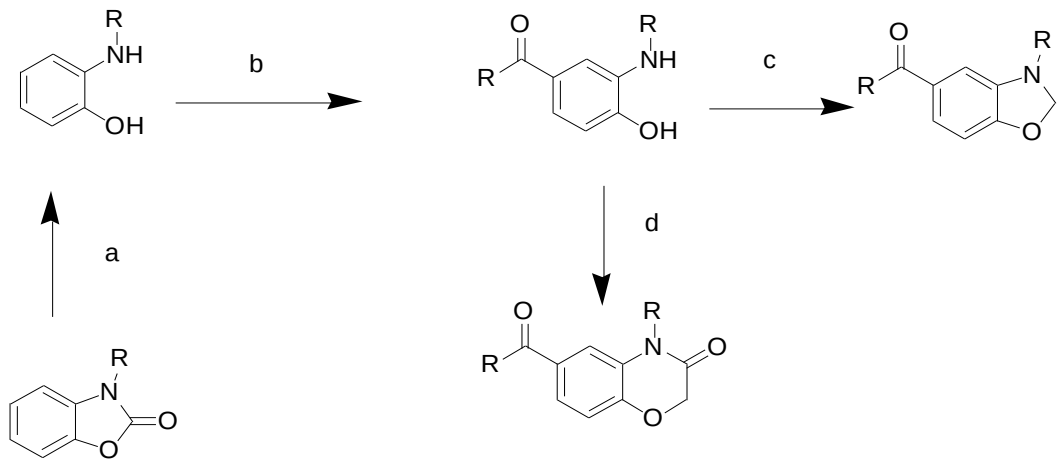


Figure 2.4 (a) Keto form (b) Enol form of Benzoxazolone

2.1.3.2 Ring Expansion and Ring Opening of Benzoxazolone

Benzoxazolone derivatives show stability in acidic medium but they are not stable in basic medium resulting in ring opening which gives 2-aminophenols [1,2]. The acylation of 2-aminophenols could be done in the 4th position followed by closure of benzoxazolone ring results benzoxazolone derivatives with acyl group on 5th position. If the ring expansion occurs, benzoxazinones can be produced from benzoxazolones with appropriate conditions as shown in figure 2.5.



a: aq.NaOH, b: R-COCl, $ALCl_3$.DMF, c: $ClCOOC_2H_5$ / TEA,

d: $BrCH_2COOC_2H_5$ / TEA

Figure 2.5 Ring expansion and ring opening of benzoxazolones [1]

2.1.3.3 Reactions of Benzoxazolone at 3rd Position

2(3H)-Benzoxazolone undergoes different types of reactions such as electrophilic substitution reactions like Friedal-Crafts acylation or alkylation, nitration, halogenation and sulfonation [1,3]. Enolizable character of the amide functional group is the main reason of many transformations occuring at the heterocyclic nitrogen atom in the 3rd position of benzoxazolone ring. N-acylation of 2(3H)-benzoxazolone under acid/base catalysis generate derivative (a) whereas base-catalyzed alkylation reaction give derivative (b) [1,3] as shown in figure 2.6.

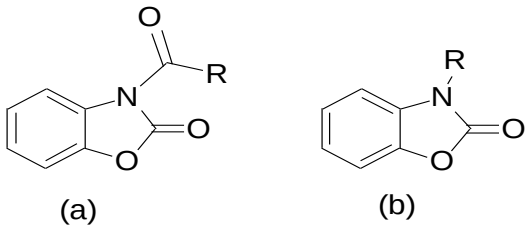


Figure 2.6 The structure of (a) N-acylation (b) N-alkylation at position 3 [1,3]

Reaction of acrylonitrile with 2(3H)-benzoxazolone under basic conditions give N-cyanoethyl derivative which is an example of Michael addition reaction. Mannich reaction (which is a condensation reaction) is formed by transformation of α-hydrogen on heteroatom (nitrogen) at position 3 [22] as shown in figure 2.7.

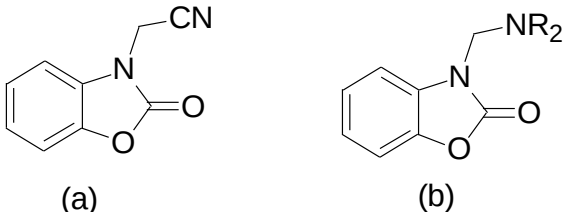


Figure 2.7 (a) Product of Michael addition (b) Product of Mannich reaction [22]

One example of Mannich reactivity at 3-position was published by Koksal *et al* [23] where a piperazine group was attached to nitrogen atom of the ring structure via a methylene bridge as shown in figure 2.8. These 5-nitro-2-benzoxazolone derivatives were tested for their possible analgesic or anti-inflammatory activities.

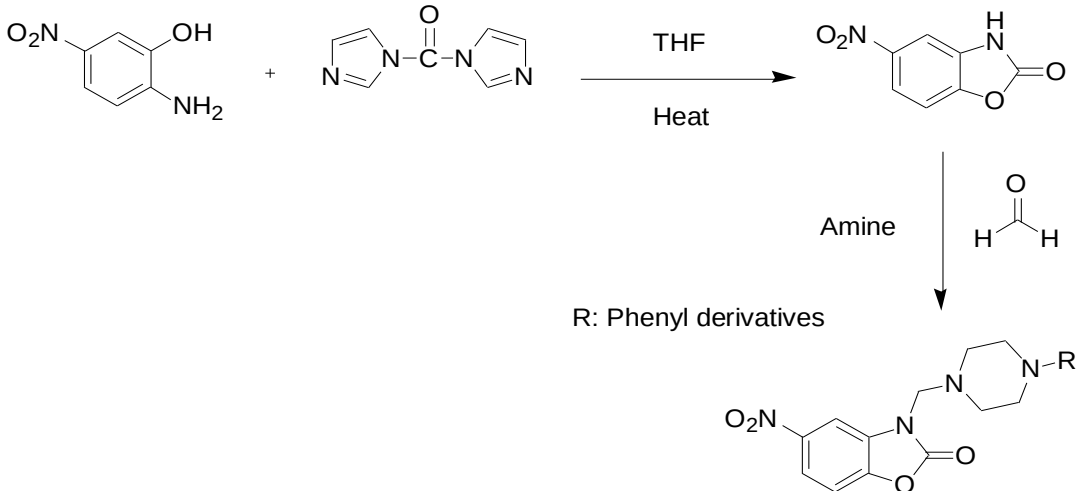


Figure 2.8 Example of Mannich reactivity at 3rd position [23]

2.1.3.4 Reactions of Benzoxazolone at 6th Position

Electrophilic substitution reactions on benzoxazolone ring happens at position 6, which is observed not only for simple nitration, halogenation and sulfonation reactions, but also for Friedal-Craft acylation [22].

In Friedal-Craft acylation reaction, since benzoxazolone ring is highly electron rich, the protonation of heterocyclic nitrogen atom present in benzoxazolone structure can occur via $AlCl_3$ present in the medium which is a Lewis acid necessary for the reaction. 2(3H)-benzoxazolone acts as a strong inactivating substrate against the electrophilic attack of the acylium ion because of extensive protonation (or complexation) experienced in the Friedal-Craft reaction. To overcome that problem, reaction is performed using the $AlCl_3$.DMF complex to obtain compound (a) [22] as shown in figure 2.9.

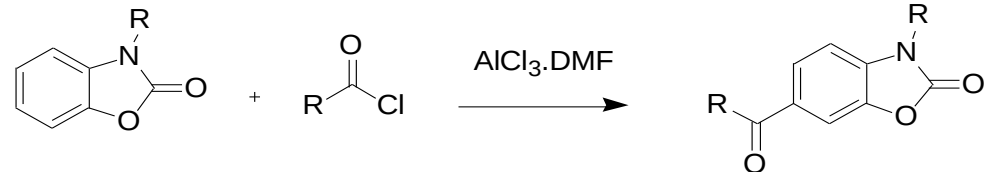


Figure 2.9 Synthesis method of 6-Acyl-2(3H)-Benzoxazolone derivative using $AlCl_3$.DMF complex [22]

The acylation can also be done at position 6 using PPA (polyphosphoric acid) to yield product (b) but the reactivity of PPA is less than $AlCl_3$.DMF complex[22,24]. Acylation reaction with PPA is shown in figure 2.10.

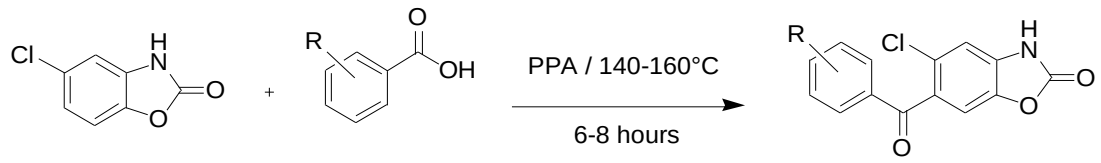
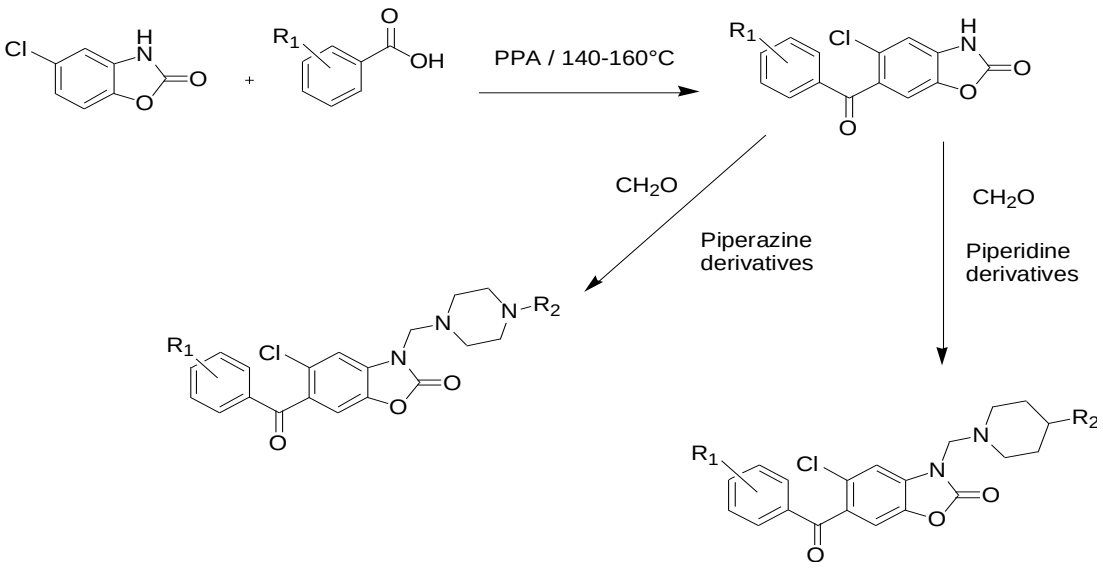


Figure 2.10 Acylation at position 6 using PPA [24]

A study including both acylation at 6th position of benzoxazolone and Mannich reactivity at 3rd position was published by Ozkanli *et al* [24]where seven compounds of chlorzoxazone derivatives acylated at position 6 using PPA in their synthesis with yields between 40-60% . Synthesis of these derivatives are shown in fig. 2.11.



R_1 : 2,6-Difluoro or 3,5-Difluoro R_2:

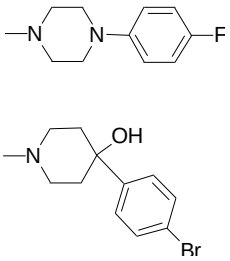


Figure 2.11 Synthesis pathway of 6-Acyl-5chloro-3-substituted-2(3H)-benzoxazolone derivatives with PPA[24]

2.1.3.5 Synthesis and Reactions of 5- Substituted Benzoxazolones

As a result of electrophilic aromatic substitution reactions such as nitration and halogenation, various derivatives of benzoxazolone can be obtained [3]. 5-chloro-2(3H)-benzoxazolone (Chlorzoxazone) is an example of benzoxazolone derivative with a chlorine substituent at 5th position which is known as a powerful muscle relaxant. Chlorzoxazone can be obtained from the reaction of 2-amino-4-chlorophenol and urea in acidic conditions, using 60% H_2SO_4 [3] as shown in figure 2.12.

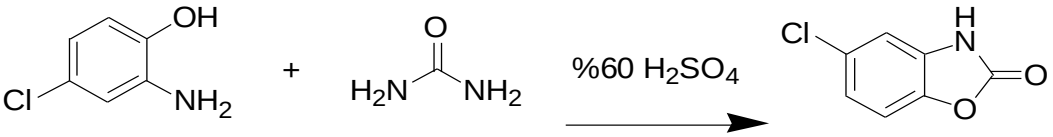


Figure 2.12 Synthesis of Chlorzoxazone from the reaction of 2-amino-4-chlorophenol and urea [3]

Chlorzoxazone can also be prepared by the reaction of 5-chlorosalicylic acid and ammonium azide in presence of $DMF.POCl_3$ known as Vilsmeier complex [3] as shown in figure 2.13.

Figure 2.13 Synthesis of 5-chloro2(3H)-benzoxazolone from 5-chlorosalicylic acid using Vilsmeier complex[3]

Various derivatives with methyl substituent at 5th position of benzoxazolone can be synthesized using Mannich reaction. Gokhan *et al* [25] prepared 5-methyl-3-piperazinomethyl-2-benzoxazolone series to illustrate their possible analgesic and anti-inflammatory activities as shown in figure 2.14.

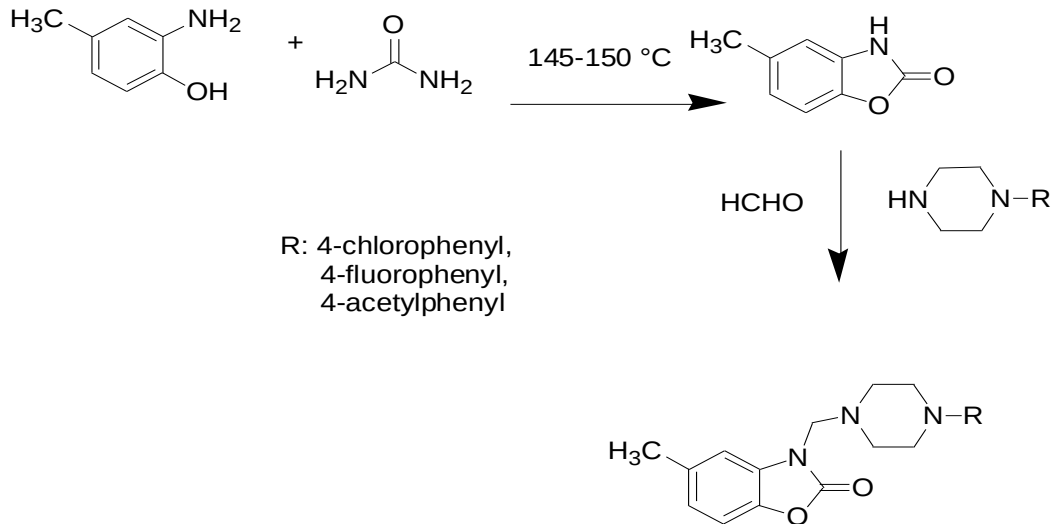


Figure 2.14 Synthesis of 5-methyl-3-substituted benzoxazolone derivatives [25]

2.2 Mannich Reaction and Mannich Bases

2.2.1 Mannich Reaction

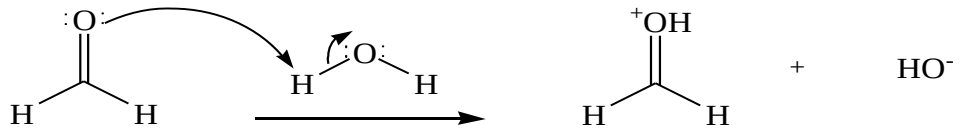
Mannich reaction is a useful procedure in order to synthesize natural and biologically active organic compounds. German chemist Carl Ulrich Franz Mannich gave his name to this reaction which he discovered in 1912 [26].

The Mannich reaction is occured by the condensation of amines mainly a primary or secondary amine into an aldehyde or usually a hydrochloride salt with a compound containing one or more active hydrogen atoms. The β-aminocarbonyl compound formed as a result of the reaction is called the Mannich base. In case of Mannich reaction, active hydrogen atoms are replaced with an aminomethyl group. Sometimes, these aminomethyl groups can be found as substituted derivatives [27].

Mannich derivatives are widely used in agriculture for crop protection and in polymer chemistry as reaction accelerators as well as crosslinkers. The popular application area to date has been in natural product synthesis and pharmaceutical chemistry [28, 29].

2.2.1.1 Mannich Reaction Mechanism of Benzoxazolone

The main mechanism of Mannich reaction has two steps; the first step is iminium ion formation and the second step is attack of iminium ion by benzoxazolone nucleus as a nucleophile as shown in fig. 2.15.



First Step: Formation of iminium ion

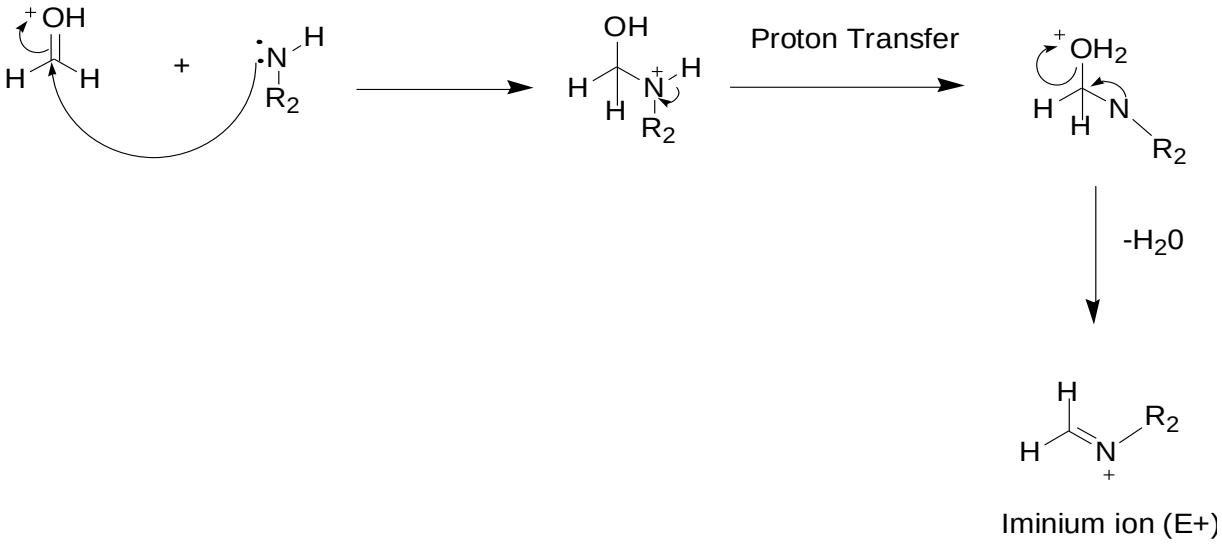


Figure 2.15 Mannich reaction mechanism of benzoxazolone derivatives (First Step)

Second Step: Attack of iminium ion by the substrate and deprotonation of active hydrogen by nucleophile to give the target product as shown in figure 2.16.

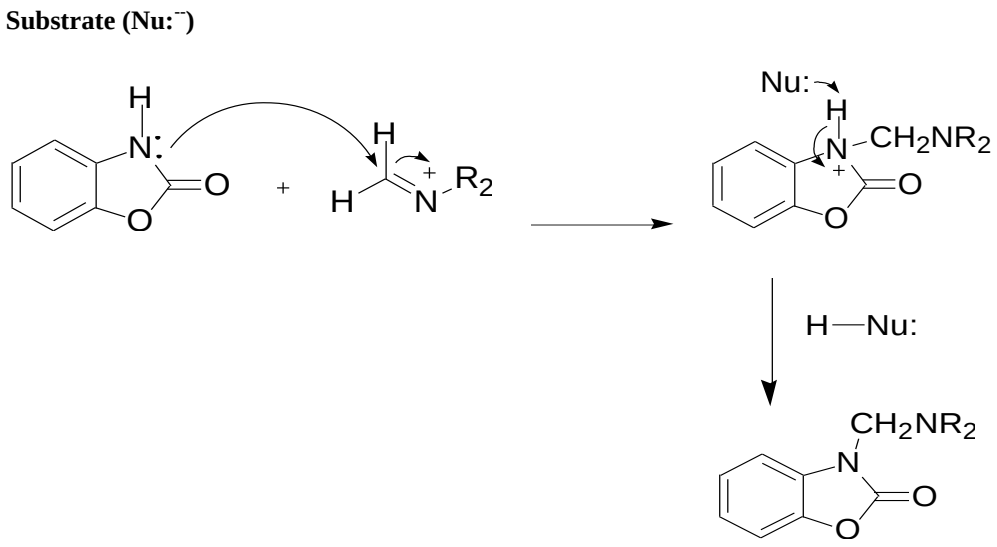


Figure 2.16 Mannich reaction mechanism of Benzoxazolone Derivatives (Second Step)

2.2.2 Mannich Bases

Mannich base is a β-amino ketone and the end product of a Mannich reaction as shown below in fig.2.17 [30]. The Mannich base acts as an active agent as well as a crosslinker for the synthesis of very important pharmacophores or various natural products. Mannich bases play an essential role in synthetic pathway developments in pharmaceutical chemistry because of their high reactivity and they have an ability to be converted into other organic molecules such as an amino alcohol [30].

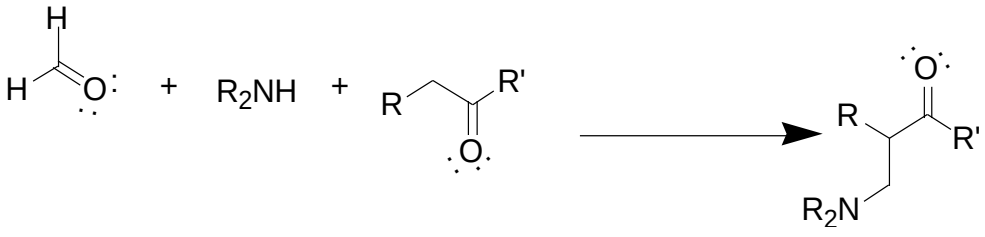


Figure 2.17 β-amino ketone end product of a Mannich reaction

2.2.3 Cytotoxicity Studies of Mannich Bases

Several Mannich bases were synthesized and screened for their cytotoxic activities. Gul *et al* studied the synthesis of Mannich bases of acetophenones to have anti-tumour and cytotoxic activities. The cytotoxic activity of these compounds was tested against various cancer cell lines such as mouse renal carcinoma and human T-lymphocyte cell lines for possible anticancer activity [31]. Linear regression analysis method was used for determination of IC_{50} values. As a result, mono-Mannich bases were converted to bis-Mannich bases and the anticancer activity was increased.

Vashishtha *et al* investigated 1-(4-aryloxyphenyl)-3-piperidin-1-yl-propan-1-one hydrochlorides and related compounds which were synthesised by Mannich reaction investigated to have anticancer properties against L1210 and Molt 4-C8 cell lines. Cytotoxicity evaluations were done using MTT method. [32].

Below are structural examples of cytotoxic Mannich bases synthesized in these studies as shown in fig.2.18 and fig. 2.19.

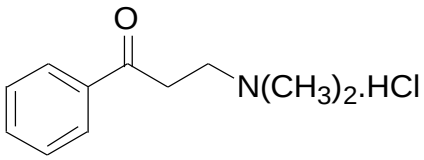


Figure 2.18 Structure of acetophenone derivative with cytotoxic activity [31]

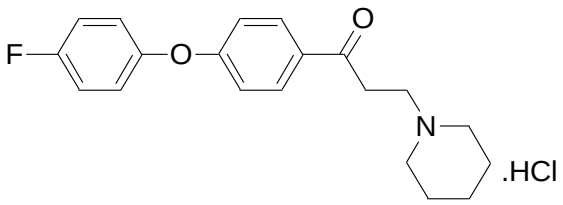


Figure 2.19 Structure of piperidinyl propanone derivative with cytotoxic activity [32]

2.3 Biologically Active Benzoxazolone Derivatives

2.3.1 Apoptosis and Anticancer Activity

2.3.1.1 Apoptosis

A programmed cell death as a result of some molecular signals in the cytosol is called apoptosis. In mammals, apoptotic pathways are divided into two types: first one is the of the external pathway mediated by death receptor on cell membranes and the internal pathway which is mediated by mitochondria [33]. Extrinsic pathway can be triggered by a stimulus from the outside of the cell. Fas ligand (FasL) binds to the extracellular domain of trans membrane receptors which then triggers an initiation of the caspase cascade [33, 34].

However, intrinsic apoptosis can be triggered by stress stimulus and cytochrome c is released from mitochondria which then activates the chain of caspase enzymes, caspase-3 and caspases-9 respectively. For both mechanism pathways, caspases-3 triggers apoptosis which also includes causes DNA damage and cleavage of the DNA strands [33, 34].

In order to maintain the balance between cell death and survival as well as genome integrity, the apoptotic signaling pathway is vital [35]. Pathways can be altered in cancer cells and a variety of strategies can be used to avoid apoptosis. In recent years, researchers have been targeting drugs with cytotoxic activities that can alter cellular apoptotic routes and eliminate cancer cells [35].

2.3.1.2 Anticancer Studies

Ivanova and co-workers studied benzoxazolone derivatives having chalcone like structures for anticancer activity against BV-173, a human pre-B-cell leukemia cell line, with molarities ranging between 4,7- 18,4 μM. The IC_{50} values were measured showing moderate activity [18]. Structure of chalcone like benzoxazolone derivatives is shown in fig. 2.20.

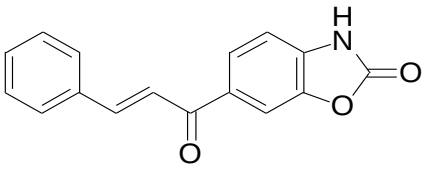


Figure 2.20 Structure of chalcone like benzoxazolone derivative [18]

Similar compounds were also tested for their cytotoxicity and carbonic anhydrase (CA) inhibitory activities as reported by Bilginer *et al* [36] as shown in fig.2.21.
3-bromophenyl propenoyl bearing benzoxazolone molecule showed the most effective activity for carbonic anhydrase inhibition and cytotoxicity. These compounds showed moderate activities at concentration ranges between 30.5 ± 11.3 to 65.5 ± 25.6 μM.

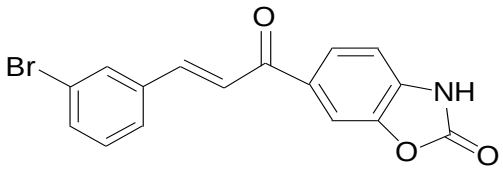
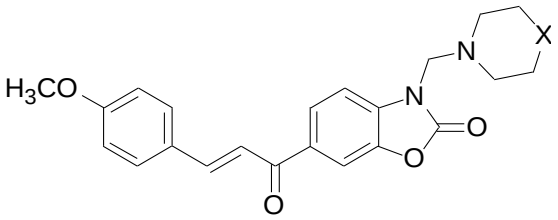


Figure 2.21 Bromophenyl propenoyl benzoxazolone derivative with carbonic anhydrase inhibitory and cytotoxic activity [36].

Another example of chalcone like benzoxazolone derivative with anticancer activitiy is shown in figure 2.22 which was reported by Ivanova *et al* [18] and screened for chronic myeloid leukemia. As a result, these derivatives were found to induce apoptosis in K-562 cells at higher concentrations and the evaluations were illustrated by DNA laddering.



X = C, O or S.

Figure 2.22 Structure of benzoxazolone derivatives with anticancer activities [18]

Soyer *et al* investigated N-substituted phenylacetamide or propionamide containing benzoxazolone derivatives for their possible leukocyte myeloperoxidase (MPO) chlorinating activity [37]. An example compound is shown in figure 2.23.

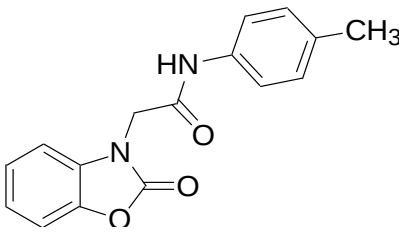


Figure 2.23 Structure of N-phenylacetamide bearing benzoxazolone derivative [37].

Bilginer *et al* also sythesized and studied benzoxazolone based chalcone derivatives via Mannich reaction [39] again by substituting 5-position of the core structure. These derivatives are found as having a higher cytotoxicity than 5-fluorouracil, a reference drug used in this study. Structure of a chalcone derivative with high cytotoxic activity is shown in figure 2.24.

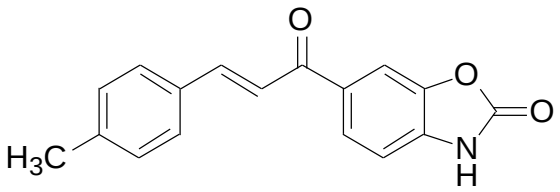


Figure 2.24 Structure of a chalcone derivative with high cytotoxic activity [39]

Ognyan and co-workers studied possible anticancer activities of benzoxazolone derivatives with trimethoxyphenyl propenoyl group at their 6th position [38]. An example of newly synthesized cytotoxic compound is shown in figure 2.25. These compounds were evaluated for their cytotoxicity against several cell lines such as BV-173, HL-60, SKW-3, MDA-MB-231 and K-562. These derivatives were found to have highest chemosensitivity in BV- 173 but they were moderately active in SKW-3 cell lines.

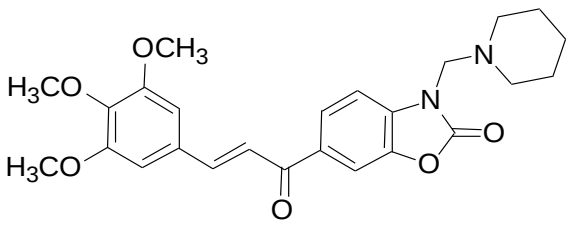


Figure 2.25 Structure a phenyl propenoyl mannich base of benzoxazolone [38].

Gerova *et al* synthesized new benzoxazolone derivatives by making modifications on the structure of natural combretastatin A-4 using benzoxazolone ring as bioisosteric replacement [40]. These derivatives were screened for their antitumor activity against HepG2 cells and their IC_{50} values were reported as between 0.19 uM – 0.28 uM. An example of synthesized derivative is shown in fig.2.26.

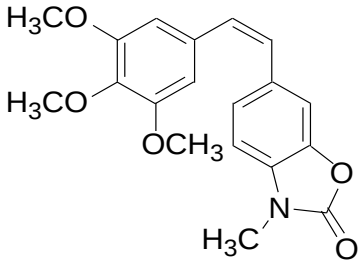


Figure 2.26 Structure of benzoxazolone derivative from combretastatin A-4 [40]

2.3.2 Antimicrobial Studies

Benzoxazolone derivatives are heterocyclic molecules widely studied in pharmaceutical chemistry because of their a wide spectrum of antimicrobial activities against different microorganisms such as bacteria as well as fungi [41-44].

Lackzowski *et al* studied antimicrobial activities of newly synthesized benzoxazolone derivatives with thiazolyl groups at position 6 against gram positive strains of *Micrococcus luteus* [19] An example of active compound sythesized in this study is shown below in fig. 2.27. Their antimicrobial tests were done by microdilution method and minimum inhibitory concentrations of these derivatives were between 500-1000 μg/mL.

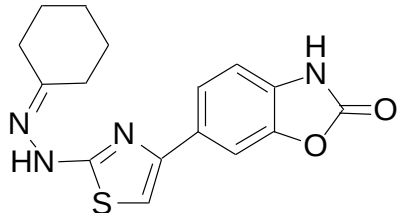


Figure 2.27 Structure of 6-thiazolyl benzoxazolone derivative [19]

Soyer and co-workers studied determination of antimicrobial activities for propanamide substituted derivatives of benzoxazolone againts gram positive bacteria and several types of fungi [20] as shown in figure 2.28. The minimum inhibitory concentrations of these compounds were evaluated by microdilution and found to have high activity against *E.coli and S.aureus.*

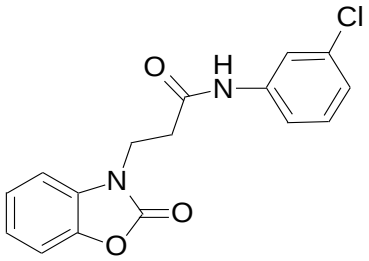


Figure 2.28 Structure of compound with benzoxazolone and propanamide derivative bearing substituent [20]

Modiya *et al* studied antimicrobial activity of triazole substituted benzoxazolone derivatives at 5^{th} position with disk diffusion method for antimicrobial susceptibility testing against gram negative bacteria followed by microdilution method in order to test minimum effective concentrations [41]. The most active compound in this study is shown in figure 2.29.

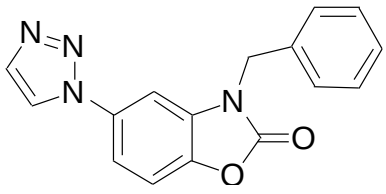


Figure 2.29 Structure of triazole substituted benzoxazolone derivative [41].

Gokhan *et al* performed investigations on benzoxazolone derivatives with acyl groups at 6th position and piperazinomethyl group at 3rd position. Other than analgesic-antiinflammatory effects, these derivatives were screened for their antimicrobial activities [42]. The structure of a compound synthesized is shown in figure 2.30. As a result, most of these compounds showed moderate activity against tested bacteria and fungi.

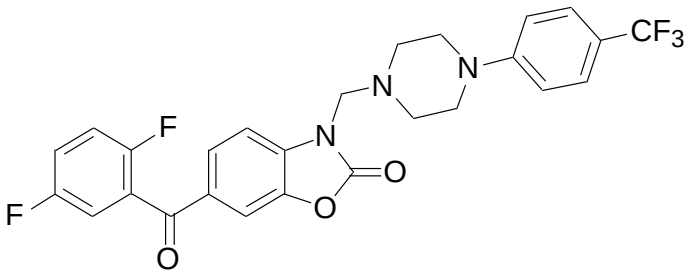
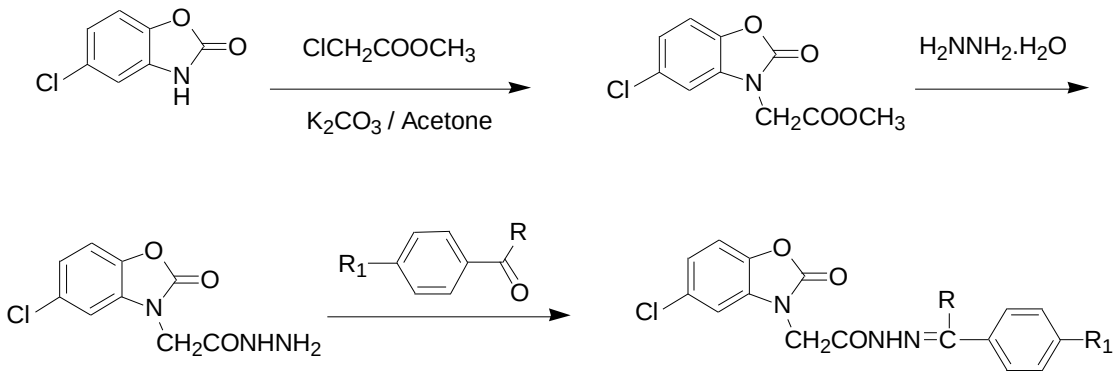


Figure 2.30 6-Acyl-3-piperazinomethyl-2-benzoxazolinone derivative with antimicrobial properties [42]

Onkol *et al* performed microwave synthesis of chlorzoxazone derivatives containing hydrazine or hydrazone moieties in third position, in order to be screened for their antimicrobial activities [43]. Some of these derivatives have activity against *Staphylococcus aureus* but all derivatives were effective against *Candida albicans* showing higher antifungal activity than reference drug used as positive control (fuconazole). The synthesis method is shown in fig.2.31.



R : CH_3 or H and R_1 : Halogens (F, Cl or Br)

Figure 2.31 Synthesis method of chlorzoxazone derivatives containing hydrazine or hydrazone moieties at 3rd position [43]

Erol *et al* studied antimicrobial activity of new benzoxazolone derivatives with thiazolinomethyl groups on their 3rd position [44]. An example for synthesized compounds in this study is shown in fig.2.32. The activities were evaluated by both disk techniques and tube dilution method. The compounds were found to have antifungal affects against *Candida albicans* with a minimum inhibitory concentrations within the range of 12,5-25 μg/ml.

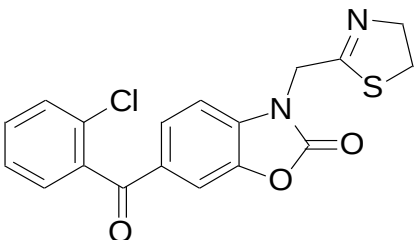
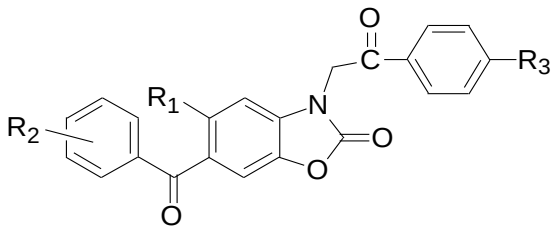


Figure 2.32 Thiazolinomethyl-2(3H)-benzoxazolone derivative with antimicrobial activity [44]

Koksal *et al* studied newly prepared benzoxazolinone derivatives having benzoylmethyl moieties substituted on 3rd position and screened for their antimicrobial activities using different strains of bacteria and three different fungi species[45]. Their minimum inhibitory concentration is determined using microdilution method. General structure of tested derivatives is shown in fig 2.33.



R_1 : Cl, R_2 : 3-Fluoro or 2-Fluoro, R_3: H, Cl or Br

Figure 2.33 General structure of benzoxazolinone derivatives with benzoylmethyl moieties at position 3 [45]

2.4 General Methods Used For Cytotoxicity Test

2.4.1 MTT Assay

Cytotoxicity determination methods can be colorimetric, luminescent or enzymatic [46]. The MTT test is a kind of colorimetric test to evaluate metabolic activity of cells [46]. Cellular oxidoreductases are NADPH dependent enzymes which can affect the number of viable cells. MTT is a tetrazolium bromide salt used as a dye which is reduced by oxidoreductase enzymes to form insoluble formazan salts with purple color. Tetrazolium salts have heterocyclic organic structures which can be reduced to a structure called formazan product by gaining electrons only in the active mitochondria where a colour change occurs only in living cells due to the formation of formazan salt products [47] as shown in figure 2.34.

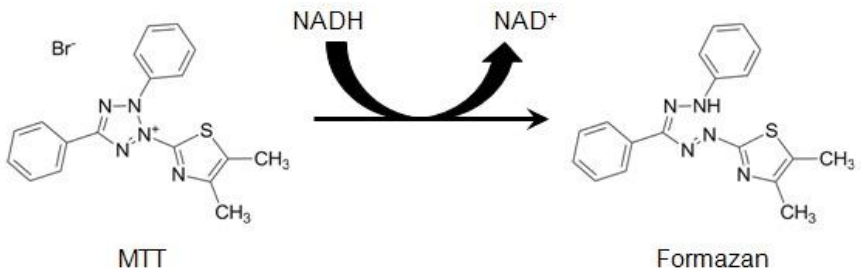


Figure 2.34 Formation of formazan products from MTT [47]

The absorbance of this colored solution can be determined by measuring it with a spectrophotometer at a range of wavelengths between 500-600 nm [48]. The degree of light absorption depends on the degree of concentration of formazan salt deposits inside the cell and on the cell surface [48].

2.4.2 TUNEL Assay

TUNEL is the abbreviation of "terminal deoxynucleotidyl transferase dUTP nick end labeling". In this method, fragmentation of DNA is detected as a result of apoptosis. In individual cells, TUNEL assay is widely used to identify programmed cell death or to detect break down of DNA strands in excess amounts [47,48]. Therminal deoxynucleotidyl transferase enzyme catalyzes the binding of labeled deoxynucleotides. They can be labelled with biological markers such fluorochromes and binds to the 3' hydroxyl endings is the main mechanism under TUNEL method. It can also label cells with damaged DNA in other ways during apoptosis [48].

2.4.3 Immunocytochemical Evaluations

Immunocytochemistry is a technique to search for a specific antigen (protein) in a cell [47,48]. In research or pathology laboratories, immunocytochemical reactions are applied in different situations.

The most important applications are histogenetic diagnosis of tumor cells, determination of tumor subtypes (such as lymphoma), characterization of the first site of the malignant tumor, investigation of therapeutic symptoms and prognostic factors in some diseases, benign and distinguishing malignant tumors, understanding the structures and materials secreted by the cell [48].

Immunocytochemical evaluations can be used to detect presence of antigens-antibody bindings of; cytochrome-c from mitochondria, caspase-3 enzyme and Fas-ligand which induce intrinsic or extrinsic pathways of programmed cell death using specific primary and secondary antibodies as shown in figure 2.35 [49].

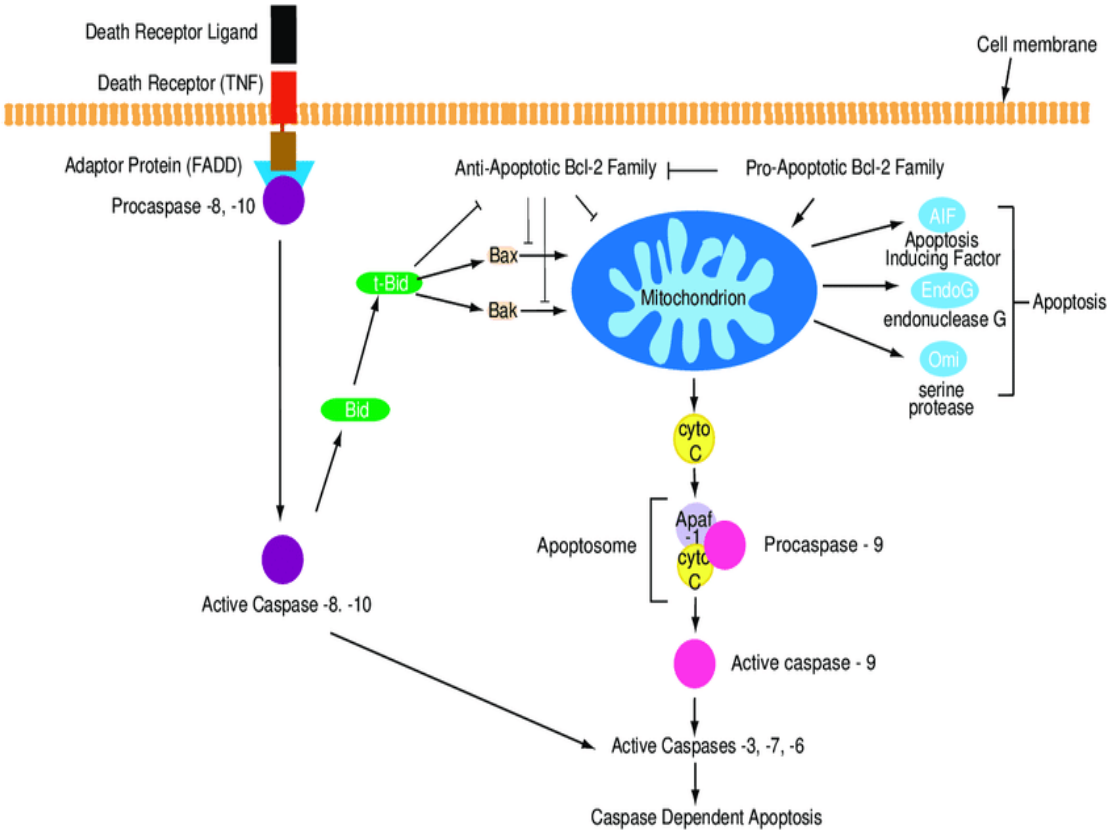


Figure 2.35 Extrinsic and intrinsic pathways of apoptosis [49]

2.5 General Methods Used for Antimicrobial Test

2.5.1 Disk diffusion test

The most common method for detecting antibiotic sensitivity in routine laboratories is disk diffusion tests as shown in fig.2.36 [50]. This method was developed by Kirby Bauer. In this test, absorbed antibiotic is applied to the medium in which the organism is inoculated. Paper discs are impregnated with certain amounts of antibiotics in which the microorganism to be tested is heavily inoculated and placed on agar medium. As the discs dissolve after a while and diffuse towards the aggregate, the inoculated microorganism also begins to multiply. The more inhibition zone formed around the disk means the more sensitive the microorganism is to the drug. The millimetric measurements of inhibition zones were done and the sensitivity status of microorganism against the antimicrobial agents used is determined.

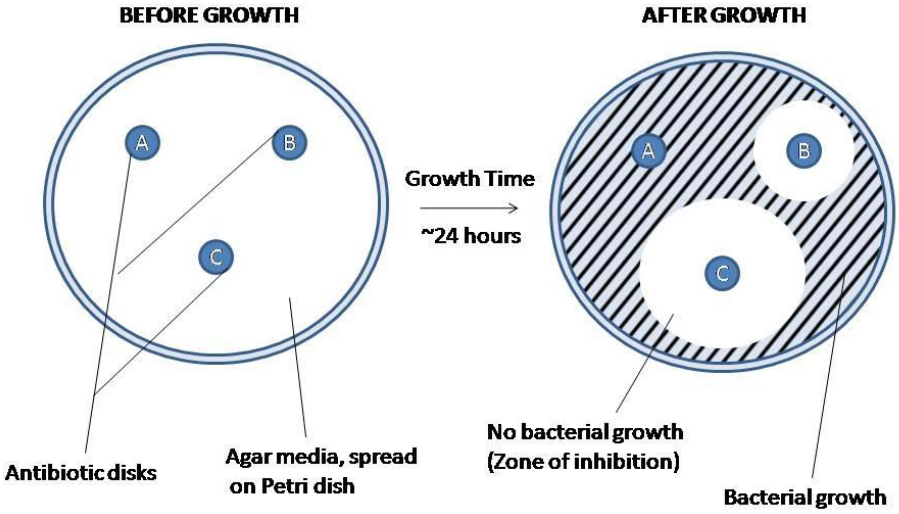


Figure 2.36 Demonstration of disk diffusion method before and after bacterial growth [50]

3. MATERIALS AND METHODS

3.1. Chemical Methods

The chemicals used for organic synthesis were obtained from Sigma Aldrich Chemical Co and the melting points of molecules were measured using Mettler Toledo FP 900 Thermo System device in the laboratory.

The attenuated reflection of each synthesized molecule was examined from Infrared spectroscopy using a spectrophotometer, Perkin Elmer Spectrum 100 shown in wave numbers (cm^{-1}). The proton and carbon nuclear magnetic resonance spectrum of each molecules were examined on NMR device of Mercury Varian 400 MHz where tetramethylsilane was used as a standard solution.

As a solvent, deuterated chloroform and dimethylsulfoxide (DMSO-d_6) were suitable solvents for analysis. Values of different types of protons and carbons on the structures were measured in parts per million (ppm) as chemical shifts (δ). Leco CHNS 932 analyzer device was used to perform the elemental analysis. For the purification of compounds, thin layer chromatography method was used on silica gel GF 254 (DC-Alufplien-Kieselgel, Germany)

3.2. General Procedure for Synthesis

In the synthesis, equal molar concentrations of 2(3*H*)-benzoxazolone or 5-chloro-2(3H)-benzoxazolone and appropriate piperazine derivatives were dissolved separately in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture followed by heating under reflux conditions for 1 hour [51]. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

3.3. Cell Line and Cell Culture

Non-metastatic MCF-7 (ATCC: HTB-22) and metastatic M4A4 (ATCC: CRL-2914) were used as breast cancer cell lines. MCF-7 and M4A4 cell lines were put in RPMI-1640 medium. 10% heat inactivated fetal bovine serum which is used to supply growth facors needed for their growth. 1% penicillin-streptomycin was used to eradicate the growth of any bacteria if contamination was present and 1% glutamine were included in cell culture which was performed at 37°C in 5% CO_2 with humidified atmosphere. As the cultured cells reached their state of confluency, the subculute was done using solution of 0.25% trypsin-EDTA. All materials were obtained from Biochrom, EMB Millipore and Capricorn Scientific.

3.4 Cell Viability and Growth Assay

The cytotoxicity of derivatives were screened by performing MTT test protocol. 100 mM of benzoxazolone compounds **1** and **2** were prepared in DMSO. RPMI-1640 medium was used for making the dilutions with concentrations between 5 μM-100 μM of each compound. Same procudures were applied for compounds **5** and **6** but ethanol was used as a solvent.

Both DMSO and ethanol final concentrations in cell lines were less than 0.05%. MCF-7 and M4A4 cells with 100 μM RPMI-1640 medium were seeded in 96-well culture dishes with 5 x 10^4/ml density of each well. The row with no cells or tested compounds was negative control, they only contain the solvent itself. On the other hand, the row which contains only living cells was used as positive control.

Compound dilutions were repeated three times and both cell lines were left for incubation for 24 hours and 48 hours. 10 µl of MTT solution which was heated to 37°C was added on each well and left for incubation for 4 hours. The conditions were kept constant at a temperature of 37°C in 5% concentration of CO_2. 50 µl of appropriate solvent was used to dissolve purple formazan products. A spectrophotometer was used at 540 nm and the absorbance values of each concentration were measured for all compounds. All of the procedures mentioned were triplicated for each compound.

3.5. TUNEL Assay for Detection of Cell Death

Fragmentation of DNA was detected by labelling apoptotic cells with specific staining while using commercial apoptosis detection kit in situ (ApoptagPlus Peroxidase In Situ Apoptosis Detection Kit, S7101, Millipore, USA).

3.6. Immunocytochemistry Evaluations

Immunocytochemical evaluations of cultured MCF-7 cells with tested compounds for binding of labelled antibodies to the antigens; cytochrome-c, caspase-3, and Fas-ligand (FasL) were performed. Fixation process of MCF-7 cells were done using 4% paraformaldehyde with PBS at 4°C. In order to enhance the permeabilization of cells, permeabilization agents were used such as Tween 20. 3% concentration of H_2O_2 was used to quench the activity of endogenous peroxidase enzyme after incubation at room temperature and after washing cells with PBS three times for 5 minutes, unlabelled primary antibodies were treated with targeting caspase-3, cytochrome-c and Fas-Ligand antigens and left for incubation to enhance binding at 4°C overnight. All the target proteins were obtained from Santa Cruz Biotechnology, USA.

Secondary antibodies which were biotinylated were added and left for incubation for half an hour followed by triplicated PBS wash. 100 µl of streptavidin-peroxidase complex was put into the cultured cells which were treated with PBS.

The cells were left for incubation to improve immuno-labelling for 5 minutes after addition of cromogen (DAB). Distilled water was used to wash DAB. Mayer's hematoxylien was used for counterstaining of cells about 5 minutes and prepared via mounting medium. All samples were illustrated using a light microscope.

Grades for the staining of target antigens were done semi quantitatively with H-SCORE= Σл (i+1) equation. The i value shows the staining with 1 for mild, 2 for moderate and 3 for severe intensity and л shows the stained cells % varying between 0-100 range for each intensity.

3.7 Statistical Anlaysis

Results are given as mean ± standard deviation which is shown as 'SD'. The results were analyzed by using a software called GraphPad Prism 7. Group differences founded as statistically appropriate were calculated using Mann-Whitney method. A *p* value lesser than 0,05 was meaning that the results obtained were considered as statistically significant.

3.8. Microbiology

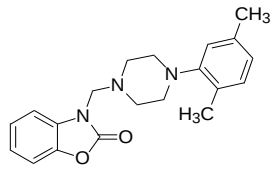
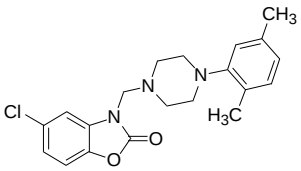
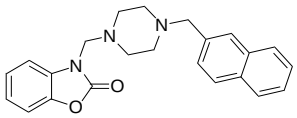
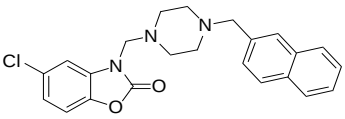
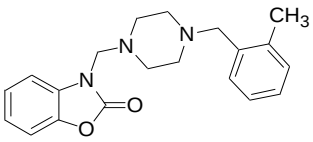
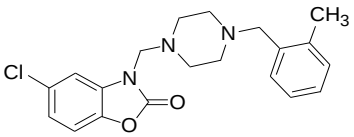
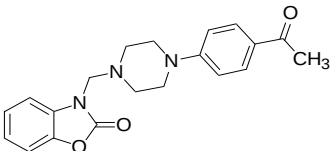
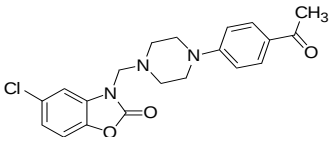
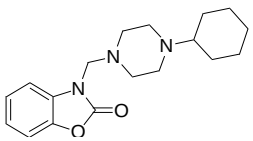
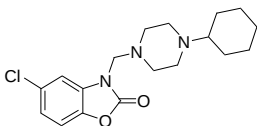
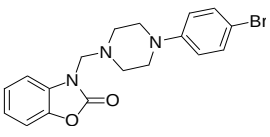
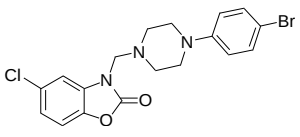
For microbiology tests, disk diffusion method was studied according to EUCAST protocol. Different strains of gram positive bacteria such as *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 14579) and *Enterococcus faecalis* (ATCC 29212), gram negative bacteria such as *Escherichia coli* (ATCC 25922) are used for antibacterial activity whereas *Candida albicans* (ATCC 90028) was used for antifungal activity study. The surface of agar with human blood for seeding bacterial strains and fungi were inoculated.

3 mL of sterile saline solution was used to prepare microbial suspensions with 0.45% NaCl and pH of 5.0-7.2 and an optical density of 0.5 according to McFarland standard scale. The 'Mueller- Hinton' type of agar plates were left for incubation at a temperature of 37 °C for 24 hours for different strains of bacteria and fungi. DMSO was used as a solvent to dissolve specified quantity of synthesized compounds. Each compound was diluted to get concentration of 1000 ug/L. Filter-paper disks were put in each agar plate which were soaked in tested compounds, in positive controls and negative controls. For positive control, tetracyclin is used for *Staphylococcus aureus, Bacillus cereus* and *Enterococcus faecalis.* Ciprofloxacin is preferred for *Escherichia coli* and for *Candida albicans*, amphotericin B is used. For the negative control, DMSO itself is used. After that procedure, the Petri dishes were left for incubation for 24 hours at a constant temperature of 37 °C and diameter of inhibition zones for each disk was measured with millimetric scale.

4. CHEMISTRY RESULTS

The characterizations of benzoxazolone derivatives were done by FT-IR spectrum, ^{1}H-NMR and ^{13}C-NMR spectra as well as elemental analysis. The list of newly synthesized derivatives are given in table 4.1.

Table 4.1 Structures of synthesized benzoxazolone derivatives

 Compound **1**	 Compound **2**	 Compound **3**
 Compound **4**	 Compound **5**	 Compound **6**
 Compound **7**	 Compound **8**	 Compound **9**
 Compound **10**	 Compound **11**	 Compound **12**

4.1. Chemical Data of Compound 1

IUPAC Name : 3-[4-(2,5-Dimethylphenyl)piperazin-1-ylmethyl]-3H-benzoxazol-2-one

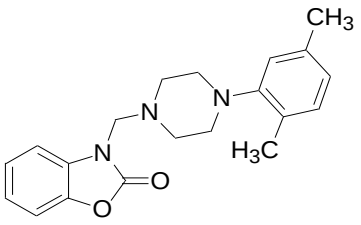


Figure 4.1 Structure of compound **1**

Equal molar concentrations of 2(3H)-benzoxazolone and 2,5-dimethylphenyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 51.3 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Spot Detection: Under UV lamp at 254 nm

Retention Factor (Rf): 0.32

Physical Appearance: Bright yellowish white powder.

Solubility: Completely soluble in chloroform and dimethylsulfoxide.

Slightly soluble in ethanol and methanol in room temperature.

Melting Point : 144 °C

Molecular Formula: $C_{20}H_{23}N_3O_2$

Molecular Weight (g/mol): 337,42

Elemental Analysis

	C	H	N
Calculated % :	71.19	6.87	12.45
Found % :	70.77	7.14	12.52

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2810-3060 (Aliphatic C-H), 1760 (C=O)

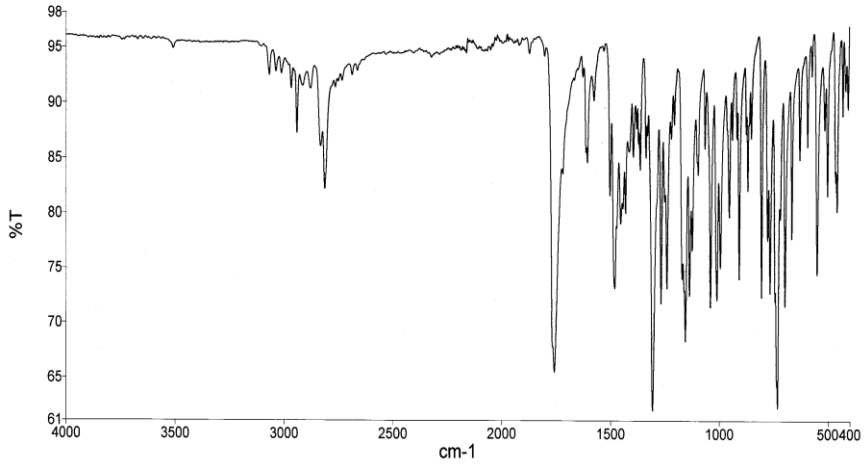


Figure 4.2 FT-IR spectrum of compound **1**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.3-6.7 (m, 7 H, Ar-CH), 4.7 (s, 2 H, CH_2), 2.9 (t, 4 H , pip-CH_2 H^2, H^6), 2.8 (t, 4 H, pip-CH_2 H^3, H^5), 2.3 (s, 3H, CH_3), 2.2 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.4, 151.1, 142.6, 136.1, 131.9, 130.9, 129.4, 123.9, 122.6, 119.8, 110.1, 109.4, (Ar-C), 64.8 (CH_2), 51.5, 51.1 (pip-C), 21.2, 17.4 (CH_3).

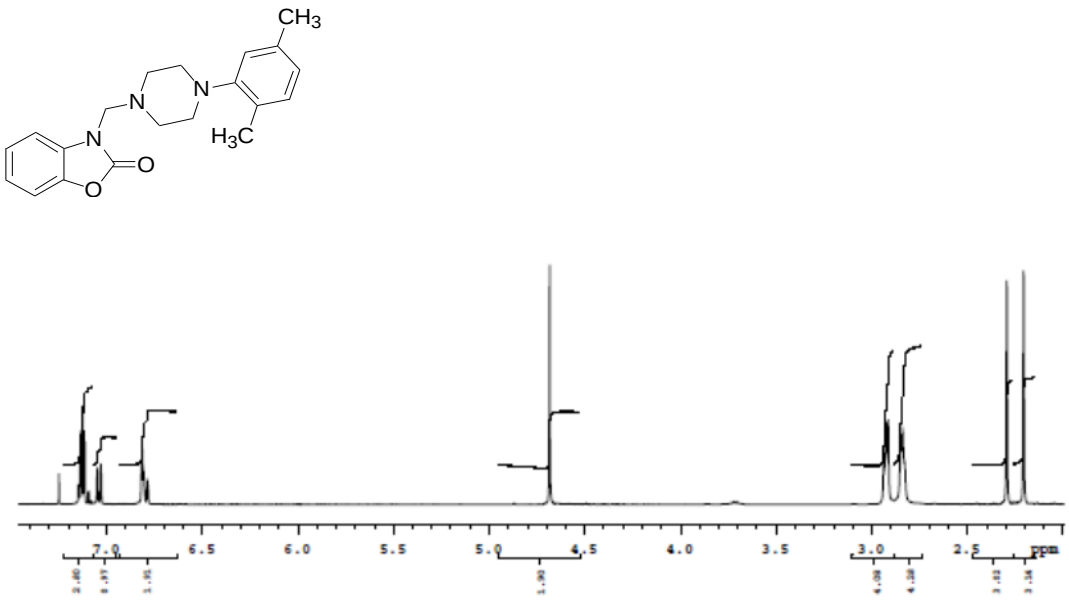


Figure 4.3 ^{1}H NMR spectrum of compound **1**

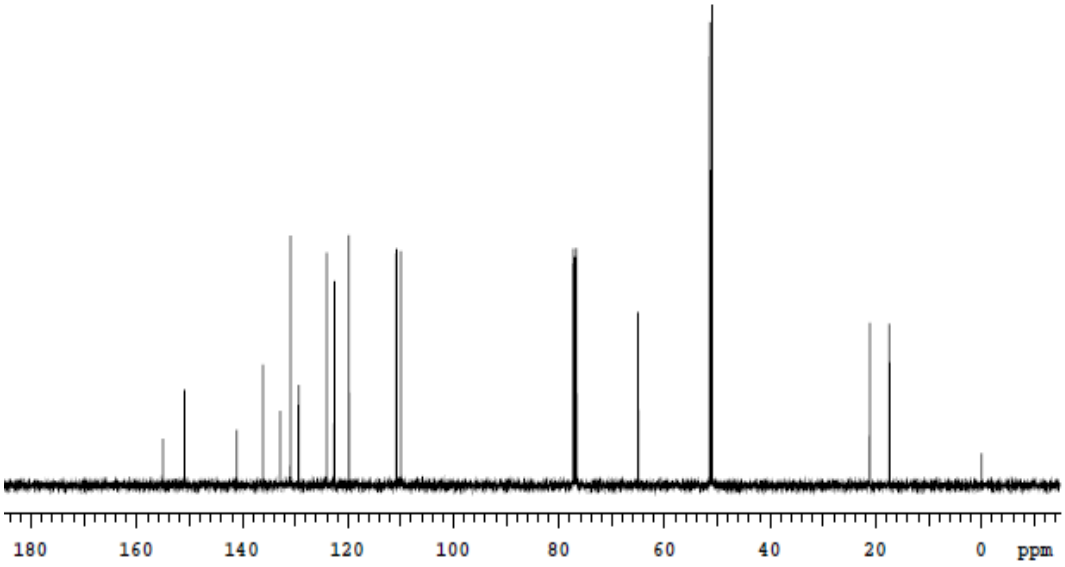


Figure 4.4 ^{13}C NMR spectrum of compound **1**

4.2. Chemical Data of Compound 2

IUPAC Name : 5-Chloro-3-[4-(2,5-dimethylphenyl)-piperazin-1-ylmethyl]-3H-benzoxazol-2-one

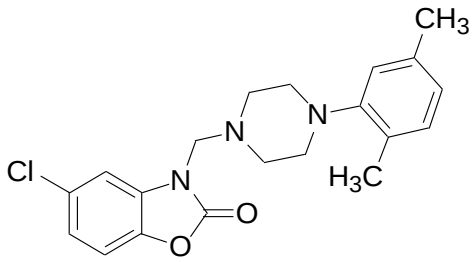


Figure 4.5 Structure of compound **2**

Equal molar concentrations of 5-chloro-2(3H)-benzoxazolone and 2,5-dimethylphenyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 30.8 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Spot Detection: Under UV lamp at 254 nm

Retention Factor (Rf): 0.34

Physical Appearance: Bright white powder.

Solubility: Completely soluble in chloroform and dimethylsulfoxide.

Slightly soluble in ethanol and methanol in room temperature.

Melting Point : 134.4 °C

Molecular Formula: $C_{20}H_{22}Cl\ N_3O_2$

Molecular Weight (g/mol): 371,86

Elemental Analysis

	C	H	N
Calculated % :	63.60	5.96	11.30
Found % :	63.49	6.09	11.35

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2810-3060 (Aliphatic C-H), 1760 (C=O).

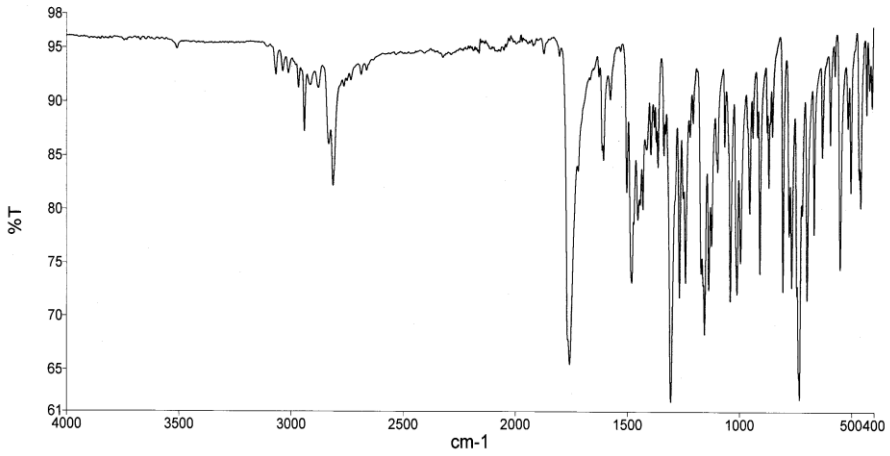


Figure 4.6 FT-IR spectrum of compound **2**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.3-6.7 (m, 6 H, Ar-CH), 4.7 (s, 2H, CH_2), 2.9 (t, 4 H, pip-CH_2 H^2, H^6), 2.8 (t, 4 H, pip-CH_2 H^3, H^5), 2.3 (s, 3H, CH_3), 2.2 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.1, 150.9, 141.1, 136.1, 132.8, 130.9, 129.4, 124.1, 122.6, 119.8, 110.8, 109.8, (Ar-C), 65.1 (CH_2), 51.5, 51.1 (pip-C), 21.2, 17.4.

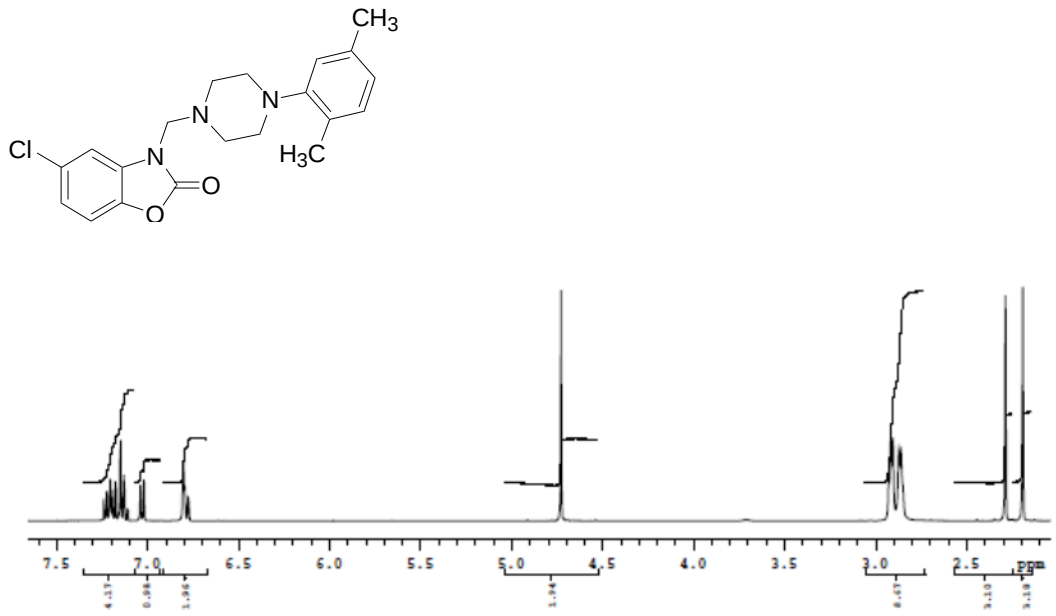


Figure 4.7 ^{1}H NMR spectrum of compound **2**

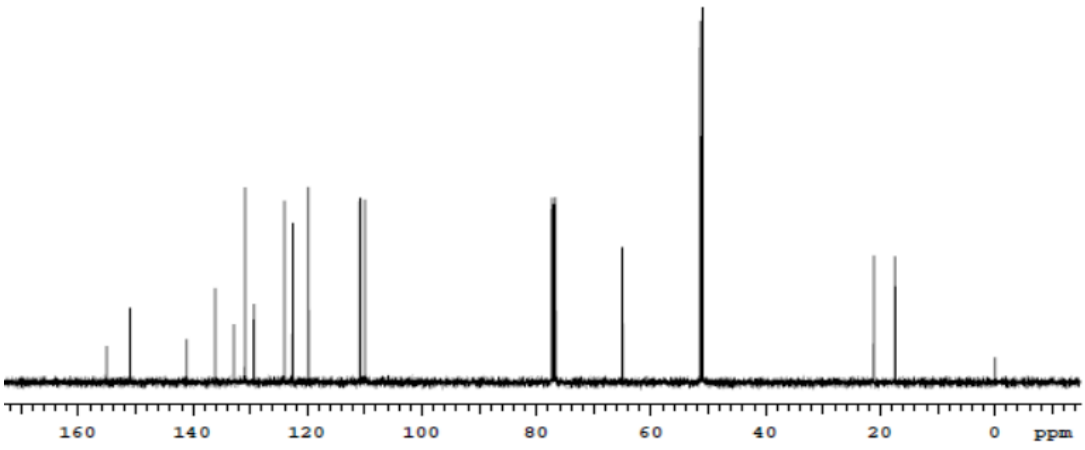


Figure 4.8 ^{13}C NMR spectrum of compound **2**

4.3. Chemical Data of Compound 3

IUPAC Name : 3-(4-Naphthalen-2-ylmethylpiperazin-1-ylmethyl)-3H-benzoxazol-2-one

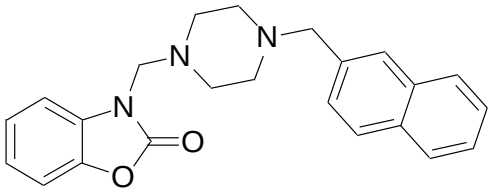


Figure 4.9 Structure of compound **3**

Equal molar concentrations (15 mmol) of 2(3H)-benzoxazolone and 2-napthylmethyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 40.7 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.27

Physical Appearance: Pinky white powder.

Solubility: Highly soluble in chloroform. Slightly soluble in ethanol and methanol in room temperature.

Melting Point : 156.5 °C

Molecular Formula: $C_{23}H_{23}N_3O_2$

Molecular Weight (g/mol): 373,45

Elemental Analysis

	C	H	N
Calculated % :	73,97	6,21	11,25
Found % :	72,81	6,15	11,18

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H), 2810-3060 (Aliphatic C-H), 1765 (C=O).

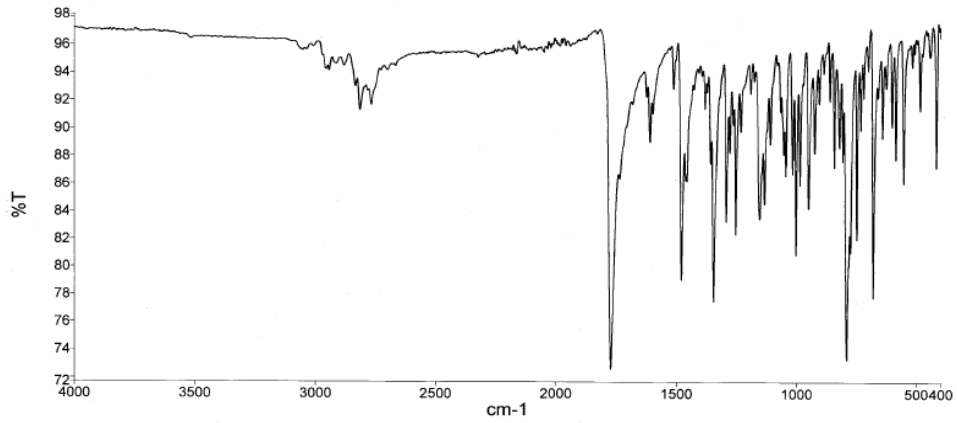


Figure 4.10 FT-IR spectrum of compound **3**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.5-6.7 (m, 11H, Ar-CH), 4.7 (s, 2H, CH_2),3.9 (s, 2H, CH_2), 2.9 (t, 4H, pip-CH_2 H^2, H^6), 2.8 (t, 4H, pip-CH_2 H^3, H^5).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.1, 150.9,142.5, 141.1, 136.1,133.8, 132.8, 130.9, 129.4,127.3,125.7, 124.1, 122.6, 119.8, 110.8, 109.8, (Ar-C), 65.1 (CH_2), 60.9(CH_2), 53.0, 50.5 (pip-C).

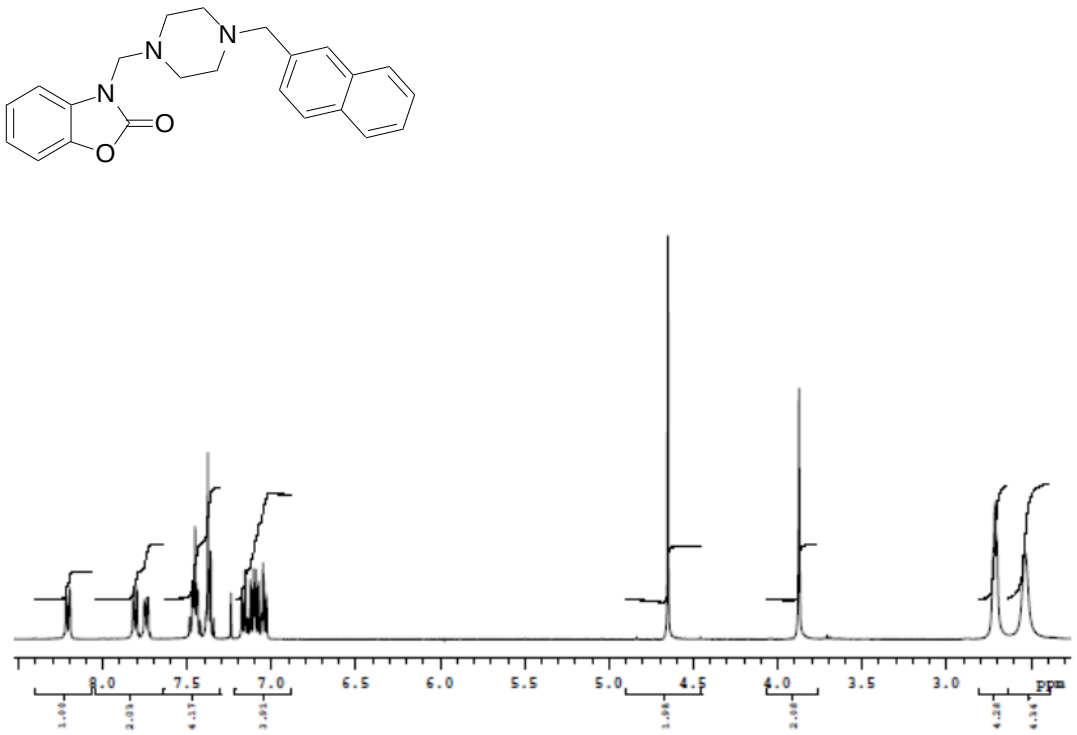


Figure 4.11 ^{1}H NMR spectrum of compound **3**

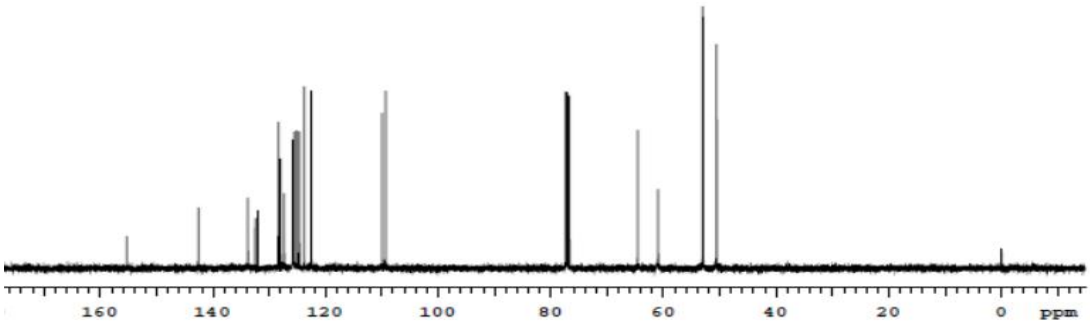


Figure 4.12 ^{13}C NMR spectrum of compound **3**

4.4. Chemical Data of Compound 4

IUPAC Name : 5-Chloro-3-(4-naphthalen-2-ylmethylpiperazin-1-ylmethyl)-3H-benzoxazol-2-one

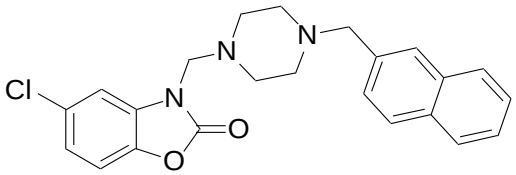


Figure 4.13 Structure of compound **4**

Equal molar concentrations (15 mmol) of 5-chloro-2(3H)-benzoxazolone and 2-napthylmethyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 50.2 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.24

Physical Appearance: White powder.

Solubility: Highly soluble in chloroform. Slightly soluble in ethanol and methanol in room temperature.

Melting Point : 151.2 °C

Molecular Formula: $C_{23}H_{22}Cl\ N_3O_2$

Molecular Weight (g/mol): 407,89

Elemental Analysis

	C	H	N
Calculated % :	67,66	5,39	10,29
Found % :	67,73	4,86	10,21

FT-IR Infrared Spectrum

Vmax (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2810-3060 (Aliphatic C-H), 1760 (C=O).

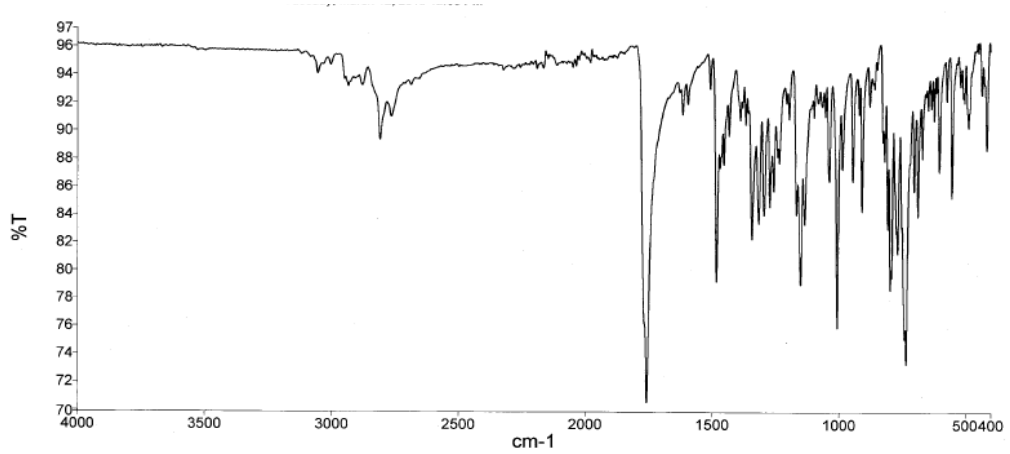


Figure 4.14 FT-IR spectrum of compound **4**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.1-6.8 (m, 10H, Ar-CH), 4.7 (s, 2H, CH_2),3.9 (s, 2H, CH_2), 2.9 (t, 4H, pip-CH_2 H^2, H^6), 2.8 (t, 4H, pip-CH_2 H^3, H^5).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.1, 150.9,142.5, 141.1, 136.1,133.8, 132.8, 130.9, 129.4,127.3,125.7, 124.1, 122.6, 119.8, 110.8, 109.8, (Ar-C), 65.1 (CH_2), 60.9(CH_2), 53.0, 50.5 (pip-C).

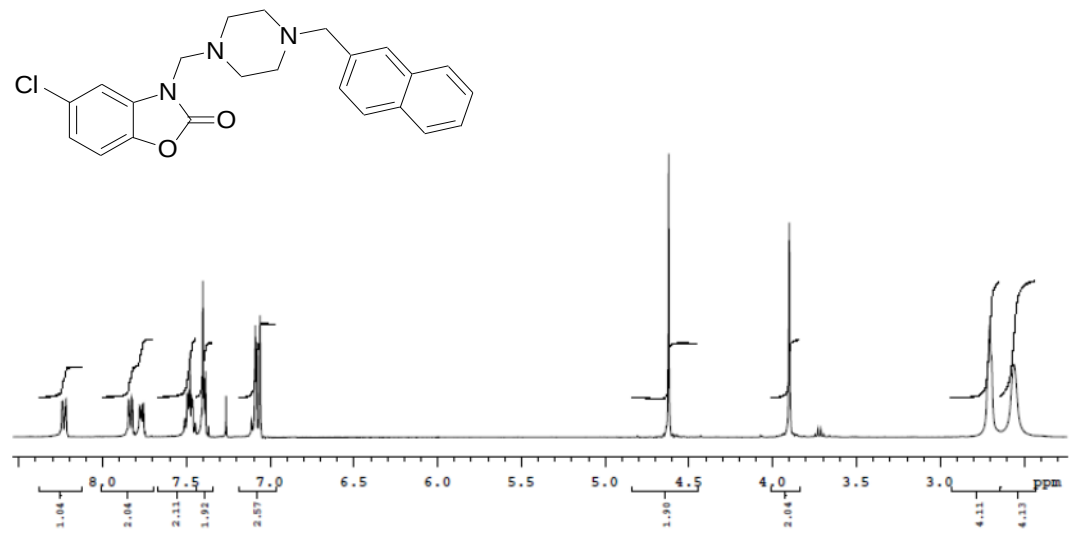


Figure 4.15 ^{1}H NMR spectrum of compound **4**

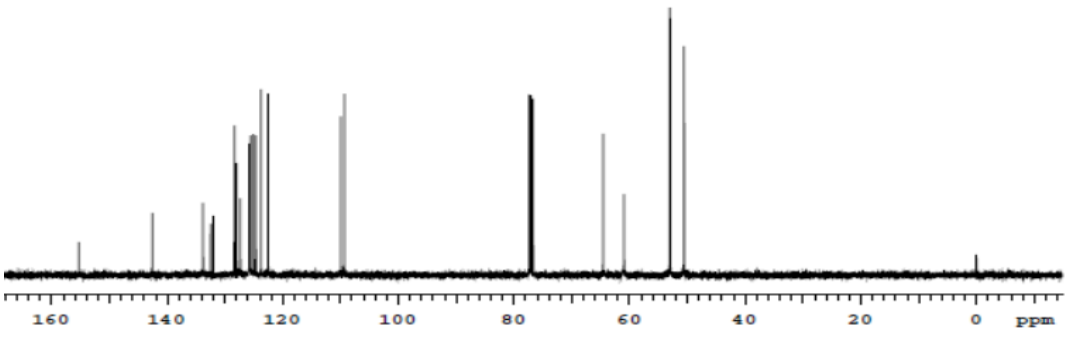


Figure 4.16 ^{13}C NMR spectrum of compound **4**

4.5. Chemical Data for Compound 5

IUPAC Name : 3-[4-(2-Methylbenzyl) piperazin-1-ylmethyl]-3H-benzoxazol-2-one

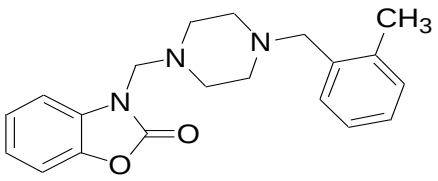


Figure 4.17 Structure of compound **5**

Equal molar concentrations (15 mmol) of 2(3H)-benzoxazolone and 2-methylbenzyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 54.2 %

Thin Layer Chromatography

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.40

Physical Appearance: Dark yellow powder.

Solubility: Soluble in ethanol, chloroform and dimethylsulfoxide.

Melting Point : 189.3 °C

Molecular Formula: $C_{20}H_{23}N_3O_2$

Molecular Weight (g/mol): 337,42

Elemental Analysis

	C	H	N
Calculated % :	71,12	6,81	12,44
Found % :	70,80	6,52	12,38

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H), 2810-3100 (Aliphatic C-H), 1753 (C=O).

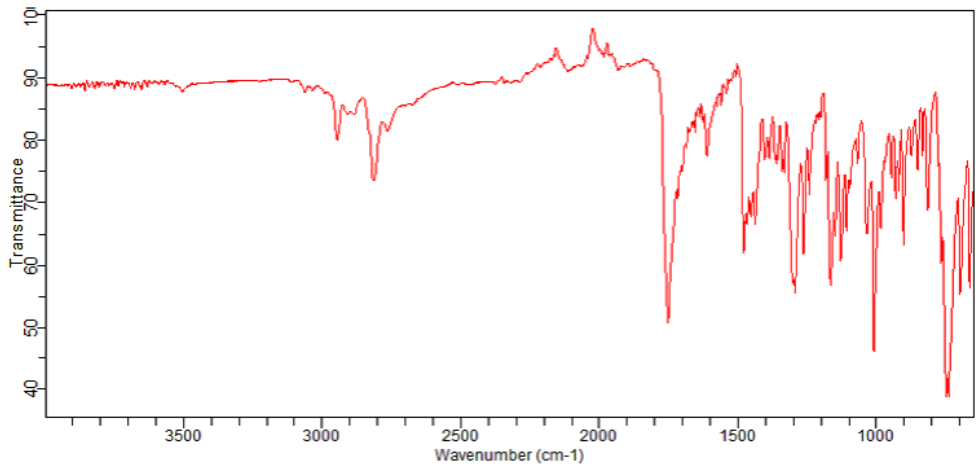


Figure 4.18 FT-IR spectrum of compound **5**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.4-6.7 (m, 8H, Ar-CH), 4.7 (s, 2H, CH_2),3.5 (s, 2H, CH_2), 2.9 (t, 4H, pip-CH_2 H^2, H^6), 2.8 (t, 4H, pip-CH_2 H^3, H^5), 2.3 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.3,142.5, 136.2,130.2, 129.7,127.0,125.4, 124.5, 123.7, 122.5, 109.9, 109.3, (Ar-C), 64.5 (CH_2), 60.6 (CH_2), 52.8, 50.6 (pip-C), 19.1 (CH_3).

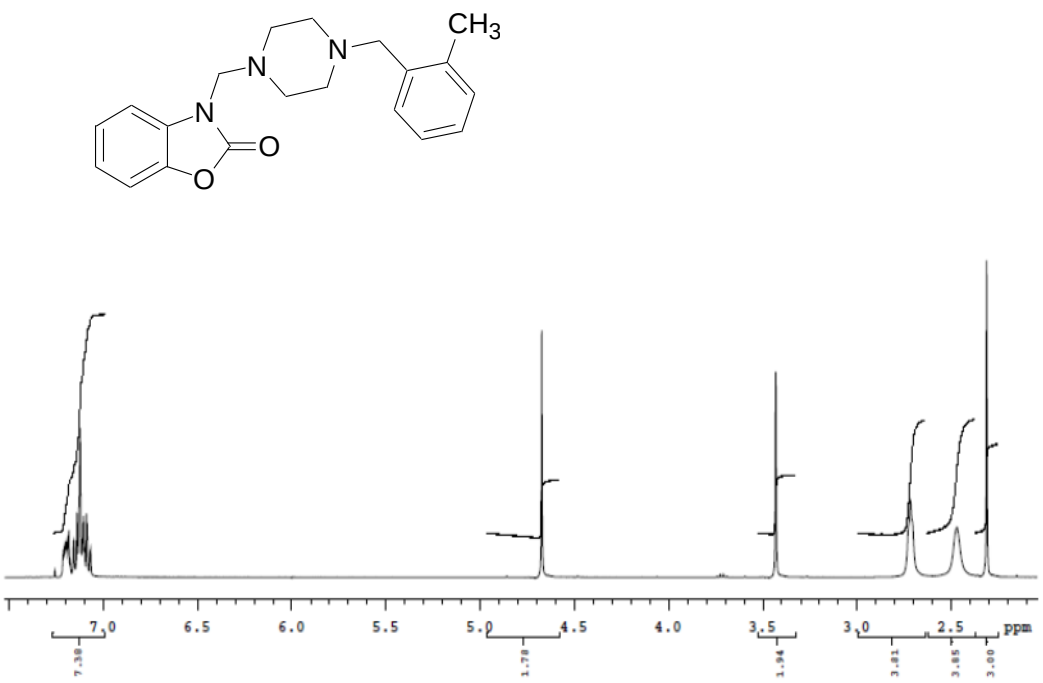


Figure 4.19 ^{1}H NMR Spectrum of Compound **5**

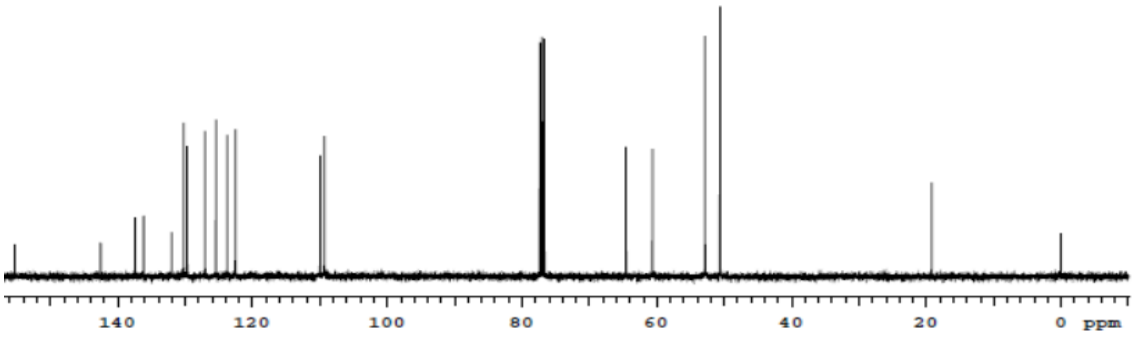


Figure 4.20 ^{13}C NMR spectrum of compound **5**

4.6. Chemical Data of Compound 6

IUPAC Name : 5-Chloro-3-[4-(2-methylbenzyl)piperazin-1-ylmethyl]-3H-benzoxazol-2-one

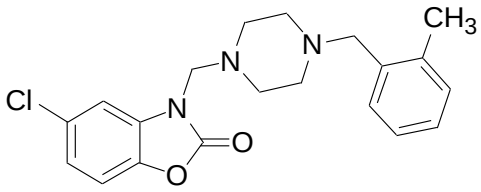


Figure 4.21 Structure of compound **6**

Equal molar concentrations (15 mmol) of 5-chloro-2(3H)-benzoxazolone and 2-methylbenzyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 45 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.47

Physical Appearance: White crystalline powder.

Solubility: Completely soluble in ethanol, chloroform and dimethylsulfoxide.

Melting Point : 197.4 °C

Molecular Formula: $C_{20}H_{22}ClN_3O_2$

Molecular Weight (g/mol): 371,86

Elemental Analysis

	C	H	N
Calculated % :	64,54	5,91	11,29
Found % :	64,71	5,64	11,30

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H), 2810-3100 (Aliphatic C-H), 1770 (C=O).

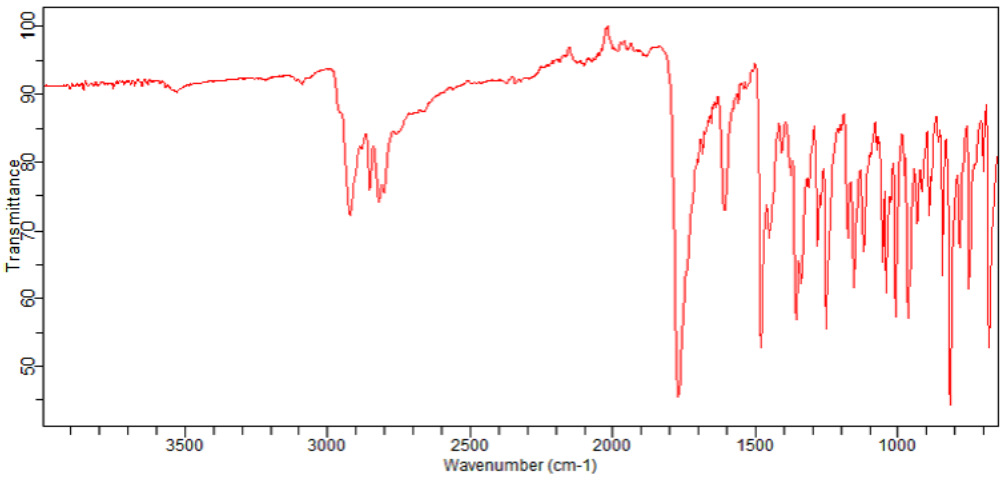


Figure 4.22 FT-IR spectrum of compound **6**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.3-6.9 (m, 7H, Ar-CH), 4.7 (s, 2H, CH_2),3.5 (s, 2H, CH_2), 2.9 (t, 4H, pip-CH_2 H^2, H^6), 2.8 (t, 4H, pip-CH_2 H^3, H^5), 2.3 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.0, 141.0,137.4, 136.1, 132.9 ,130.2, 129.3,127.1,125.4, 122.5, 110.7, 109.3, (Ar-C), 64.8 (CH_2), 60.6 (CH_2), 52.7, 50.6 (pip-C), 19.1 (CH_3).

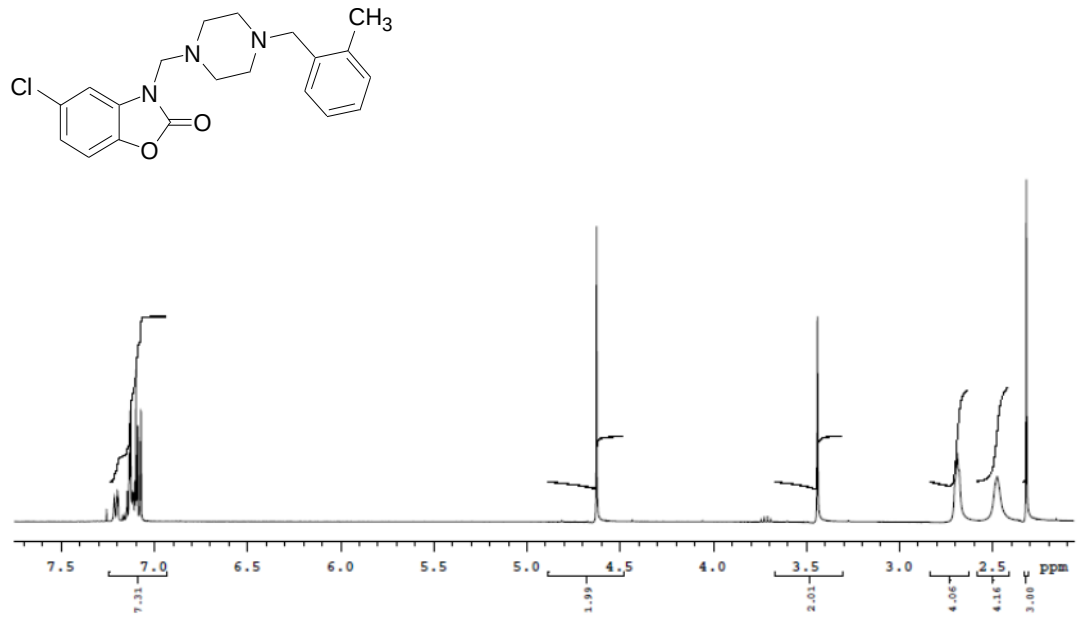


Figure 4.23 ^{1}H NMR spectrum of compound **6**

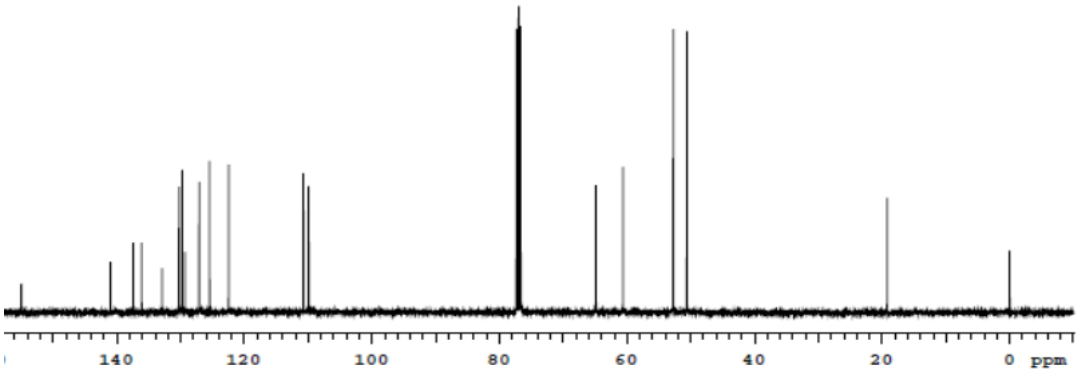


Figure 4.24 ^{13}C NMR spectrum of compound **6**

4.7. Chemical Data of Compound 7

IUPAC Name: 3-[4-(4-Acetylphenyl)piperazin-1-ylmethyl]-3H-benzoxazol-2-one

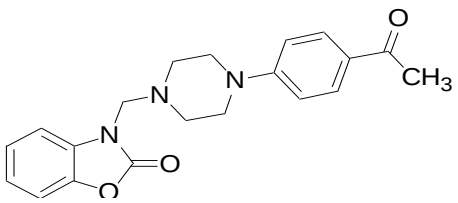


Figure 4.25 Structure of compound **7**

Equal molar concentrations (15 mmol) of 2(3H)-benzoxazolone and 4-acetylphenyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 21 %

Thin Layer Chromatography

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.42

Physical Appearance: Light yellow powder.

Solubility: Highly soluble in chloroform and dimethylsulfoxide. Slightly soluble in ethanol and methanol in room temperature.

Melting Point : 191.2 °C

Molecular Formula: $C_{20}H_{21}N_3O_3$

Molecular Weight (g/mol): 351,40

Elemental Analysis

	C	H	N
Calculated % :	68,29	5,97	11,95
Found % :	68,36	6,02	11,90

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2810-3100 (Aliphatic C-H), 1751 (C=O).

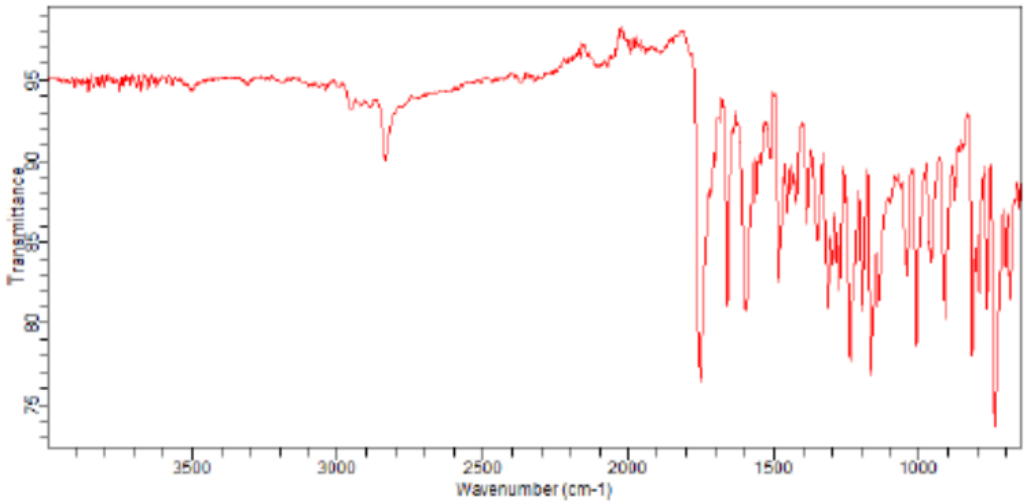


Figure 4.26 FT-IR spectrum of compound **7**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.8-6.8 (m, 8H, Ar-CH), 4.7 (s, 2H, CH_2), 2.9 (t, 4H, pip-CH_2 H^2, H^6), 3.4 (t, 4H, pip-CH_2 H^3, H^5), 2.8 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.2, 153.8,142.5, 131.6, 130.3,129.4,127.9,123.9,122.8, 113.6, 110.1, 109.2, (Ar-C), 64.4 (CH_2), 50.1, 47.2 (pip-C), 26.1 (CH_3).

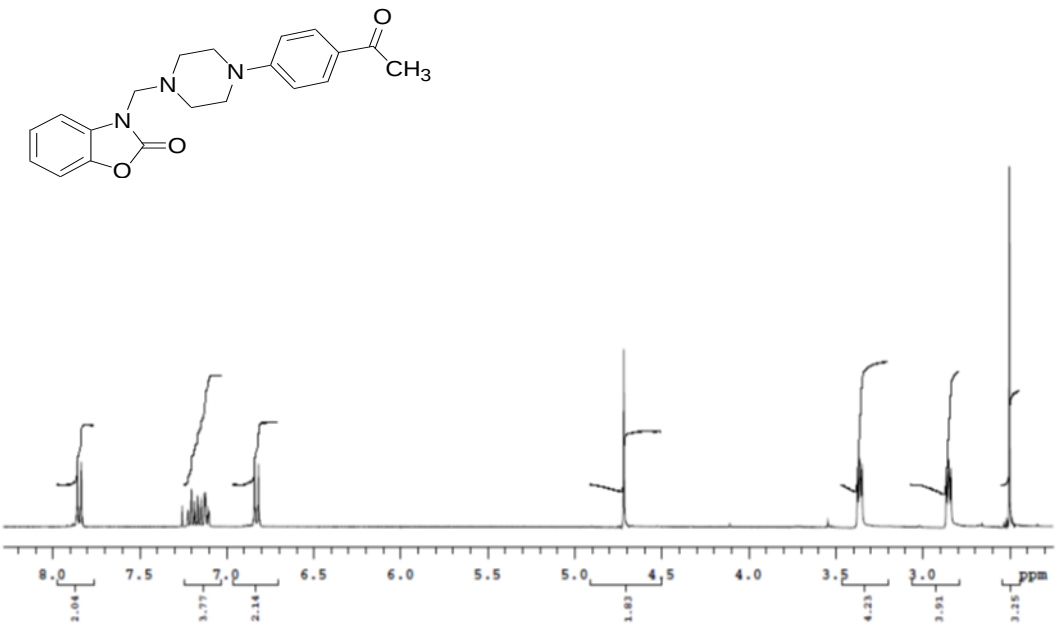


Figure 4.27 ^{1}H NMR spectrum of compound **7**

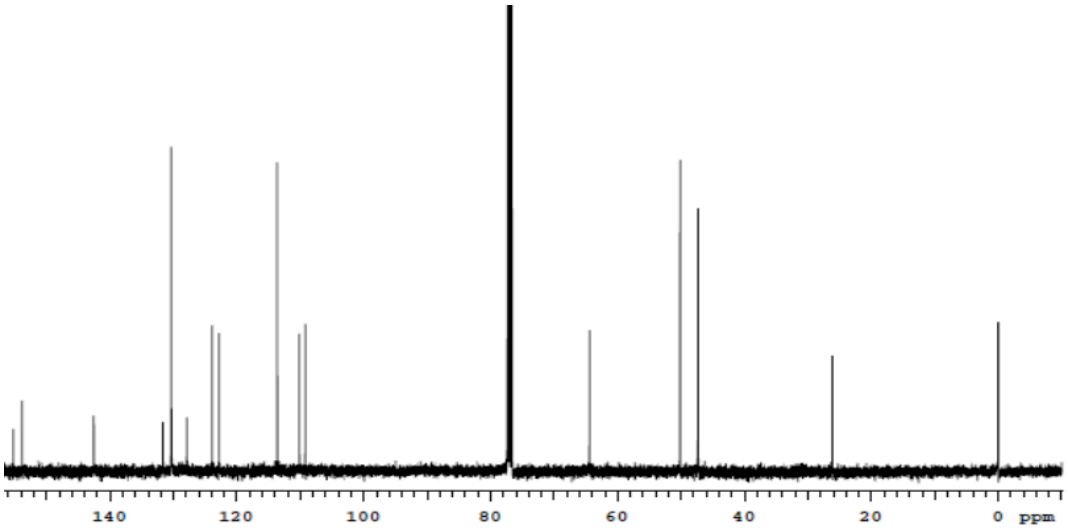


Figure 4.28 ^{13}C NMR spectrum of compound **7**

4.8. Chemical Data of Compound 8

IUPAC Name: 3-[4-(4-Acetylphenyl)piperazin-1-ylmethyl]-5-chloro-3H-benzoxazol-2-one

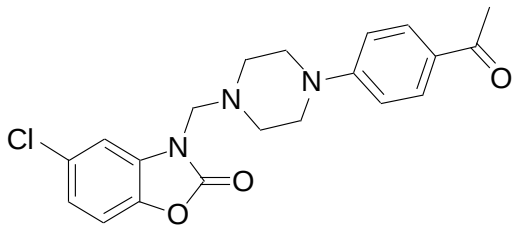


Figure 4.29 Structure of compound **8**

Equal molar concentrations (15 mmol) of 5-chloro-2(3H)-benzoxazolone and 4-acetylphenyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 16 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.56

Physical Appearance: White powder.

Solubility: Completely soluble in chloroform and dimethylsulfoxide. Slightly soluble in ethanol and methanol in room temperature.

Melting Point : 198.3 °C

Molecular Formula: $C_{20}H_{20}ClN_3O_3$
Molecular Weight (g/mol): 385,84
Elemental Analysis

	C	H	N
Calculated % :	62,20	5,18	10,88
Found % :	62,26	4,89	10,80

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H), 2800-3100 (Aliphatic C-H), 1783 (C=O).

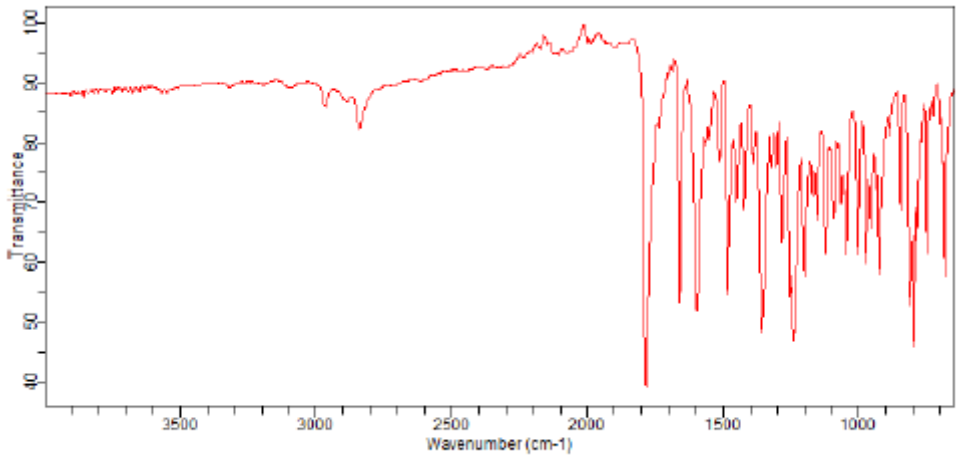


Figure 4.30 FT-IR spectrum of compound **8**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.8-6.8 (m, 7H, Ar-CH), 4.7 (s, 2H, CH_2), 2.9 (t, 4H, pip-CH_2 H^2, H^6), 3.4 (t, 4H, pip-CH_2 H^3, H^5), 2.5 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 154.9, 153.8,141.0, 132.5, 130.3,129.4,127.9,123.9,122.7, 113.6, 110.9, 109.8, (Ar-C), 64.4 (CH_2), 50.1, 47.2 (pip-C), 26.1 (CH_3).

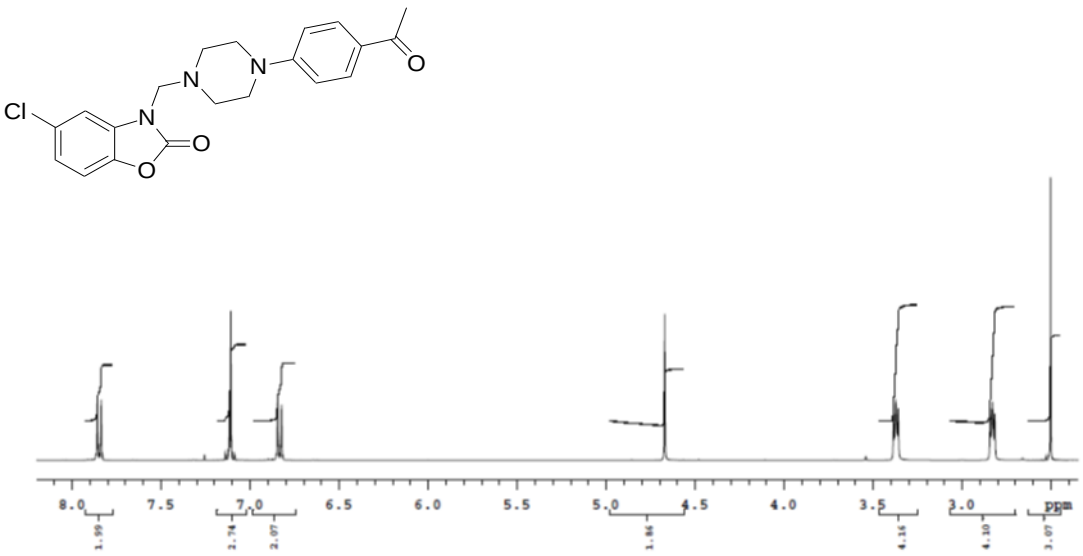


Figure 4.31 ^{1}H NMR spectrum of compound **8**

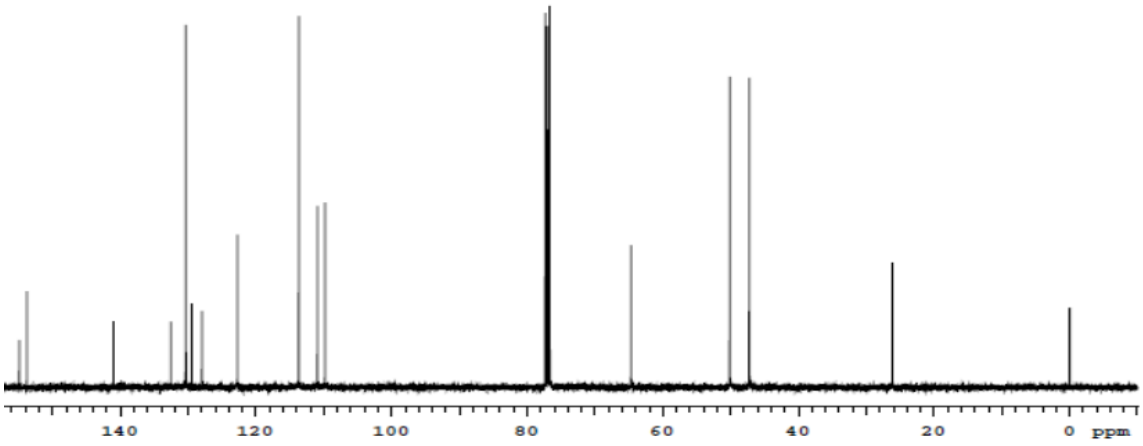


Figure 4.32 ^{13}C NMR spectrum of compound **8**

4.9. Chemical Data of Compound 9

IUPAC Name: 3-(4-Cyclohexylpiperazin-1-ylmethyl)-3H-benzoxazol-2-one

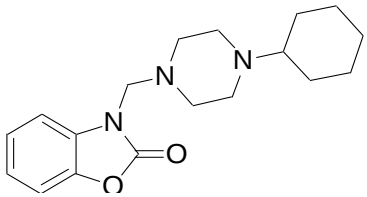


Figure 4.33 Structure of compound **9**

Equal molar concentrations (15 mmol) of 2(3H)-benzoxazolone and 4-cyclohexylpiperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 47 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.35

Physical Appearance: Yellowish white powder.

Solubility: Completely soluble in chloroform and dimethylsulfoxide.

Melting Point : 165.5 °C

Molecular Formula: $C_{18}H_{25}N_3O_2$

Molecular Weight (g/mol): 315,41

Elemental Analysis

	C	H	N
Calculated % :	68,48	7,92	13,17
Found % :	68,54	7,86	13,32

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2800-3000 (Aliphatic C-H), 1783 (C=O).

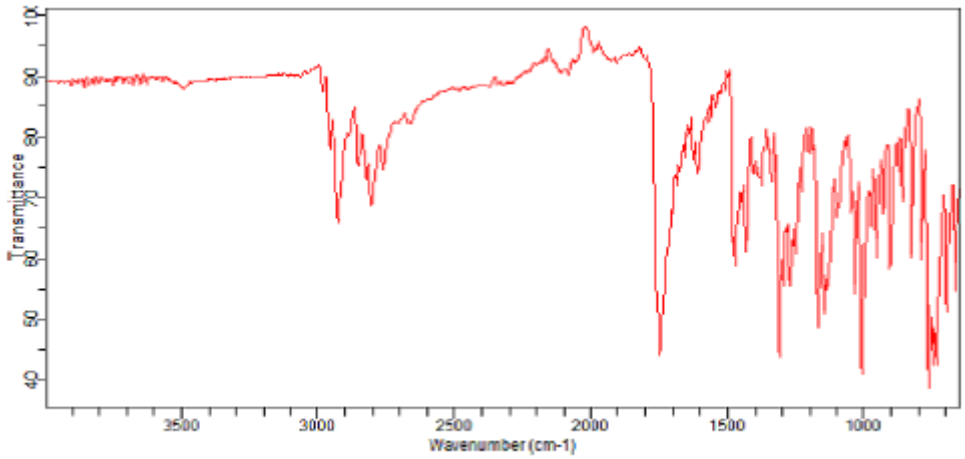


Figure 4.34 FT-IR spectrum of compound **9**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.3-7.1 (m, 4H, Ar-CH), 4.7 (s, 2H, CH_2), 2.7 (t, 4H, pip-CH_2 H^2, H^6), 2.5 (t, 4H, pip-CH_2 H^3, H^5), 2.2 (m, 1H, cyclohexyl- CH H^1), 1.8 (q, 4H, cyclohexyl-CH_2 H^2,H^6), 1.2 (m, 4H, cyclohexyl-CH_2 H^3,H^5) 1.1 (m, 1H, cyclohexyl-CH H^4)

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.3,142.5, 132.0, 123.7,122.5, 109.9, (Ar-C), 64.4 (CH_2), 63.3 (cyclohexyl-CH), 50.9, 48.6 (pip-C), 28.9, 26.2, 25.8 (cyclohexyl- CH_2).

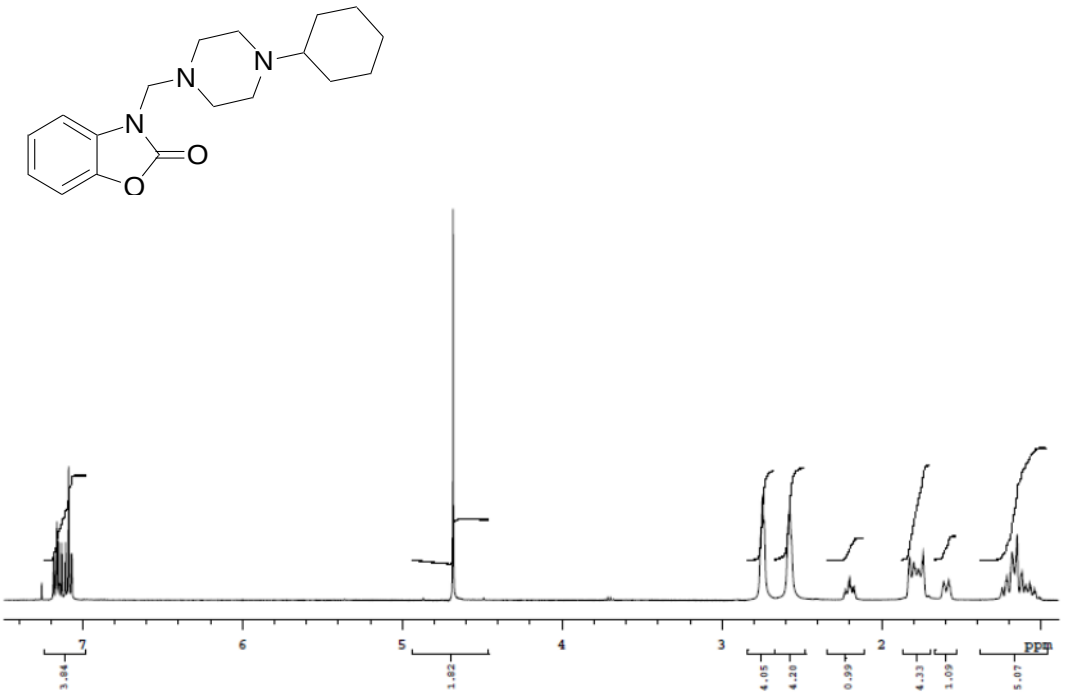


Figure 4.35 ^{1}H NMR spectrum of compound **9**

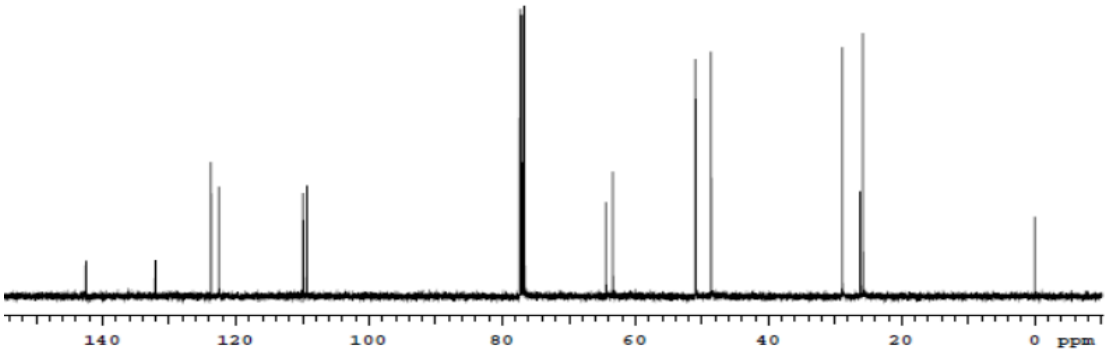


Figure 4.36 ^{13}C NMR spectrum of compound **9**

4.10. Chemical Data of Compound 10

IUPAC Name : 5-Chloro-3-(4-cyclohexylpiperazin-1-ylmethyl)-3H-benzoxazol-2-one

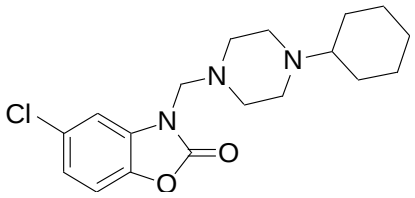


Figure 4.37 Structure of compound **10**

Equal molar concentrations (15 mmol) of 5-chloro-2(3H)-benzoxazolone and 4-cyclohexylpiperazine were dissolved separately in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 32 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.28

Physical Appearance: White powder.

Solubility: Completely soluble in chloroform. Slightly soluble in ethanol and methanol in room temperature.

Melting Point : 162.1 °C

Molecular Formula: $C_{18}H_{24}ClN_3O_2$
Molecular Weight (g/mol): 349,86
Elemental Analysis

	C	H	N
Calculated % :	61,73	6,85	12,00
Found % :	61,65	6,91	12,04

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2803-2922 (Aliphatic C-H), 1772 (C=O).

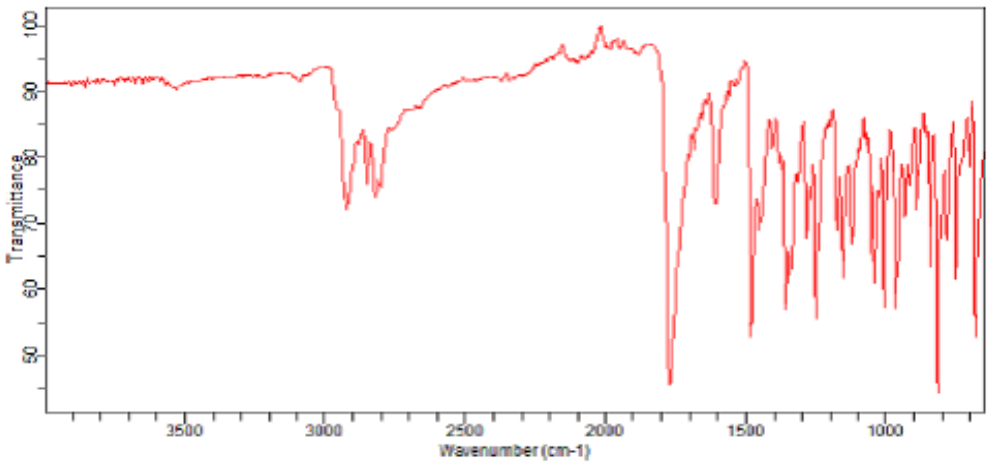


Figure 4.38 FT-IR Spectrum of Compound **10**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.3-7.0 (m, 3H, Ar-CH), 4.7 (s, 2H, CH_2), 2.7 (t, 4H, pip-CH_2 H^2, H^6), 2.5 (t, 4H, pip-CH_2 H^3, H^5), 2.2 (m, 1H, cyclohexyl- CH H^1), 1.8 (q, 4H, cyclohexyl-CH_2 H^2,H^6), 1.2 (m, 4H, cyclohexyl-CH_2 H^3,H^5) 1.1 (m, 1H, cyclohexyl-CH H^4)

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.3,142.5, 132.0, 123.7,122.5, 109.9, (Ar-C), 64.4 (CH_2), 63.3 (cyclohexyl-CH), 50.9, 48.6 (pip-C), 28.9, 26.2, 25.8 (cyclohexyl- CH_2).

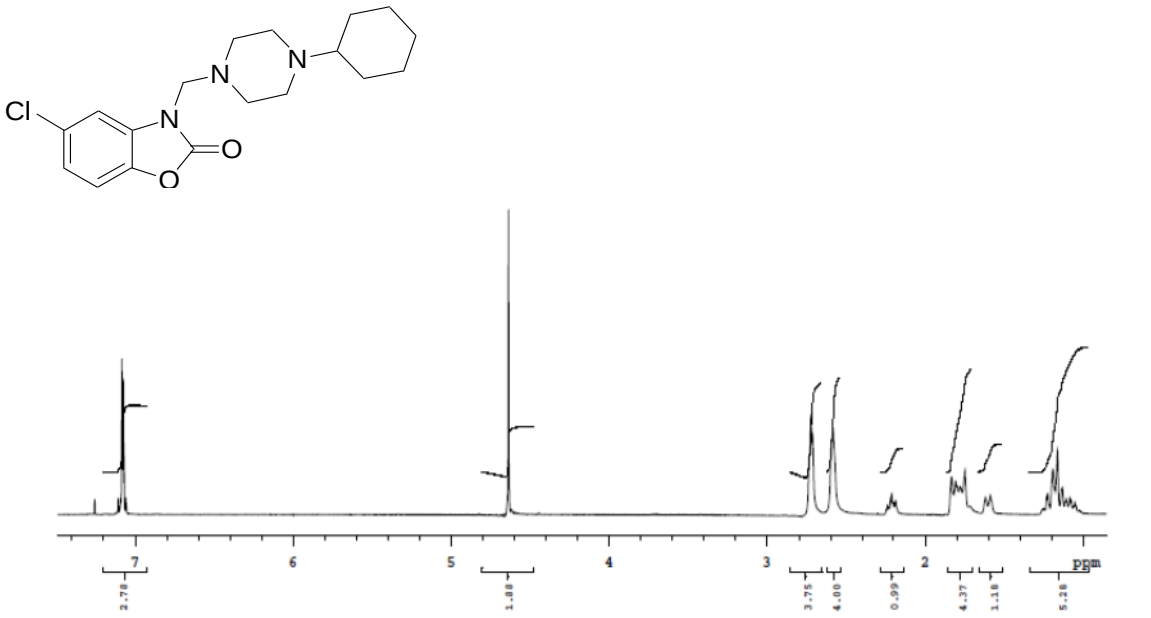


Figure 4.39 ^{1}H NMR spectrum of compound **10**

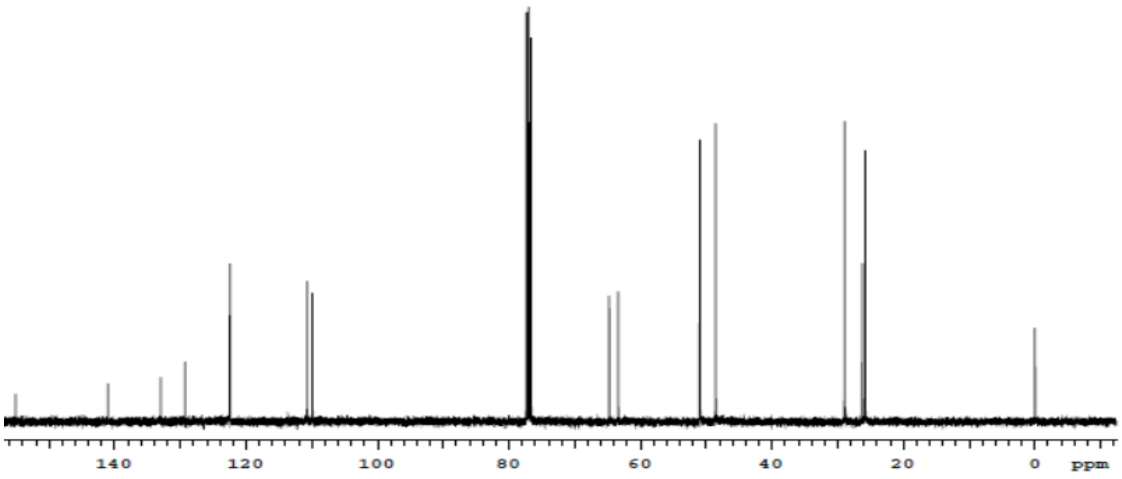


Figure 4.40 ^{13}C NMR spectrum of compound **10**

4.11. Chemical Data of Compound 11

IUPAC Name : 3-[4-(4-Bromophenyl) piperazin-1-ylmethyl]-3H-benzoxazol-2-one

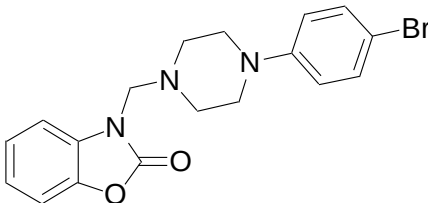


Figure 4.41 Structure of compound **11**

Equal molar concentrations (15 mmol) of 2(3H)-benzoxazolone and 4-bromophenyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 52 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.43

Physical Appearance: Brownish white powder.

Solubility: Completely soluble in dimethylformamide and dimethylsulfoxide.

Melting Point : 176.2 °C

Molecular Formula: $C_{18}H_{18}BrN_3O_2$

Molecular Weight (g/mol): 388,26

Elemental Analysis

	C	H	N
Calculated % :	55,63	4,63	10,81
Found % :	55,49	4,36	10,77

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2838-2905 (Aliphatic C-H), 1783 (C=O).

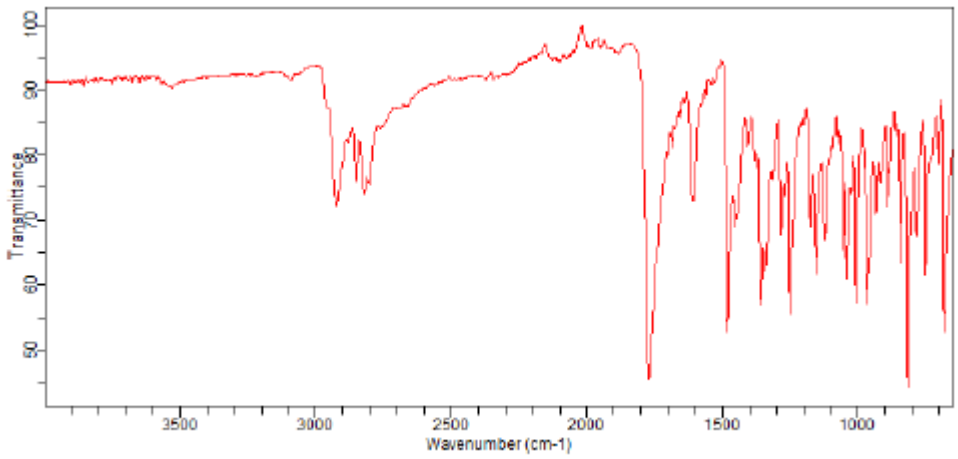


Figure 4.42 FT- IR spectrum of compound **11**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 7.4-6.8 (m, 8H, Ar-CH), 4.7 (s, 2H, CH_2), 3.2 (t, 4H, pip-CH_2 H^2, H^6), 2.5 (t, 4H, pip-CH_2 H^3, H^5).

^{13}C NMR (100 MHz, DMSO-d_6) δ 155.0, 150.5, 149.3, 131.9, 122.9, 121.5, 117.9, 112.4, 110.6, 110.0, 109.7, 109.0, (Ar-C), 64.2 (CH_2), 50.2, 48.3 (pip-C).

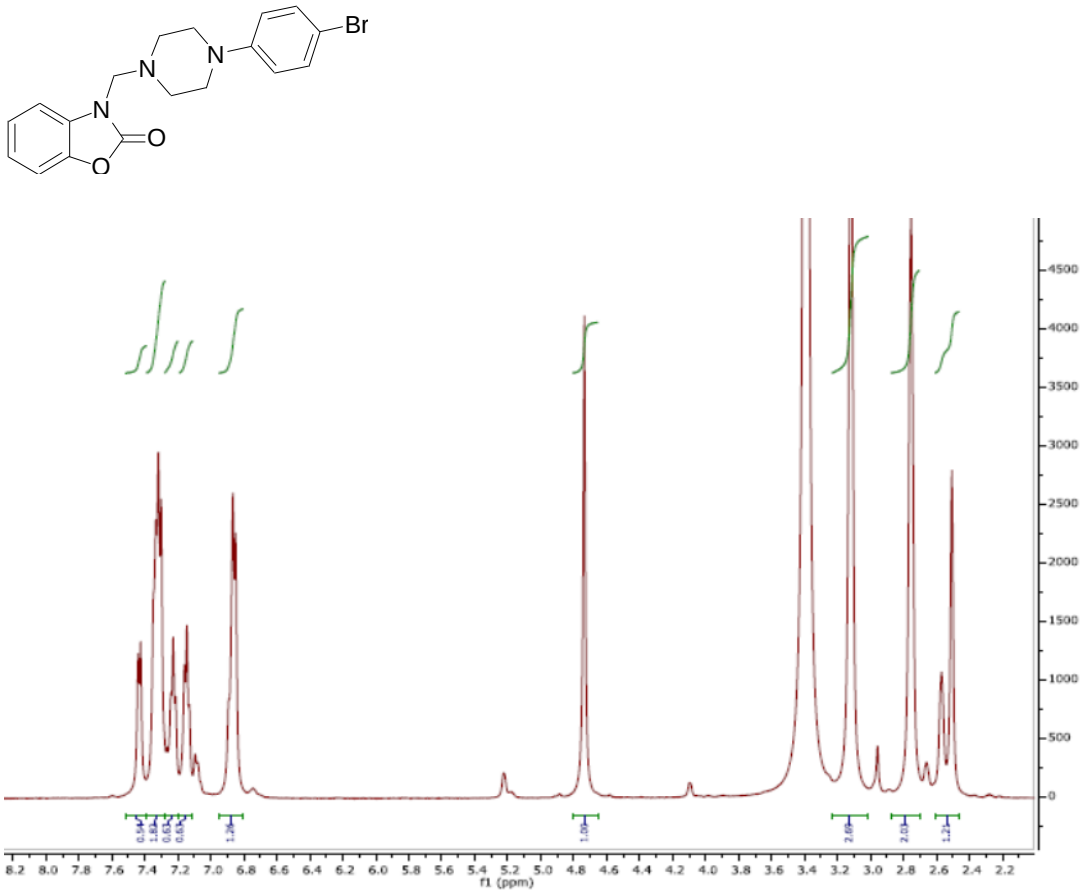


Figure 4.43 ^{1}H NMR spectrum of compound **11**

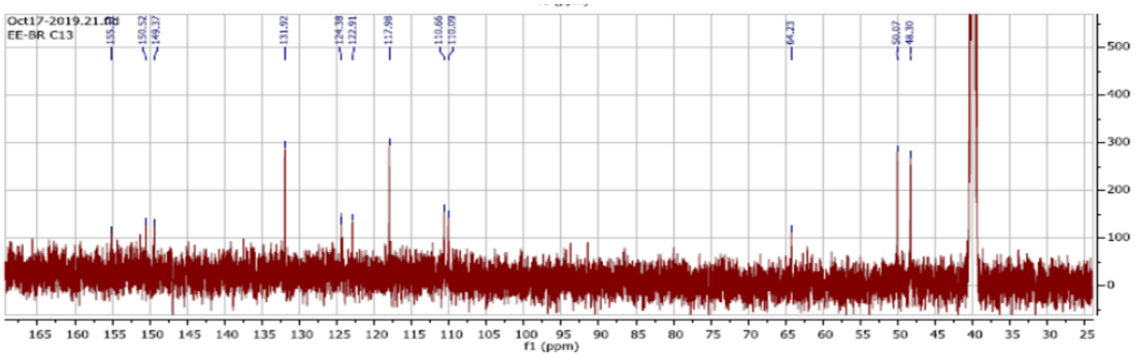


Figure 4.44 ^{13}C NMR spectrum of compound **11**

4.12. Chemical Data of Compound 12

IUPAC Name : 3-[4-(4-Bromophenyl)piperazin-1-ylmethyl]-5-chloro-3H-benzoxazol-2-one

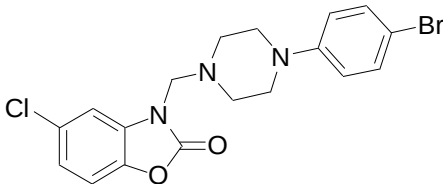


Figure 4.45 Structure of compound **12**

Equal molar concentrations (15 mmol) of 5-chloro-2(3H)-benzoxazolone and 4-bromophenyl piperazine were separately dissolved in methanol and mixed after addition of appropriate amount of formalin (35% w/v) into the reaction mixture and heated under reflux for 1 hour. The reaction mixture was put into an ice bath after reflux and solid precipitate was dried after vacuum filtration and then purified by recrystallization method with an appropriate solvent.

Yield (%) : 57 %

For TLC (Thin Layer Chromatography)

Stationary Phase: Silica gel GF 254

Mobile Phase: Ethyl Acetate : Hexane (2:1)

Detection of spots: Under UV lamp at 254 nm

Retention Factor (Rf): 0.41

Physical Appearance: Shiny white powder.

Solubility: Completely soluble in dimethylformamide and dimethylsulfoxide.

Melting Point : 181.1 °C

Molecular Formula: $C_{18}H_{17}BrClN_3O_2$

Molecular Weight (g/mol): 422,70

Elemental Analysis

	C	H	N
Calculated % :	51,10	4,02	9,93
Found % :	51,15	3,82	9,89

FT-IR Infrared Spectrum

ν_{max} (KBr, cm^{-1}) : Above 3000 (Aromatic C-H)**,** 2838-2905 (Aliphatic C-H), 1783 (C=O).

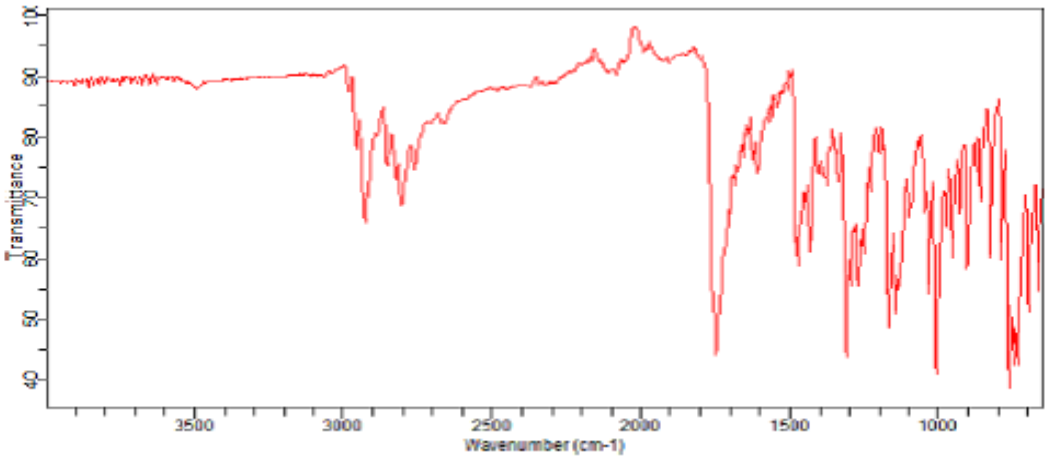


Figure 4.46 FT-IR spectrum of compound **12**

^{1}H NMR and ^{13}C NMR Spectra

^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 7.4-6.8 (m, 7H, Ar-CH), 4.7 (s, 2H, CH_2), 3.2 (t, 4H, pip-CH_2 H^2, H^6), 2.5 (t, 4H, pip-CH_2 H^3, H^5).

^{13}C NMR (100 MHz, DMSO-d_6) δ 155.0, 150.5,149.3, 131.9, 122.9,121.5, 117.9,112.4, 110.6, 110.0,109.7, 109.0, (Ar-C), 64.2 (CH_2), 50.2, 48.3 (pip-C).

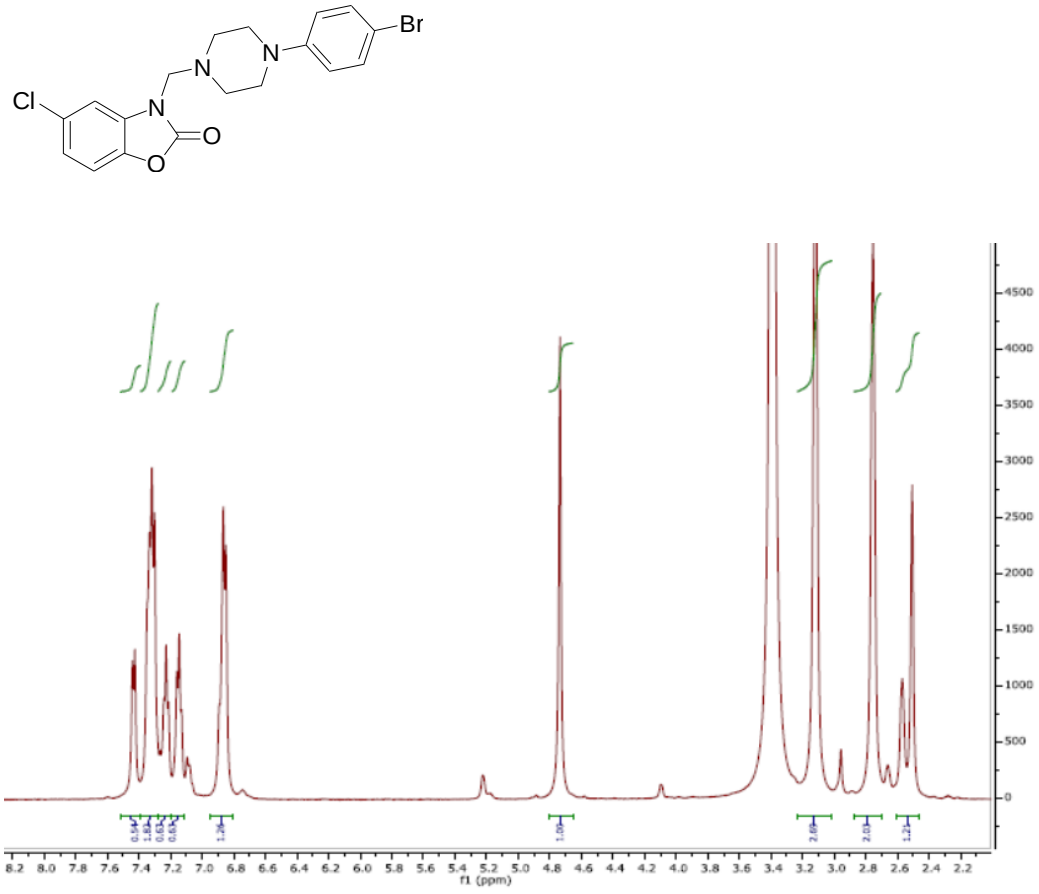


Figure 4.47 ^{1}H NMR spectrum of compound **12**

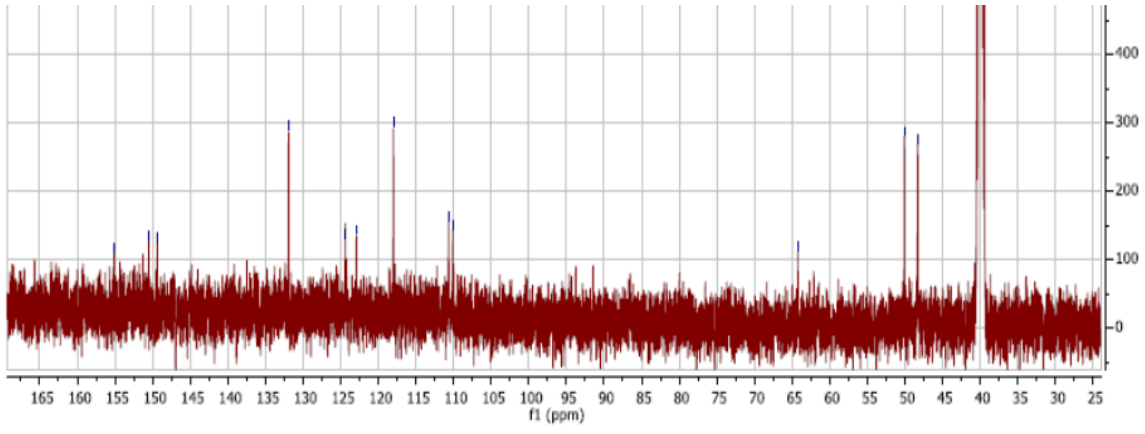


Figure 4.48 ^{13}C NMR spectrum of compound **12**

5. BIOLOGICAL ACTIVITY RESULTS

5.1. Cytotoxicity Results of Compounds 1, 2, 5, 6

5.1.1 MTT results of compound 1 on MCF-7 cells

For all concentrations of compound **1**, there was a drastic decrease in percentage MCF-7 cell viability and proliferation as the dose and duration of incubation increased. According to the results, compound **1** at 50 μM concentration was more effective for decreasing MCF-7 cell viability in contrast to other diluted concentrations left for 48 hours incubation (Figure 5.1).

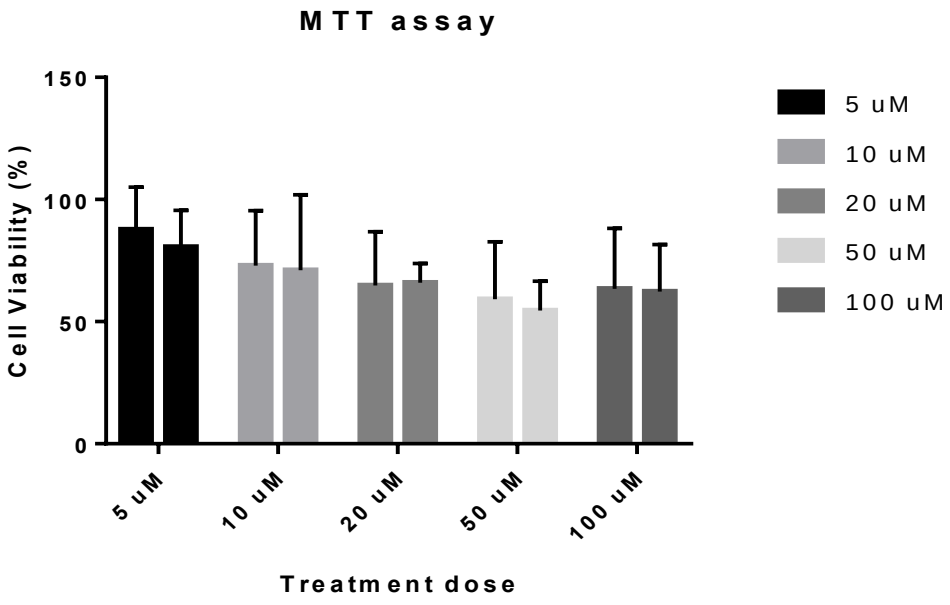


Figure 5.1 MTT results of compound **1** on cell viability of MCF-7 cells.

5.1.2 MTT results of compound 2 on MCF-7 cells

For all concentrations of compound **2**, there was a drastic decrease in MCF-7 cell viability and proliferation and as the dose and duration of incubation increased. According to the results, compound **2** at 100 μM concentration was more effective for decreasing MCF-7 cell viability in contrast to other diluted concentrations left for 48 hours incubation (Figure 5.2).

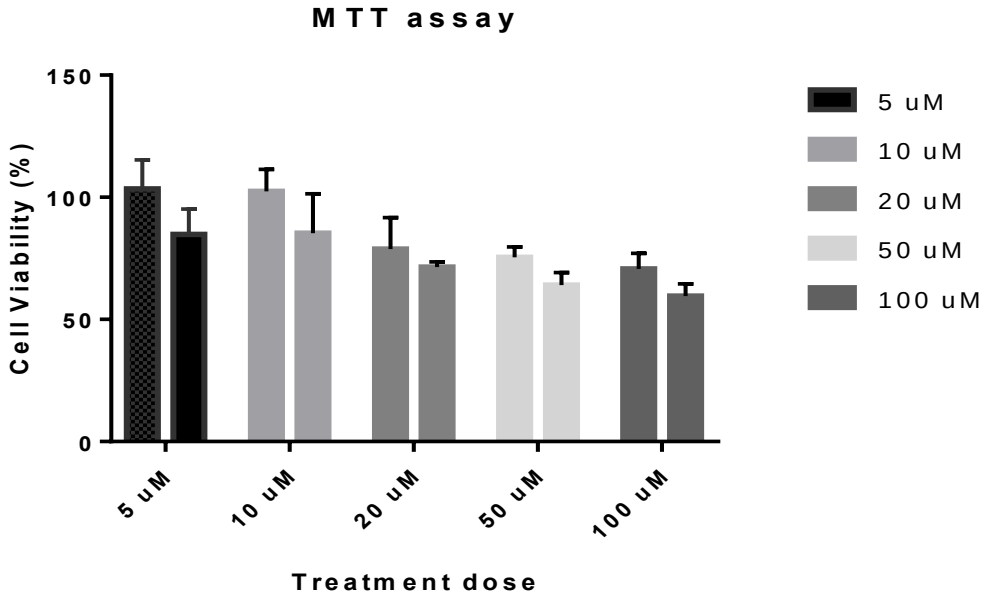


Figure 5.2 MTT results of compound **2** on cell viability of MCF-7 cells.

5.1.3 MTT Results of Compound 5 on MCF-7 Cells

The cells were cultured with five different concentrations of compound **5** for 24 and 48 hours incubation. According to the results, all concentrations of compound **5** showed no significant decrease in cell viability percentage when compared with MTT assay of ethanol used as a negative control since the percentage of cell viability for all concentrations was higher than 80% as shown in figure 5.3.

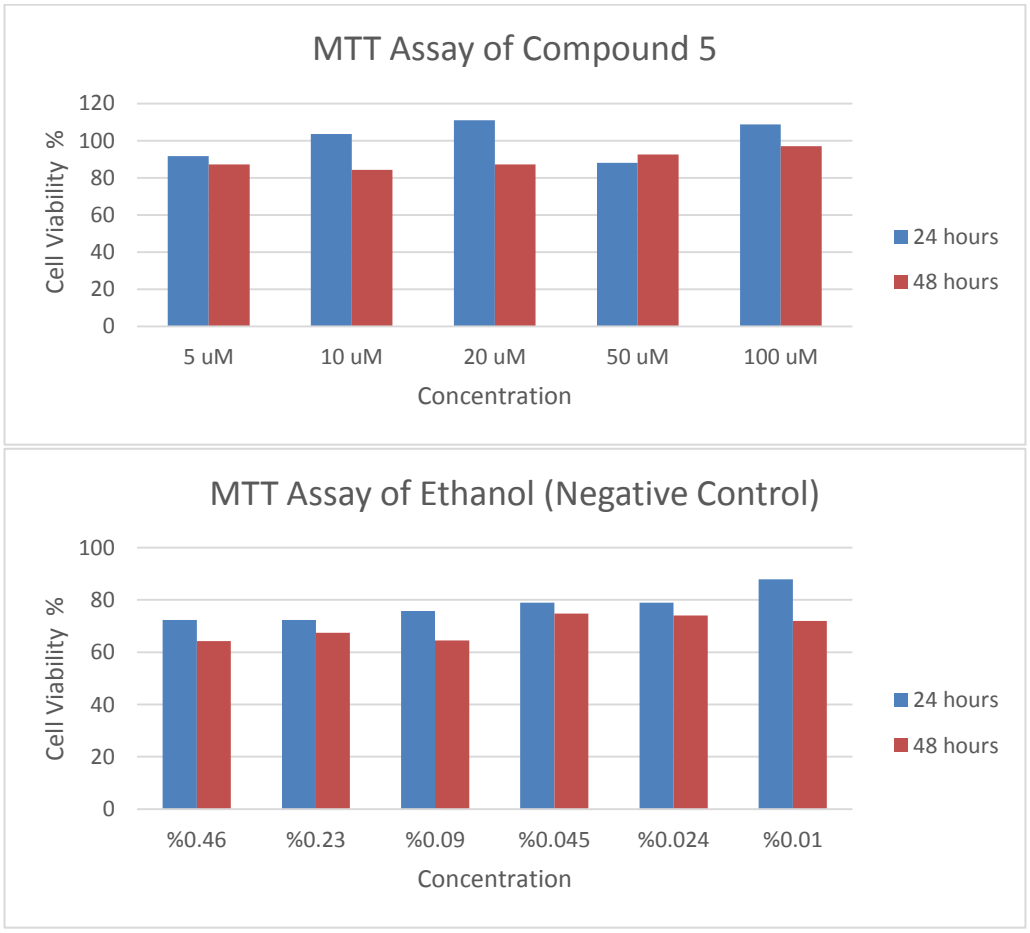


Figure 5.3 Effect of compound **5** on cell viability of MCF-7 cells and MTT assay of ethanol as negative control

5.1.4 MTT Results of Compound 6 on MCF-7 Cells

The cells were cultured with five different concentrations of compound **6** for 24 and 48 hours incubation. According to the results, all concentrations of compound **6** showed no significant decrease in cell viability percentage when compared with MTT assay of ethanol used as a negative control since the percentage of cell viability for all concentrations was higher than 80% as shown in figure 5.4.

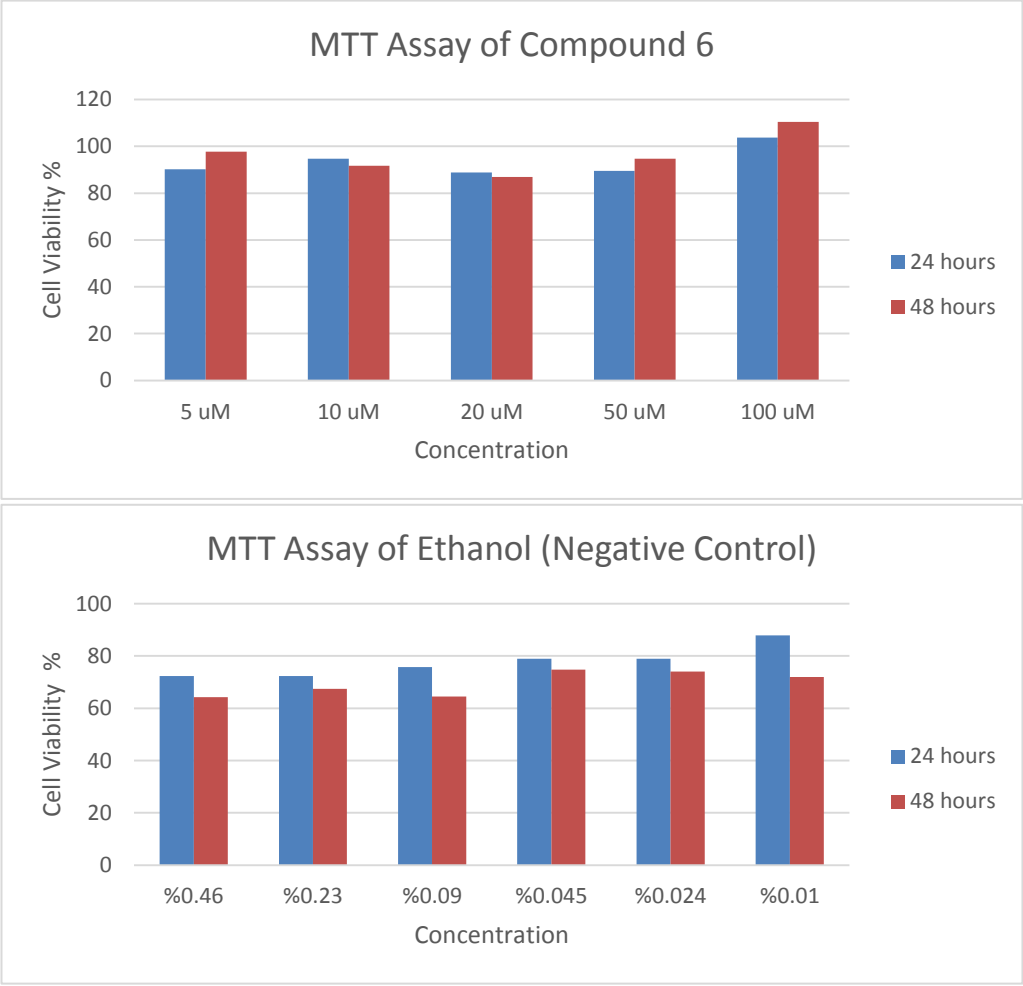


Figure 5.4 Effect of compound **6** on cell viability of MCF-7 cells and MTT assay of ethanol as negative control

5.2 Cytotoxicity Results of Compounds on Metastatic M4A4 Cells

5.2.1 MTT Results of Compound 1 on M4A4 Cells

M4A4 cells were cultured with five different concentrations of compound **1**. All concentrations of compound **1** resulted in no significant decrease in cell viability but they increased cell proliferation significantly at concentrations of 5 µM and 10 µM at 48 hours incubation respectively when compared with MTT results of ethanol as a negative control as shown in figure 5.5.

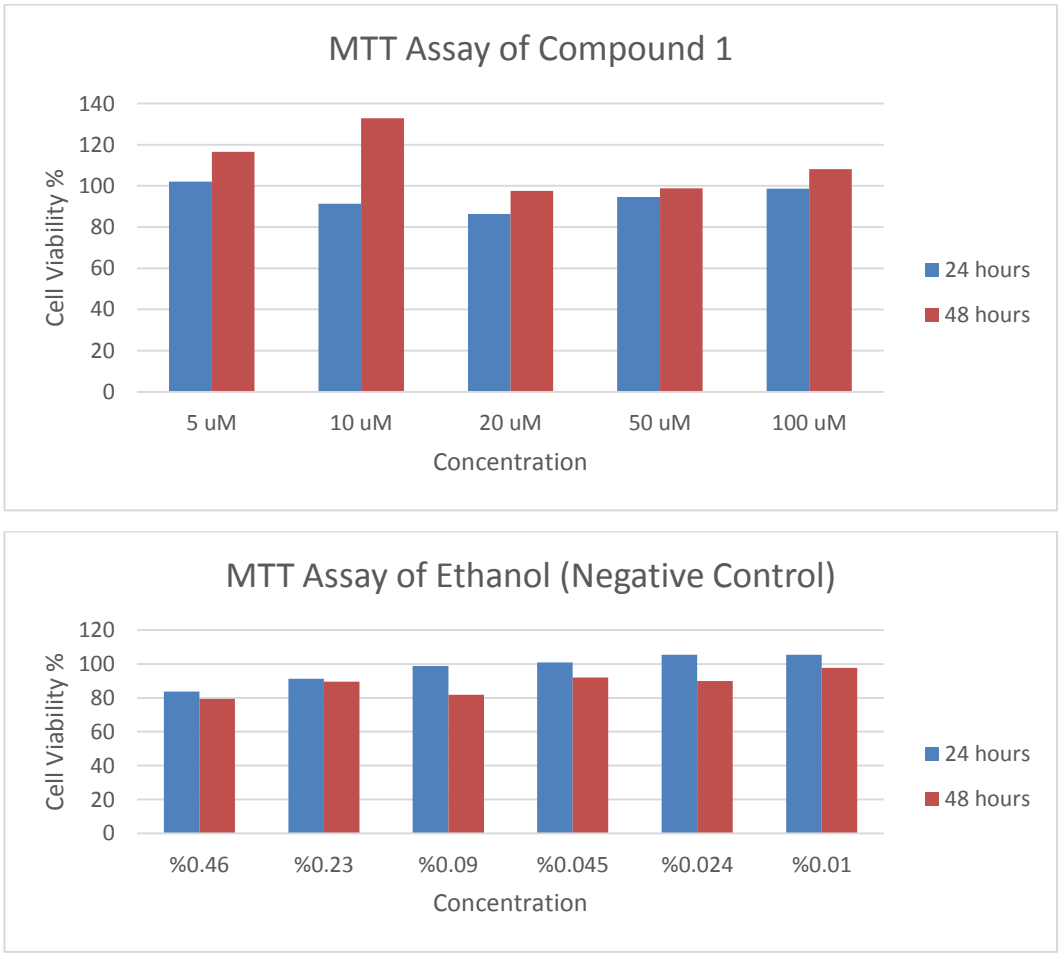


Figure 5.5 Effect of compound **1** on cell viability of M4A4 cells and MTT assay of ethanol as negative control

5.2.2 MTT Results of Compound 2 on M4A4 Cells

M4A4 cells were cultured with five different concentrations of compound **2** for 24 and 48 hours incubation period. All concentrations of compound **2** resulted in no significant decrease in cell viability percentage when compared with MTT results of ethanol as a negative control left for 24 hours and 48 hours incubation as shown in figure 5.6.

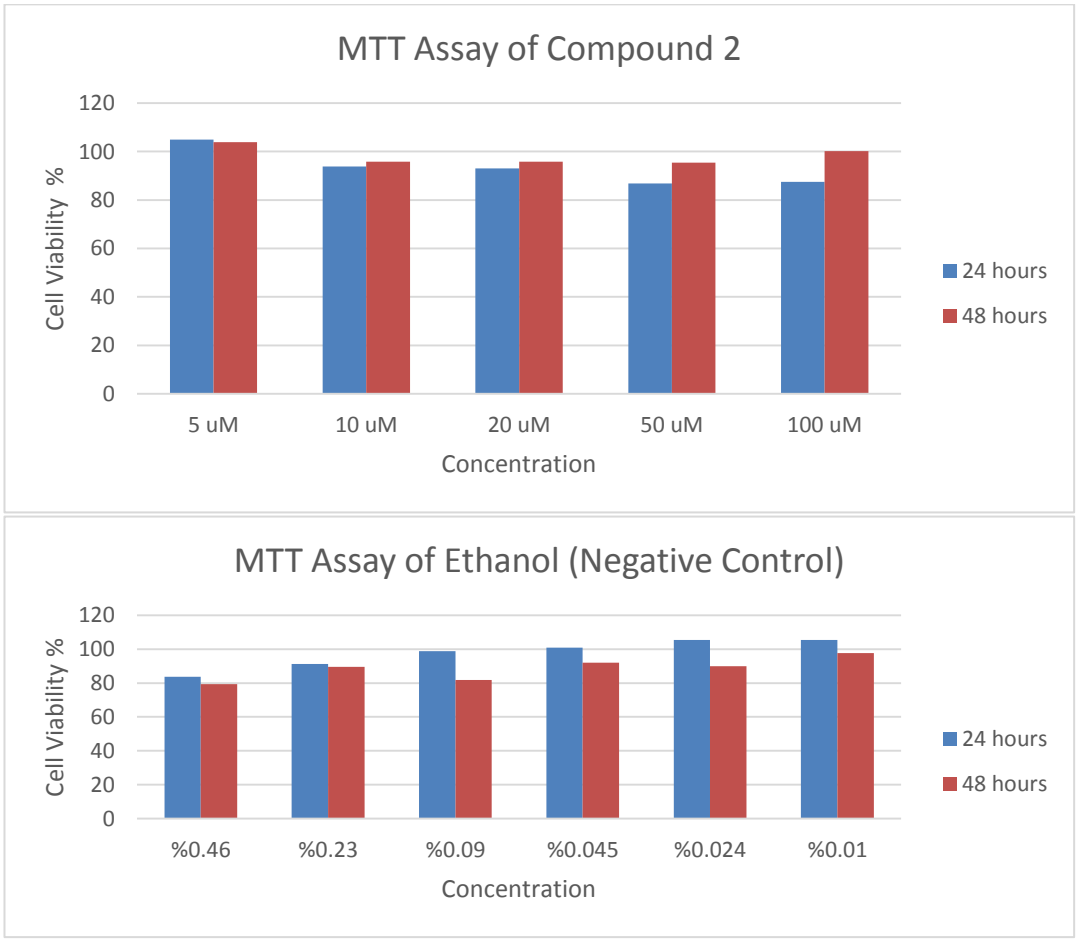


Figure 5.6 Effect of compound **2** on cell viability of M4A4 cells and MTT assay of ethanol as negative control

5.2.3 MTT Results of Compound 5 on M4A4 Cells

M4A4 cells were cultured with different concentrations of compound **5**. All concentrations of compound **5** showed no significant decrease in cell viability but they significantly enhance cell proliferation at 48 hours incubation especially between concentrations of 10-100µM since the cell viability is increased above 100% when compared with MTT results of ethanol as a negative control as shown in figure 5.7.

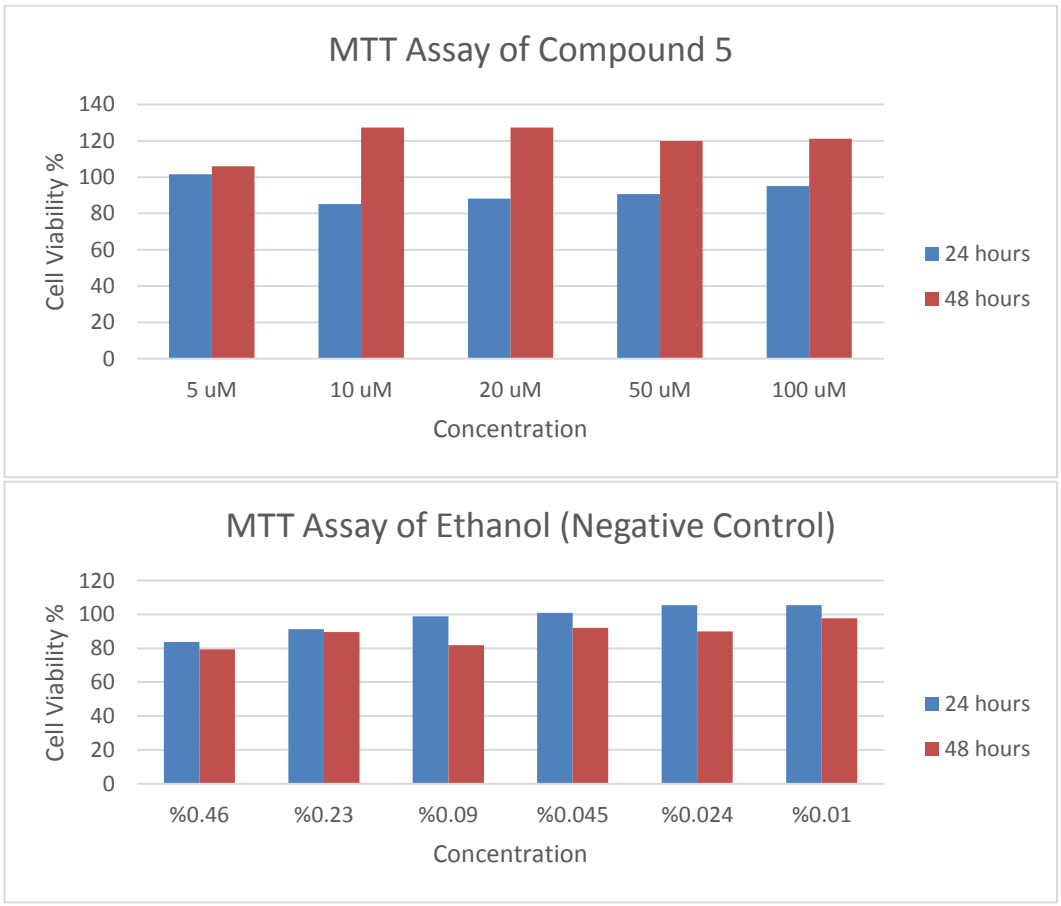


Figure 5.7 Effect of compound **5** on cell viability of M4A4 cells and MTT assay of ethanol as negative control

5.2.4 MTT Results of Compound 6 on M4A4 Cells

M4A4 cells were cultured with different concentrations of compound **6**. All concentrations of compound **6** showed no significant decrease in cell viability but they enhance cell proliferation at 48 hours incubation since the cell viability is increased above 100% for all concentrations when compared with MTT results of ethanol as a negative control as shown in figure 5.8.

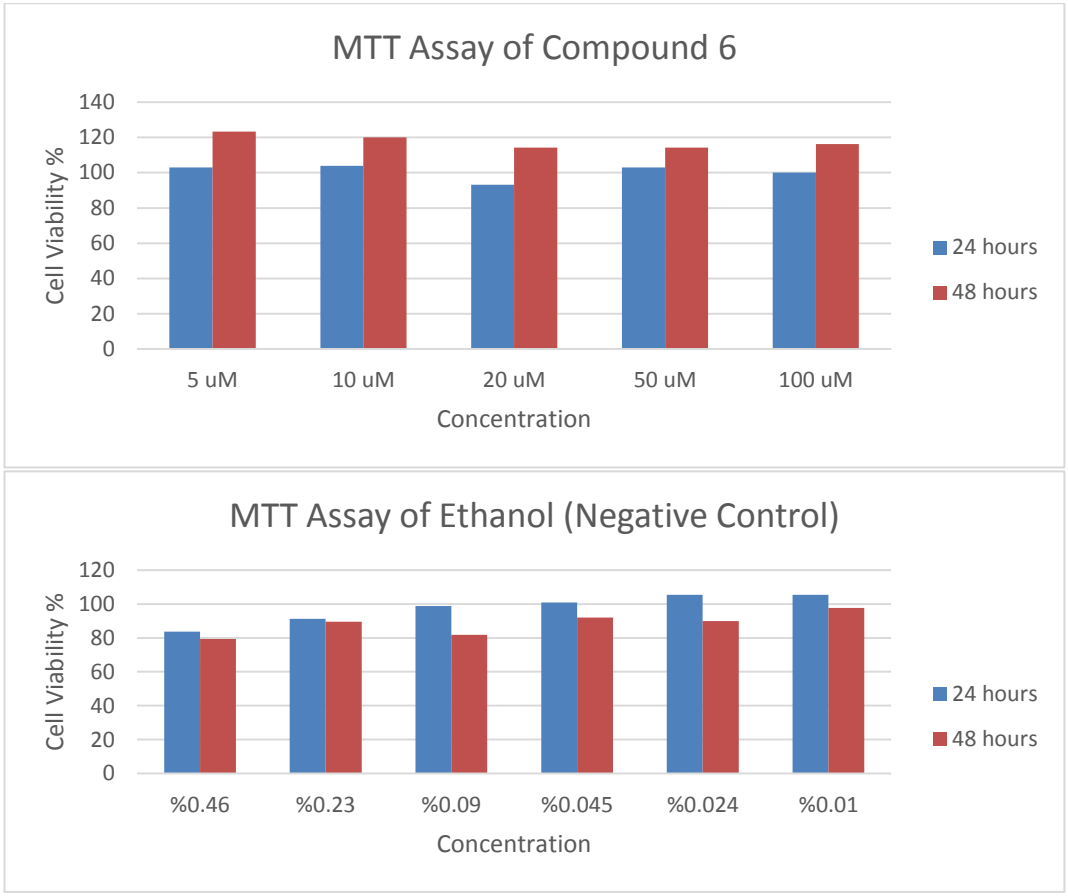


Figure 5.8 Effect of compound **6** on cell viability of M4A4 cells and MTT assay of ethanol as negative control

5.3 TUNEL Assay and Immunocytochemical Evaluations

5.3.1 Apoptotic Effects of Compound 1and Compound 2 in MCF-7 Cells

TUNEL assay was applied to the cells incubated with 50 μM concentration of compound **1** for 48 hours time period. The number of TUNEL positive cells that were treated with compound **1** were considered as highly significant in MCF-7 cells in contrast to the value of control group as shown in table 5.1.

Table 5.1 The percentage of TUNEL positive cells cultured with compound **1**

Compound 1	**Control**
85 ± 7 [a]	35.67 ± 4.04

[a] The data was considered as significant in contrast to the control group since $p<0.001$. Data were given as means ± standard deviation and were compared by Mann-Whitney.

The target cells were incubated with 100 μM concentration of compound **2** for 48 hours time period. The number of TUNEL positive cells that were cultured with compound **2** were highly significant in tested cells in contrast to the value of control group as shown in table 5.2.

Table 5.2 The percentage of TUNEL positive cells cultured with compound **2**

Compound 2	**Control group**
72.66 ± 11.38[a]	35.67 ± 4.04

[a] The data was considered as significant in contrast to the control group since $p<0.01$. Data are given as means ± standard deviation and were compared by Mann-Whitney.

5.3.2 Immunocytochemical Evaluation of Compound 1and Compound 2

The intensity of immunostaining for cytochrome c was found to be moderate to strong in MCF-7 cells. Immunoreactivity of cytochrome-c was found higher in MCF-7 cells cultured with compound **1** in contrast to the control group, showing a significant rise in H-SCORE compared to the values of control group.

Caspase-3 immunostaining in MCF-7 cells was stronger for compound **1** compared to the control group. The H-SCORE results revealed that immunolabeling was significantly higher in compound **1** in MCF-7 cells in contrast to the control group. The data is given as means ± standard deviation and were compared by Mann-Whitney as shown in table 5.3.

Table 5.3 The H-SCORE of target antigens in cells cultured with compound **1**

	Compound 1	**Control group**
Fas-L	277.4 ± 18.93	235.6 ± 33.54
Cytochrome-c	278.4 ± 20.49[a]	234.7 ± 11.33
Caspase-3	262.6 ± 34.15[b]	112.6 ± 9.379

[a] The data was considered as significant in contarst to the control group ($p<0.01$).

[b] The data was considered as significant in contarst to the control group ($p<0.01$).

Immunostaining of Fas ligand was strong in MCF-7 cells cultured with compound **2** and H-SCORE results were significantly higher compared to the control group. Cytochrome-c immunoreactivity was higher in MCF-7 cells cultured with compound **2** than values obtained from control group. Immunostaining of caspase-3 in MCF-7 cells was potent for compound **2**. The immunoreactivity of caspase-3 in MCF-7 cells was higher in cells treated with compound **2** than in the control group. According to the H-SCORE results, for MCF-7 cells cultured with compound **2** showed higher immunolabelling in contrast to the control group. The values of antigen immunolabelling in MCF-7 cells cultured with compound **2** according to the H-SCORE shown in table 5.4

Table 5.4 The H-SCORE of target antigens in cells cultured with compound **2**

	Compound 2	**Control group**
Fas-L	287.2 ± 22.77[a]	235.6 ± 33.54
Cytochrome-c	275.8 ± 29.62	234.7 ± 11.33
Caspase-3	274.± 28.94[b]	112.6 ± 9.379

[a] The data was considered as significant in contrast to control group ($p<0.05$).

[b] The data was considered as significant in contrast to control group ($p<0.01$).

5.4 Microbiology Results

The zone of inhibition values for antibacterial and antifungal activities are shown below on table 5.5.

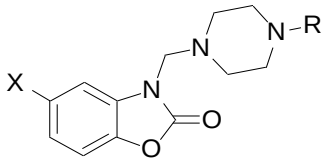


Table 5.5 Zone of inhibition (mm) measured for antibacterial and antifungal activity of compounds

Name of Compound	**X**	**R**	**Zone of Inhibition (mm)**				
			S.aureus [1]	***E.coli*** [3]	***B.cereus***[1]	***E.faecalis***[1]	***Candida albicans***[2]
Compound **3**	H	-napthylmethyl	0 mm	10 mm	0 mm	0 mm	0 mm
Compound **4**	Cl	-napthylmethyl	0 mm	12 mm	0 mm	16 mm	0 mm
Compound **5**	H	-2-methylbenzyl	0 mm	10 mm	0 mm	0 mm	0 mm
Compound **6**	Cl	-2-methylbenzyl	0 mm	12 mm	0 mm	0 mm	0 mm
Compound **7**	H	-p-acetylphenyl	15 mm	14 mm	0 mm	0 mm	0 mm
Compound **8**	Cl	-p-acetylphenyl	15 mm	15 mm	0 mm	0 mm	0 mm
Compound **9**	H	-cyclohexyl	14 mm	14 mm	0 mm	0 mm	0 mm
Compound **10**	Cl	-cyclohexyl	20 mm	16 mm	0 mm	0 mm	0 mm
Compound **11**	H	p-bromophenyl	17 mm	14 mm	0 mm	0 mm	0 mm
Compound **12**	Cl	p-bromophenyl	17 mm	15 mm	0 mm	0 mm	0 mm
Positive Controls[1,2,3]			25 mm	40 mm	17 mm	21mm	14 mm

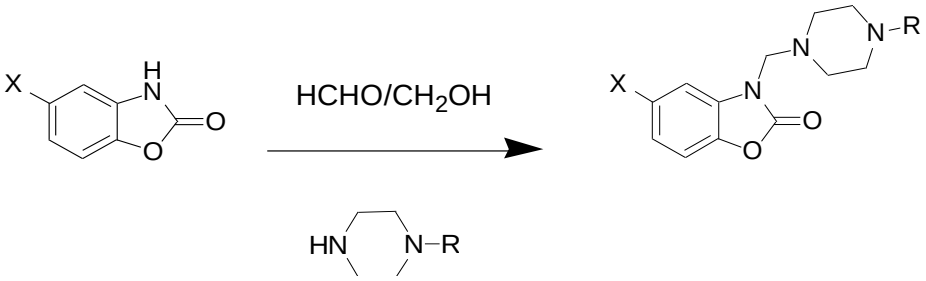
[1]Tetracycline , [2]Amphotericine B, [3]Ciprofloxacin

6. DISCUSSION

6.1. Synthesis of Derivatives

In this thesis, twelve different benzoxazolone derivatives were synthesized where the molecules either have a different piperazine moeity at the 3-position or/and a chlorine atom at the 5-position of the core structure.

These derivatives given in this thesis contain different piperazine derivatives than the ones studied before [51]. In Mannich reaction between benzoxazolone derivatives and appropriate piperazine derivatives, there is a condensation between active hydrogen of benzoxazolone ring at 3rd position and the active hydrogen of piperazine ring in presence of formalin (35% w/v) and a methylene bridge is formed between these two structures as shown in figure 6.1. The reactions were carried out under reflux conditions for 1 hour.



X : H or Cl

Figure 6.1 Synthesis Pathway of Benzoxazolone Derivatives [51]

In general, the yield values are about 50% except for *p*-acetylphenyl piperazine derivatives in which case low yield have been observed.The list of synthesized benzoxazolone derivatives and their yields are listed in table 6.1.

Table 6.1 Synthesized derivatives and their yields

Compound Name	**X**	**R**	**Yield**
Compound **1**	H	-2,5-dimethylphenyl	51.3%
Compound **2**	Cl	-2,5-dimethylphenyl	30.8 %
Compound **3**	H	-napthylmethyl	40.7%
Compound **4**	Cl	-napthylmethyl	54%
Compound **5**	H	-2-methylbenzyl	50.2%
Compound **6**	Cl	-2-methylbenzyl	45%
Compound **7**	H	-p-acetylphenyl	21%
Compound **8**	Cl	-p-acetylphenyl	16%
Compound **9**	H	-cyclohexyl	47%
Compound **10**	Cl	-cyclohexyl	32%
Compound **11**	H	p-bromophenyl	52%
Compound **12**	Cl	p-bromophenyl	57%

These newly synthesized molecules were divided into two groups for activity studies. One for cytotoxicity studies and the other for antimicrobial/antifungal studies due to the solubility issues. Only four of these compounds were screened for cytotoxicity since the others are either not very soluble in DMSO or gelation occurs upon addition of the PBS buffer and RPMI medium after dissolving them in DMSO. Ethanol was also used as a solvent for MTT studies.

6.2 FT-IR and NMR Analysis

From the IR spectra it is clear that, the N-H band of benzoxazolone and piperazine derivative at 3100-3400 cm^{-1} has disappeared for all compounds. This absence of the absorption band confirms that the reaction took place between the nitrogen atom of the benzoxazolone ring and nitrogen atom of piperazine moeity. In all compounds, the (C=O) stretching band of the lactam ring appear at 1765 cm^{-1}.

From the ^{1}H NMR spectra in general, the hydrogen atoms of the methylene bridge between benzoxazolone nitrogen and piperazine nitrogen was appeared as a 'singlet' at 4.7 ppm on the ^{1}H NMR spectra for all compounds which is the evidence of formation of Mannich reaction. The piperazine protons (H^{6} and H^{2}) and (H^{3} and H^{5}) appeared as 'triplets' at about 2.8-2.5 ppm range for all compounds. The aromatic hydrogens appeared as 'multiplets' between 6.7 to 7.5 ppm on ^{1}H-NMR spectra as expected for all compounds. The integral values of the aromatic protons match to the proposed structures.

For compound **9** and compound **10** which have cyclohexyl group on their piperazine ring, the cyclohexyl protons appeared as three pentets and a quartet between 1.1- 2.2 ppm at aliphatic proton region. In their ^{13}C NMR spectra, aliphatic carbons of cyclohexyl group appear between 25- 29 ppm.

For compounds **5** and **6**, they have 2-methyl benzyl substituents on their piperazine ring. In their ^{1}H NMR spectra, there are two sets of methylene hydrogen peaks as singlets at 4.7 ppm and 3.5 ppm. In addition, the peak at 2.3 ppm shows aliphatic hydrogens of methyl group. Benzyl carbon seem to appear at about 60.6 ppm on their ^{13}C NMR spectrum.

6.3. Cytotoxicity

There are only a few reports in the literature on the cytotoxic activities of different derivatives of benzoxazolones [31-40]. The compounds presented in this study appear to be smaller only with a substitution at the 3-position compared the ones published before. MTT results showed that compound **1** and **2** both having 2,5-dimethylphenyl piperazine substituents at the 3-position of benzoxazolone, are effective in non metastatic MCF-7 cell line and induce apoptosis via different pathways but compounds **5** and **6** which have 2-methylbenzyl piperazine group on 3rd position of benzoxazolone structure, seem to be not effective against MCF-7 cells.

Moreover, all tested compounds (**1, 2, 5, 6**) did not show an effective activity against metastatic M4A4 cell line as measured in terms of reduction in cell viability.

In immunocytochemistry studies, the presence of FasL in apoptosis is a clear sign of exogenously induced apoptosis. The results showed that, Fas ligand immunoreactivity was higher in compound **1** treated MCF-7 cells significantly in contrast to the control group. However, compound **2** has been shown to significantly increase the immunoreactivity of cytochrome c in MCF-7 cells. The results also show that compound **2** is effective in inducing the endogenous pathway in MCF-7 cells.

Caspase-3 immunoreactivity was significantly higher in MCF-7 cells treated with compounds **1** and **2**, indicating that the apoptotic pathway was reached. Also, these results were confirmed by the TUNEL test. According to the TUNEL assay results, both compounds **1** and **2** were effective in inducing apoptotic DNA fragmentation in MCF-7 cells.

It appears that compound **1** without a chlorine atom at position 5 of the benzoxazolone structure can be characterized by effective extrinsic apoptotic activity but contrastingly, compound **2** with a chlorine substituent at the 5 position appears to initiate intrinsic proapoptotic activity in MCF-7 cells. Immunoreactivity antigens in MCF-7 cells cultured with compound **1** and compound **2** is shown in figure 6.2.

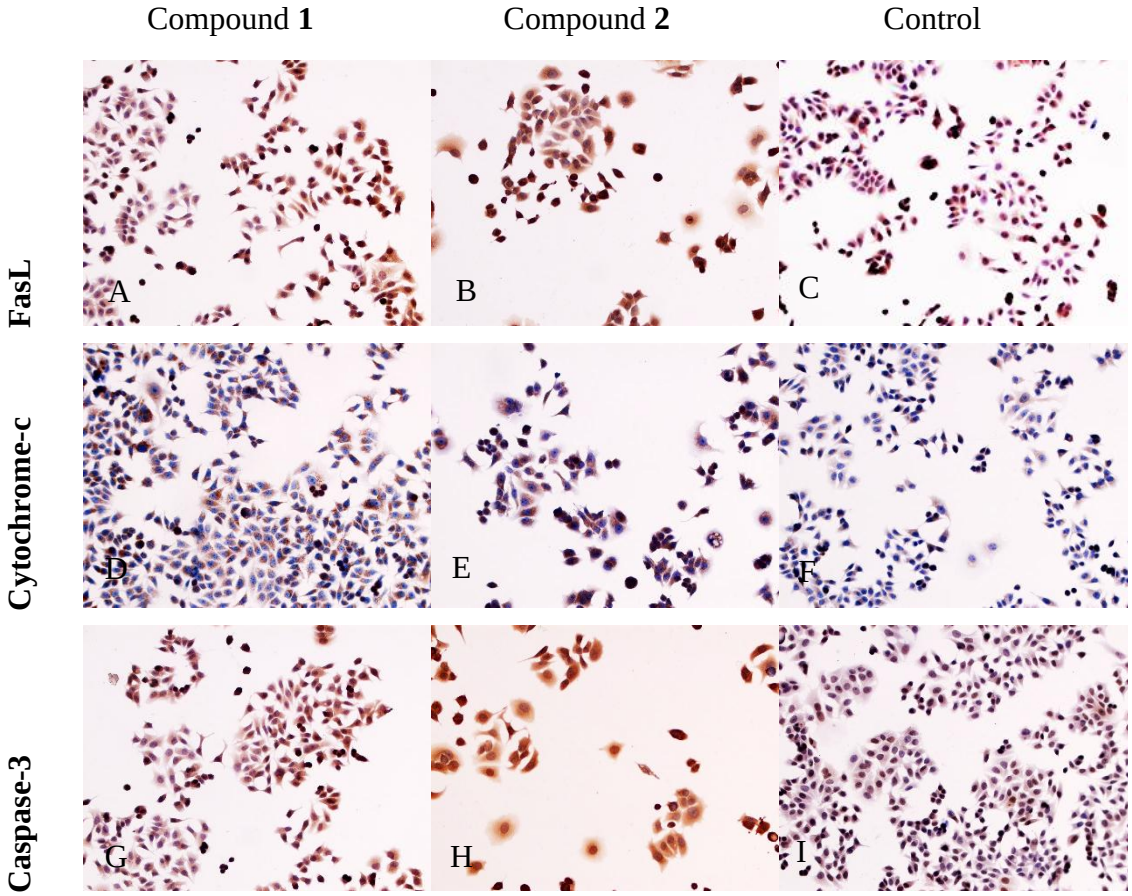


Figure 6.2 Immunoreactivity of target antigens in MCF-7 cells cultured with compound **1** and compound **2** at 100 µM and 50 µM concentration for 48hours, incubation respectively. (A, D, G) compound 1 cultured MCF-7 cells, (B, E, H) compound **2** cultured MCF-7 cells, (C, F, I) only MCF-7 cells.

6.4 Antibacterial and Antifungal Activities

Previously, antimicrobial and antifungal activities of similar compounds have been reported where some were substituted at 3- and some at 6-position of the benzoxazolone core structure [41-45].

The zone of inhibition of sythesized molecules (Compounds **3-12**) were measured in millimeters (mm) and screened for their antibacterial and antifungal activity by disk diffusion method. From the results of disk diffusion test, we can say that our newly synthesized benzoxazolone derivatives in general are not effective against gram positive bacteria *B.cereus*, *E.faecalis* and also they have no antifungal affect against *Candida albicans* since they have no zone of inhibition (0 mm) formation after incubation.

Compounds **3-6**, have no inhibition zones against any strains of bacteria except E.coli. All tested compounds do not have any antimicrobial and antifungal activity against *B.cereus* and *Candida albicans* as shown in figure 6.3.

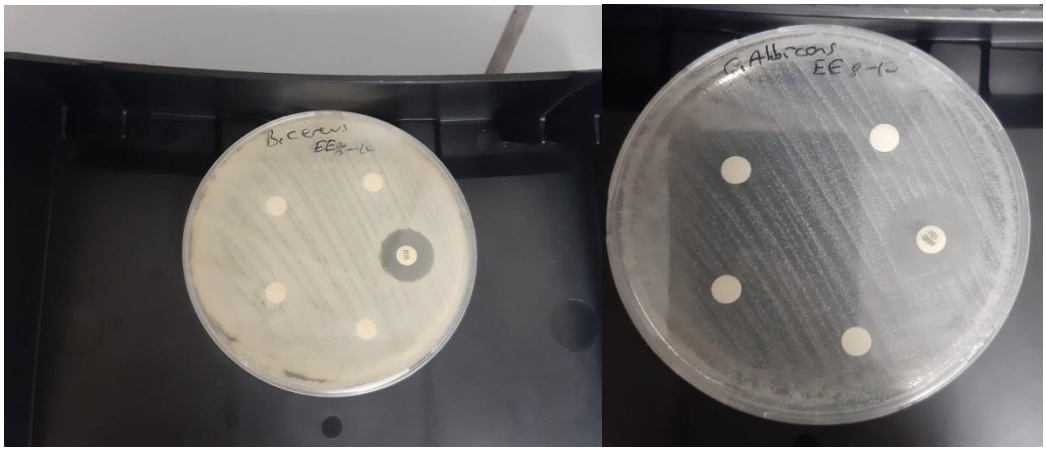


Figure 6.3 Zone of inhibition tests against *B.cereus* and *Candida albicans* respectively.

On the other hand as we can see from table **5.4.1**, the only compound which shows antimicrobial activity agaist *E.faecalis* is compound **4** which has a chlorine group at the5th position and napthylmethyl piperazine ring on the nitrogen atom of its structure. Each tested compound has a very little antimicrobial affect against *E.coli* when compared with the inhibition zone of positive control disk containing ciprofloxacin antibiotic.

Compounds **7-12** have slight antimicrobial affects on *S.aureus* when compared with the inhibition zone of positive control disk containing tetracycline antibiotic.

When we compare the results of zone of inhibition for all compounds tested, the most effective compound against both gram negative *E.coli* and gram positive *S.aureus* is compound **10** with chlorine substituent at 5th position and cyclohexyl substituent as R group on the piperazine ring.

Zone inhibitions of compound **10-12** against *S.aureus* is shown in figure 6.4.

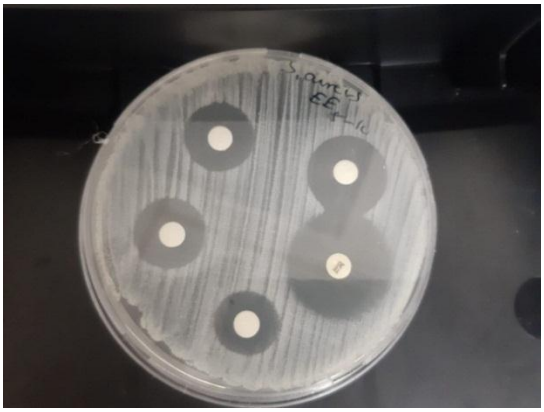


Figure 6.4 Zone inhibition of compound **10-12** against *S.aureus*

7. CONCLUSION

New Mannich bases of 3-substituted benzoxazolone derivatives have been prepared with moderate yields and investigated for either cytotoxic or antimicrobial/antifungal activities.

MTT results clearly indicate that molecules bearing benzoxazolone structure could have cytotoxic effects on MCF-7 cells. According to the results, compound **1** was much more effective than compound **2** in terms of inhibiting growth, proliferation and induction of apoptosis of breast cancer cells.

Another interesting outcome was that compound **1** and compound **2** trigger apoptosis in MCF-7 cells by two different pathways which are extrinsic and intrinsic pathways. Presence of FasL in apoptosis is a clear sign of apoptosis triggered by extrinsic pathway. Immunoreactivity of FasL was significantly higher in MCF-7 cells cultured with compound **1**. However, compound **2** had shown to significantly increase immunoreactivity of cytochrome-c in MCF-7 cells showing that compound **2** was effective in triggering intrinsic pathway. Caspase-3 immunoreactivity was significantly higher both in compound **1** and compound **2** treated MCF-7 cells meaning that the downstream of caspase chain in the apoptotic mechanism was reached.

The presence of the chlorine substituent at the 5-position appear to have an import effect on the proapoptotic mechanism. It will be promising to study further modifications on the 3rd position with other piperazine or amine group as well as different substituents at 5-position in the development of effective anti-cancer agents.

Even though there are reports of antimicrobial activity on similar compounds our results observed for these molecules were not high enough or nonexistent. It can be concluded that both chlorine substituent at 5th position and presence of an aliphatic group as R group on piperazine ring may enhance the antibacterial activity when compared with the aromatic R groups.

Further testing for antimicrobial activity against different gram negative strains appear to be interesting since all synthesized compounds show a slight zone of inhibition against *E.coli* which is also a gram negative bacteria.

These results suggest that gram positive strains have higher resistance against tested compounds than gram negative strains. We can also say that there is no antifungal activity of tested compounds against *Candida* species. The compounds would be further tested as potential antimicrobial agents especially against different gram negative strains.

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APPENDICES

Appendix-1: Synthesis and apoptotic activities of new 2(3H)-benzoxazolone derivatives in breast cancer cells. (Anticancer Agents in Medicinal Chemistry-Article)

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Abstract

***Background*:** 2(3H)-Benzoxazolone derivatives are preferential structural blocks in pharmacological probe designing with possibility of modifications at various positions on the core structure. Benzoxazolones showed various biological activities such as analgesics, anti-inflammatory and anti-cancer.

Objective: In the present work, we have prepared new Mannich bases of 2(3H)-benzoxazolone derivatives and evaluated their cytotoxicities and proapoptotic properties in MCF-7 breast cancer cell line.

***Methods*:** The structures of these compounds were characterized by FT-IR, elemental analysis, ^{1}H and ^{13}C NMR. Cytotoxicities of all the target compounds were investigated by MTT assay. Apoptotic properties of compounds were evaluated by immunocytochemistry using antibodies against to caspase-3, cytochrome-c, FasL and TUNEL assay.

***Results*:** These two novel compounds, **1** and **2**, both have the same piperazine substituent on the nitrogen atom of benzoxazolone and the main difference in the

structures of these compounds is the presence of Cl substituent at the 5- position of the benzoxazolone ring.

MTT results showed that compound **1** and **2** were effective in terms of reduction of cell viability at 100 μM and 50 μM concentration for 48 h, respectively. As a result of immunohistochemical staining, Fas L and caspase-3 immunoreactivities were significantly increased in MCF-7 cells after treated with compound **1**. Additionally, caspase-3 and cytochrome-c immunoreactivities were also increased significantly in MCF-7 cells after treated with compound **2**. The number of TUNEL positive cells was significantly higher in MCF-7 cells when compared with control group after treated with both compound **1** and **2**.

***Conclusion*:** It could be concluded that N-substituted benzoxazolone derivatives increase potential anti-cancer effects and they could be promising novel therapeutic agents for chemotherapy.

Keywords: 2(3H)-benzoxazolone, Mannich reaction, cytotoxicity, apoptosis, breast cancer, MCF-7

1. INTRODUCTION

With the drastic increase in the number of cancer cases worldwide, the need to design selective anti-cancer drugs has become eminent. It is well understood that, in order to develop effective anti-cancer drugs, it is essential to study the mechanism of how the cell death is controlled and/or induced. 2(3H)-Benzoxazolone derivatives have attracted great interest in designing new drug candidates due to the possibility of modifications at various positions on the core structure. The ease of chemical modifications allows fine tuning of the biological activity by controlling the nature and the position of the substituent [1]. Some of the pharmacological activities of 2(3H)-Benzoxazolone derivatives include anti-bacterial, anti-fungal, analgesics-anti-inflammatory, anti-nociceptive and anti-cancer [2-5].

Apoptosis is a well known molecular signaling pathway of programmed cell death. In mammals, there are two apoptotic pathways: death receptor mediated extrinsic pathway and the mitochondrial mediated intrinsic pathway.

Extrinsic pathway that could be triggered by a stimulus from the outside of the cell is caused by binding of the Fas ligand (FasL) to the extracellular domain of trans membrane receptors which then leads to an initiation of the caspase cascade.
On the other hand, intrinsic apoptosis can be triggered by various stress stimuli and causes the release of cytochrome c from the mitochondria which then subsequently results in the activation of caspases-3 and caspases-9. Eventually, for both mechanism pathways, caspases-3 triggers apoptosis which also causes damage and cleavage of the DNA strand [6-7].

Apoptotic signaling pathway is very important in preserving a balance between cell death, cell survival as well as in maintaining genome integrity [8]. In cancer cells, apoptotic pathways can be altered and diverse strategies may be used to evade apoptosis. In recent years, researchers target anti-cancer drugs that can modulate apoptotic pathways and eliminate cancer cells. Although, there are many publications on the diverse biological activity [2-5] of substituted 2(3H)-benzoxazolone molecules, cytotoxic activity studies are rare and published only recently. Ivanova and co-workers studied benzoxazolone derivatives having chalcone like structures for cytotoxicity in the human pre-B-cell leukemia cell line, BV-173 [9-10]. Similar compounds were also tested for their cytotoxicity and carbonic anhydrase (CA) inhibitory activities as reported by Bilginer *et al*. [11]. These previous results clearly indicate that molecules bearing benzoxazolone structure have strong cytotoxic effects on cancer cells and further studies involving cytotoxic activities of different derivatives of benzoxazolones appear promising in the development of effective anti-cancer agents.

There are no reports addressing the proapoptotic effects of 2(3H)-benzoxazolone molecules on breast cancer cells. In this study, we have prepared new Mannich bases of benzoxazolone derivatives. These molecules have dimethylphenylpiperazine substituent at the third position of the benzoxazolone core structure. These new compounds were screened for their cytotoxicity toward MCF-7 breast cancer cell line by using MTT assay and the possible mechanism of the apoptosis induced by these compounds was investigated by indirect immunocytochemistry using antibodies against to caspase-3, cytochrome-c, Fas ligand (FasL) and also TUNEL assay.

2. MATERIALS AND METHODS

2.1. Chemical Methods

All chemicals and reagents were obtained from Sigma Aldrich Chemical Co. or Riedel Chemical Co. and were used without further purification. Melting point of the compounds was recorded on the Mettler Toledo FP 900 Thermo System Digital melting point apparatus and the values are uncorrected. The FT-IR spectra of the compounds were recorded on a Perkin Elmer Spectrum 100 spectrophotometer with attenuated total reflection (ATR) (in wave numbers) in cm^{-1}. The ^{1}H and ^{13}C-NMR spectra of the compounds were recorded on a Mercury Varian 400 MHz NMR Spectrometer using deuterated chloroform ($CDCl_3$) as solvent. Chemical shifts (δ) values were reported in parts per million (ppm). Elemental analyses (C, H, N) were performed on Leco CHNS 932 analyzer. The purity of the compounds was assessed by TLC on silica gel GF 254 (DC-Alufplien-Kieselgel, Germany).

2.2. General procedures for the synthesis of piperazine derivatives

Synthesis was carried out according to the previously published procedure [4]. 15 mmol 2(3*H*)-benzoxazolone derivative and 15 mmol of 2,5-dimethylphenylpiperazine derivative were dissolved in 10 ml of methanol followed by the addition of 20 mmol formalin (35% w/v). The reaction mixture was then refluxed in a water bath for 1 hour. The mixture was poured onto crushed ice and the resulting precipitate was filtered off, washed with cold methanol, dried and purified by recrystallization using ethanol as a solvent.

5-Chloro-3-{[4-(2,5-Dimethylphenyl)]piperazino-1-yl}-2-benzoxazolone (1)

White solid (30.8, yield %); mp 134.4 ºC. IR (cm^{-1}) 2800-3060 (C-H), 1760 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.2-6.8 (m, 6 H, Ar-C*H*), 4.7 (s, 2 H, CH_2), 2.9 (t, 4 H, pip-CH_2 H^2, H^6), 2.8 (t, 4 H, pip-CH_2 H^3, H^5), 2.3 (s, 3H, CH_3), 2.2 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ155.1, 150.9, 141.1, 136.1, 132.8, 130.9, 129.4, 124.1, 122.6, 119.8, 110.8, 109.8, (Ar-*C*), 65.1 (*C*H_2), 51.5, 51.1 (pip-*C*), 21.2, 17.4 (CH_3). Anal. Calc. for $C_{20}H_{22}ClN_3O_2$ C, 63.6; H, 5.96; N, 11.30; Found C, 63.49; H, 6.09; N, 11.35.

3-{[4-(2,5-Dimethylphenyl)]piperazino-1-yl}-2-benzoxazolone (2)

White solid (51.3, yield %); mp 144 ºC. IR (cm^{-1}) 2800-3060 (C-H), 1760 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.2-6.8 (m, 7 H, Ar-C*H*), 4.7 (s, 2 H, CH_2), 2.9 (t, 4 H, pip-CH_2 H^2, H^6), 2.8 (t, 4 H, pip-CH_2 H^3, H^5), 2.3 (s, 3H, CH_3), 2.2 (s, 3H, CH_3);^{13}C NMR (100 MHz, $CDCl_3$) δ155.4, 151.1, 142.6, 136.1, 131.9, 130.9, 129.4, 123.9, 122.6, 119.8, 110.1, 109.4, (Ar-*C*), 64.8 (*C*H_2), 51.5, 51.1 (pip-*C*), 21.2, 17.4 (CH_3). Anal. Calc. for $C_{20}H_{23}N_3O_2$ C, 71.19; H, 6.87; N, 12.45; Found C, 70.77; H, 7.14; N, 12.52.

2.3. Cell line and cell culture

Human breast cancer cells were used MCF-7 (ATCC: HTB-22). MCF-7 cells were maintained in media containing RPMI-1640 (Biochrom, FG 1215), 10% heat inactivated fetal bovine serum (FBS) (Capricorn Scientific, FBS-11B), 1% penicillin-streptomisin (Biochrom, A2213) and 1% L-glutamine (EMD Millipore, K0282). Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2. As the cultured cells reached confluency state, they were sub-cultured using 0.25% trypsin-EDTA solution (Biochrom, L 2143).

2.4. Cell viability and growth assay

The cytotoxicities were measured using an MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Biotium, #30006). MTT is based on colorimetric measurement of reduction of 3-(4, 5-dimethylthialzol-2-yl)-2, 5-diphenyltetrazoliumbromide which is reduced by living cells to yield purple formazan product. Stock compound **1** and compound **2** were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich), 100 mM and diluted in culture medium with five different concentrations (5 μM, 10 μM, 20 μM, 50 μM and 100 μM). DMSO final concentration in cell line was less than 0.05%.

MCF-7 cells were collected, suspended in medium and seeded in 96-well culture dishes at a density of 5 x 10^4/ml cells in each well with 100 µl medium. Negative control row neither contained any cells nor extracts and positive control row only had cells seeded in. Compound dilutions were triplicated and both cell lines were incubated for 24 and 48 h. After incubation MTT solution was heated to 37°C and then 10 µl were added to the each well. After 4 h incubation at 37°C in 5% CO_2, 50 µl DMSO was added to dissolve the formazan salts. The absorbance was measured at 540 nm with spectrophotometer (Versa Max, Molecular Device, Sunnyvale, USA). All experiments were performed in triplicate for each compound.

2.5. TUNEL assay for detection of cell death

DNA fragmentation was detected by labeling apoptotic cells with specific staining while using commercial *in situ* apoptosis detection kit (ApoptagPlus Peroxidase *In Situ* Apoptosis Detection Kit, S7101, Millipore, USA). After fixation of cells with 4% paraformaldehyde in PBS at 4°C for 30 minutes, they were washed with PBS for 2 times and permeabilized with 0.1% Tween 20, they were washed with equilibrium buffer and incubated 1 hour at 37°C with tdt solution. After washing with stop solution, anti-peroxidase solution was added and after 30 min incubation, slides were washed with PBS and diaminobenzidine (DAB) was added for 5 min. It was washed with distilled water and the cells were counterstained with Mayer's hematoxylien for 5 min and mounted with mounting medium (Merck Millipore, 107961, Germany). All specimens were examined using a light microscope (Olympus BX40, Tokyo, Japan).

2.6. Indirect Immunocytochemistry

Cultured MCF-7 cells were assessed immunocytochemically for binding of antibodies against caspase-3, cytochrome-c and Fas-ligand (FasL). MCF-7 cells were fixed with 4% paraformaldehyde in PBS at 4°C for 30 minutes. 0.1 % Tween 20 (Sigma-Aldich) solution was added for permeabilization for 15 minutes. The cells were washed with PBS and endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 5 minutes at room temperature.

After washing cells with PBS three times for 5 minutes, primary antibodies anti-caspase-3 (sc-7272, SantaCruz Biotechnology, Inc., USA), anti-cytochrome-c (sc-13156, Santa Cruz Biotechnology, Inc., USA) and anti-Fas-Ligand (sc-834 Santa Cruz Biotechnology, Inc., USA) were added and incubated overnight at 4°C. Biotinylated secondary antibody (Histostain-Plus, IHC Kit, HRP, 859043, Thermo Fischer) was added and incubated for 30 minutes followed by PBS wash (x3) for 5 minutes. Strepavidin-peroxidase complex (100 µl) was added to cultured cells. Cells then washed by PBS and DAB was added and incubated for 5 minutes for enhancement of immuno-labelling. DAB was washed with distilled water. Cells were counterstained with Mayer's hematoxylien for 5 minutes and mounted with mounting medium (Merck Millipore, 107961, Germany). All specimens were examined using a light microscope (Olympus BX40, Tokyo, Japan).

Staining of caspase-3, cytochrome-c and Fas-ligand was also graded semi quantitatively using the H-SCORE that was calculated with the following equation: HSCORE=Σл (i+1), where i is the intensity of staining with a value of 1, 2 or 3 (mild, moderate, or strong, respectively) and л is the percentage of cells stained with each intensity, varying between 0 and 100%.

2.7. Statistical analysis

Results were expressed as mean ± standard deviation (SD). The results were analyzed using GraphPad Prism 7 software. Differences among groups were analyzed statistically with Mann-Whitney where appropriate. A p value of <0.05 was considered as statistically significant.

3. RESULTS

3.1. Chemistry

2(3*H*)-benzoxazolone derivatives were reacted with 2,5-dimethylphenylpiperazine through Mannich reaction to form 3-substituted-2(3*H*)-benzoxazolone compounds as shown in Figure **1** by employing the synthesis method reported in the literature [4]. The structures of these compounds obtained were characterized by FT-IR, ^{1}H and ^{13}C NMR and elemental analysis.

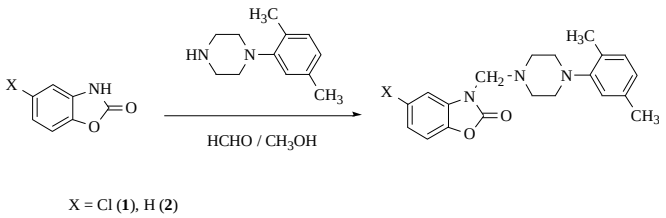

Fig. (1). Synthesis scheme of title compounds

Both compounds have the same piperazine substituent and the main difference in the structures of these compounds is the presence of Cl substituent at the 5- position of the benzoxazolone ring of compound **1**. The chemical structures of both molecules were deduced by the spectral data. From the IR spectra it is clear that, the N-H band of the 2(3H)-benzoxazolone ring and the piperazine derivative at 3100-3400 cm^{-1} have disappeared. This absence of the absorption band confirms that the reaction took place at the N atom of the benzoxazolone ring and piperazine upon Mannich reaction. In both compounds, the (C=O) stretching band of the lactam ring appears at 1760 cm^{-1}, as reported for similar molecules.

The hydrogen atoms of the methylene bridge between two nitrogen atoms appear as a singlet at about 4.7 ppm on the ^{1}H NMR spectra for the two compounds. The piperazine protons (H^{6} and H^{2}) and (H^{3} and H^{5}) were seen as triplets at 2.9 and 2.8 ppm, respectively for compounds **1** and **2**. Additionally, the two methyl groups on the phenyl ring are evident as two singlets at 2.3 ppm and 2.2 ppm on the ^{1}H NMR spectra.

The aromatic protons appear as multiplets between 6.8 to 7.2 ppm on ^{1}H-NMR spectra as expected for compounds **1** and **2**. The integral values of the aromatic protons match to the proposed structures of the two molecules.

3.2. Cell viability and cytotoxicity

MCF-7 cells were treated with five different concentrations of (5, 10, 20, 50 and 100 μM) compound **1** and compound **2** for 24 and 48 hours. The cell viability was determined as described above by MTT assay. All concentrations of compound **1** and compound **2** resulted in a dramatic decrease of MCF-7 cell proliferation and had toxic effect in a dose and time-dependent manner. Our results showed that, compound **1** at 100 μM concentration was more effective at inhibiting MCF-7 cell growth when compared with other dilutions for 48 h incubation period (Fig. **2**). Compound **2** at 50 μM concentration was more effective at inhibiting MCF-7 cell growth when compared with other dilutions for 48 h incubation period (Fig. **3**).

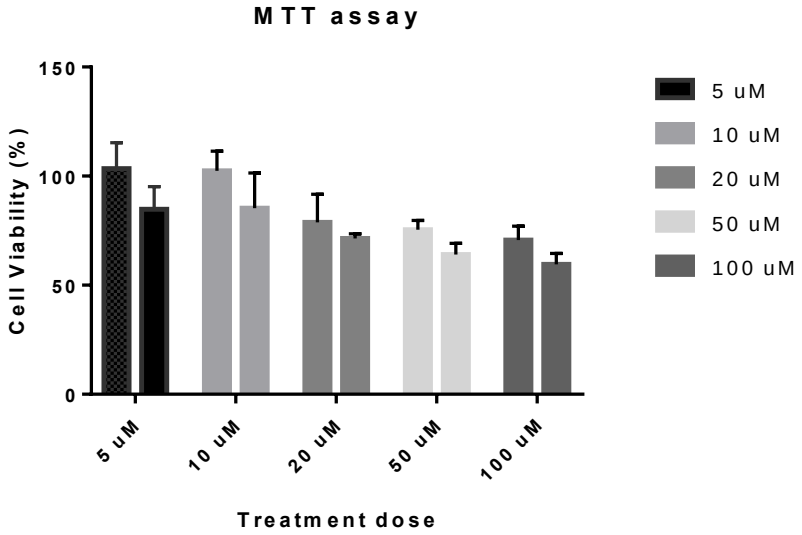

Fig. (2). Effect of compound **1** on cell viability of MCF-7 cells. MCF-7 cells were treated with different concentrations of compound **1** for 24 and 48 h. Viability was quantitated by the MTT assay.

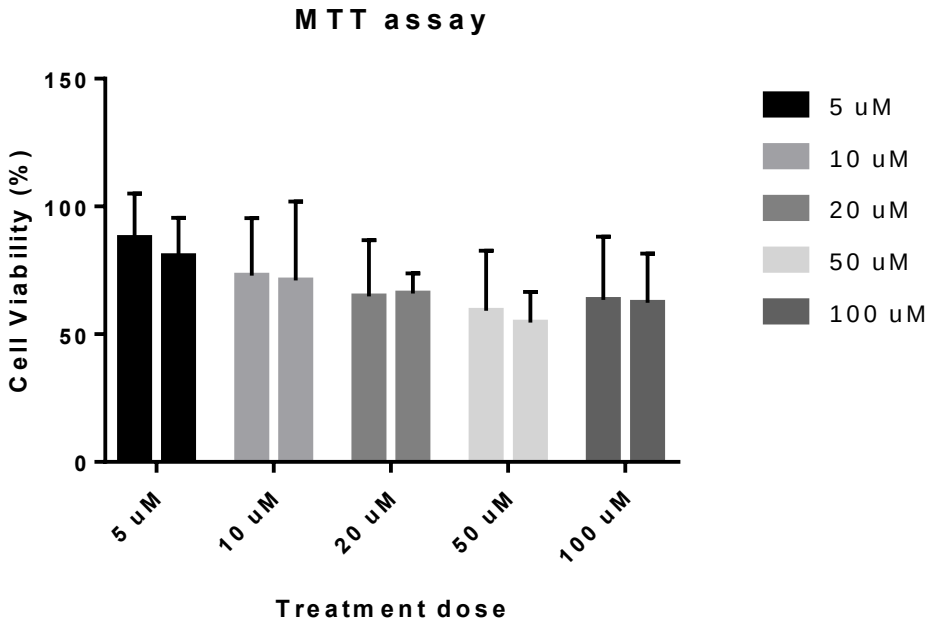

Fig. (3). Effect of compound **2** on cell viability of MCF-7 cells. MCF-7 cells were treated with different concentrations of compound **2** for 24 and 48 h. Viability was quantitated by the MTT assay.

3.3. Cell morphology

MCF-7 cells are adherent epithelial cells. After treated with compound **1**, vacuoles were detected in the cytoplasm of MCF-7 cells and number of the cell decreased (Fig. **4A** and **4B**). The morphology of MCF-7 cells treated with compound **2** were similar to control (Fig. **4C**). Inverted microscope images of the compounds clearly indicate that compound **1** has more pronounced effect on the morphology of the MCF-7 cells.

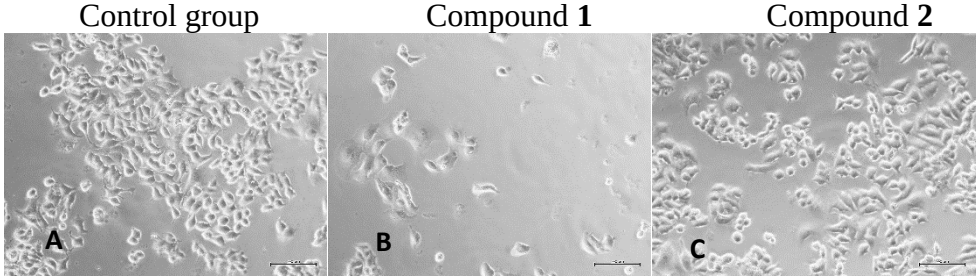

Fig. (4). MCF-7cells imaged under the inverted microscope: (**A**) MCF-7 cells and (**B**) Compound **1** treated MCF-7 cells (**C**) Compound **2** treated MCF-7 cells. Scale bars= 100 μm.

3.4. Apoptotic effects of compound 1 and compound 2 in MCF-7 cells

TUNEL assay was used in MCF-7 cells that were incubated with 100 μM and 50 μM concentrations of compound **1** and compound **2** for 48 h, respectively. The number of TUNEL positive cells that were treated with both compound **1** and compound **2** were highly significant in MCF-7 cells when compared with both control group (Table 1) (Fig. **5 A-C**).

Table 1. The percentage of TUNEL positive MCF-7 cells treated with compound **1** and compound **2** at 100 μM and 50 μM concentrations for 48h, respectively.

Compound 1	Compound 2	Control group
72.66± 11.38[a]	85± 7 [b]	35.67 ± 4.04

Data are expressed as means ± SD and were compared by Mann-Whitney.

[a] The data was significant when compared with control group ($p<0.01$)

[b] The data was significant when compared with control group ($p<0.001$)

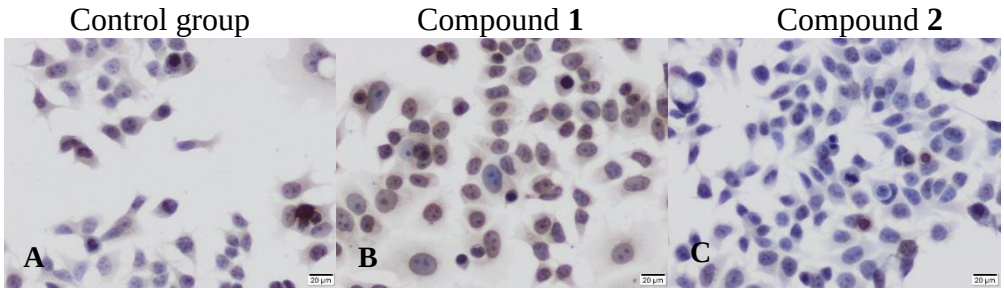

Fig. (5). Apoptotic effects of compound **1** and compound **2** in MCF-7 cell line: (**A**) MCF-7 control cells, (**B**) Compound **1** treated MCF-7 cells, (**C**) Compound **2** treated MCF-7 cells. Scale bars= 20 μm.

3.5. Immunocytochemical evaluation

FasL immunostaining was strong in MCF-7 cells treated with compound **1** and H-SCORE results was significantly higher when compared with control group ($p<0.05$, Table 2) (Fig. **6A**). On the other hand, H-SCORE for compound **2** treated MCF-7 cells was not significant when compared with control group ($p>0.05$, Table 2).

Immunostaining intensity for cytochrome-c was moderate to strong for both compounds in MCF-7 cells (Fig. **6D**, **6E**). As shown in Table 2, cytochrome-c immunoreactivity was higher in both compound **1** and compound **2** treated MCF-7 cells than in control group. Compound **2** treated MCF-7 cells had shown significant increase in H-SCORE than control group ($p<0.05$, Table 2). On the other hand, H-SCORE for compound **1** treated MCF-7 cells was not significant when compared with control group ($p>0.05$, Table 2).

Immunostaining of caspase-3 in MCF-7 cells was strong for compound **1** and compound **2**. As shown in Figure **6**, immunoreactivity of caspase-3 in MCF-7 cells was higher in compound **1** and compound **2** treated cells than control group. H-SCORE results revealed that, immunolabelling was significantly higher in both compounds in comparison with control group respectively ($p<0.01$, $p<0.01$, Table 2) in MCF-7 cells.

Table 2. The H-SCORE of FasL, cytochrome-c and caspase-3 immuno labelling in MCF-7 cells treated with Compound 1 and Compound 2at 100 µM and 50 µM concentration for 48h, respectively.

	Compound 1	**Compound 2**	**Control group**
Fas-L	287.2 ± 22.77^{a}	277.4 ± 18.93	235.6 ± 33.54
Cytochrome-c	275.8 ± 29.62	278.4 ± 20.49^{b}	234.7 ± 11.33
Caspase-3	274.5 ± 28.94^{c}	262.6 ± 34.15^{d}	112.6 ± 9.379

Data is expressed as means ± SD and were compared by Mann-Whitney.

[a] The data was significant when compared with control group ($p<0.05$)
[b] The data was significant when compared with control group ($p<0.01$)
[c] The data was significant when compared with control group ($p<0.01$)
[b] The data was significant when compared with control group ($p<0.01$)

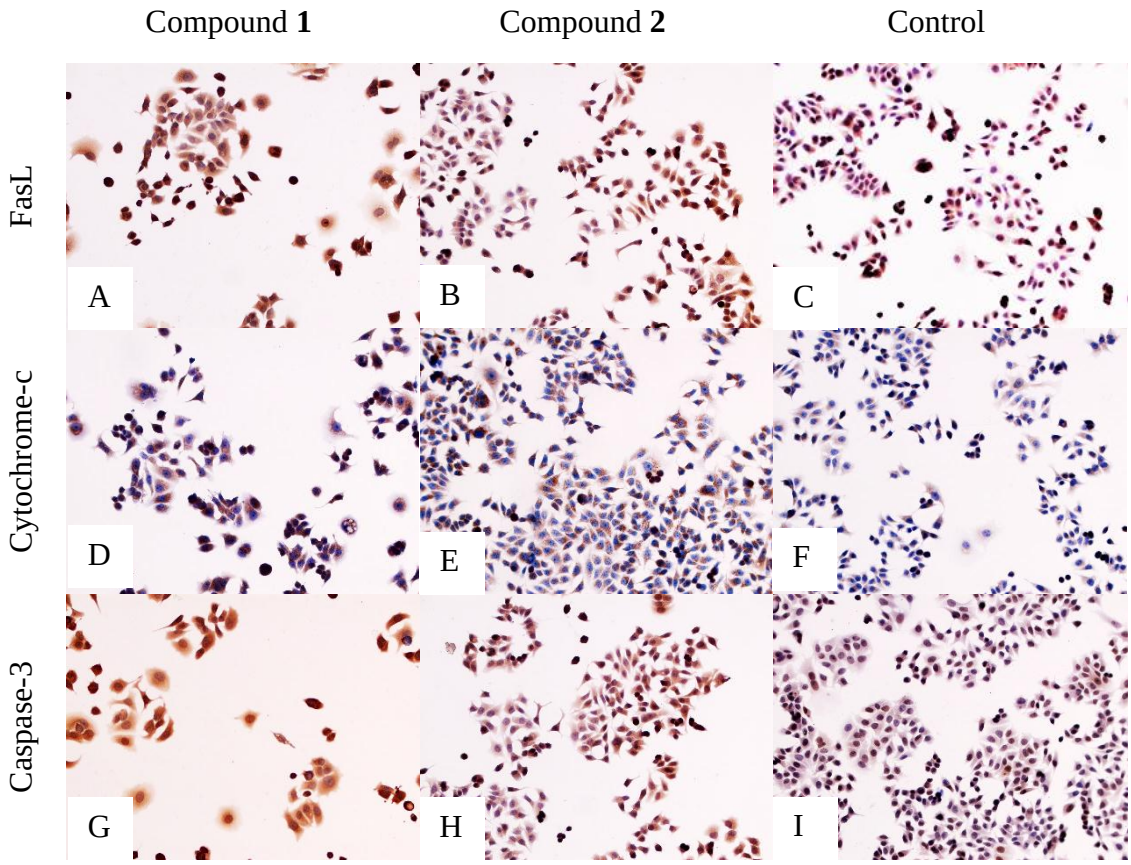

Fig. (6). Immunoreactivity of FasL (**A, B, C**), cytochrome-c (**D, E, F**) and caspase-3 (**G, H, I**) in MCF-7 cells treated with compound **1** (**A, D, G**) and compound **2** (**B, E, H**) at 100 µM and 50 µM concentration for 48h, respectively. (**C, F, I**) MCF-7 cells culture with standard culture conditions. Scale Bars=20µm

4. DISCUSSION

Newly synthesized, Mannich bases of 2(3H)-benzoxazolone derivatives **1** and **2**, were reported for the first time with their cytotoxic and proapoptotic properties in our study. Compound **1** was rather more effective than compound **2** in terms of inhibiting breast cancer cells growth, proliferation and induction of apoptosis. Interestingly, the results indicate that compound **1** and compound **2** induced apoptosis via different pathways in MCF-7 cells.

Apoptosis, programmed cell death, is a vital process for cell turnover, embryonic development and immune system functions. Also, it occurs as a damage response in cells that is stimulated by variety of physiological and pathological stimuli and conditions in diseases. For over decades, a major goal of clinical oncology has been the development of targeted therapies for prompting the elimination of cancer cells by apoptosis. However, neoplastic cells become resistant to apoptosis by the genetic and epigenetic alterations [12]. In recent years, many scientists have been focused on the synthesis new molecules such as benzoxazolone and its derivatives which is shown to stimulate apoptosis and have antitumor activities. Ivanova et al studied chalcone like benzoxazolone derivatives which were found to have caused DNA laddering in BV-173 cell line suggesting programmed cell death [9]. In a related study, similar molecules were tested in–BV-173 (human B cell precursor leukemia), MCF-7 and MDA-MB-231 cell lines and was reported to have promoted genomic DNA to fragment into mono and oligonucleosomes [13]. El-Helby et al. [14] demonstrated that benzoxazole derivatives had anticancer activities in hepatocellular carcinoma (HepG2), colorectal carcinoma (HCT-116), and breast cancer (MCF-7) through inhibition of VEGFR-2 enzyme. Omar et al. [15] showed that novel benzoxazole derivatives which include amide and dithiocarbamate moieties, exerted anti-cancer activities via inhibition of EGFR and ARO enzymes in MCF-7 and MDA-MB-231 cell lines. Additionally, these new compounds increased expression of caspase-9 protein, elicited apoptosis at preG1 phase and arrested cell cycle at G2/M phase in MCF-7 and MDA-MB-231 cell lines. Lately reported azepane ring containing benzoxazole compounds' cytotoxicity were evaluated for MCF-7 cell line and shown to stimulate caspase-3 activity for apoptotic pathway [16].

In contrast to the other benzoxazolone derivatives that are reported in the literature, compounds **1** and **2** are smaller in size and have no lipophilic groups at position 6. It is evident from the literature that even a small change in the structure can alter the apoptotic pathway, therefore the mechanism of apaptosis for these two new compounds was elucidated to get a better insight into structure activity relationships of these derivatives. Considering our results, caspase-3 immunoreactivities were significantly higher in compound **1** and compound **2** treated MCF-7 cells than control group.

Caspase-3 immunoreactivity was significantly higher in compounds **1** and **2** treated MCF-7 cells indicating that downstream of apoptotic pathway had been reached. Moreover, these results were verified by TUNEL assay study. According to the TUNEL assay results, both compounds **1** and **2** were effective in triggering apoptotic DNA fragmentation in MCF-7 cells. The results indicated that both compound **1** and **2** could stimulate apoptosis in MCF-7 cells.

Apoptosis can be stimulated by one of two separate pathways, the extrinsic and intrinsic pathways. Extrinsic apoptotic pathway is triggered by stimulation of death receptors and activates the initiator caspase-8 which can propagate the apoptosis signal by direct cleavage of downstream effector caspases, caspase-3. In contrast, FasL is a death receptor protein and also its binding with its receptor triggers extrinsic apoptosis pathway [17]. The intrinsic apoptotic pathway, mitochondrial pathway, is stimulated by the release of apoptogenic factors such as cytochrome c from the mitochondrial intermembrane space. The elevated levels of cytochrome c in the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex. Both extrinsic and intrinsic apoptotic pathways lead to cell death execution via caspase-3 activation [8]. Our results showed that immunoreactivity of FasL was significantly higher in compound **1** treated MCF-7 cells than control group. FasL is a good marker for extrinsic apoptotic pathway. Presence of FasL in apoptosis is a clear sign of apoptosis triggered by extrinsic pathway. However, compound **2** had shown to significantly increase immunoreactivity of cytochrome-c in MCF-7 cells. The results also suggest that compound **2** was effective in triggering intrinsic pathway in MCF-7 cells. Although the precise reasons of apoptotic action of tested compounds is yet to be determined, it appears that compound **1**, with a Cl substituent at the 5-position, may be characterized by effective extrinsic apoptotic activity. Contrarily, compound **2** with no substituent at the 5-position of the 2(3H)-benzoxazolone structure appear to initiate intrinsic proapoptotic activity in MCF-7 cells. From these results, it is evident that the presence of a substituent at the 5-position is affecting the proapoptotic mechanism.

In conclusion, our study demonstrated that novel 2(3H)-benzoxazolone derivatives **1** and **2** induced apoptosis via extrinsic and intrinsic pathways in MCF-7 cells, respectively. It will be worthwhile to do a systematic study on the effect of the type and the size of substituents on all possible carcinogenesis mechanism by the related derivatives of these compounds to further assess the different signaling pathways.

LIST OF ABBREVIATIONS

NMR = Nuclear Magnetic Resonance

FT-IR = Fourier-transform infrared spectroscopy

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling

MCF-7 = Michigan Cancer Foundation-7

TLC = Thin Layer Chromatography

PBS = Phosphate-buffered saline

H-SCORE = Histo score

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No humans and animals were used in this study.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

Not applicable.

Appendix-2 : Studies on new 2(3H)-benzoxazolone derivatives: synthesis, characterization and cytotoxic effects. (European Biotechnology 2019 Congress April 11-13 2019 – Valencia /Spain- Poster Presentation).

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Abstract

2(3H)-Benzoxazolone derivatives have been reported to show diverse biological activities depending on the position and type of the substituent. Recent studies show that these types of molecules could also show promising anticancer activities. A series of new Mannich bases of 2(3H)-benzoxazolone derivatives have been prepared. These molecules have piperazine group at the third position of the core structure. The structural characterization of these compounds was performed by FT-IR, 1H NMR and ESI-MS analysis. These 2(3H)-benzoxazolone derivatives were analyzed for their cytotoxicity toward MCF-7 cancer cell line. Different concentrations of these molecules were incubated for 24 h and 48 h. MTT assays were employed to measure cytotoxicity and cell growth. Our results showed that, among all concentrations of 2(3H)-Benzoxazolone derivatives studied (5, 10, 20, 50 and 100 μg/ml), dimethylphenylpiperazine substituted derivative, at 50 μM concentration was more effective at inhibiting MCF-7 cell growth compared with other dilutions for 24 h and 48 h incubation period.

Keywords: 2(3H)-benzoxazolone, Mannich reaction, breast cancer, cytotoxicity

Appendix-3 : Cytotoxicity and apoptotic effects of new 2(3H)-benzoxazolone derivatives on MCF-7 cells. (EurasianBioChem 2020 Congress 19-20 March 2020 – Ankara/ Türkiye)

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Abstract

2(3H)-Benzoxazolone derivatives are new drug candidates due to the possibility of modifications at various positions on the core structure. In this study, we have prepared new Mannich bases of 2(3H)-benzoxazolone derivatives. These molecules have dimethylphenylpiperazine substituent at the third position of the benzoxazolone core structure. These new compounds were screened for their cytotoxicity toward MCF-7 breast cancer cell line by employing MTT assays and the possible mechanism of the apoptosis. The structures of these compounds were characterized by FT-IR and H^1 NMR. Cytotoxic effect of 2(3H)-benzoxazolone derivatives on the MCF-7 cells was measured by MTT assay. MCF-7 cells were treated with different concentrations of (5-100μM) benzoxazolone derivatives for 24 and 48 hours. Apoptotic properties of benzoxazolone derivatives were determined by immunocytochemistry using antibodies (caspase-3, cytochrome-c and FasL) and TUNEL assay. MTT results showed that compound 1 and 2 were effective in terms of reduction of cell viability at 100 μM and 50 μM concentration for 48 h, respectively. As a result of immunohistochemical staining, Fas L and caspase-3 immunoreactivities were significantly increased in MCF-7 cells after treated with compound 1. Additionally, caspase-3 and cytochrome-c immunoreactivities were also increased significantly in MCF-7 cells after treated with compound 2. The number of TUNEL positive cells was significantly higher in MCF-7 cells when compared with control group after treated with both compound 1 and 2. The results suggest that compound 1 and 2 might have potential anticancer effects and they could be potential novel therapeutic agents for chemotherapy.

Keywords: 2(3H)-Benzoxazolone, cytotoxicity, mannich reaction, breast cancer

Appendix-4 : Curriculum Vitae (CV)

Name	Emine	**Surname**	Erdağ
Place of Birth	Nicosia	**Date of Birth**	21.10.1993
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E-mail	emine.erdag@neu.edu.tr		

Educational Level	**Name of the Institution Graduated**	**Year of Graduation**
Ph.D	Near East University Faculty of Pharmacy	2020
Master	Near East University Faculty of Pharmacy	2016
High School	Near East College	2011

Work Experience

Duty	**Institution**	**Duration**
Research Assistant	Near East University Faculty of Pharmacy	(2016- 2020)

Foreign Languages	Reading Comprehension*	Speaking*	Writing*
English	Good	Good	Good
French	Good	Good	Good

Foreign Language Exam Grade #								
YDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE

	Quantitative	Counterweigh	Verbal
ALES Grade Spring (2016)	56,92677	58,09492	61,35998

Computer Knowledge

Program	Ability to Use
Microsoft Word, Microsoft Excell	Good