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BIOLOGICAL SCREENING OF SYNTHESIZED OXALAMIDE DERIVATIVES FOR ANTIOXIDANT AND ANTICANCER PROPERTIES

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ABSTRACT

In the present study oxalamide derivatives were synthesized by treating oxalyl chloride with different derivatives of aniline (namely aniline, 5-bromo, 2,5-dimethyl, 3-bromo, 4-bromo, 3-chloro, 4-chloro, and 4-(Trifluoromethyl) aniline). The synthesized compounds were characterized using H^1-NMR, EI-MS, and FTIR spectrometry. Synthesized oxalamides were also evaluated for their antioxidant and anticancer activities. The antioxidant activity was studied using DPPH radical scavenging assay whereas the anticancer activity was studied against blood cancer cell lines, namely RAJI and $DOHH_2$. The oxalamide derivatives containing bis (2, 5-dimethylphenyl), bis (3-bromophenyl), and bis (4-bromophenyl) substitutions showed significant antioxidant properties which were comparable with butylated hydroxyanisole; standard drug. However, none of the synthesized oxalamide derivatives showed any significant anticancer activity.

Keywords: oxalamide, antioxidant, anticancer, characterization, synthesis, biological-screening, amides

INTRODUCTION

Oxygenated species possessing unpaired electron, commonly known as reactive oxygen species ROS, are continuously released in the body during different enzymatic and non-enzymatic biological reactions (Dickinson and Chang, 2011; Mahmoud *et al.*, 2011). Reactive Oxygen species are involved in signal transmissions to brain and body defense mechanism against pathogens (Dickinson and Chang, 2011). However, overproduction of ROS can cause oxidative stress and can damage biomolecules such as proteins, nucleic acid, lipids. Oxidative stress eventually give rise to inflammation and promote autoimmune diseases (Balmus *et al.*, 2016; Sznarkowska *et al.*, 2017; Tian *et al.*, 2017; Zuo *et al.*, 2016) such as cancer, cardiovascular disorders, atherosclerosis, malaria, rheumatoid arthritis, Alzheimer's disease, Parkinson's disease, neurodegenerative diseases (Raziq *et al.*, 2017), cataracts, chronic inflammation, and pre-mature aging (Aruoma, 1998; Fang *et al.*, 2002; Finkel and Holbrook, 2000; Waris and Ahsan, 2006).

Antioxidants are compound that reduce the free radicals and ROS and subsequently decrease oxygen stress. Although a number of ROS inhibitors (aspirin, ibuprofen, and naproxen) have been reported (Khalaf *et al.*, 2008; Lee *et al.*, 1998) most of them have their associated side effects including allergies, non-selectivity and effects related to central nervous system (Sostres *et al.*, 2010).

Cancer is rapid, uncontrolled multiplication of abnormal cells. There are different types of cancer based on part of body affected. Common types include cancer of bladder, bone, bowel, brain, breast, cervical, eye, gallbladder, head and neck, kidney, liver, lung, anal, skin, esophageal, ovarian, pancreatic, penile, prostate, intestine, soft tissue, stomach, testicular, thyroid, uterine, vaginal, etc. Some other types of cancer are Hodgkin lymphoma, Leukemia, Mesothelioma, Myeloma, Non-Hodgkin lymphoma. It is known that cancer is affecting millions of people per annum around the globe as reported by cancer research UK (2019). In recent years, many laboratories are extensively involved in the synthesis of new anticancer agents and designing cancer therapies with least side effects, less drug resistance and having high efficacies (Giordano and Petrelli, 2008). Although there are many successes the absolute effectiveness has not been achieved against any disseminated cancer (Hanikoglu *et al.*, 2019; Nepali *et al.*, 2014).

Lymphoma is a type of cancer in which white blood cells (lymphocytes) proliferate abnormally. 90% of the lymphomas are Beta cell lymphomas. Here, we selected two beta cell lymphoma cell lines, RAJI and $DOHH_2$. The clonal proliferations of B-cells show a high degree of variation in terms of clinical and presenting features, histopathology, immunophenotype, and genetics.

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ), propyl gallate (PG) are commonly used synthetic antioxidants. They have limited scope because of their low thermal stability, unaesthetic features and harmful health effects. Amides have great pharmacological history and possess diverse range of applications. (Rajput and Sharma, 2018). They are famous for their anti-bacterial, anti-fungicidal, anti-convulsant, lipoxygenase inhibition, anesthetic, and platelet aggregation (Fazal-ur-Rehman *et al.,* 2019; Malki *et al.,* 2017). Amides can be synthesized from oxalic acid are diamides which are classified as oxalamides. Being diamides, they can act as bidentate ligand donating two pairs of lone pairs to a single metal and can also act as bis bidentate ligand donating their lone pairs to two different metal ions. Oxamides, as bis-bidentate ligands, can be used to synthesize supra-molecules. In pharmaceutical field, study of antioxidants, antibacterial and enzyme inhibitions remain a vital area of research as these can depreciate human skin, body, can be the reason of different disease or even can cause death.

Present work comprised of the synthesis of selected amides, their characterization, and evaluation of their antioxidant and anticancer activities. The amides synthesized were diphenyloxalamide, bis (2,5-dimethylphenyl)oxalamide, bis (3-bromophenyl)oxalamide, bis (4-bromophenyl)oxalamide, bis (3-chlorophenyl)oxalamide, bis (4-chlorophenyl)oxalamide, and bis (4-(trifluoromethyl)phenyl)oxalamide. The antioxidant activity of synthesized amides was evaluated and compared to standard antioxidants butylated hydroxyanisole (BHA). The anticancer activity of amides against RAJI and $DAHH_2$ cancer cells was compared to standard anticancer drugs, Imatinib and Dasatinib.

MATERIAL AND METHODS

General Experimental:

All chemicals used in this study were Reagent grade and purchased from sigma, BDH or Merck. Oxalyl chloride (Merck cat # 8070660025), triethylamine (Merck cat # 8083520100), tetrahydrofuran (Merck cat # 1097312500), n-hexane (Merck cat # 1007952500), ethyl acetate (Merck cat # 1007892500), aniline (Sigma Aldrich cat # 8222561000), 2,5-dimethylaniline (Sigma Aldrich cat # 102253), 3-bromoaniline (Sigma Aldrich cat # 180025), 4-bromoaniline (Sigma Aldrich cat # 8016000100), 3-chloroaniline (Sigma Aldrich cat # 8026120250), 4-chloroaniline (Sigma Aldrich cat # 8026130100), 4-trifluoromethylaniline (Sigma Aldrich cat # 224936), butylated hydorxyanisole (Sigma Aldrich cat # W218308), DMSO, 2, 2'-diphenyl-1-picryl hydrazyl (Sigma Aldrich cat # D9132). Aluminum plates pre coated with silica gel were used for TLC (Supelco cat # 99569). Shimadzu 8900 IR spectrophotometer, Avane Bruker 400MHz ^{1}H NMR spectrometer and JEOL MS Route 600H EI Mass spectrometer were used for characterization.

General procedure for the Synthesis of ligands:

Ten mMole of selected amine and 0.5mL of triethyl amine were dissolved in 80ml of tetrahydrofuran (THF), the resulting solution was cooled in ice bath (≈ 5°C). To this solution 5.0mmole oxalyl chloride were added and the mixture was stirred at room temperature. The progress of the reaction was monitored using TLC. After 24 hours of stirring, reaction mixture was dried on rotavapor. The product formed was washed off using sodium bicarbonate solution and water was used to wash resulting solid. After washing solid was dried under vacuum. For further purification of oxalamides column chromatography was performed on silica gel column using n-hexane: ethyl acetate (7:3) as eluent (Fazal-ur-Rehman *et al.,* 2019) .

BIOLOGICAL SCREENING

Antioxidant Activity

2, 2'-diphenyl-1-picryl hydrazyl (DPPH) Radical Scavenging Activity:

The antioxidant activity of synthesized oxamides was evaluated for DPPH free radical scavenging. The method is based on the formation of colorless hydrazine by the protonation of DPPH free radicals. A stock solution of 0.3 mM DPPH was prepared using ethanol as solvent. A series of solution for each of the synthesized oxamide was prepared using DMSO as solvent. The concentrations used were 500, 250, 150, 125, 62.5, 31.2, 15.6, 7.8 µM oxamide. A pit of a 96-well plate was used as a reaction vessel. To each of the eight wells 10 µl of respective oxamide solution was transferred followed by addition of 90 µl of DPPH stock solution in each well. A separate well was used for control. The incubation temperature was 37˚C and the stirring time was 30 minutes. Absorbance of solutions at the end of reaction time was recorded at 517nm using microtiter plate reader (Spectra max plus 384 Molecular devices USA). The method was standardized using Butylated hydroxyanisole (BHA). Percent radical

scavenging activity was calculated using DMSO treated control. IC_{50} values were determined using EZ fit software (Perrella Software, USA) (Ali *et al.*, 2009; Siddiq *et al.*, 2012).

$$DPPH\ radical\ scavenging\ effect\ (\%) = \left(Ac - \frac{As}{Ac}\right) \times 100$$

Where, As is the absorbance of sample and Ac is the absorbance of control.

Alamar blue assay for cell cytotoxicity analysis

Alamar blue assay was used to determine cytotoxicity of synthesized oxalamide derivatives. For each oxalamide derivatives, two cancer cell lines RAJI and $DOHH_2$ (The two B lymphoma cancer cell lines highly malignant Raji (Burkitt's lymphoma) and low malignant $DOHH_2$ (follicular lymphoma); were kindly gifted from Dr A. Kluin-Nelemans, Groningen, the Netherlands). The cell lines were seeded in 10×10^5 cells per well with 200µL final reaction volume. Medium used for this was RPMI-1640 medium which contained 10% heat inactivated fetal bovine serum (PAA laboratories). Different concentrations of oxalamides were then added in cells and incubated for 48 hours. After this time, the cells were incubated for 4 hours at 37^0C in humidified atmosphere containing 5% CO_2 along with addition of 22 µL Alamar blue dye 10%. After this incubation time fluorescence recording were taken at (λex = 560 nm, λem = 590 nm) in fluorometer (O'brien *et al.*, 2000).

RESULTS AND DISCUSSION

Chemistry

The summarized synthesis scheme is shown below (Fig. **1**). Oxalamide derivatives were white solid, non-hygroscopic, amorphous, stable and soluble only in DMSO at room temperature but dissolve in acetonitrile, ethanol and ethyl acetate on heating.

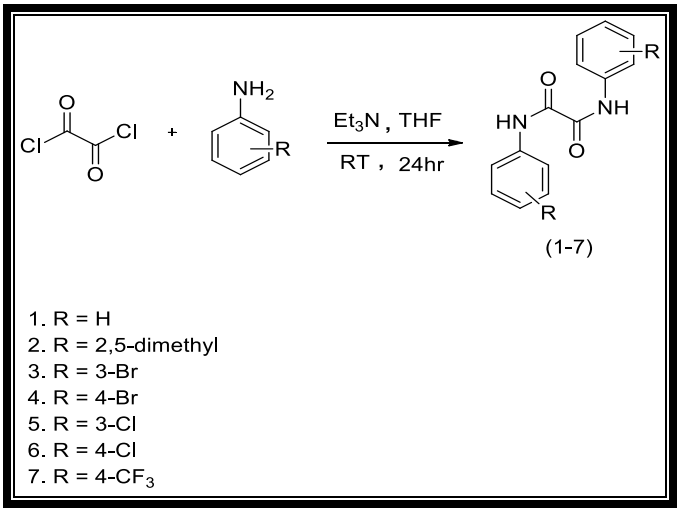
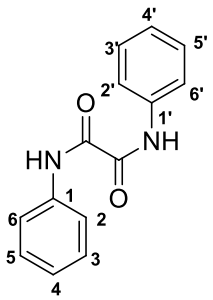


Fig. 1. Synthsis of oxalamides.

Characterization

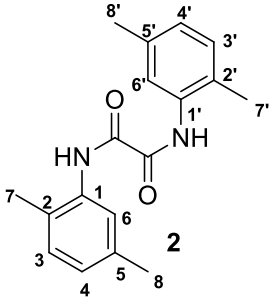
The characterization and physicochemical data obtained from spectroscopic techniques, namely, NMR, EI-MS, and IR for the synthesized oxalamides is shown as follows.

N1,N2-diphenyloxalamide (1)



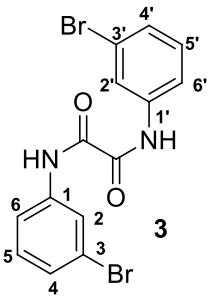
Yield: (38%), White solid.
M.p. : 252-252℃.
^{1}H-NMR (DMSO, 300 MHz) δ 10.83 (s, 2H, N-H), 7.87 (d, 4H, J = 8.4 Hz, H-2, H-2', H-6' H-6'), 7.37 (t, 4H, J = 7.5 Hz, H-3, H-3', H-5, H-5'), 715 (t, 4H, J = 7.2 Hz, H-4, H-4').
MS (EI): 240.0[M^+], 120.93.
IR (KBr) υmax cm^{-1}: 3303.8 (NH), 3055.0 (sp2-CH), 1668.3 (C=O), 1596.9, 1523.7 (C=N, C=C), 1436.9 (CH bending aromatic), 1311.5 (C-N).

N1,N2-bis(2,5-dimethylphenyl)oxalamide (2)



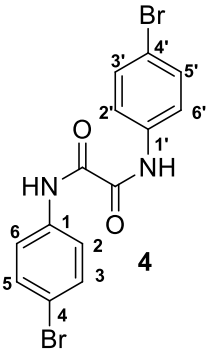
Yield: (39%), White solid.
M.p.: 228℃.
^{1}H-NMR (DMSO, 300 MHz) δ 10.16 (s, 2H, N-H), 7.33 (s, 2H, H-6, H-6‘), 7.16 (d, 2H, *J* = 7.8Hz, H-4, H-4’), 7.00 (d, 2H, J = 7.8Hz, H-3, H-3’), 2.28 (s, 6H, 2-CH_3), 2.28 (s, 6H, 2-CH_3).
MS (EI): 296.1[M^+, 100%], 148.0, 121.0.
IR (KBr) υmax cm^{-1}: 3255.6 (NH), 3050.6 (sp2-CH), 2920.0 (sp3-CH), 1670.2 (C=O), 1577.1 (C=N), 1521.7 (C=C), 1471.6 (C-H bending aromatic), 1217.4 (C-N)

N1,N2-bis(3-bromophenyl)oxalamide (3)



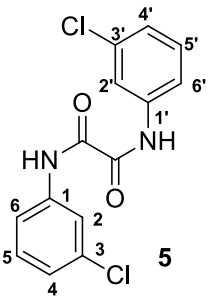
Yield: (49.8%), White solid.
M.p. : 217-219℃.
^{1}H-NMR (DMSO, 300 MHz) δ 11.05 (s, 2H, N-H), 8.13 (s, 2H, H-2, H-2’), 7.86 (t, 2H, *J* = 2.4 Hz, H-5, H-5’), 7.35 (m, 4H, H-6, H-4’, H-6’).
MS (EI): 398.3[M^+], 399.3[M+1], 400.3[M+2], 199.1, 171.0[100%].
IR (KBr) υmax cm^{-1}: 3340.5 (NH), 3055.0 (sp2-CH), 1691.5 (C=O), 1583.4 (C=N), 1525.6 (C=C), 1400.2 (C-H bending aromatic), 1294.1 (C-N).

N1,N2-bis(4-bromophenyl)oxalamide (4)



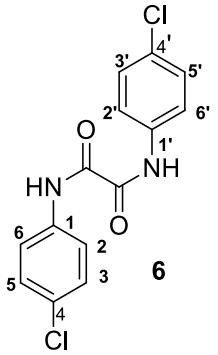
Yield: (79.3%), White solid.
M.p. : 260℃.
^{1}H-NMR (DMSO, 300 MHz) δ 10.97 (s, 2H, *N-H*), 7.84 (d, 4H, *J* = 6.6 Hz, H-3, H-5, H-3’, H-5’), 7.57 (d, 4H, *J* = 6.6 Hz, H-2, H-6, H-2’, H-6’).
MS (EI): 398.1[M^+], 399.1[M+1], 400.0[M+2], 199.1, 171.1[100%].
IR (KBr) υmax cm^{-1}: 3292.3 (NH), 3045.4 (sp2-CH), 1662.5 (C=O), 1589.2 (C=N), 1510.2 (C=C), 1390.6 (C-H bending aromatic), 1294.1 (C-N).

N1,N2-bis(2-chlorophenyl)oxalamide (5)



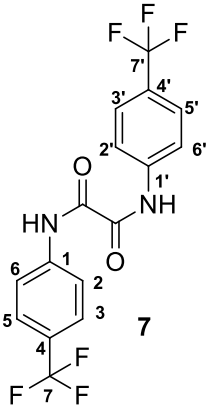
Yield: (31%), White solid.
M.p. : 199-201℃.
^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.29 (s, 2H, N-H),7.78 (s, 2H, H-2, H-2’), 7.49 (d, 2H, *J* = 6.0 Hz, H-4, H-4’), 7.31 (t, 2H, *J* = 6.0 Hz, H-5, H-5’), 7.19 (d, 2H, *J* = 6.0 Hz, H-6, H-6’).
MS (EI): 308.2[M^+], 309.3[M+1], 310.3[M+2], 154.1, 127.1[100%].
IR (KBr) υmax cm^{-1}: 3305.8 (NH), 3076.2 (sp2-CH), 1668.3 (C=O), 1589.2 (C=N), 1521.7(C=C), 1425.3(Aromatic CH bending), 1272.9 (C-N).

N1, N2-bis(4-chlorophenyl)oxalamide (6)



Yield: (72%), White solid.
M.p. : 293-295℃.
^{1}H-NMR ($CDCl_3$, 300 MHz) δ 10.96 (s, 2H, N-H), 7.89 (d, 4H, J = 6.6Hz, H-3, H-5, H-3', H-5'), 7.44 (d, 4H, *J* = 6.3 Hz, H-2, H-6, H-2', H-6').
MS (EI): 308.0[M^+], 308.9[M+1], 310.0[M+2], 154.9, 127.0[100%].
IR (KBr) υmax cm^{-1}: 3296.1 (NH), 3099.4 (sp2-CH), 1662.5 (C=O), 1589.2 (C=N), 1514.0 (C=C), 1392.5(C-H bending aromatic), 1291.6 (C-N).

N1,N2-bis(4-(trifluoromethyl) phenyl)oxalamide (7)



Yield: (60%), White solid.
M.p. : 305-307℃.
^{1}H-NMR (DMSO, 300 MHz) δ 11.21 (s, 2H, *N-H*), 8.09 (d, 4H, *J* = 6.3Hz, H-3, H-5, H-3', H-5'), 7.77 (d, 4H, *J* = 6.3 Hz, H-2, H-6, H-2', H-6').
MS (EI): 376.0[M^+], 377[M+1], 188.0, 159.9[100%].
IR (KBr) υmax cm^{-1}: 3357.8 (NH), 3294.2 (sp2-CH), 1679.9 (C=O), 1600.8 (C=N), 1423.4 (C-H bending aromatic), 1249.8 (C-N).
Results of all spectroscopic analysis confirmed the synthesis of oxalamide and its purity. ^{1}H-NMR, EI-MS and IR spectra of compound 3 are shown in (Fig. **2**).

BIOLOGICAL SCREENING

Antioxidant Studies:

Results for antioxidant screening of oxalamide derivatives are shown in Table 1. DPPH radical scavenging assay showed that three out of the seven synthesized oxalamide derivatives exhibited significant antioxidant activity which was close to the activity of standard drug (BHA). The half maximal inhibitory concentration for compound 2, 3, and 4 was 51.2, 55.4, and 47.3 µM, whereas, for BHA the IC_{50} was 44.2 µM. The efficiency of compound 2, 3, and 4 was 86, 80, and 93% of the standard BHA. Four oxalamide derivatives were found to be inactive in this screening. These results revealed that substitution on oxalamide effected the antioxidant property of compound. This may be due to difference in electronegativity of the substituents. Compounds 5, 6, and 7 substituted with highly electronegative group showed no activity while compounds 2, 3 and 4 showed good activities. The unsubstituted oxalamides showed no antioxidant properties.

DPPH scavenging activity of the compounds synthesized in this study was also compared with some of the reported analogues like anilines, phenylenediamines, aminophenols, polyanilines and HCl doped anilines. It was reported that polyanilines doped with hydrochloric acid showed higher efficiency as compared to polyanilines while the later were more efficient than anilines (Gizdavic-Nikolaidis *et al.*, 2004). The IC_{50} of the compounds synthesized in this study were bis(2,5-dimethylphenyl)oxalamide (51.2 µM), bis(3-bromophenyl)oxalamide (55.4 µM), and bis(4-bromophenyl)oxalamide (47.3 µM) which is much better when compared to o-phenylenediamine (590µM), p-phenylenedamine (600 µM), 2-aminophenol (400 µM), 3-aminophenol (330 µM), and 4-aminophenol (300 µM) (Bendary *et al.*, 2013; Wright *et al.*, 2001). The high efficiency of diamines is attributed to the resonance stability of the product formed after radical scavenging (Wright *et al.*, 2001). The compounds synthesized in this study had higher resonance stability due to their high aromaticity which resulted in low IC_{50} values. The compounds which didn't show significant antioxidant activity had electron withdrawing substitutions and their negative inductive effect would have affected their resonance stabilization.

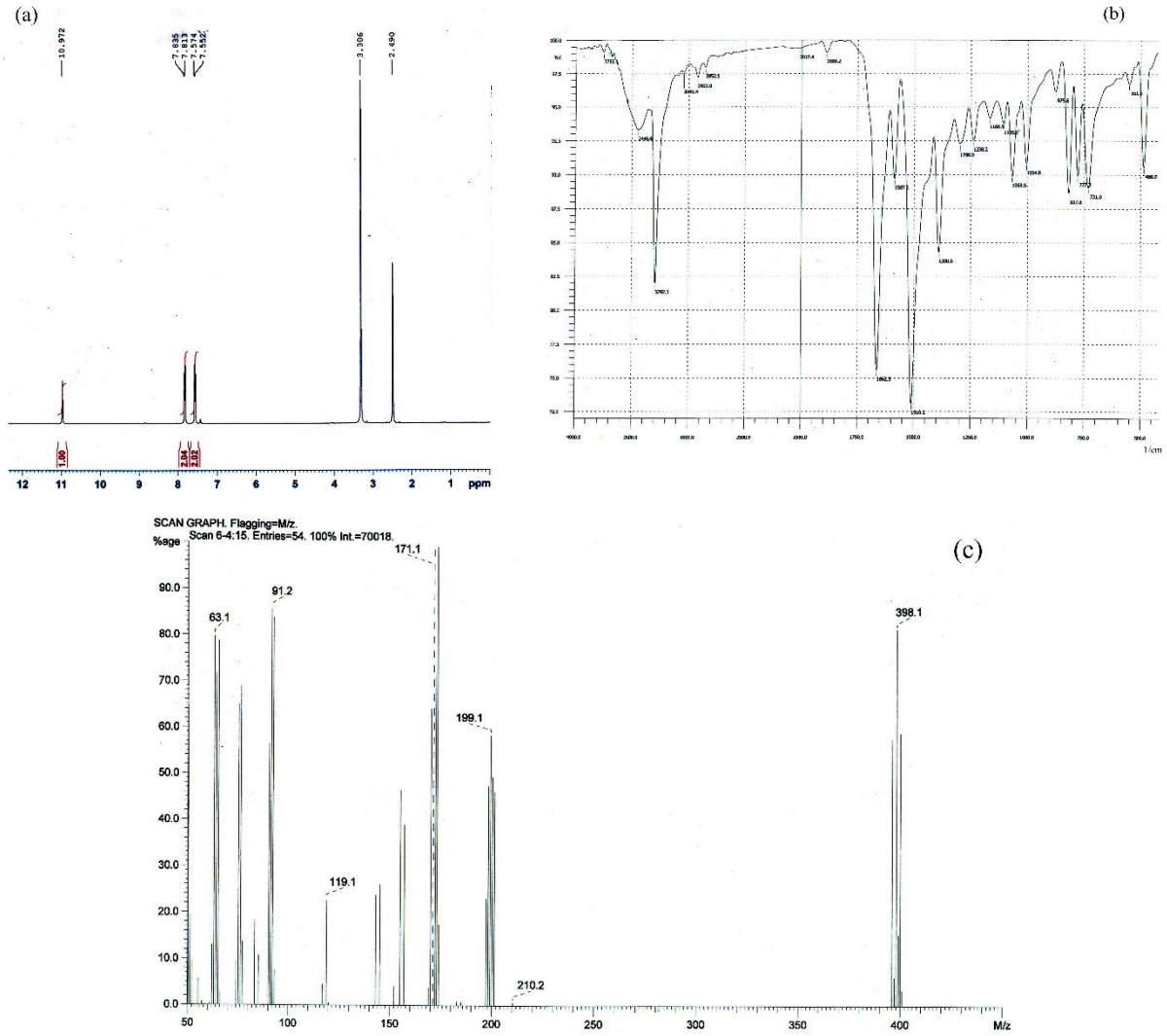


Fig. 2. Characterization of synthesized oxamides a) NMR Spectrum, b) IR Spectrum, and c) EI-MS spectrum.

Table 1. Antioxidant activity screening of synthesized oxalamides.

Sample ID	R	Antioxidant Activity IC_{50} (μM± SEM)
1	H	N.A.
2	2,5-dimethyl	51.2±0.34
3	3-Br	55.4 ± 0.38
4	4-Br	47.3 ± 0.24
5	3-Cl	N.A
6	4-Cl	N.A
7	4-CF_3	N.A.
BHA (standard)	-	44.2 ± 0.04

N.A. = No Activity

Anticancer studies:

Anticancer activities of oxalamides are shown in Table 2. Oxalamide derivatives were found to be inactive against cancer cell lines and hence don't possess anticancer activity against beta lymphoma.

In this study a series of N,N-bis substituted oxalamide derivatives were synthesized by reacting amine with oxalyl chloride in the presence of triethylamine. Synthesized oxalamides were characterized using different spectroscopic techniques. The synthesized compounds were screened for antioxidant properties. It was observed that bis (2,5-dimethylphenyl)oxalamide, bis (3-bromophenyl)oxalamide, and bis (4-bromophenyl)oxalamide had good antioxidant properties. The compounds have potential to undergo further pharmacological studies. However, synthesized oxalamide derivatives did not show any anticancer properties.

Table 2. Anticancer activity studies for synthesized oxalamides.

Sample ID	R	Raji (Burkitt's Lymphoma)	$DOHH_2$ (Follicular lymphoma)
1	H	N.A.	N.A.
2	2,5-dimethyl	N.A.	N.A.
3	4-Br	N.A.	N.A.
4	3-Br	N.A.	N.A.
5	4-Cl	N.A	N.A
6	3-Cl	N.A	N.A
7	4-CF_3	N.A.	N.A.
(Standard) Imatinib	-	65.44 ± 3.76	53.10 ± 1.37
(Standard) Dasatinib	-	33.64 ± 1.9	47.08± 0.75

N.A. = No Activity

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